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The effect of psychrotrophic bacteria on the quality of UHT milk

A thesis presented in partial fulfilment of the requirements for the degree of

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Abstract

The psychrotrophic bacterial contamination of raw milk is one of the key factors determining the quality of processed dairy products due to the heat-stable enzymes produced by these bacteria. However, routine testing of raw milk only tests for total culturable mesophilic bacteria, not specifically the psychrotrophic bacteria. Another limitation of routine testing is the lack of any indication of the non-culturable bacterial types present, which may also influence milk quality. Studying the impact of this un-cultural component is a challenge but being able to detect them is useful in relating their presence to milk quality.

In Chapter 3, 16S rDNA high-throughput sequencing and MALDI-TOF MS were used to identify the culturable and non-culturable psychrotrophic bacteria population of NZ raw milk, which had not been done before. Both methods showed that *Pseudomonas* is the predominant genus while high-throughput sequencing revealed a more diverse population than MALDI-TOF MS. The combination of these two methods can provide us a better picture of the psychrotrophic bacteria population of raw milk compared with the traditional methods. A seasonal and regional variation in the psychotrophic bacterial composition in raw milk was observed. This information is also valuable to the NZ dairy industry, to enable the selection of the best quality milk for specific applications. The aim of Chapter 4 was to investigate the effect of extended chilled storage on the diversity of psychrotrophic bacteria in raw milk under the genus and species levels. The results showed that the psychrotrophic bacterial composition of chilled enriched milk is less diverse than fresh raw milk. The proportions of different bacteria also changed. For example, *P. fragi* was the predominant species in fresh raw milk while *P. lundensis* became the predominant species after 5 days of chilled storage. Changes in the microbial composition of raw milk to specific bacteria can potentially affect the quality of final milk products as different bacteria vary in their ability to produce enzymes that spoil milk. This chapter also evaluated the heat-stability of proteolytic and lipolytic enzymes from the psychrotrophic bacteria. The heat stability of the enzymes produced by some bacteria, such as *Acinetobacter* species isolated from milk, has not been reported previously. To maintain the quality of dairy products, understanding the growth of

Pseudomonas and their protease activity in raw milk before any heat treatment is important. In Chapter 5, six dominant *Pseudomonas* showing heat-stable protease activity from Chapter 4 were grown in TSB, Skim UHT milk, and Whole UHT milk at 7°C for 7 days. There were higher levels of proteolytic activity in milk media compared to TSB, with the whole milk showing the highest level. This leads to a hypothesis that dairy ingredients influence protease activity. The proteolytic activity in TSB medium enriched with different dairy ingredients showed that the presence of milkfat increased proteolytic activity. This was an unexpected result, not reported previously. The results based on an azocasein method were further confirmed by zymographic analysis. Multiple and stronger bands were observed in whole milk compared with skim milk and other media. Stronger bands were also observed in TSB enriched with fat.

This is the first report showing that milk fat can induce the protease activity of some *Pseudomonas* strains. One of the key determinants of the quality of UHT milk is the quality of the raw milk used to manufacture this product. However, the definition of “quality” of raw milk is not well defined. Total bacterial count, somatic cell counts, and antibiotic levels do not necessarily relate to the amount enzyme-producing bacteria nor the heat resistance of the enzymes produced. The aim of Chapter 6 was to replicate the effect of specific, high protease enzyme producing bacteria in raw milk used for UHT milk manufacture and predict the shelf life from the numbers of these bacteria raw milk used for UHT milk manufacture. Bacterial isolates (both single and mixed isolates) showing heat-stable proteolytic activity and commonly found in raw milk were grown to different numbers (10^4 - 10^7 cfu/mL) in farm-fresh UHT treated milk then UHT treatment was repeated. The storage life of this product was monitored over different temperatures (20, 30, and 55°C) likely to be found in the supply chain for up to 9 months. The results provide, for the first time, a guideline for UHT manufacturers to predict shelf life based on the initial microbiological content of the raw milk. Improvements in selecting the high microbiological quality of raw milk may reduce economic loss in the dairy industry.

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Declaration

The presented thesis is comprised of 7 chapters. Partial results of chapters 3, 4, 5, and 6 are structured as manuscripts that have either been published or submitted. Thus, sections in material and methods are repeated in some chapters, however, results and discussion are different for each chapter.

The contributions of each of the authors in the publications have been addressed in the DRC 16 forms attached at the end of each thesis chapter.

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List of Publications

Published Manuscripts

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3. **Dong Zhang**, Siqu Li, Jon Palmer, Koon Hoong Teh, Sharon Leow, and Steve Flint. (2020). "The relationship between numbers of *Pseudomonas* bacteria in milk used to manufacture UHT milk and the effect on product quality." *International Dairy Journal*, 105.

Submitted Manuscripts

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1. Dong Zhang. The effect of raw milk quality on final dairy product quality. Postgraduate Student Symposium, Massey University, Palmerston North, New Zealand, 2016 Feb. Oral.
2. Dong Zhang. Isolation, identification, and characterization of heat-stable enzyme-producing psychrotrophic bacteria from raw milk using 16S rDNA high-throughput sequencing and MALDI-TOF MS. NZMS Conference, Christchurch, 2016 Nov. Poster.
3. Dong Zhang. Next-generation sequencing reveals the seasonal variation of psychrotrophic bacteria in New Zealand raw milk. NZMS Conference, Auckland, 2017 Nov. Poster.
4. Dong Zhang. Effect of single and mixed isolates of protease-producing *Pseudomonas* spp. in milk used for UHT manufacture. CAAS Conference, Beijing, China, 2019 May. Oral.
5. Dong Zhang. Milk fat influences proteolytic enzyme activity of dairy *Pseudomonas* species. NZMS Conference, Palmerston North, 2019 Nov. Oral.

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Chapter 1. Introduction

1.1 Background

Raw milk is a fragile ingredient in dairy product manufacture, and the quality of the final product may be affected by many variables associated with raw milk handling. From a microbiological perspective, it is thought that the bacteria, in terms of the numbers and types, present in raw milk at the start of dairy product manufacture, have a big impact on the quality of the final product. Although the combination of refrigeration ($\leq 7^{\circ}\text{C}$), reduced transportation and storage time and heat treatments (72°C , 15 s or 140°C , 1-10 s) of raw milk can significantly extend the shelf life of dairy products, heat-stable bacterial enzymes produced by psychrotrophic bacteria (psychrotrophs) have the potential to harm final dairy products. For example, in ultra-high-temperature (UHT) dairy products, the shelf life is affected mostly by psychrotrophic bacterial proteolytic enzymes (Marchand et al., 2017). Estimates of the spoilage rate of UHT packs vary from 1 to 4 per 10,000, with a rate of 1 in 10,000 being considered a reasonable commercial standard (Deeth, 2010). It is well-known that the dairy industry plays a vital role in New Zealand economics. 95% of the New Zealand dairy products exports overseas, including UHT milk. New Zealand is a quite different type of dairy industry compared to many other countries all over the world, particularly compared to Europe and the USA where there is a greater reliance on supplementary feeding. Pasture feed is the major feed source for animals in New Zealand, which may cause a different microbial make-up of raw milk used for UHT milk. One of the key issues that we became aware of when starting this project is that we were informed by Tetra Pak that consumer complaints on the UHT milk quality during shelf life have been increasing recently, which costs the dairy industry money in New Zealand. However, the science that underpins this issue is lacking. Routine quality indicator tests such as titratable acidity, pH, and total bacteria count used for raw liquid dairy streams before processing, are not reliable predictors of the customer complaints (Tetra Pak, 2014). The ultimate goal of this project is to find detailed information and scientific evidence that could explain this observation.

This project used both DNA-based and culture-based technologies to profile the whole psychrotrophic bacterial community of raw milk, which is very important to the New Zealand industry and hasn't been done previously. Storage conditions and microbiological factors that lead to damage of raw milk components have been defined in the study, and links between numbers and types of proteolytic psychrotrophs in raw milk and UHT milk quality have been shown, thus providing fundamental information of potential value to the dairy industry in selecting top-grade raw material used for UHT milk.

1.2 Research questions

- What are the psychrotrophs of raw milk in New Zealand dairy manufacturing plants?
 - a) What are the culturable and non-culturable psychrotrophs in raw milk?
 - b) How do different seasons and regions affect the psychrotrophic bacterial composition of raw milk in New Zealand?
- What are the enzymatic activities of psychrotrophs isolated from raw milk?
 - a) Can these bacteria produce enzymes at 7°C and are these enzymes active at 7°C?
 - b) Can the isolated bacteria produce heat-stable enzymes?
- What environmental factors influence the growth and proteolytic activity of psychrotrophs from raw milk?
 - a) Are the bacterial growth and proteolytic activity different in the dairy environment and non-dairy environments?
 - b) Can dairy components affect bacterial proteolytic activity?
- Can the deterioration of UHT milk be predicted before processing?
 - a) How can we determine if the quality of raw milk is good enough to be processed?

- b) What more appropriate test methods for dairy ingredients prior to processing can be used to predict more accurate measures of final product quality?

1.3 Hypotheses

- The combination of culture-independent and culture-dependent analysis will improve our understanding of the psychrotrophic bacterial profile of New Zealand raw milk across seasons and regions.
- Prolonged refrigeration of raw milk will change the psychrotrophic bacterial population at both the genus and species levels. The extracellular enzymes of some psychrotrophs that have not reported previously maybe heat stable.
- Different dairy components will influence bacterial proteolytic activity, especially milk fat.
- Raw milk containing less than 10^6 cfu/mL of protease-producing psycrotrophs may not be suitable for UHT manufacture.

1.4 Objectives

- To investigate the seasonal and regional psychrotrophic microbial communities of raw milk in New Zealand dairy manufacture using culture-independent and culture-dependent methods
- To investigate the effect of extended refrigeration on the changes of psychrotrophic bacterial composition of raw milk and select the heat-stable extracellular enzyme-secreting (protease and lipase) isolates for further studies
- To demonstrate bacterial growth and proteolytic activity of 6 selected dairy isolates in different conditions at 7°C for 7 days
- To create a worst-case scenario of raw milk psychrotrophic bacterial composition (based on high enzyme secreting bacteria) for UHT manufacture, and therefore to understand

how the numbers and types of protease-secreting bacteria in raw milk impact final UHT milk quality

1.5 References

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Chapter 2. Literature review

2.1 Introduction

Bovine milk is an excellent nutritious food that is used for human consumption. However, such an environment, which contains carbohydrates, proteins, fats, vitamins, minerals with high water activity, and a neutral pH, supports the growth of many microorganisms (Claeys et al., 2013). The contamination and growth of these microorganisms present in raw milk may occur on the farm, during transportation and in the processing plant. They can negatively affect the quality of dairy products. For example, milk containing high numbers of bacteria will have a short shelf life, resulting in off flavours and smells, and also increase fouling in heat exchangers and thus reduce the efficiency of processing equipment. To extend the shelf life of products, different heat treatments, such as pasteurization and ultra-high-temperature (UHT) processing, are applied to destroy the majority of the bacteria present in raw milk. However, some bacteria such as psychrotrophic bacteria (psychrotrophs), can produce heat-stable enzymes (proteases and lipases) before the heat treatment. These heat-stable enzymes can remain active following the heat treatment and then hydrolyse the milk proteins and fats during storage, leading to the spoilage of final products. The specific composition of the raw milk microbiota can influence on the quality and shelf life of final dairy products in the market.

In this study, the main bacterial groups naturally present in raw milk will be discussed, providing some indication of the main sources of contamination and how they can influence milk quality before processing. This review will also look at the role of the bacteria in the dairy industry and how their heat-stable enzymes affect final dairy product quality during storage.

2.2 Microbiology of raw milk

Raw milk is free of microorganisms when it is in a healthy cow's udder. However, milk starts to become contaminated by many microorganisms when it is released from the teat channel. After secretion, faecal contamination and general dirt from the animal surface and microorganisms in the environment will find their way into the milk (Angelidis, 2015). Not all

bacteria found in raw milk represent the same threat to milk quality. The activity of a select group of microorganisms (acid-producing, extracellular enzyme-secreting, spore-forming bacteria, etc.) are most likely to have the greatest impact on product quality. This section reviews the major bacteria involved in the spoilage of dairy products.

2.2.1 Psychrotrophs

Keeping raw milk under refrigeration is a global practice to maintain the microbiological quality of raw milk because it can effectively decrease the growth of mesophilic and thermophilic bacteria in raw milk. The refrigerated storage of raw milk can be up to 5 days before heat treatment (Lafarge et al., 2004). However, these conditions can also provide a selective advantage for the growth of psychrotrophs. The term psychrotrophs refers to the microbes that can grow or even thrive at cold temperatures ($\leq 7^{\circ}\text{C}$), although the optimal growth temperature is between 20 to 30°C (Quigley et al., 2013). Psychrotrophs are capable of synthesis of phospholipids and lipids, resulting in an increasing proportion of polyunsaturated fatty acids in their cells which protect the bacteria in a refrigerated environment (Oliveira et al., 2015). These bacteria primarily exist in the water and soil (Yuan et al., 2019).

One of the major technological problems in the dairy industry is the heat-stable enzymes produced by psychrotrophs that can attack milk components. Although these bacteria can be killed by pasteurization and UHT processing, heat-stable enzymes produced by these bacteria during milk storage and transportation under refrigeration can remain active after heat treatment. These heat-stable enzymes can degrade milk proteins and fats, increasing the concentration of free fatty acids and amino acids, resulting in a trace of bitterness, off-flavour and gelation of final products (Chen et al., 1997; Yuan et al., 2019).

Psychrotrophs are biological indicators to assess the microbial quality of raw milk. When the level of psychrotrophs exceeds 10^6 cfu/mL, the quality of final dairy products can be severely affected (Marchand et al., 2009). The European Union (EU) standard for top-grade raw milk recommends that the population of psychrotrophs is less than 4.22 log cfu/mL (Mühlherr et al.,

2003). The initial level of bacterial counts and storage conditions affect psychrotrophic bacterial populations. Under good hygiene conditions, psychrotrophs generally account for 10% of the microbiota of raw milk, but when milk is stored under poor hygienic conditions, the psychrotrophic bacterial population can make up 75% of the total microbial load of raw milk (Angelidis, 2015). Poor hygienic farming practices and animal infection (mastitis), will increase the initial bacterial counts and result in high bacterial numbers being reached sooner than milk with lower initial counts (Fig. 2.1). The levels of the contamination are closely linked to the on-farm environment, the milking equipment and the microbiological quality of bedding, feed, air and water.

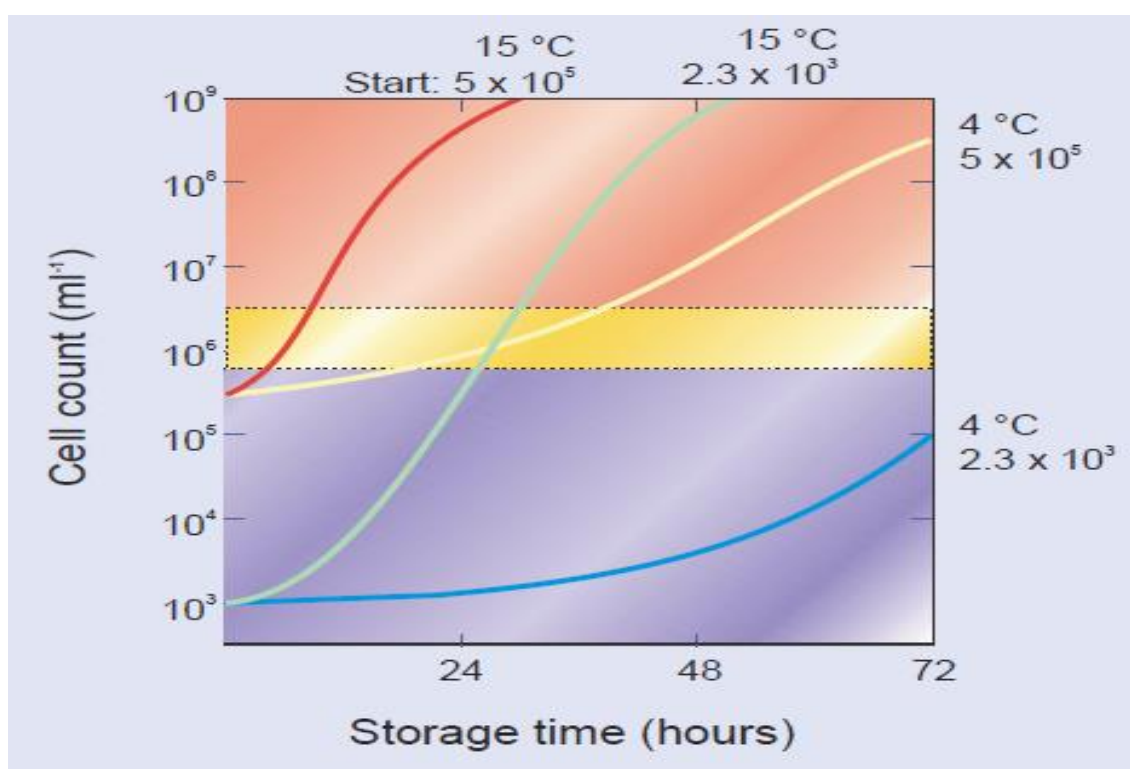


Figure 2.1 Bacterial growth from different initial counts at two different temperatures (Delaval, 2000). *Copyright © Delaval belongs to Tetra Pak, who were part sponsors of this project. The copyright is automatically authorized.

Fresh raw milk drawn from a cow's udder does not contain detectable numbers of culturable psychrotrophs (Oliveira et al., 2015). However, these populations develop over time during cold storage raw milk. The faster the milk is cooled after milking, the better the quality when it is received from the farm. The influence of temperature on bacterial development in raw milk is shown in Figure 2.1. Starting from 3.0×10^3 cfu/mL, cooling to below 4 °C can significantly contribute to the quality of the milk on the farm. For example, Griffiths et al. (1987) compared

the storage of raw milk under what they describe as “deep cooling” of the milk to 2°C under active refrigeration with storage described as “normal conditions” in a commercial silo at 6°C. An increase (2 to 3.9 days) in the storage life of the milk (based on the time to reach 10⁶ cfu/mL) was achieved when the farm bulk milk was stored at 2°C rather than 6°C. Stored raw milk at ≤ 7°C before processing is generally accepted by many countries and dairy companies, but different countries have different standards for milk cooling:

- a) The EU regulations state that when the milk enters the processing plant, the temperature of the cooled milk must not exceed 10°C unless the milk has been collected within 2 h of milking (EU, 1992).
- b) In the United States, the Food and Drug Administration recommends raw milk for pasteurisation, ultra-pasteurisation or aseptic processing must be cooled to 7°C or less within 2 h after milking, provided the blend temperature after the first and subsequent milking does not exceed 10°C. This temperature (7°C) must be maintained until further processing occurs (Food and Drug Administration, 1989).
- c) In Australia, milk must be cooled to 4°C, or below, within 3.5 h of the start of milking (Standards Australia, 1996).
- d) In New Zealand, the latest raw milk cooling regulation introduced by the Ministry for Primary Industries New Zealand in August, 2017 states that “raw milk must be cooled to 10°C or below within 4 h of the commencement of milking, be cooled to 6°C or below within 6 h from the commencement of milking, or 2 h from the completion of milking, be held at or below 6°C without freezing until collection or the next milking and must not exceed 10°C during subsequent milking” (Ministry of Primary Industries, 2017).

Both Gram-negative and Gram-positive psychrotrophs have been isolated from raw milk. Gram-positive bacteria found in raw milk include the following genera: *Bacillus*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Arthrobacter*, *Staphylococcus*, and *Carnobacterium* (Oliveira et al., 2015; Quigley et al., 2013; Yuan et al., 2019). *Bacillus* spp. are

the most common Gram-positive psychrotrophs in raw milk, mainly consisting of *B. cereus*, *B. subtilis*, and *B. polymyxa* (Magnusson, Christiansson, & Svensson, 2007). Because of the longer lag phase at temperatures of 0-7°C, *Bacillus* spp. are less common in raw milk than *Pseudomonas* spp. However, *Bacillus* predominates in marginal cooling temperatures, from 8°C to 10°C (Angelidis, 2015). Between 40-84% of *Bacillus* spp. are capable of producing both proteases and lipases (Samaržija, Zamberlin, & Pogačić, 2012b). *B. cereus* is an important psychrotrophic pathogenic bacterium in the dairy industry as it produces endospores and survives after heat treatment. The majority of *B. cereus* are not able to degrade lactose but can ferment glucose, fructose, trehalose, N-acetyl glucosamine, and mannose (Angelidis, 2015). The vegetative cells of most strains produce proteases when the cell counts are more than 10⁶ cfu/mL, resulting in sweet curdling of milk. They also produce phospholipases acting against phospholipids causing a fat accumulation defect in cream, also called “bitty cream” whereas most of the Gram-negative psychrotrophs do not cause the bitty cream defect in dairy products (Chen et al., 2003).

The Gram-negative bacteria include the following genera: *Pseudomonas*, *Aeromonas*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter*, and *Flavobacterium*. The majority of Gram-negative psychrotrophs isolated from raw milk are *Pseudomonas* spp., which usually comprise 65-70% of the whole psychrotropic bacterial population (Ledenbach & Marshall, 2010). *Pseudomonas* is the most heterogeneous and ecologically important genus in the dairy industry (Marchand et al., 2009). They originate from most natural habitats, but primarily water and soil (Marchand et al., 2009). They tend to outgrow other bacteria present in refrigerated raw milk due to their simple nutritional requirements and relative short generation time (<4 h) at 0-7°C (De Jonghe et al., 2011). One cell of a *Pseudomonas* spp. can reach 1.0x10⁶ cfu/ml within 8 days at 4°C (Dogan & Boor, 2003). More than 50% of *Pseudomonas* spp. in raw milk are *P. fluorescens*. Other *Pseudomonas* spp., such as *P. fragi* and *P. lundensis*, are also commonly found in raw milk and involved in the degradation and spoilage of dairy products (Dogan & Boor, 2003; Marchand et al., 2009).

If the milking equipment is improperly cleaned, psychrotrophs can attach to the surface of the stainless pipes or equipment and form a biofilm (Teh et al., 2015). Biofilms pose serious challenges to the dairy industry because they contaminate various surfaces, including stainless steel, plastic, and rubber, and release bacteria into milk passing over those surfaces (Marchand et al., 2012). Once the biofilms are formed, they are very difficult to control, resulting in the production of more bacteria and their enzymes (Teh et al., 2011). Regular cleaning is important to minimise the biofilm contamination of milking equipment and control the contamination of raw milk.

2.2.2 Lactic acid bacteria

Lactic acid bacteria (LAB) are facultative anaerobic microbes found in cow's milk. They exist on the skin of the cow, on bedding material and feed (Widyastuti & Febrisiantosa, 2014). This group includes *Lactococcus* spp. (8.2×10^1 to 1.4×10^4 cfu/mL), *Streptococcus* spp. (1.4×10^1 to 1.5×10^4 cfu/mL), *Lactobacillus* spp. (1.0×10^2 to 3.2×10^4 cfu/mL), *Leuconostoc* spp. (9.8×10^1 to 2.5×10^3 cfu/mL), and *Enterococcus* spp. (2.5×10^1 to 1.5×10^3 cfu/mL) in fresh cow's milk (Delbès, Ali-Mandjee, & Montel, 2007; Masoud et al., 2011; Quigley et al., 2013; Randazzo et al., 2002). Selected strains of LAB are widely used as starter cultures and probiotics to make fermented dairy products, such as cheese, yogurt, and kefir (Leroy & De Vuyst, 2004). While LAB do not grow and produce heat-stable enzymes at 7°C, and can be eliminated by pasteurization or UHT processing, they can ferment sugars (e.g. lactose to lactic acid), resulting in a lowering of the pH of the milk. If raw milk drops 0.1 to 0.2 pH units, the acidified milk is difficult to heat without precipitation and coagulation occurring. Milk deposits are poor thermal conductors and restrict both heat transfer and fluid flow, which result in fouling in the heat exchanger and shortened production time. If the pH of raw milk drops from 6.9 to 6.5, the milk cannot be used in pasteurized or UHT milk manufacture as it can precipitate or coagulate in the heat exchanger and package (Tetra Pak, 2014).

2.2.3 Spore-forming bacteria

Spore-forming bacteria can survive harsh environments such as nutrient limitation, heat treatments, UV light, and osmotic pressure for extremely long periods - even years (Buehner, Anand, & Djira, 2015). The spores can germinate to vegetative cells once the environment is suitable (i.e. availability of water, appropriate nutrients, and growth temperature). In milk, they originate mainly from the cow's skin, soil, silage, and forage (McHugh et al., 2017).

The number of spore-forming bacteria in raw milk is rarely over $5.0 \times 10^3/\text{mL}$. However, there are bacterial spores that can remain active after pasteurization and limit the shelf life of pasteurized milk (Tetra Pak, 2014). The most common aerobic spore-forming bacteria cultured from raw milk are *B. licheniformis*, *B. cereus*, *B. subtilis*, *B. mycoides* and *B. megaterium* (Griffiths & Phillips, 1990). *Bacillus* spp. are known to cause dairy product spoilage by producing heat-stable enzymes during cold storage (Doyle et al., 2015). Additionally, Ranieri et al. (2012) reported that the number of *Paenibacillus* spp. in pasteurized milk comprised over 95% of the total microbial load after 10-day cold storage, which may lead to a serious deterioration of milk products.

2.2.4 Thermotolerant bacteria

Frank & Yousef (2004) define thermotolerant as bacteria that can survive low-temperature long time (LTLT, 63°C, 30 min) pasteurization. The most prevalent of thermotolerant bacteria are *Bacillus* spp. and *Paenibacillus* spp. Other genera such as *Clostridium* spp., *Corynebacterium* spp., *Kocuria* spp. and *Staphylococcus* spp. also have been isolated from pasteurized milk (Coorevits et al., 2008). The main sources of contamination of thermotolerant bacteria in raw milk are the skin of the cow, bedding material, and feed (Tetra Pak, 2014).

The population of thermotolerant bacteria is usually very low in raw milk ($<5.0 \times 10^2 \text{ cfu/mL}$). However, they can form biofilms, which can lead to the post-pasteurisation contamination (Tetra Pak, 2014). *Geobacillus sporothermodurans* is one thermotolerant contaminant causing the concern in dairy products, particularly in UHT milk (Vaerewijck et al., 2001). The highly heat-stable

spores of *G. sporothermodurans* were first detected in UHT milk in 1985 in southern Europe (Rupesh et al., 2011). The number of this bacterium exceeded the sterility criterion of 10^2 cfu/mL according to the EU criteria (Scheldeman et al., 2006). *G. sporothermodurans* can produce acid during storage, resulting in the “flat sour” defect in canned milk products. Also, the biofilm formation of *G. sporothermodurans* in the dairy industry can reduce flow rates and heat transfer (Lang et al., 2011; Simões, Simões, & Vieira, 2010). However, the contamination and biofilm formation of *G. sporothermodurans* is not well understood in dairy manufacture (Scheldeman et al., 2006).

2.2.5 Coliforms

Coliform bacteria have been defined as facultative anaerobic, Gram-negative, rod-shaped bacteria with the optimum growth temperature of 30-37°C that can ferment lactose to lactic acid with the production of gas. Coliforms are present in the soil, dirty water, milking equipment, and faeces. *Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter* are the most common genera isolated from milk that belong to the coliform group. As coliforms can be eliminated by high-temperature short time (HTST, 72°C, 15 s) pasteurisation, they are commonly used as indicator microorganisms for hygiene issues after pasteurization. If there are no coliform bacteria present, this is one indication of successful pasteurization. Coliform bacteria are also used to indicate successful cleaning in the dairy manufacturing plants (Angelidis, 2015). Coliform contamination can result in economic loss in cheese making. They can break down milk proteins, ferment lactose to lactic acid and produce gas, resulting in an unacceptable flavour, smell, and texture in cheese. Although the formation of lactic acid by the starter bacteria reduces the pH, the coliform bacteria can survive the early stages of cheese making (Alalade & Adeneye, 2006). They can also affect the quality of buttermilk and sour cream by the production of diacetyl, leading to a yogurt-like flavour (Ledenbach & Marshall, 2010).

2.2.6 Mastitis bacteria

Some bacteria cause mastitis, an intra-mammary infection (IMI) in cows. *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma* spp. are typical causes of mastitis (Barbano, Ma, & Santos, 2006). Mastitis can change chemical composition and reduce the yield of milk. The changes in the chemical composition of milk include changes in whey protein and salt content that enhance the spoilage of milk. Bacteria that cause mastitis can increase of the somatic cell count (SCC) in the milk. The SCC is associated with protease enzymes in raw milk called plasmin, which can break down milk proteins in a similar way to microbial proteases. Although mild heat treatments are enough to kill all the mastitis bacteria, an excess of SCC can contribute to the plasmin, leading to the bitterness, gelation, and sedimentation of milk (Gröhn et al., 2004; Raubertas & Shook, 1982). Therefore, SSC is a key indicator of milk quality, reflecting the health status of the mammary gland and the quality of final products. The SCC in milk from uninfected cows is less than 100,000 cells/mL and greater than 250,000 cells/mL for cows infected with mastitis (Schukken, et al., 2003). The threshold of SCC in raw milk allowed for processing varies in different countries. In EU, Canada, Austria and New Zealand, the maximum number of SCC in raw milk is 400,000 cells/mL, whereas in the US, the maximum number is 750,000 cells/ mL (More et al., 2009; Tetra Pak, 2014).

2.3 Methods to identify bacteria in raw milk

Milk has a very complex microbial community as described above, and understanding this community is important to determine the risks to milk quality. Several techniques have been applied to detect the diversity of bacteria in raw milk. Traditionally, bacteria were identified according to their colony morphology, cell structure, metabolism, Gram-staining, and biochemical profiling (Jost et al., 2013; Miguel et al., 2010). Milk samples are plated on an agar medium and incubated for a few days to allow colony formation. Single colonies are selected for subculture and biochemical and phenotypic identification (e.g. API, Microbact, and Biolog systems). However, the growth of bacteria on different agars can lead to different morphological characteristics, resulting in ambiguous results. Furthermore, these traditional methods are time-

consuming, laborious, and imprecise. Recently, DNA-based methods, such as specific polymerase chain reaction (PCR) and 16S rDNA sequencing, have been used for rapid and accurate identification of bacterial colonies (Cremonesi et al., 2005; Leskelä et al., 2005). However, these culture-based methods do not reveal the fastidious and non-culturable bacteria that are important in the overall ecosystem of raw milk.

2.3.1 MALDI-TOF MS (Culture-dependent)

An alternative method to identify culturable microorganisms is matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). This is a cheap, rapid, simple, and powerful tool first invented in 1988 by Franz Hillenkamp (Biswas & Rolain, 2013). Although the first use of MALDI-TOF MS for bacteria identification was at the end of the 1990s, it was not widely available for commercial use in laboratories until 2000 due to the lack of sufficient databases (Tshikhudo et al., 2013). MALDI-TOF MS allows reliable discrimination of psychrotrophic bacterial isolates to genus and species level by comparing the spectrum of housekeeping protein profiles of reference strains in a spectral library (Vithanage et al., 2014). In MALDI-TOF MS analysis, a bacterial colony is initially dissolved in a suitable matrix compound and applied to a steel plate then dried. The mixture is exposed to a pulsed laser in a measuring chamber. The mixture containing the bacteria's protein is then vaporized by the laser energy, which leads to the ionization of the sample. The mixture is separated in an electromagnetic field and the time of flight of the ions is accurately measured at the end of the flight tube by a detector. Finally, a mass spectrum is carried out and analyzed by specific software and compared with existing databases (Croxatto, Prod'homme, & Greub, 2012; Sogawa et al., 2011). Fig. 2.2 shows the mechanism and process of MALDI-TOF MS.

Some scientific papers have reported that MALDI-TOF MS can be used as a reliable and rapid method for routine microbial identification in laboratories (Burgess, Flint, & Lindsay, 2014; Jadhav et al., 2014; Masoud et al., 2011). However, Public Health UK has announced some weaknesses in this technology. First, some microbes like *Burkholderia* spp. are hard to identify because of genetic similarity. Secondly, this system is not able to distinguish closely related

bacteria like *Escherichia coli* and *Shigella* spp.. Finally, the identification accuracy is affected by the culture conditions and reference database (Giebel et al., 2010).

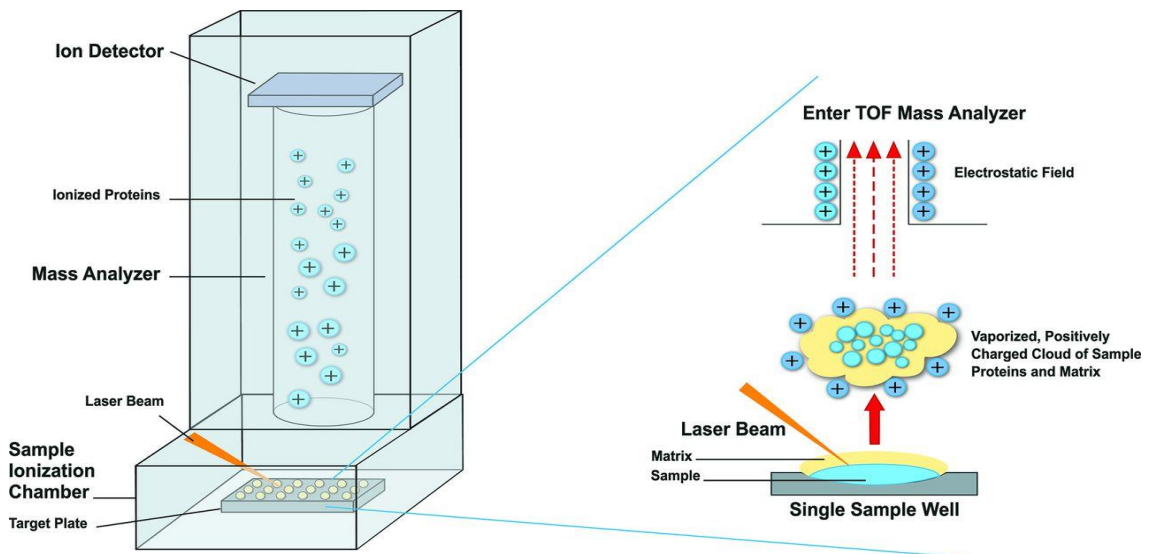


Figure 2.2 Simplified diagram of the MALDI-TOF MS process (Patel, 2015). *Copyright © Reuse of the diagram in the thesis have been permitted by the corresponding author.

2.3.2 Metagenomics sequencing (Culture-independent)

To overcome the limitations of culture-based identification, metagenomics 16S amplicon sequencing is a new way to investigate whole microbial communities using next-generation sequencing platforms (Illumina Miseq or Roche 454 FLX platform). These methods are based on the extraction of all bacterial gDNA from a sample without the cultivation followed by amplification of the 16S rDNA gene by the PCR. There are many benefits of using 16S rDNA to detect bacteria species in phylogeny and taxonomy. All bacteria contain the 16S rDNA gene, which has 10 conserved regions and 9 hypervariable regions that are used to identify different cultures. The 16S rDNA gene is about 1500 bp, which is long enough for bioinformatics (Janda & Abbott, 2007; Jost et al., 2013). Next-generation sequencing can analyse entire microbial communities in varying conditions and time points without cultivation of samples. Recently, the microbial composition of cow's raw milk has been extensively studied by DNA sequencing technologies, which revealed a greater bacterial diversity than the culture-dependent approaches (Derakhshani, Tun, & Khafipour, 2016; Mayo et al., 2014; Solieri, Dakal, & Giudici, 2013). This technology provides benefits in terms of less labour and the analysis of both culturable and non-

culturable bacteria which makes the results of microbial ecology analysis more accurate, representative, and reproducible than just culture-based methods alone. The weaknesses of this technology are the cost and the inability to distinguish between viable and dead cells.

2.4 Enzymes

Raw milk contains many enzymes, and some are of microbial origin, however, many others naturally come from the cow. Enzymes have been defined as a group of substances secreted by living organisms that act as catalysts to accelerate specific biochemical reactions. As the actions of enzymes are very specific, each kind of enzyme specific to one type of substrate. Enzymes increase the rates of chemical reactions, but do not alter the equilibrium. In practice, protease only catalyzes a forward reaction rather than a reverse reaction while lipase is unknown. Enzymatic activity is sometimes dependant on substrate availability and conditions. For example, the activity may be affected by pH and temperature (Bylund, 2003). Enzymes are important in the dairy industry. For instance, rennet is used to curdle the casein in cheese making. Bacteria can produce both desirable enzymes that add to the flavour of cheese and undesirable enzymes that reduce the yield and affect the quality of dairy products (Wilkinson & Kilcawley, 2005). The enzymes found in raw milk are classified into two main groups, indigenous enzymes, and bacterial enzymes.

2.4.1 Enzymes (indigenous)

Over 60 types of indigenous enzymes naturally exist in cow's milk (Fox, 2003). Among these, the most common are plasmin, peroxidases, catalases, and phosphatases. The indigenous milk enzymes are generally heat-labile and have no significant effect on the nutritional and organoleptic properties of dairy products as they are normally inactivated by heat treatment, such as lipoprotein lipase, which can be completely inactivated by pasteurisation (Deeth, 2003). Phosphatases are extensively used to determine the efficiency of HTST pasteurization in dairy industries (Bylund, 2003). However, some heat-stable indigenous enzymes can cause the deterioration of dairy products. For example, proteolysis by plasmin is the major heat-stable indigenous milk protease that can greatly affect the quality of dairy products. Plasmin is generally

the major protease activity in milk. Plasmin, plasminogen, and plasminogen activators are associated with the casein micelles and milk fat globule membranes in milk. The activity of this enzyme is controlled by certain inhibitors such as plasmin inhibitors, or inhibitors of plasminogen activators which inhibit the activity of plasminogen activators so that plasminogen cannot be converted into plasmin. Most plasmin inhibitors are present in the serum phase of milk, which exhibits optimal activity at pH 7.5 and 37°C. One of the key differences between plasmin and bacterial protease is that bacterial proteases predominantly affect κ -casein over β -casein and α -casein, while plasmin readily hydrolyzes β -casein, and α -casein than κ -casein (Bastian, & Brown, 1996). It is a heat-stable indigenous enzyme that can remain after pasteurization or even UHT. Severe heat treatment of milk (e.g. 115°C for 20 min or 120°C for 15 min) is required to deactivate the plasmin induced proteolysis in milk products (Rauh et al., 2014).

2.4.2 Bacterial proteases

Bacteria can produce heat-stable proteases that can hydrolyse peptide bonds in milk proteins, resulting in the alteration of physicochemical, functional and sensory properties of milk and milk products. More specifically, proteolysis can result in bitter flavour, gelation, and spoilage in milk (Lafarge et al., 2004). Fig. 2.3 illustrates the hypothetical mechanism of the destabilisation of UHT milk from proteolysis. The secretion of proteases is commonly at a maximum in late-log or early stationary phase of bacterial growth (Chen et al., 2003). Different types of bacteria can secrete different types of enzymes with a range of molecular weight. For instance, most *Pseudomonas* strains can produce only one type of proteases, which is a neutral zinc metalloprotease with a molecular weight ranging from 47-50 kDa with pH optima of 6.5-8 (Fairbairn & Law, 1986). *Bacillus* species can produce more diverse types of proteases. For example, *G. stearothermophilus* are capable of secreting two metalloproteases with a molecular weight of approximately 67.6 and 20 kDa (Chopra & Mathur, 1984). Most proteases from psychrotrophs preferentially attack casein over whey proteins. Among casein proteins, κ -casein are more susceptible than α -casein and β -casein (Fairbairn & Law, 1986). The hydrolysis of casein

micelles by spoilage proteases causes casein micelle aggregation and sedimentation (Capodifoglio et al., 2016).

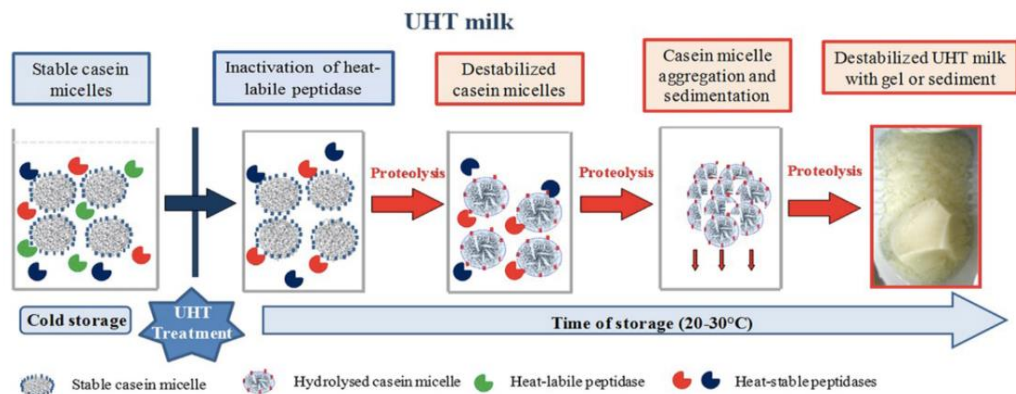


Figure 2.3 Hypothetical mechanism of proteolysis from heat-stable proteases (Machado et al., 2017). *Copyright © 2017 Machado, Baglinière, Marchand, Van Coillie, Vanetti, De Block & Heyndrickx. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, under accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Many bacterial proteases are reported to remain active after pasteurization and UHT processing. For instance, a protease from *Pseudomonas* strains retain 55-65% of the initial activity after HTST and 20-40% activity after heat treatment at 140°C, 5 s (Chen et al., 2011; Datta et al., 2003). According to Adams et al. (1975), proteases from 10 different *Pseudomonas* strains all remained active after 149°C, 10 s heat treatment. Crude proteases produced by *G. stearothermophilus* and *B. licheniformis* had no loss of activity after 10 min heat treatment (90°C) at pH 7 (Chopra & Mathur, 1984). The heat stability of protease is even higher in a synthetic milk salt solution and in milk rather than in buffer solution (Kumura, Mikawa, & Saito, 1993). For example, the metalloprotease, AprX, is the main heat-stable protease produced by *Pseudomonas* species. The D-value for the deactivation of AprX in milk was recorded to be 124 s at 140°C allowing it to survive most UHT processing (Kroll & Klostermeyer, 1984). AprX can rapidly and accurately refold with the formation of calcium salt when the temperature is lowered. Due to its flexible tertiary structure, the heat stability is enhanced in the presence of calcium, which makes it hard to thermally eliminate in milk (Baur et al., 2015; Dufour et al., 2008; Marchand et al., 2009). In general, AprX shows activity over a wide temperature range (0-55°C), but is at its optimum from 37 to 47°C (Baglinière et al., 2013; Baur et al., 2015; Zhang & Lv, 2014). There

are three mechanisms by which heat causes loss of activity of proteases. First, heat results in the conformational unfolding of proteases, known as denaturation. Secondly, heat enhances the self-digestion of proteases, resulting in autoproteolysis. Thirdly, heat may cause irreversible non-enzymatic covalent modification (Daniel, Toogood, & Bergquist, 1996). However, low-temperature inactivation has been observed for some bacterial proteolytic activity showing sensitivity to low-temperature treatment (50-60°C) because of the formation of enzyme-casein complexes (Sørhaug & Stepaniak, 1997).

Proteolysis in milk is usually determined by tests that detect proteolytic activity. Some examples of these tests are agar-based methods, electrophoresis, high-performance liquid chromatography (HPLC), immunological methods, zymographic analysis and spectrophotometric, and fluorometric methods (Němečková, Pechačová, & Roubal, 2009). In this study, three methods were used calcium caseinate agar, spectrophotometry, and zymography.

The use of calcium caseinate agar, originally formulated by Frazier & Rupp in 1928, is used for the selection and enumeration of protease-producing bacteria in the dairy industry. Casein enzymatic hydrolysis provides nitrogen, carbon, vitamins (the source of the vitamins is from the meat extract), and amino acids as nutrients for microbial growth. Phosphates and sodium chloride are added to the buffer used in the formulation of the medium to maintain pH and osmotic equilibrium, respectively. The microorganisms that show proteolytic activity can degrade the caseinate around the colonies to soluble components with the formation of a clear inner zone (Frazier & Rupp, 1928). This method is rapid, cheap and easy to select protease-producing bacteria. However, this method cannot be used for quantitative analysis.

In spectrophotometric methods, synthetic chromogenic substrates are used to measure low amounts of proteolytic activity (Chen et al., 2003). These substrates react with proteases to produce coloured products, for example with the use of azocasein (casein coupled with diazotized aryl amines) for the quantification of proteolytic activity, first developed by Charney & Tomarelli in 1947. The digestion of a solution of such proteins releases the chromophoric group, which is soluble in trichloroacetic acid and gives it a red-orange colour. The method itself relies on the

reaction between the substrate and an enzyme under its optimum temperature/pH for a given time. The solution colour intensity, read at 400 nm, is a function of the amount of azocasein digested, since all proteins form a precipitate after the addition of trichloroacetic acid. The method is still one of the most reliable methods to quantify the proteolytic activity of enzymes due to its colour stability (Andreani et al., 2016).

Zymography has been widely used for the analysis of proteolytic activity due to its simple, sensitive, and quantifiable nature. It has been extensively adapted for research on bacterial proteases. Polyacrylamide gels are infused with protein substrates (gelatin or casein) and the solution containing protease is added to separate the proteases using electrophoresis (Cathcart, 2016). The substrates are then degraded by the proteases of different molecular weights, degrading different zones in the gel. The proteolytic activities in zymography gels are visualized by the presence of clear bands indicating protease degradation of casein and lack of staining with Coomassie brilliant blue stain (Ranzato et al., 2017). Zymography methods have been proven to have the ability to detect total proteolytic activity from different samples at a low cost (Leber & Balkwill, 1997). The limitation of this method is the use of hazardous chemicals and low sensitivity of detection for small molecular size peptides.

2.4.3 Bacterial lipases

Lipolytic enzymes (esterase and lipase) are the carboxylesterases that hydrolyse acylglycerols (Deeth, 2006). The focus of this project is on bacterial lipases in raw milk. The reason is fats in milk mainly consists of triglycerides, in the formation of fat globules. The key difference between esterase and lipase depends on their solubility. Since lipases are active towards aggregated substrates, lipase activity is directly correlated with the total substrate surface area in an emulsion, and not with the substrate concentration. Esterase activity is found to be highest towards more water-soluble substrates and is not as active as lipases in milk (Chen et al., 2003). The majority of bacterial lipases are extracellular and produced during the late log and early stationary phases of growth. In general, they have molecular sizes ranging from 30 to 50 kDa with pH optima between 7 and 9. Most *Bacillus* lipases show the highest catalytic activities at temperatures

ranging from 60°C to 75°C, which is 30°C above the highest activity for the *Pseudomonas* lipases (30–45°C) (Chen et al., 2003). The types of extracellular lipases produced by bacteria are very diverse in their properties and substrate specificities and bacteria usually produce more than one type of lipase. The action of lipases is to hydrolyse triglycerides to release free fatty acids (FFAs) and corresponding glycerides. The chain length of fatty acids released can vary with the specificity of the lipase (Chen et al., 2003). Most of them have specificity for the sn-1 and sn-3 positions of triacylglycerols. The released FFAs can lead to sharp, cheesy or rancid flavours of milk products (Macrae, 1983).

The lipolytic activity of *Pseudomonas* species is relatively heat-stable. For example, the crude lipase secreted from a psychrotrophic *Pseudomonas* species isolated from raw milk remained active, retaining 55-100% of activity after LTLT pasteurization in raw milk and 75-100% activity after heat treatment at 100°C for 30 s in skim milk (Fitz-Gerald, Deeth, & Coghill, 1982; Law, Sharpe, & Chapman, 1976). Andersson et al. (1979) reported that the lipase produced by *P. fluorescens* SIK W1 had a D-value of 23.5 min (a calculated $t_{1/2}$ of 7.0 min) at 100°C in skim milk. A crude lipase produced by *B. stearothermophilus* isolated from a milk powder plant had a $t_{1/2}$ of 690 min at 70°C in buffer at pH 7.0 (Chen et al., 2003). The overall heat stability of lipase in the milk medium is more than in phosphate buffer. A combination of polysaccharides and divalent cations (such as Ca^{2+}) increases the heat stability of the enzyme in solution. Fox & Stepaniak (1983) confirmed that the extracellular lipase from *P. fluorescens* AFT36 was more heat-stable in a synthetic milk salts solution than in phosphate buffer.

Although butterfat and some glycerides such as triolein have been reported to select lipase-producing bacteria, tributyrin is the most common substrate used for the detection and enumeration of lipolytic producing microorganisms in food material (Caro et al., 2000; Shelley, Deeth, & MacRae, 1987). In tributyrin agar, special peptone and yeast extract can provide organic nitrogen, carbon compounds and vitamins for the growth of these microorganisms (Rapp & Olivecrona, 1978). Tributyrin, the most commonly used emulsified lipid substrate incorporated into agar media, shows zones of clarification as lipid hydrolysis reactions occur. Lipases

hydrolyse tributyrin to dibutylin, monobutylin, glycerol, and butyric acid, all of which are water-soluble, to produce a clear zone around lipolytic colonies in the opaque emulsion (Shelley, Deeth, & MacRae, 1987). Although some researchers have reported a poor correlation between tributyrinase activity and butterfat hydrolysis (Hugo, & Beveridge, 1962; Jones, & Richards, 1952), there have also been several reports of good outcomes using tributyrin as a lipase substrate (Lawrence, 1967; Mourey, & Kilbertus, 1976). Butterfat is logically the perfect substrate for the detection of lipolytic microorganisms of importance to the dairy industry, but it is difficult to obtain a fine emulsion of butterfat in agar and it is difficult to generate opacity in the medium after hydrolysis by lipases, which limits its use in the dairy industry (Lawrence, 1967). Therefore, tributyrin agar is recommended as one of the most useful and convenient media that can be used as a screening procedure for lipolytic micro-organisms (Law, Sharpe, & Chapman, 1976).

The quality and shelf life of milk powder can be affected by lipases. Although bacteria are not always the main factor responsible for the spoilage of whole milk powder (WMP) due to the low water content, the lipases, which can remain active in conditions of low water activity, can damage the quality of milk powder during storage. For example, Clelston et al., (1997) reported that lipolysis could occur in WMP with a moisture content of < 3 g/100g.

2.4.4 Factors affecting bacterial enzymatic activity

While the scope of this study includes the determination of the effects of dairy ingredients on the proteolytic activity of psychrotrophs, few studies have covered this topic. Enzymatic activity of a microorganism is dependent on various factors like the media composition, presence of metal ions, and physical factors like pH, temperature, inoculum density, and incubation time (Rajendran & Thangavelu, 2008). For example, milk is a good medium for inducing lipolytic activity, which can be stimulated by the presence of lipids, such as milk fat and olive oil. Polysaccharides, such as glycogen, hyaluronate, laminarin, pectin B, and gum arabic, can also stimulate the activity of bacterial lipases (Aravindan, Anbumathi, & Viruthagiri, 2007).

Triki-Ellouz et al. (2003) reported that *Pseudomonas aeruginosa* MN7 showed greater proteolytic activity when cultivated on a fish substrate, which acts as its carbon and nitrogen source. Similarly, the secretion of alkaline proteases from *Actinomycetes* was reported to depend on the growth rate and the availability of carbon and nitrogen sources in the medium (Mehta, Thumar, & Singh, 2006). According to a study by Akhavan & Jabalameli (2011), starch and maltose are the best substrates amongst the simple sugars used in their study (fructose, glucose, and sucrose) for proteolytic activity from *Bacillus* species. They also stated that corn steep liquor was the best nitrogen source for *Bacillus* species' proteolytic activity. Apart from the availability of carbon and nitrogen sources, Hosseini et al. (2016) reported that the addition of zinc sulfate to the culture medium enhanced the proteolytic activity of *S. griseovarus* PTCC1130. However, the complexity and specificity of different bacterial strains pose a limit on drawing a definitive relationship between the species and their proteolytic activity.

2.5 UHT milk quality

UHT milk manufacture is a continuous process using high-temperature sterilization at 135-150°C for 1-10 s (indirect or direct) and aseptic packaging to produce a commercially-sterile product with a 6 to 9-month shelf-life at ambient temperature (Lu et al., 2013). UHT dairy products are important in countries that do not have an efficient cold chain. UHT products are also popular in many developed dairy markets. The market share of UHT milk is more than 70% of the total dairy market in China, France, Germany and Spain. Many other developed markets, such as Australia, the USA, and New Zealand, are also witnessing the growth of UHT milk due to the popularity of flavoured milk, nutritional protein shakes, school milk programs, and high demand for export (Fuller et al., 2006). The aim of the UHT process is to inactivate all vegetative cells, bacterial spores, and most enzymes while minimizing the chemical changes in the products to produce a commercially sterile product. However, there are some problems with UHT milk, such as changes in flavour, proteolysis, age gelation, and microbial spoilage by their heat-stable enzymes (Tetra Pak, 2014).

2.5.1 Factors affecting UHT milk quality

2.5.1.1 Raw milk quality

The quality of raw milk is critical for producing high-quality UHT milk. The poor quality of raw milk not only adversely affects the final products but also the processing. Raw milk with high microbial numbers is more susceptible to gelation than milk with low microbial numbers. Law et al. (1977) pointed out that in UHT milk produced from raw milk with a psychrotrophic bacterial count exceeding 5.0×10^6 and 8.0×10^6 cfu/mL, the gelation time of UHT milk was less than 62 and 12 days, respectively. Raw milk that has been stored at high temperature ($> 10^\circ\text{C}$) or under refrigeration for long periods is unsuitable for the UHT processing due to the lactic acid-producing bacteria and heat-stable enzymes produced by psychrotrophs. Raw milk containing high numbers of lactic acid bacteria is not suitable for UHT processing because the lactic acid bacteria can drop the pH of milk, leading to fouling in heat exchangers or direct steam injectors. The fouling results in a reduction of production capacity and extended cleaning time (Celestino, Iyer, & Roginski, 1997; Tetra Pak, 2014). Poor quality material can bring out potential hazards due to the microbial spoilage caused by heat-stable enzymes produced before UHT processing, and this has been a subject of some researchers (Bremer, Fillery, & McQuillan, 2006; Burgess, Lindsay, & Flint, 2010; Teh et al., 2014).

2.5.1.2 Storage temperature

Age gelation of UHT milk is related to storage temperature. It is known that at least two mechanisms can cause gelation during shelf life. One mechanism involves proteolytic degradation of the milk proteins through heat-stable indigenous or extracellular enzymes, destabilizing milk, and ultimately forming a gel. The other mechanism is referred to as a physico-chemical mechanism. In proteolytic age gelation mechanisms, the enzymatic degradation of the proteins may be accelerated by increasing storage temperatures. For instance, in a study by Malmgren et al. (2017), UHT milk samples that were kept at temperatures from 5 to 40°C for up to 6 months and at monthly intervals were analysed for gelation and proteolysis by HPLC and electrophoresis. The samples stored at 5°C and 40°C did not gel over the 6 months storage period whereas the

samples stored at 22°C and 30°C gelled after 5 and 3 months, respectively. The samples stored at 5°C had only low levels of proteolysis by indigenous enzymes, whereas the samples stored at 22, 30 or 40°C showed high proteolysis, with the extent of proteolysis at each month increasing with storage temperature. This indicated that proteolysis could be enhanced by increasing the storage temperature, while gelation was not observed on storage at 40°C.

2.5.2 Sedimentation in UHT milk

Sedimentation of UHT milk can be described as the formation of a layer of protein-based material at the bottom of a pack of the product. When the level of sediment is small, most consumers may not notice. However, when the sediment layer starts to get soft and gelatinous, which is likely to be in the stages of early gelation, it becomes a problem. Sedimentation can generally be considered as the natural settling of colloidal particles in milk. The settled particles naturally fuse to form a network. When sedimentation is excessive and occurs faster than the initial particle sizes would predict, this indicates some aggregation phenomenon is occurring, increasing the particle size and accelerating sedimentation (Anema, 2019). This usually occurs in the first weeks after the manufacture of the UHT milk and is thus different from age gelation, which takes a few months for visible signs to appear. The pH of milk has a major impact on sedimentation. A low pH (< 6.7) of milk can markedly increase sedimentation levels in both direct (steam is briefly injected into the product) and indirect (product is heated through a heat exchanger) UHT milk. Sedimentation can become excessive at pH values below 6.6 for milk at its normal concentration or at a pH below about 6.5 for milk at twice its original concentration (Zadow & Hardham, 1981). The increased temperature on storage may also accelerate sedimentation due to lowered viscosity of the continuous phase (Ratnayake, Gallmann, & Jelen, 1997). Many other factors can affect the sedimentation UHT milk, such as the method used to heat milk (indirect and direct), fat content, whey protein ratio etc. (Anema, 2019). However, the effect of bacterial proteases has rarely been reported.

2.5.3 Gelation in UHT milk

Milk gelation is the formation of a voluminous 3D protein network that leads to the loss of fluidity in UHT milk. The mechanistic pathway of age gelation caused by bacterial enzymes is usually attributed to the hydrolysis of κ -casein, producing “para- κ -casein-like” micelles and a “glyco-macro-peptide-like” by-product in the serum phase (Anema, 2019). This hydrolysis of casein may be affected by hydrolysis of α -casein and β -casein, but this is usually less extensive than that of κ -casein. On longer storage, further hydrolysis occurs, forming smaller peptides derived from all caseins in milk, resulting in the gelling phenomenon (Zhang, Bijl, & Hettinga, 2018).

The main factor causing milk gelation is bacterial proteolytic degradation. Good quality milk that is quickly collected from the farm, transported, and processed to UHT milk should not have issues with degradation and gelation through these bacterial proteases. As the psychrotrophs can thrive and produce heat-stable proteases at cool temperatures, issues may arise when the raw milk is stored chilled for long periods before pasteurization or UHT processing, as these conditions will enhance the formation of bacterial proteases that are responsible for age gelation. The high heat stability of the protease from these psychrotrophs indicates that it would require substantial increases in the intensity of the UHT heat treatments to deactivate the proteases, and this will eventually have adverse effects on the organoleptic and nutritional qualities of the milk. Thus, it is better to avoid accumulating these proteases in the milk rather than trying to destroy them through heat processing.

2.5.4 Ways to control spoilage enzymes in UHT milk

2.5.4.1 Hygiene

To achieve a superior microbiological quality, good hygiene practices have to be implemented at the farm and all stages leading to the manufacture of the final product (Martin, Boor, & Wiedmann, 2018). Bacteria are ubiquitous on the dairy farm. Unclean milking equipment, lines, and utensil surfaces that come into contact with dirt, manure, and milk will increase the

chance of bacterial contamination of raw milk. Biofilms are mainly blamed for promoting the growth of heat-stable enzyme-producing psychrotrophs, because biofilms can act as a niche for these bacteria, protecting them from the activity of detergents and sanitizers (Teh et al., 2014). High densities of bacterial cells will provoke quorum sensing and induce the production/activity of enzymes (Dong & Zhang, 2005). For this reason, uncontaminated raw milk is not synonymous with the unspoiled final product, and all of the equipment coming in contact with the raw milk should be adequately cleaned and disinfected to prevent the formation of biofilms.

2.5.4.2 Cooling

High bacterial counts and improper storage temperatures are prerequisites for the production of bacterial enzymes, causing poor quality raw milk. Therefore, controlling the level of psychrotrophs is of utmost importance to minimize enzyme production and activity. Rapid precooling of raw milk is necessary because milk leaves the udder at approximately 35°C, which is a perfect temperature for bacterial growth. If the milk is not precooled before it enters the bulk tank, the warm milk will increase the temperature of the tank and thereby induce bacterial growth and enzyme production/activity. Alves et al. (2018) reported that the refrigeration temperatures at which raw milk is usually stored can decrease most bacterial enzymatic activity, but still be insufficient to prevent enzyme production if the initial psychrotrophic bacteria count is more than 10^3 cfu/mL. Thus, if the bacterial quality is low, shorter collection intervals may be needed to prevent enzyme synthesis.

2.5.4.3 Thermalization

Modern large dairy companies usually keep raw milk in silos under refrigeration for several hours or days before processing into dairy products. Some companies use a heat pre-treatment called thermalization (57 to 68°C for 15 to 30 s or 63 to 65°C for 15 s) to inactivate psychrotrophs before refrigerated storage. This pre-heating treatment can eliminate most of the psychrotrophs in raw milk, thereby inhibiting the proliferation of psychrotrophs as well as the production of their spoilage enzymes (Zhang, Bijl, & Hettinga, 2018). Rapid cooling of raw milk

to $\leq 7^{\circ}\text{C}$ should follow the heat treatment. The combination of pre-heating and rapid cooling can effectively maintain the quality of stored raw materials for several days before further processing and eventually improve the quality of final products.

2.6 Conclusions

The general concept that the microbial quality of raw milk influences the quality of final dairy products is widely accepted. However, the complete whole psychrotrophic microbiota that is responsible for spoilage in raw milk is not well understood. The growth of psychrotrophs and their proteolytic activity in dairy and non-dairy environments before heat treatment has not been well studied. Moreover, although many scientific papers have been published claiming heat-stable bacterial proteases are responsible for limiting the shelf-life of UHT milk, there are only a limited number of papers to suggest the link between the microbial population in raw milk, the type of heat-stable enzyme-producing bacteria and the quality of UHT milk.

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Chapter 3. 16S rDNA high-throughput sequencing and MALDI-TOF MS are complementary when studying psychrotrophic bacterial diversity of raw cow's milk

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Abstract

Refrigeration is used to control the microbiological quality of raw milk before processing. However, this favours the growth of psychrotrophic bacteria (psychrotrophs) that can produce spoilage enzymes, which may deteriorate final dairy products. This study aimed to identify the psychrotrophs from raw milk across four seasons and six regions using culture-independent and culture-dependent approaches. Raw milk across four seasons was obtained from six different geographical milking regions in New Zealand. After exposing raw milk to 7°C for 5 days, milk samples were analysed using 16S rDNA high-throughput sequencing (HTS) and MALDI-TOF MS. Both approaches showed the *Pseudomonas* genus to predominate in all samples. HTS revealed more than 11 genera and 18 species, with *P. psychrophila* (39.02%) and *P. fluorescens* (37.53%) to be the most prevalent species in all the samples. MALDI-TOF MS identified 9 genera and 20 species and 93.68% of all isolates tested were found to be *Pseudomonas*, with *P. lundensis* (24.41%) and *P. fragi* (20.47%) predominating. The proportions of species in milk samples across seasons and regions was observed by HTS. Both seasonal and regional variations in microbiota were observed by HTS. The use of HTS and MALDI-TOF MS was complementary in describing the psychrotrophic bacterial diversity of raw milk and provides an understanding of the raw milk microflora that may influence milk quality. This is the first report to compare data obtained from 16S rDNA high-throughput sequencing and MALDI-TOF analysis to assess the psychrotrophic microbial quality of refrigerated raw milk in New Zealand.

Keywords: Lipase, protease, spoilage, milk quality, raw milk handling

3.1 Introduction

Milk provides an excellent environment for the growth of a variety of microorganisms due to its neutral pH, high water activity, and richness in nutrients (Champagne et al., 1994). The microbiological quality of raw milk is closely linked to the health of the cow, the teat surfaces, the feed, bedding material, milking equipment, bulk tank storage, and transportation conditions (Mangia, Fancello, & Deiana, 2016; Vithanage et al., 2016). Refrigeration of raw milk immediately after milking and maintaining chilled temperatures ($\leq 7^{\circ}\text{C}$) before processing is a common international practice to delay the growth of bacteria and maintain the quality (Law, 1979). However, it is well known that prolonged refrigerated storage of raw milk can favour the growth of psychrotrophs, which can produce heat-stable proteolytic and lipolytic enzymes. These heat-stable enzymes can remain active following heat treatment (including pasteurization and UHT processing) and then hydrolyse the milk proteins and fats during storage, leading to the spoilage and reduced shelf-life of commercial dairy products (Samaržija, Zamberlin, & Pogačić, 2012).

The majority of the bacteria found in fresh raw milk directly isolated from the udder are Gram-positive mesophilic aerobic bacteria with only 10% being psychrotrophs. The populations of psychrotrophs in raw milk can then increase to 50% of the whole microbial load after 1-day cold storage and can finally increase to more than 90% of the whole microbial load after 2-day cold storage (Lafarge et al., 2004; Magan, et al., 2001). They include the following genera: *Acinetobacter*, *Achromobacter*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, and *Serratia*, with *Pseudomonas* being the predominant genus (Cousin, 1982; Ercolini et al., 2009). Different psychrotrophs can secrete different types of enzymes, such as proteases, lipases, and phospholipases with different molecular sizes. For example, *Pseudomonas fluorescens* can produce protease, lipase, and phospholipase whereas *Ancinetobacter guillouiae* were reported to be only lipolytic (Vithanage et al., 2016). Most *Pseudomonas* strains can produce only one type of protease, a neutral zinc metalloproteinase with molecular weight about 47-50 kDa (Marchand et al., 2009). *Bacillus* species can produce many diverse types of proteinases with

different molecular weights (Fairbairn & Law, 1986). Stoeckel et al. (2016) reported that the proteases produced by different *Pseudomonas* species have various effects on protein destabilization and flavour defects of UHT milk. Since psychrotrophs are capable of producing a variety of enzymes that can cause defects in dairy products, it is important to determine the diversity of psychrotrophs in raw milk to ensure effective storage practices for milk before processing to minimise the growth of these bacteria.

Studies investigating the microbial diversity in raw milk have used culture-based methods, which may only detect a proportion of total microbiota. Molecular based methods, such as 16S rDNA high-throughput sequencing (HTS), are useful new technologies to study raw milk microbial communities that capture both the culturable and non-culturable populations. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is a relatively new method for the rapid identification of culturable microorganisms that is simpler and easier than API, Biolog, and culture-based 16S rDNA gene sequencing (Weber et al., 2014). In this study, both culture-independent (HTS) and culture-dependent (MALDI-TOF MS) approaches were used to determine the diversity of psychrotrophs in raw milk collected from New Zealand in different seasons and regions.

3.2 Materials and methods

3.2.1 Raw milk sampling

All the milk samples were provided by Milk Test NZ, Hamilton. The milk samples had been stored at 7°C for 12 h (samples from North Island) up to 24 h (samples from South Island) before arriving at Milk Test NZ. Raw milk samples were obtained during each season (autumn, winter, spring, and summer) and represented the main raw milk collection regions of New Zealand (Northland, Waikato, Bay of Plenty, Hawkes Bay, Clandeboye, and Southland; Fig. 3.1). Raw milk samples (35 mL) were randomly obtained from 10 different farms in each region (n=60 per season, total n=240) and transferred to Massey University within 12 h. The 10 samples from within each region were commingled and then stored at 7°C for 5 days before analysis to select

for the predominant psychrotrophic populations. In total, 24 individual samples across 6 regions and 4 seasons were analysed.

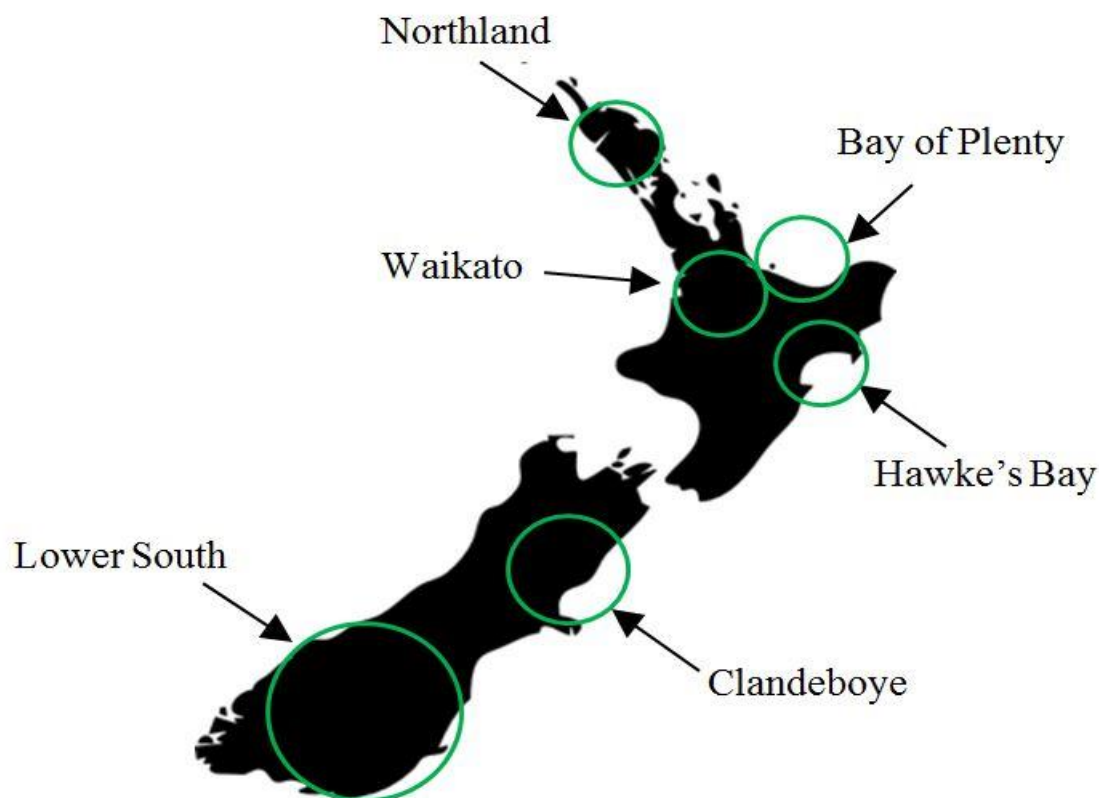


Figure 3.1 Sampling map.

3.2.2 DNA extraction and 16S rDNA high-throughput sequencing

The total bacterial gDNA of raw milk was extracted using the Presto™ Mini gDNA Bacteria kit (Geneaid, Thailand) following the manufacturer's instructions. The kit was directly applied to raw milk without any treatment beforehand. The quality and concentration of extracted DNA were assessed by 2% agarose gel electrophoresis (Invitrogen, Kiryat Shmona, Israel) and Colibri spectrophotometry (optical density at 260/280 nm ratio; Berthold Detection Systems, Germany). The DNA samples were then sent to New Zealand Genomics Ltd (NZGL; Massey Genome Service at Massey University, Palmerston North) for sequencing on the Illumina MiSeq Sequencing Platform. Sterile distilled water was used as the negative control.

16S rRNA gene libraries were constructed using PCR to amplify the variable regions V3 and V4 using the forward 16Sf (5'-CCTACGGGAGGCAGCAG-3') and the reverse 16Sr (5'-GGACTACHVGGGTWTCTAAT-3') primers. Amplicons were generated using a high-fidelity polymerase (AccuPrime; Invitrogen) and then were purified using a magnetic bead capture kit (Ampure; Agencourt) and quantified using a fluorometric kit (QuantIT PicoGreen; Invitrogen). The purified amplicons were then pooled in equimolar concentrations using a SequalPrep plate normalization kit (Invitrogen), and the final concentration of the library was determined using a SYBR green quantitative PCR (qPCR) assay with primers specific to the Illumina adapters (Kappa). The 24 libraries were pooled by equal molarity and run on 25% of one 2X250 base PE run.

The sequence reads from the 24 samples were analysed inside the QIIME2 environment (version 2018.2) (Caporaso et al., 2010). The paired sequences were imported as the type 'SampleData[PairedEndSequencesWithQuality]' using a manifest file. The sequences had quality trimming and analysis performed on them using the incorporated dada2 method (Callahan et al., 2016). No samples with low reads were discarded, as the full set of 24 samples were needed for the analysis. Within the QIIME2 environment, the quality plots were manually inspected using the qiime2view viewer (www.view.qiime2.org/) after running "qiime tools import" and "qiime demux summarize". The trimming was then performed in the dada2 denoise-paired command as "qiime dada2 denoise-paired --i-demultiplexed-seqs demux-paired-end.qza --o-table table_tf10_tr10_truf250_trur240 --o-representative-sequences rep-seqs_tf10_tr10_truf250_trur240 --p-trim-left-f 10 --p-trim-left-r 10 --p-trunc-len-f 250 --p-trunc-len-r 240". In other words, after visual inspection, both read sequence files had the first 10 bases removed, and only read2 sequence files had the last 10 bases removed. The data were summarised using a metadata file and also tabulated. Alpha (Shannon, chao1, and observed_otus) and beta (Euclidean and Jaccard) diversity metrics were calculated on the individual samples, and also when grouped based on the metadata factors "season" and "region". The samples were classified taxonomically using the QIIME2 feature-classifier with a modified version of the NCBI 16S

rRNA BLAST database (“16SMicrobial.tar.gz”; downloaded and processed in August 2018) that had been programmatically modified to remove some of the intervening taxonomic levels from the classification.

3.2.3 Bacterial isolation and procedure for MALDI-TOF MS identification

Each of the 24 individual milk samples was serially diluted using 10-fold volumes of 0.1% (w/v) sterile peptone water (GranuCult™, Merck, Germany) and cultured on milk plate count agar (MPCA; Oxoid, Basingstoke, UK), using the spread plate technique (the minimum numbers that can be detected by spread plate technique is 10^1 cfu/mL), in triplicate. The plates were then incubated at 7°C for 7 days to enumerate the populations of psychrotrophs (Hantsis-Zacharov & Halpern, 2007). Bacterial colonies with unique morphologies (colour, form, size, elevation, and margin) were selected from a readable plate (10-100 colonies) and streaked on MPCA to obtain pure cultures. The isolated bacteria were grown in nutrient broth (NB; Becton Dickson, USA) overnight and then were kept on Cryopreserved beads (Thermo Fisher Scientific, USA) at -80°C for future work. The percentage of each bacterial genus and species were determined by the identified each genus and species divided by the total isolates.

The MALDI-TOF MS method was used to rapidly identify the selected bacteria. Because the ionization efficiency of analytes depends on the matrix, analyte-to-matrix ratio, crystal morphology, laser energy, the ratio of the matrix to the cell is very important. The condition was standardised by TSA. A reference strains *E. coli* grown on TSA was used as a standard compared to the database every time. An overnight colony on tryptic soy agar (TSA; Becton Dickinson, Cockeysville, MD, USA) was transferred into a 1.5 mL Eppendorf tube with 300 µL of sterile water and 900 uL of 100% ethanol (Sigma, New Zealand) and vortex mixed for at least 1 min, followed by centrifugation for 2 min at 13, 000 x g. The suspension (the supernatant fluid from the Eppendorf tube) was mixed with 30 µL of 70% formic acid (Sigma, St. Louis. MO, USA) and acetonitrile (Merck, Darmstadt, Germany) by vortex mixing. The mixture was again centrifuged for 2 min at 13, 000 x g, and 1 µL of the supernatant was dipped onto the MALDI stainless steel target plate to be air dried for 1 min. 1 µL of the matrix solution (HCCA, cyano-4-

hydroxycinnamic acid, Bruker Daltonik) was then overlaid on the dried samples. The HCCA was made by a mixture of 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma, St. Louis, MO, USA). The dried target steel was loaded in the Microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The resulting spectra were analysed using the Bruker Daltonik MALDI Biotyper 3.0 Real-Time Classification (RTC) program (Lindsay, Hill, & Venter, 2014). Each sample was tested in triplicate.

3.3 Results

3.3.1 Comparison between HTS and MALDI-TOF MS

The average psychrotrophic bacteria count from unenriched milk was in the range of 2-3 log cfu/mL. The population of psychrotrophs from the milk after 5-day enrichment at 7°C reached 6-7 log cfu/mL. From Milk Test New Zealand, the origin of the milk samples before enrichment, the somatic cells count for all milk samples were less than 100,000 cells/mL. To compare the differences in the microbial composition of the enriched milk using HTS and MALDI-TOF MS, a taxonomic analysis of 24 milk samples at the genus and species level are provided in Table 3.1. HTS generated a total of 4,334,304 raw reads from 24 raw milk samples, with a minimum of 66,095 and a maximum of 296,169 reads. HTS identified more than 11 genera, namely *Acinetobacter*, *Carnobacterium*, *Chryseobacterium*, *Erwinia*, *Flavobacterium*, *Hafnia*, *Kluyvera*, *Lactococcus*, *Leuconostoc*, *Pseudomonas* and *Serratia*. *Pseudomonas* appeared to be the predominant genus among all 24 samples. *Serratia*, *Lactococcus* and *Acinetobacter* were the second most prevalent genera in all other samples, whereas the remaining 7 genera together only represented a small proportion of the population. At the species level, 9 species showed higher than 1% prevalence, with *P. psychrophila* (39.02%) and *P. fluorescens* (37.53%) dominant.

MALDI-TOF MS was used as a rapid method to identify culturable bacteria based on the spectra produced from protein extracts. A total of 127 psychrotrophs were cultured in this study and identified by MALDI-TOF MS, representing 9 genera and 20 species. MALDI-TOF MS analysis showed similarities in all the microbial communities and agreed with the HTS results

with *Pseudomonas* dominating. The dominant genus was *Pseudomonas* (93.68%), with other genera making up 6.32% of the isolates comprising *Acinetobacter*, *Bacillus*, *Buttiauxella*, *Carnobacterium*, *Lactococcus*, *Hafnia*, *Rahnella* and *Serratia*. Among the 21 species belonging to *Pseudomonas*, *P. lundensis* was the predominant species (24.41%), followed by *P. fragi* (20.47%) and *P. fluorescens* (5.51%). A total of 28.33% (36/127) of *Pseudomonas* were not identifiable to the species level ($1.7 < \text{score} < 2.0$).

Table 3.1 Bacteria identified by HTS and MALDI-TOF to genus level and species level.

Genus- HTS	Abundance (%)	Genus- MALDI	Abundance (%)	Species- HTS	Abundance (%)	Species- MALDI	Abundance (%)
<i>Acinetobacter</i>	5.37	<i>Acinetobacter</i>	0.79	<i>Acinetobacter</i>	2.03	<i>Acinetobacter</i>	0.79
				<i>guillouiae</i>		<i>guillouiae</i>	
<i>Carnobacterium</i>	0.23	<i>Bacillus</i>	0.79	<i>Acinetobacter</i>	0.91	<i>Bacillus</i>	0.79
				<i>johnsonii</i>		<i>cereus</i>	
<i>Chryseobacterium</i>	0.12	<i>Buttiauxella</i>	0.79	<i>Acinetobacter</i>	0.73	<i>Buttiauxella</i>	0.79
				<i>radioresistens</i>		<i>brennerae</i>	
<i>Erwinia</i>	0.50	<i>Carnobacterium</i>	0.79	<i>Carnobacterium</i>	0.55	<i>Carnobacterium</i>	0.79
				<i>maltaromaticum</i>		<i>maltaromaticum</i>	
<i>Flavobacterium</i>	0.22	<i>Lactococcus</i>	0.79	<i>Chryseobacterium</i>	0.10	<i>Lactococcus</i>	0.79
				<i>carnipullorum</i>		<i>raffinolactis</i>	
<i>Hafnia</i>	0.25	<i>Hafnia</i>	0.79	<i>Erwinia</i>	0.44	<i>Hafnia</i>	0.79
				<i>aphidicola</i>		<i>alvei</i>	
<i>Kluyvera</i>	1.59	<i>Rahnella</i>	0.79	<i>Flavobacterium</i>	0.15	<i>Rahnella</i>	0.79
				<i>hibernum</i>		<i>aquatillis</i>	
<i>Lactococcus</i>	5.33	<i>Pseudomonas</i>	93.68	<i>Hafnia</i>	1.21	<i>Pseudomonas</i>	0.79
				<i>psychrotolerans</i>		<i>antarctica</i>	
<i>Leuconostoc</i>	0.34	<i>Serratia</i>	0.79	<i>Kluyvera</i>	1.01	<i>Pseudomonas</i>	1.57
				<i>cryocrescens</i>		<i>extremorientis</i>	
<i>Pseudomonas</i>	78.67			<i>Lactococcus</i>	1.52	<i>Pseudomonas</i>	5.51
				<i>lactis</i>		<i>fluorescens</i>	
<i>Serratia</i>	4.18			<i>Lactococcus</i>	3.03	<i>Pseudomonas</i>	20.47
				<i>raffinolactis</i>		<i>fragi</i>	
Others	3.20			<i>Pseudomonas</i>	0.01	<i>Pseudomonas</i>	2.36
				<i>abietaniphila</i>		<i>gessardii</i>	
				<i>Pseudomonas</i>	0.05	<i>Pseudomonas</i>	2.36
				<i>chlororaphis</i>		<i>koreensis</i>	

<i>Pseudomonas fluorescens</i>	37.53	<i>Pseudomonas libanensis</i>	0.79
<i>Pseudomonas graminis</i>	0.77	<i>Pseudomonas lundensis</i>	24.41
<i>Pseudomonas koreensis</i>	1.21	<i>Pseudomonas protegens</i>	0.79
<i>Pseudomonas psychrophila</i>	39.02	<i>Pseudomonas rhodesiae</i>	3.15
<i>Serratia proteamaculans</i>	3.43	<i>Pseudomonas synxantha</i>	0.79
Others	6.30	<i>Pseudomonas taetrolens</i>	2.36
		<i>Serratia liquefaciens</i>	0.79
		<i>Pseudomonas spp.</i>	28.33

3.3.2 Seasonal and regional variation

Differences in the psychrotrophic bacterial community were seen in raw milk across the seasons and regions in New Zealand using HTS (Table 3.2 and 3.3). *Pseudomonas* spp. were the most abundant species detected in all samples, with winter and Waikato showing the highest abundance. HTS showed that *P. psychrophila* and *P. fluorescens* were the two most prevalent *Pseudomonas* species in all samples across all four seasons and six regions. The abundance of *P. psychrophila* was higher than *P. fluorescens* in spring, whereas the opposite was observed across the other three seasons. The abundance of the second dominant species varied in summer (*Acinetobacter guillouiae*), autumn (*Serratia proteamaculans*), winter (*Lactococcus raffinolactis*) and spring (*Lactococcus lactis*). The abundance of *P. psychrophila* was higher than *P. fluorescens* in Northland and Waikato, whereas the opposite was observed across the other regions. The abundance of the second dominant species varied in Northland (*Lactococcus raffinolactis*), Waikato and Clondeboyne (*Serratia proteamaculans*), Bay of Plenty and Southland (*Lactococcus*

lactis), and Hawkes Bay (*Acinetobacter guillouiae*). Other than the most and second dominant species, the percentages of the remaining species were very low and data are not shown.

Table 3.2 Seasonal variation in the predominant isolates detected by HTS at the species level.

Species	Abundance (%)			
	Autumn	Winter	Spring	Summer
<i>Pseudomonas psychrophila</i>	38.44	37.50	49.26	30.86
<i>Pseudomonas fluorescens</i>	37.15	50.49	28.92	33.54
<i>Serratia proteamaculans</i>	9.51			
<i>Lactococcus raffinolactis</i>		3.04		
<i>Lactococcus lactis</i>			5.13	
<i>Acinetobacter guillouiae</i>				6.60

Table 3.3 Regional variation in the predominant isolates detected by HTS at the species level.

Species	Abundance (%)					
	Northland	Waikato	Bay of Plenty	Hawkes Bay	Clandeboye	Southland
<i>Pseudomonas psychrophila</i>	53.18	71.12	31.17	29.56	14.46	33.22
<i>Pseudomonas fluorescens</i>	23.35	20.50	39.74	48.56	61.41	35.96
<i>Serratia proteamaculans</i>		2.79			4.57	
<i>Lactococcus raffinolactis</i>	10.75					
<i>Lactococcus lactis</i>			6.25			4.08
<i>Acinetobacter guillouiae</i>				8.34		

3.4 Discussion

The maintenance of high microbiological quality of raw milk before processing is essential to ensure the quality of processed dairy products. Although the combination of refrigeration and pasteurization of raw milk extends the shelf life of dairy products, psychrotrophic bacterial growth before pasteurization and the resultant spoilage of milk before or after pasteurisation has the potential to damage the quality of dairy products. The objective of this present study was to investigate the psychrotrophic bacterial communities of raw milk across seasons and regions, based on culture-independent and culture-dependent approaches.

In the present work, the milk samples differed in their microbial ecology, however, both culture-independent and culture-dependent approaches showed that *Pseudomonas* were the predominant microbiota in all raw milk samples after a few days refrigeration, which follows many other studies (Champagne et al., 1994; Xin et al., 2017; Yuan et al., 2017). The reason for the high prevalence of *Pseudomonas* spp. may be due to the relatively short generation time (< 4 h) at 0-7°C of these bacteria in comparison with other psychrotrophs, which implies that one single cell can reach 10⁶ cfu/mL after 8-day storage (Dogan & Boor, 2003). Lei et al. (2017) stated that *Pseudomonas* require very limited nutrients to survive and grow even in unfavourable conditions. This might also account for the dominance of *Pseudomonas* in refrigerated raw milk. *Pseudomonas* spp. are naturally present in raw milk having their origin in the cow's feed, drinking water, bulk milk storage tank, milking machine and the farm environment (Carloni et al., 2016). Poor farm hygiene and contamination of cow's feed and water utensils may be the origins for the *Pseudomonas* in the raw milk. *Pseudomonas* spp. present in raw milk are potentially responsible for the defects in final products, such as UHT milk, due to the production of heat-stable spoilage protease and lipase (Stoeckel et al., 2016). The secretion of these two enzymes is commonly at a maximum in late log or early stationary phase of bacterial growth (Chen, Daniel, & Coolbear, 2003). Proteases can be defined as the enzymes that hydrolyse peptide bonds, and lipases are the carboxylesterases that hydrolyse acylglycerol. The bacterial protease present in milk preferentially degrades casein micelles, especially κ -casein, over whey proteins. The hydrolysis of κ -casein may form a 3D network of cross-linked protein with some other hydrolysed caseins (α -casein and β -casein). The mixed protein can accelerate the off-flavours and early gelation of UHT milk during storage (Datta & Deeth, 2003). Lipase can hydrolyse milk triacylglycerol into short-chain free fatty acids (FFAs), such as butyric acid, caproic acid, and caprylic acid, resulting in sharp, cheesy and rancid taste in final products (Chen et al., 2003). The enzymes produced by *Pseudomonas* spp. can be very heat-stable. For example, a protease from *Pseudomonas* strains retained 55-65% of the initial activity after heat treatment at 72°C, 15 s, and 20-40% activity after heat treatment at 140°C, 5 s (Haryani et al., 2003). According to Adams et al. (1975), proteases from 10 different *Pseudomonas* strains all remained active after 149°C, 10 s heat treatment. The

heat-stable protease is a major concern in UHT milk since it can survive the heat treatment used to produce commercially sterile milk. Even a small amount of active proteases can lead to a shortened shelf life of dairy products. Normally, the shelf life of UHT milk is about 6 to 9 months. Richardson & Newstead (1979) pointed out that UHT milk containing as little as 1ng bacterial protease per mL had only a 3-month shelf life. Therefore, to ensure the quality of final products, raw milk should be maintained in the appropriate condition (chilled in clean vessels) before processing and processed as soon as possible to minimize the outgrowth and selection of *Pseudomonas* in raw milk.

The subdominant bacterial genera found in raw milk by both HTS and MALDI-TOF were *Acinetobacter*, *Carnobacterium*, *Hafnia*, *Lactococcus*, and *Serratia*, which agrees with previous studies using both culture-independent and culture-dependent approaches to observe the psychrotrophic microbiota in raw milk. Other genera detected in this study, *Bacillus*, *Buttiauxella*, *Chryseobacterium*, *Erwinia*, *Kluyvera* and *Rahnella* have been isolated from raw cow's milk in many other studies (Quigley et al., 2013; Vithanage et al., 2016; Yuan et al., 2017). *Acinetobacter* was the second most prevalent genus detected by both HTS and MALDI-TOF MS methods. *Acinetobacter* is frequently found in refrigerated milk samples. *Acinetobacter guillouiae* is a strong lipolytic enzyme producer but less of a protease producer. The heat stability of the enzymes produced by *Acinetobacter* is not well studied (Ercolini et al., 2009; Hantsis-Zacharov & Halpern, 2007; Vithanage et al., 2016; Xin et al., 2017; Yuan et al., 2017). *Bacillus*, *Hafnia*, and *Serratia* have also been reported to produce spoilage enzymes, which influence the quality of dairy products (Vithanage et al., 2016; Xin et al., 2017; Yuan et al., 2017). *Bacillus* spp. especially *B. cereus*, have been reported to be the most common Gram-positive psychrotrophs in raw milk. 40-84% of *Bacillus* spp. can produce both protease and lipase (Samaržija, Zamberlin, & Pogačić, 2012). *Chryseobacterium* frequently occurs in raw milk and has been associated with bovine mastitis in previous studies (Kuang et al., 2009). The *Buttiauxella* genus has been found to remain active after pasteurization (Kuang et al., 2009). The *Rahnella* genus can grow rapidly in milk, cream, and cheese (Baruzzi et al., 2012). Other genera haven't been reported for their spoilage

enzyme-producing ability, but they also have the potential for limiting dairy product quality, such as high cell number, acid-producing ability and biofilm formation (Kives et al., 2005; Millière et al., 1994; Xin et al., 2017).

HTS revealed only 6 *Pseudomonas* spp. whereas MALDI-TOF MS revealed 13 different *Pseudomonas* spp. including 28.33% only identified to the genus level. This is due to the selection of the isolates based on their different morphologies, which can lead to a bias. Furthermore, a relatively large proportion of *Acinetobacter*, *Lactococcus*, and *Serratia* were revealed by HTS compared to MALDI-TOF MS analysis. One of the limitations of the culture-dependent method is the reliance on the selection of the colony on agar, which is not able to get representative figures for all the microorganisms. Less than 2% of microorganisms on this planet can be cultivated *in vitro* (Wade, 2002). Some bacteria may not be able to grow on an agar plate due to their specialised growth requirements, but they still can exist and grow in milk that could cause product issues. For example, in this study, HTS results showed that *P. psychrophila*, a protease producer reported by Matéos et al. (2015), was the predominant species whereas it was not detected by MALDI-TOF MS. One possible reason is that it does not grow optimally on MPCA. The reason for the lack of detection of 28.33% culturable *Pseudomonas* spp. is most likely due to the species not being included in the MALDI-TOF MS database (Weber et al., 2014). The overlook of “non-culturable protease-producing microorganisms in raw milk” by culture-dependent methods may limit the ability to predict the quality of final dairy products.

No identification method is ideal. The use of HTS will detect both live and dead bacteria so it may not be relevant to some studies that rely on the viable cell population. Also, milk is a complex matrix of fats, proteins, carbohydrates, and minerals, which can interfere with the PCR process with overloading with biomass, and so the total DNA might overburden the PCR reaction (Jost et al., 2016; Šuranská et al., 2016). Both approaches (HTS and MALDI-TOF) present unique challenges for identification and interpretation of biologically meaningful information, and for the moment, the high costs associated with high-throughput sequencing limit full exploitation. Although HTS can provide a full picture for profiling the microbial ecology of raw milk, for

single bacterial isolation and study, MALDI-TOF MS is a relatively cheap, reliable, and efficient method for identifying culturable bacteria. Lots of dairy companies already invested in this equipment, including Fonterra and Synlait. It costs 0.50\$ per sample. Combined approaches may offer the best possibility for achieving an understanding of complex microbial communities. Thus, the sequencing of the 16S rDNA gene and MALDI-TOF MS continue to offer powerful and economical methods to gain insight into the bacterial community composition in large numbers of samples.

The effects of seasonal variation on the microbial quality of raw milk have been extensively explored in many other studies but there is little information on psychrotrophs (Doyle et al., 2017; Kable et al., 2016; Mallet et al., 2012). In the present study, the changes in the microbial ecology of milk due to seasonality revealed by HTS can potentially be important for milk suppliers and dairy manufacturers. There is a clear trend in the abundance of *Pseudomonas* spp. according to the season, with most being found in winter, followed by spring, autumn, and fewest in summer. According to the National Institute of Water and Atmospheric Research (1981-2010) (www.niwa.co.nz), the average temperatures in winter, spring, autumn, and summer in New Zealand, are 14, 17, 19 and 22°C respectively. The rainfall is 114.5, 118.0, 117.7, and 105.2 mm respectively. In New Zealand, dairy cows are mainly fed year-round by grazing pastures, which comprise 96% of the total diet (De Klein, Smith, & Monaghan, 2006). As *Pseudomonas* thrive in wet and cold environments, the seasonal climate changes may influence microbial composition in the farm environment. The increased growth of *Pseudomonas* in the farm environment will increase the chance of raw milk contamination.

HTS shows the bacterial species dominance differed across the seasons of the year with *P. psychrophila* predominating in spring and *P. fluorescens* in the other three seasons. The prevalence of *P. fluorescens* and *P. psychrophila* in raw milk samples has been widely reported (Munsch-Alatossava & Alatossava, 2006; Samaržija, Zamberlin, & Pogačić, 2012). *P. fluorescens* and *P. psychrophila* are both believed to be proteolytic producers due to the presence of the AprX gene encoding heat-stable protease (Malmgren et al., 2017; Matéos et al., 2015; Xin

et al., 2017). *P. fluorescens* also produces lipase. Xin et al. (2017) reported that *P. psychrophila* was able to produce protease but did not mention lipase. Previous studies suggested that the fluctuation of microbiota may be linked to the optimum growth temperature of these bacteria (Griffiths, Phillips, & Muir, 1987). The optimum growth conditions for *P. psychrophila* are mild temperatures and wet conditions, which can favour the growth of *P. psychrophila* on the farm even before milking. With the high numbers of *P. psychrophila* on the farm, the milk will have a higher chance of contamination. As New Zealand dairy cows are pasture-fed, the feed during spring is relatively wet, which creates a niche for them to grow. Also, contamination of drinking water and uncleaned utensils used on the farm will provide additional opportunities for milk contamination. This result suggests that samples of milk taken in spring appear to favour a different predominant *Pseudomonas* spp. than the other three seasons. HTS showed an increase in the relative proportion of bacteria belonging to *Acinetobacter guillouiae* in summer. Kable et al. (2016) reported that the environment in which the herd was kept was the primary driver of the milk microbiota. The variations in microbial populations observed in this study are likely to be due to warmer temperatures and less precipitation in summer, which favours the growth of *Acinetobacter*. Lei et al. (2017) stated that *Acinetobacter* spp. is characterized by its tendency to tolerate drying. Another possible reason is the temperature abuse of milk during transportation and storage at the processing plant. According to the Ministry for Primary Industries New Zealand Code of Practice for the design and operation of farm dairies (www.foodsafety.govt.nz), raw milk must enter the bulk tank at 6°C or below. However, in New Zealand, raw milk is transported from the dairy farm to the dairy processing plant by the milk tanker. Most of the milk tankers in New Zealand are single-skinned and without refrigeration. There is a concern in the dairy industry that while milk is collected from the farm at temperatures below 6°C but may be transported and stored at temperatures higher than 6°C before processing. Failure to keep raw milk under appropriate refrigeration may lead to a shift in the raw milk microbiota. *Serratia proteamaculans* was the third predominant species in autumn followed by two *Pseudomonas* spp. They have been isolated from previous studies and able to produce protease but their growth characterisation and effect on dairy product has not been well reported (Machado et al., 2016). A relatively small abundance

of *Lactococcus raffinolactis* and *Lactococcus lactis* were found in winter and spring, respectively. They are mesophilic lactic acid bacteria that naturally thrive in the environment and mostly found on cow teats. They are not able to produce spoilage enzymes and can be eliminated through pasteurization. There is some published evidence on the effect of seasonal variation effects on milk quality (Hantsis-Zacharov & Halpern, 2007; Kable et al., 2016; Vithanage et al., 2016). However, seasonal variation cannot be controlled, and therefore controlling farm hygiene and avoiding raw milk temperature abuse continue to be important management tools in controlling raw milk quality on the farm.

A regional variation was also observed in this study. According to the National Institute of Water and Atmospheric Research (1981-2010), Waikato has a combination of warmer temperatures and higher rainfall than many other regions in New Zealand across all seasons. This may explain the greater abundance of *P. psychrophilia* in this region. However, there is no clear trend that could be attributed to geographical changes. Because New Zealand is an island in the southern hemisphere, the temperature gets warmer from south to north and the rainfall is higher in the coastal areas compared with the central regions. In this study, milk samples were combined farm samples from each region, not individual samples, to make some attempt at providing a representative regional sample. On-farm problems at each farm may also contribute to the changes in the microbial composition of raw milk.

3.5 Conclusions

This study demonstrated the complementarity of culture-independent (HTS) and culture-dependent (MALDI-TOF MS) methods for evaluating the psychrotrophic populations that may be present in raw milk. Although MALDI-TOF MS can be used as a rapid and accurate method to identify cultured isolates, combining this technique with HTS might provide a more comprehensive insight into the microbial composition of raw milk. Studies profiling the bacterial communities of raw milk can demonstrate changes in the microbial populations through a dairy season. This understanding may assist in selecting milk supplies that are best suited to the manufacture of specific products with the highest microbial quality requirements.

3.6 Links between Chapter 3 and Chapter 4

The quality of dairy product is highly related to the microbiological quality of raw milk. Chapter 3 was conducted as the first part of the research project of this thesis, giving us an overview picture of both the culturable and non-culturable psychrotrophic bacteria populations of NZ raw milk. With the information, we were able to know the microbiological factors that might cause product quality issues, which guided us to isolate the microbes and investigate their spoilage potential in Chapter 4.

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3.8 References

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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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Please indicate:		
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<ul style="list-style-type: none"> Describe the contribution that the candidate has made to the Manuscript/Published Work: 		
The candidate carried out the laboratory work and data analysis (the bioinformatics analysis for 16S rDNA sequencing was carried out by Dr. Patrick Biggs) and prepared the manuscript with input in guidance of direction and editorial help from the co-authors and supervisors.		
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Chapter 4. Identification and selection of heat-stable protease and lipase-producing psychrotrophic bacteria from fresh and chilled raw milk

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Abstract

Refrigerated storage of raw milk favours the growth of psychrotrophic bacteria (psychrotrophs). Many of these psychrotrophs can produce heat-stable proteases and lipases that remain active after heat treatment, with the potential to spoil dairy products. Raw milk samples were collected from Milk Test NZ over four seasons and six main dairy regions in New Zealand. All samples were analysed for psychrotrophic and mesophilic bacterial counts immediately and after 5-day chilled storage at 7°C. A total of 246 isolates from both fresh and chilled milk were identified by MALDI-TOF MS and examined for their production of heat-stable protease and lipase. In fresh raw milk, 119 isolates were identified, representing 12 different genera and 23 species. An increased ratio of PBC to MBC was observed after 5-day chilled storage. *Pseudomonas* spp. were predominant in both fresh and chilled milk, with a prevalence of 74.79% and 93.73%, respectively. *P. fragi* (10.92%) was the predominant species in fresh raw milk, while *P. lundensis* (24.41%) was the predominant species in chilled raw milk, followed by *P. fragi* (20.47%) and *P. fluorescens* (5.51%). Among the 246 isolates, 110 isolates showed proteolytic activity, and 94 of these isolates were able to produce heat-stable proteases. Ninety-one of these isolates were able to produce lipases, and 81 of them were heat stable. This is the first report to compare the raw milk microbiota before and after extended refrigeration under the genus and species level. This study also characterized these bacterial isolates, some of which have never been reported for their potential to spoil dairy products.

Keywords: psychrotrophic bacteria, *Pseudomonas*, heat-stable, proteases, lipases

4.1 Introduction

The microbiological quality of raw milk before processing is essential to ensure the quality of final dairy products. This includes keeping raw milk in the chilled on-farm bulk tank and efficient transportation to the manufacturing plant to minimise microbial growth. However, prolonged refrigerated storage of raw milk, which may occur on the farm, during transportation or manufacture, allows for the growth of psychrotrophs, which can produce heat-stable enzymes (proteases and lipases) (Baur et al., 2015). These heat-stable enzymes can resist pasteurization (72°C, 15 s) and even ultra-high temperature (135°C-150°C, 1-10 s). Proteases, hydrolyse casein, causing bitterness and gelation of UHT milk. Lipases degrade triglycerides and are associated with flavour defects in cream, butter, cheese, and UHT milk (Hantsis-Zacharov & Halpern, 2007).

The European Union (EU) standards for top quality milk require that the psychrotrophic bacteria count (PBC) and mesophilic bacteria count (MBC, also known as standard plate count or aerobic plate count) are not allowed to exceed 4.22 log cfu/mL and 5.00 log cfu/mL, respectively (Cempírková, 2002). Under sanitary conditions, the proportion of psychrotrophs in the total bacterial population of fresh raw milk is usually less than 10% and includes Gram-negative genera *Pseudomonas*, *Aeromonas*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter* and *Flavobacterium*, and the Gram-positive genera *Bacillus*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Arthbacter*, *Staphylococcus*, and *Carnobacterium* (Quigley et al., 2013). However, after 2-5 days of cold storage (< 7°C), the population of psychrotrophs can account for more than 90% of the total bacterial population in raw milk, with *Pseudomonas* predominating (Ercolini et al., 2009; Vithanage et al., 2016). In most countries, raw milk is not processed immediately after milking and is therefore kept refrigerated until it is shipped to the processing plant. The whole process may take up to 5 days depending on the milk collection intervals and transportation distances, which results in psychrotrophs being isolated and identified as members of the genus *Pseudomonas*. According to previous studies of psychrotrophs in raw milk (Ercolini et al., 2009; Hantsis-Zacharov & Halpern, 2007; Vithanage et al., 2016), *Pseudomonas fluorescens* are the most frequently isolated species among the *Pseudomonas*,

followed by *Pseudomonas fragi* and *Pseudomonas lundensis*. This is due to the generation time and stationary phase of these *Pseudomonas* species at 0-7°C being shorter than other psychrotrophs (Cempírková, 2002). These *Pseudomonas* species are the predominant enzyme producers present in raw milk but other psychrotrophs, such as *Serratia* and *Bacillus* have also been reported to produce spoilage heat-stable enzymes that are responsible for degradation of milk products (Hantsis-Zacharov & Halpern, 2007; Olajuyigbe & Ajele, 2005; Romero et al., 2001). The initial psychrotrophic bacterial ecology of raw milk is very important to determine the final microbial quality of chilled milk reaching the dairy processing plant. However, the shift in the numbers and types of psychrotrophs before and after refrigeration identified under the genus and species levels is not clear. It is therefore important to investigate the diversity and dynamics of psychrotrophs in fresh and chilled cow's milk and to determine the dominant enzyme-producing bacterial species to enable strategies to be developed for their control.

The present work studied the effect of extended chilled storage on the diversity of psychrotrophs in raw cow's milk by comparing samples with and without refrigerated enrichment. This study also assessed the heat-stability of proteolytic and lipolytic enzymes from the psychrotrophs that could influence the quality of the milk and the shelf life of dairy products.

4.2 Materials and methods

4.2.1 Sampling

The sampling processing was as same as Chapter 3. Twenty-four raw milk samples (commingled from about 10 different farms in each region)-(Northland, Waikato, Bay of Plenty, Hawkes Bay, Clandeboye and Lower South) representing the main milk collection areas of New Zealand, were aseptically collected from Milk Test NZ (Hamilton, NZ) across four seasons (autumn, winter, spring, and summer). The samples were kept in a chiller (< 7°C) and transferred to Massey University within 12 h. The samples were then stored at 7°C for analysis. According to current laws and practices in many countries, raw milk is usually kept below 7°C for up to 5 days until reaching a processing plant (Scatamburlo et al., 2015; von Neubeck et al., 2015).

Therefore, storing raw milk at 7°C for 5 days was chosen to study the changes in the microbial diversity of raw milk.

4.2.2 Enumeration of psychrotrophs and mesophiles (mesophilic bacteria)

Immediately and after 5-day incubation at 7°C, the samples were analysed using the spread plate method, in triplicate, according to Hantsis-Zacharov & Halpern (2007) on milk plate count agar (MPCA; Oxoid, Basingstoke, UK) and incubated at 7°C for 7 days for PBC and at 30°C for 48 h for MBC. Although many of psychrotrophs grow well at both 7°C and 30°C, mesophiles are the bacteria that only grow at 30°C. Hence, the ratio of PBC to MPC is a very important indicator monitoring the changes in microbial quality of raw milk.

4.2.3 Isolation and identification of bacteria

Psychrotrophs with unique morphologies were selected and streaked on MPCA to obtain pure cultures. The isolated bacteria were grown in nutrient broth (NB; Becton Dickson, USA) overnight and then were kept in Cryopreserved beads (Thermo Fisher Scientific, USA) at -80°C for future work. Two hundred and forty-six isolates were selected and identified using MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany) described by Zhang et al. (2019).

4.2.4 Enzyme production assays

To determine the enzyme-producing ability of the select isolates at 7°C, a fresh culture was streak-plated on calcium caseinate agar (CCA; Conda Pronadisa, Madrid, Spain) for proteolytic activity and tributyrin agar (TA; Fort Richard, New Zealand) for lipolytic activity and incubated at 7°C for up to 7 days. The plates were checked daily, and the presence of a clear zone along the streak indicates enzyme activity (Kabadjova-Hristova et al., 2006; Sangeetha, Geetha, & Arulpanandi, 2010). A known protease-producing bacterium (*P. fluorescens* C224) was used as a positive control. A known lipase-producing bacterium (*Pseudomonas aeruginosa* ATCC 27853) was used as a positive control and a non-protease and lipase producing bacterium (*Escherichia coli* ATCC 25922) was used as a negative control.

4.2.5 Screening the psychrotrophs showing heat-stable enzymatic activity - (HTST)

A loop of each enzyme-producing isolate was grown in 10 mL of sterile reconstituted skim milk (RSM, 100 g gamma-irradiated milk skim milk powder in 910 mL sterile distilled water) for 72 h at 20°C (the optimum growth temperature for most psychrotrophs in milk) in an orbital shaker at 200 rpm. 10 mL of the cultured RSM was centrifuged at 13, 000 x g for 10 min. The supernatant was heated at 72°C for 15 s using an immersed coil apparatus (Protrol, Techne 18TE-8A) followed by the addition of 1 mL of 0.1% (w/v) sodium azide (Thermo Fisher Scientific, USA) to prevent any subsequent microbial growth.

A hole was dug in CCA by a sterile hollow stainless steel tube with a diameter of about 6 mm. 0.1 ml aliquot of the heated-treated supernatant was added into the hole and incubated at 37°C (the optimum temperature for most psychrotrophic bacterial enzymes in milk) for up to 5 days (Abdou, 2003; Zhang et al., 2019). The clear zone surrounding the hole indicates proteolytic activity. The unheated supernatant was used as a positive control and un-inoculated RSM was used as a negative control.

A hole was dug on TA by a sterile hollow stainless steel tube with a diameter of about 6 mm. 0.1 ml aliquot of the heated-treated supernatant was drop-plated into the hole. The plate was incubated at 37°C for up to 5 days. The clear zone surrounding the hole indicates lipolytic activity. The unheated supernatant was used as a positive control and un-inoculated RSM was used as a negative control.

4.3 Results

4.3.1 Enumeration of psychrotrophs and mesophiles

Twenty-four fresh raw milk samples collected from different regions across four seasons and six regions were analysed for PBC and MBC. To explore the effect of extended refrigeration on the microbiological composition of raw milk, PBC and MBC of each milk sample stored at

7°C for 5 days were also investigated. The results are shown in Figs. 4.1 and 4.2. The values of PBC and MBC before storage ranged from 2.36 ± 0.10 to 3.87 ± 0.39 log cfu/mL and 3.42 ± 0.10 to 4.94 ± 0.16 log cfu/mL, respectively. The mean for the PBC and MBC were 3.30 ± 0.26 and 4.29 ± 0.21 log cfu/mL, respectively. The PBC and MBC after 5 days storage reached a maximum of 7.94 ± 0.08 and 8.26 ± 0.08 log cfu/mL, respectively. The mean for the MBC and PBC after 5 days storage was 7.60 ± 0.20 and 7.78 ± 0.16 log cfu/mL, respectively.

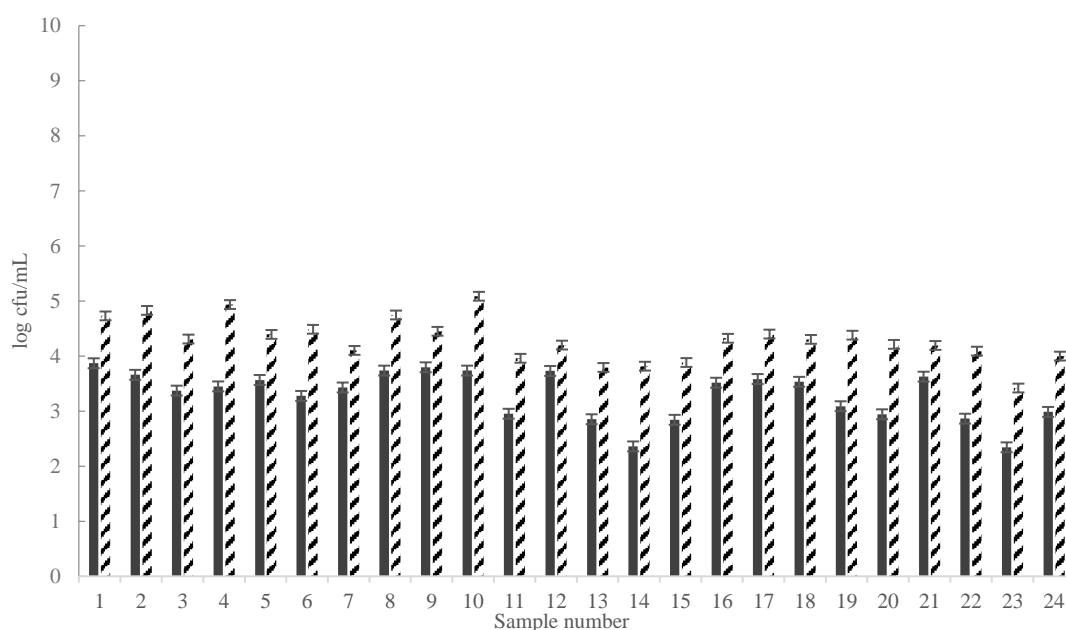


Figure 4.1 The population of psychrotrophs (black bars) and mesophiles (black and grey bars) in fresh raw milk (mean and standard deviation).

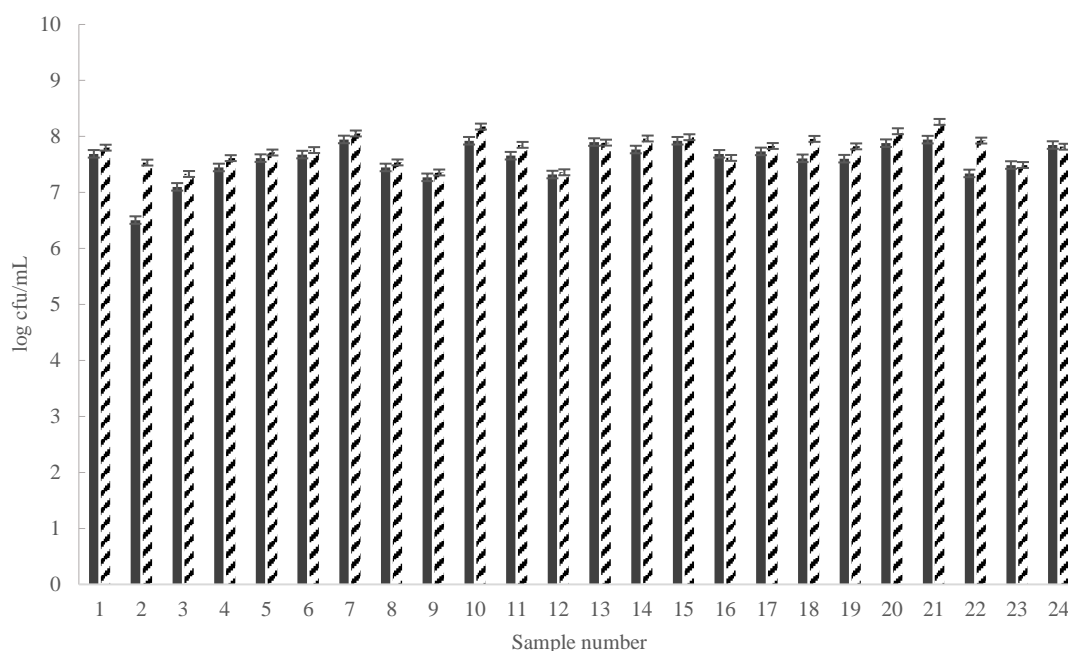


Figure 4.2 The population of psychrotrophs (black bar) and mesophiles (black and grey bar) in raw milk after 5 days of storage at 7°C (mean and standard deviation).

4.3.2 The proportion of psychrotrophs in fresh and chilled raw milk

Two hundred and forty-six psychrotrophs from fresh and chilled raw milk were identified using MALDI-TOF MS and the results are summarized in Figs.4.3-4.6. In fresh raw milk, one hundred and nineteen isolates were identified, representing 12 different genera and 23 species. *Pseudomonas* had the highest relative abundance comprising 74.79% of the total isolates while the other 11 genera *Acinetobacter*, *Candida*, *Chryseobacterium*, *Carnobacterium*, *Enterobacter*, *Enterococcus*, *Raoultella*, *Lactococcus*, *Hafnia*, *Filobasidium*, and *Serratia* were isolated in very small numbers (25.21% in total). Among the 25 species belonging to *Pseudomonas* (including unidentified *Pseudomonas* spp.), *P. fragi* (10.92%) was the predominant species, followed by *P. lundensis* (6.72%) and *P. fluorescens* (6.72%).

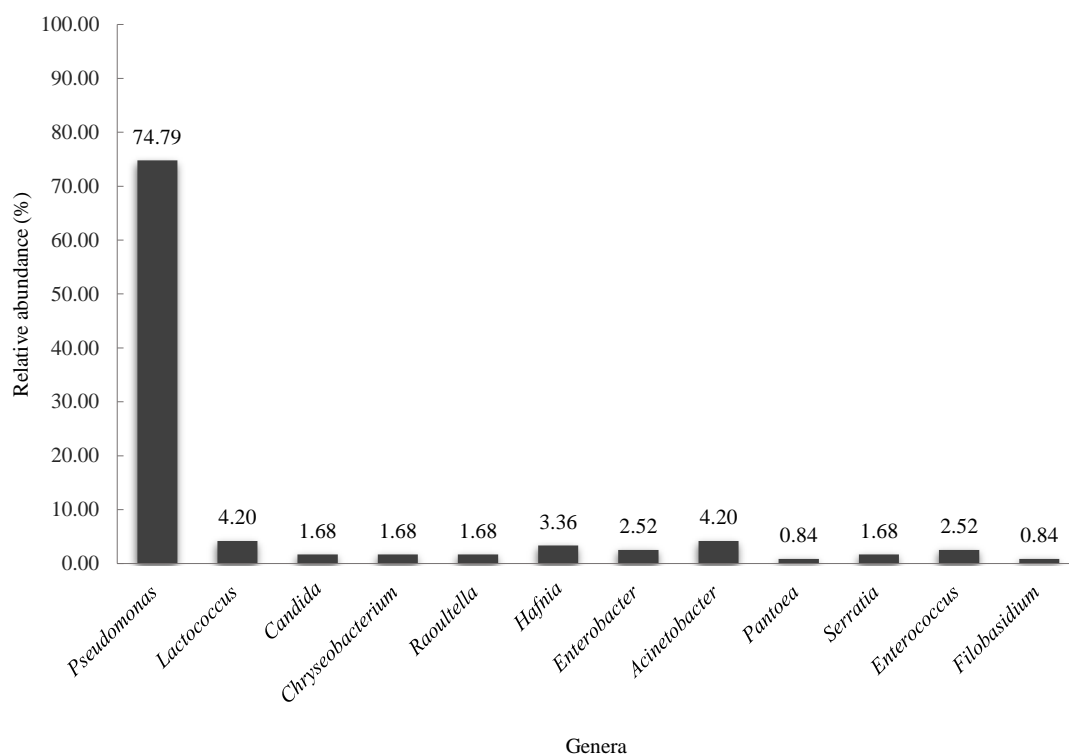


Figure 4.3 Relative abundance of psychrotrophs isolated from fresh raw milk identified by MALDI-TOF MS to genus level.

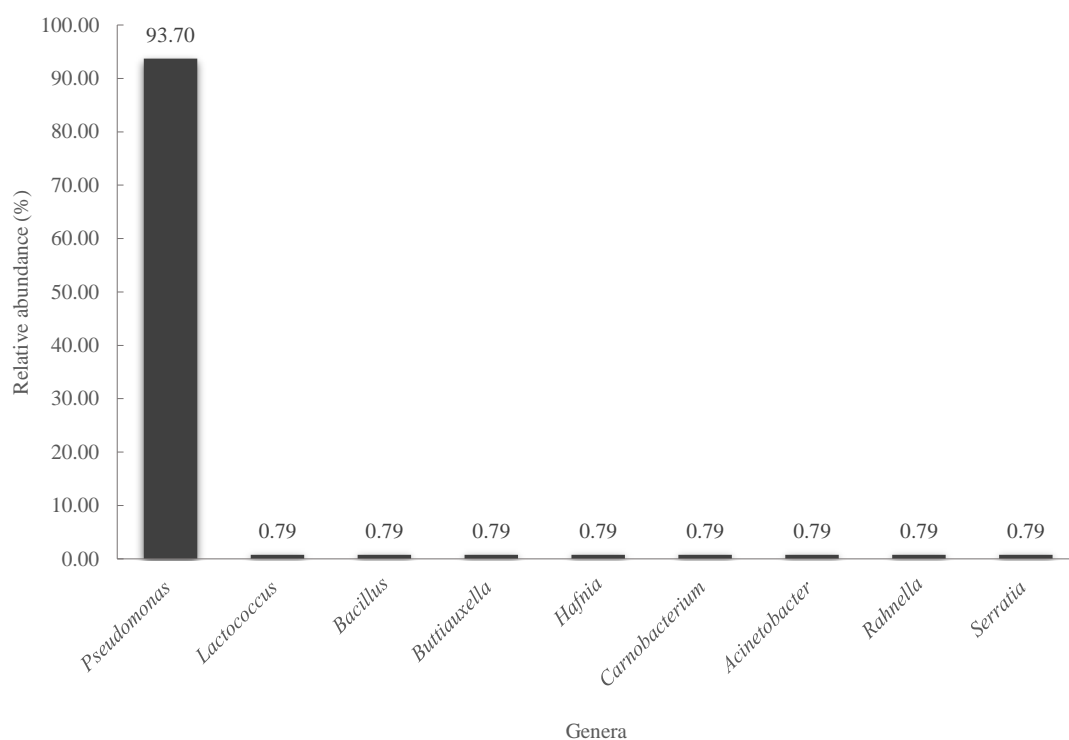


Figure 4.4 Relative abundance of psychrotrophs isolated from raw milk after 5 days of storage at 7°C identified by MALDI-TOF to genus level.

In the chilled milk, stored for 5 days, 127 isolates were identified, representing 9 genera and 20 species, indicating that the psychrotrophic bacterial composition of chilled stored milk is less diverse than that of fresh raw milk. The predominant genus was *Pseudomonas* (93.68%), while

the other 8 genera *Acinetobacter*, *Bacillus*, *Buttiauxella*, *Carnobacterium*, *Lactococcus*, *Hafnia*, *Rahnella*, and *Serratia* contributed only 6.32%, one isolate for each. Four of them were also isolated from fresh raw milk, whereas the rest (*Bacillus*, *Buttiauxella*, *Carnobacterium*, and *Rahnella*) genera were not found previously. Among the 21 species belonging to *Pseudomonas* (including unidentified *Pseudomonas* spp.), *P. lundensis* was the predominant species (24.41%), followed by *P. fragi* (20.47%) and *P. fluorescens* (5.51%). A total of 21.48% (26/121) and 28.33% (36/127) of *Pseudomonas* from fresh and stored raw milk respectively were not identifiable to the species level ($1.7 < \text{score} < 2.0$).

Some genera were absent after chilled storage. For example, *Pseudomonas frederiksbergensis* and *Pseudomonas tolaasii* were not isolated from chilled stored raw milk and only found in fresh raw milk. On the contrary, *Pseudomonas koreensis* and *Pseudomonas antarctica* were found in chilled raw milk but not fresh raw milk.

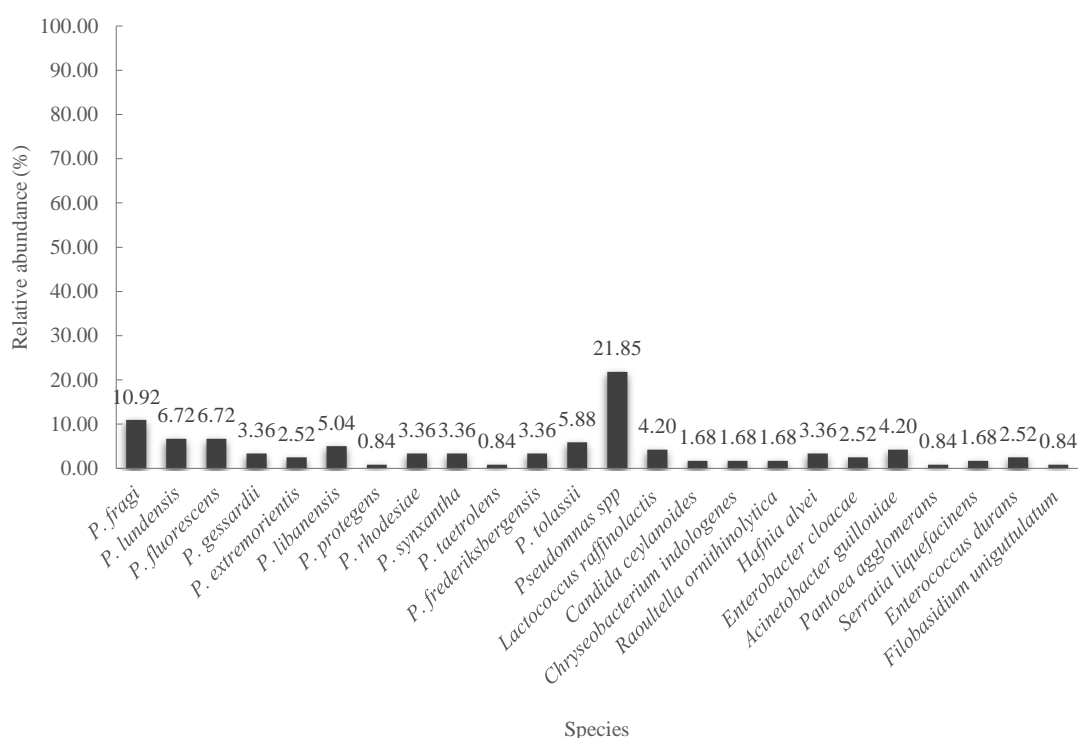


Figure 4.5 Relative abundance of psychrotrophs isolated from fresh raw milk identified by MALDI-TOF to species level.

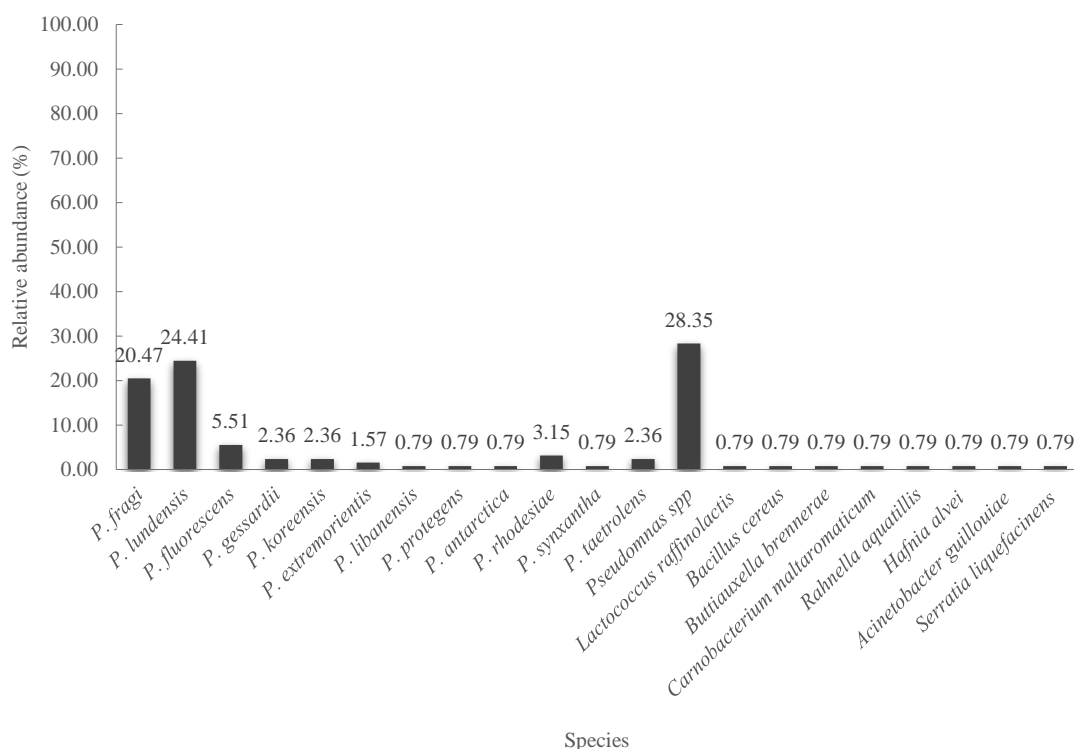


Figure 4.6 Relative abundance of psychrotrophs isolated from raw milk after 5 days of storage at 7°C identified by MALDI-TOF to species level.

4.3.3 The heat stability of proteolytic and lipolytic activity of isolated psychrotrophs

All 246 isolates were examined for their ability to produce proteolytic and lipolytic enzymes at 7°C by agar diffusion assays and the results are shown in Table 4.1. In this Chapter, the heat stability of the enzymes secreted from all the isolated psychrotrophs was examined at pasteurisation temperature, which was a pre-screening process for the isolates showing heat-stable enzymatic activity. The most predominant isolates showing heat-stable enzymatic activity under pasteurisation temperature were then tested for heat stability under UHT process conditions using a quantitative analysis detailed in Chapter 6. Among the 246 isolates, 110 isolates showed proteolytic activity at 7°C and 94 of these isolates were able to produce heat-stable proteases. Ninety-one of these isolates were able to produce lipases and 81 of them were heat stable. *Pseudomonas* has a tendency to secrete proteases alone or in combination with lipases rather than lipases alone. For other bacteria, like lactic acid bacteria (*Carnobacterium* and *Enterococcus*), they showed no extracellular enzymatic activity. *Acinetobacter* isolates displayed a very strong tendency to produce lipase, but not protease.

Table 4.1 Bacteria isolated from both fresh and chilled raw milk and their proteolytic and lipolytic activity.

Isolates identification	Numbers of isolates				
	Total	Proteolytic	Heat stable	Lipolytic	Heat stable
			proteolytic		lipolytic
<i>P. fragi</i>	39	19	15	15	14
<i>P. lundensis</i>	39	25	23	21	17
<i>P. fluorescens</i>	15	9	8	5	3
<i>P. gessardii</i>	7	3	3	2	2
<i>P. extremorientis</i>	5	0	0	0	0
<i>P. libanensis</i>	7	2	1	0	0
<i>P. protegens</i>	2	2	2	0	0
<i>P. rhodesiae</i>	8	7	6	6	6
<i>P. synxantha</i>	5	5	5	5	5
<i>P. taetrolens</i>	4	0	0	0	0
<i>P. frederiksbergensis</i>	4	0	0	0	0
<i>P. tolassii</i>	7	3	3	1	0
<i>P. koreensis</i>	3	3	3	3	3
<i>P. antarctica</i>	1	1	1	1	1
<i>Pseudomonas spp.</i>	62	27	20	22	20
<i>Lactococcus raffinolactis</i>	6	0	0	0	0
<i>Candida ceylanoides</i>	2	0	0	0	0
<i>Chryseobacterium indologenes</i>	2	0	0	0	0
<i>Raoultella ornithinolytica</i>	2	0	0	0	0
<i>Hafnia alvei</i>	5	0	0	4	4
<i>Enterobacter cloacae</i>	3	0	0	0	0
<i>Acinetobacter guillouiae</i>	6	0	0	6	6
<i>Pantoea agglomerans</i>	1	0	0	0	0
<i>Serratia liquefaciens</i>	3	3	3	0	0
<i>Enterococcus durans</i>	3	0	0	0	0
<i>Filobasidium uniguttulatum</i>	1	0	0	0	0
<i>Bacillus cereus</i>	1	1	1	0	0
<i>Buttiauxella brennerae</i>	1	0	0	0	0
<i>Carnobacterium maltaromaticum</i>	1	0	0	0	0
<i>Rahnella aquatilis</i>	1	0	0	0	0
Total	246	110	94	93	83

4.4 Discussion

Prolonged storage of raw milk under refrigeration favours the growth of psychrotrophs and the heat-stable protease and lipase produced by those psychrotrophs potentially play a major role in the spoilage of dairy products. This study aimed to analyse and compare the psychrotrophic bacterial composition and enzymatic activity of raw milk under refrigeration and to select the heat-stable enzyme-producing isolates that are most critical for investigations into the spoilage of milk products.

PBC and MBC are two important indicators of raw milk quality and ultimately the quality of final milk products. The initial microbiological quality of the raw milk in this study was considered as top-grade, according to the EU criteria (Cempírková, 2002), as the PBC and MBC were less than 4.22 log cfu/mL of and 5.00 log cfu/mL, respectively. Mesophiles were found to be the predominant bacteria in all the fresh milk samples and most samples analysed had a PBC to MBC of 1:10, which agrees with many other studies (Fricker et al., 2011; Mallet et al., 2012; Porcellato et al., 2018). However, samples stored at 7°C reached an average PBC and MBC of 7.60 ± 0.20 and 7.78 ± 0.16 log cfu/mL, respectively. An increased ratio of PBC to MBC before and after chilled incubation was observed, which clearly shows psychrotrophs can grow rapidly under refrigeration. According to Muir & Kjaerbye (1996), raw milk with a PBC of 6 log cfu/mL is not recommended for the manufacture of UHT milk products, as high levels of PBC result in short shelf life, which is due to the production of heat-stable protease and lipase. In the dairy industry, raw milk is usually collected and held under refrigeration for a few days (2-5 days) before processing, which potentially leads to a considerable increase in PBC in raw milk used for the manufacture of dairy products. The extended time may be associated with intervals between the collection of raw milk and transport distances. This reinforces the importance of minimising the storage of raw milk.

MALDI-TOF MS is a fast, cheap, and easy method to identify bacteria that has been successfully applied to study the microbiota of raw milk (Zhang et al., 2019). The agar diffusion assays (calcium caseinate and tributyrin) have also been widely used for detection and isolation

of culturable proteolytic and lipolytic microorganisms in both the academic field and the dairy industry (Müller et al., 2016; O'Connell et al., 2016; Shelley, Deeth, & MacRae, 1987). To select, identify and compare the heat-stable enzyme-producing psychrotrophs from raw milk before and after extended chilled storage, a combination of a culture-dependent identification method (MALDI-TOF MS) and agar diffusion enzyme assays were used in this study. An alternative method for detecting proteolytic psychrotrophic bacteria in raw milk based on polymerase chain reaction (PCR) has been reported by some researchers (Machado et al., 2013; Marchand et al., 2009). The principle of this method is to extract total bacterial gDNA from raw milk and the DNA template containing AprX gene, which encodes a metalloprotease, can be amplified using multiplex PCR (Martins et al., 2005). It is an alternative and rapid approach for evaluating the quality of refrigerated raw milk by detecting the AprX gene existing in the microorganism, however, the limitations of this method cannot be overlooked. First, the presence of Ca⁺, proteins, and fats can act as inhibitory substances to affect the sensitivity of the PCR process (Defour et al., 2008). It is more difficult to detect low numbers of bacterial cells by PCR in the presence of these milk components, especially fats. For example, the PCR detection limit for *P. fluorescens* in raw milk was 10⁶ cfu/mL (Marchand et al., 2009; Martins et al., 2005). Also, the simultaneous detection of the proteolytic bacteria can only be carried by amplifying the protease-encoding gene (note that some bacteria containing the AprX gene are not proteolytic) from those known protease producers, such as *P. fluorescens*, *P. fragi*, and *Aeromonas hydrophila* (Machado et al., 2013). Other species of psychrotrophic proteolytic microorganisms associated with milk spoilage may be overlooked. Moreover, the identification of lipase-producing bacteria by this method has not been reported. Compared to this method, our method can provide a broad view of assessing the microbiological quality of milk.

Pseudomonas was the predominant genus among the microbiota of fresh raw milk, accounting for 74.79% of the total psychrotrophs. After 5 days of chilled incubation at 7°C, this proportion reached 93.68%. The reason for this growth is likely to be the short generation time (< 4 h) for these bacteria at 0-7°C in comparison with other psychrotrophs (Leser, Boye, &

Hendriksen, 1995). Additionally, *Pseudomonas* need only limited nutrients to survive and grow, which may help them to become the most competitive psychrotrophs (Yuan et al., 2017). Isolates belonging to *Pseudomonas* revealed 15 different profiles in MALDI-TOF MS analysis from isolates obtained in both fresh and chilled raw milk, which indicates a high microbial diversity in fresh raw milk. *P. fluorescens* is the most frequent milk spoiler isolated from raw milk by many other researchers (De Jonghe et al., 2011; Dogan & Boor, 2003; Meng et al., 2017; Yuan et al., 2017). *P. fragi* and *P. lundensis* have also been reported as important microbial contaminants playing an important role in the spoilage of dairy products (Ercolini et al., 2009; Marchand et al., 2009). In this study, *P. fragi* was the most predominant species in fresh raw milk and *P. lundensis* became the predominant species in chilled raw milk. *P. fluorescens* also constituted a relatively large proportion in both fresh and chilled raw milk but did not predominate. This can be explained by three possible reasons. First, in our study, the isolates were picked from the agar plates based on their unique morphology and identified by MALDI-TOF MS, whereas Yuan et al. (2017), who reported that *P. fluorescens* was dominant in milk, randomly selected the isolates from the agar plates and identify the isolates using 16S rDNA gene sequence analysis. The different selection methods for bacteria and the fact that MALDI-TOF MS was unable to determine the species of some *Pseudomonas* isolates would have resulted in some bias in the present study. An improvement in the MALDI spectra databases would help ensure more isolates could be identified to species level. Secondly, the growth rate of *Pseudomonas* may vary from species to species at 2-25°C. Although the growth rates of *P. fluorescens* and *P. lundensis* at 7°C have not been reported, Lebert et al. (1998) stated that *P. fragi* grows faster than *P. fluorescens* in both UHT milk and raw milk at 7°C. Our results suggest that *P. lundensis* may grow faster than *P. fluorescens* at 7°C, so it could successfully compete with other bacteria to become dominant. Thirdly, these differences could be from variation between countries and different dairy systems. For example, cow's feed varies from country to country, and this may be a possible contamination route for *P. fragi* in fresh raw milk. Although *Pseudomonas* are ubiquitous in raw milk, Marchand et al. (2009) pointed out farms that rely on grass feed, such as New Zealand farms, could be at high risk for contamination with *P. fragi*.

A large proportion of *Pseudomonas* was able to grow and produce protease and lipase at 7°C. Their enzymatic activity also was observed at 7°C, indicating that these bacterial enzymes can break down the milk protein and fat during chilled storage before processing. The degradation of milk before processing can cause an increase in fouling in heat exchangers and thus reduce production time (e.g. takes longer for UHT processing to reach 140°C) (Datta & Deeth, 2001). Some isolates did not produce enzymes based on the agar diffusion assays in this study, indicating that either they are not able to produce extracellular enzymes or their enzymes are not active at 7°C. For those bacteria that did produce protease and lipase, these enzymes remained active after pasteurization and therefore can cause deterioration and eventually spoilage of dairy products reducing their shelf life. Not all isolates of the same species showed the same enzyme activity at 7°C, and this agrees with the results reported by Meng et al. (2018) and von Neubeck et al. (2015). Isolates of *P. fluorescens* showed lipase activity less frequently (5 out of 15), while isolates of *P. lundensis* and *P. fragi* showed a relatively higher frequency of lipase producers, 36 of 78 isolates. A similar result has been reported by von Neubeck et al. (2015). This could reflect the presence or absence of lipase genes in the bacteria (von Neubeck et al., 2015; Zhang et al., 2019). Some other *Pseudomonas* species, *P. koreensis*, *P. rhodesiae*, *P. gessardii*, *P. antarctica* and *P. synxantha* found in this study, were also able to produce heat-stable protease and lipase at 7°C and this appears to be the first report of these species isolated from milk-producing heat-stable enzymes. Our data only partially agree with Decimo et al. (2014) who found that *P. libanensis* were able to produce both protease and lipase, while the strains isolated in this study only showed proteolysis. *P. frederiksborgensis*, *P. extremorientis*, and *P. taetrolens* showed no enzymatic activity, and this has not been reported in dairy isolates of these bacteria. There are a few possible reasons that these microorganisms were not detected previously. Firstly, the population of these bacteria is usually very low in raw milk, which might be overlooked. Secondly, traditional methods (e.g. API) might not be accurate enough to identify these bacteria while they could be detected by a more sensitive method like MALDI-TOF (with an updated database). Lastly, the NZ dairy industry is quite special compared to other countries, which might cause a region-specific. Their effect on dairy products needs to be investigated in the future.

Although *Pseudomonas* spp. are the main concern regarding proteolytic and lipolytic degradation of milk, other microorganisms have also been isolated from both fresh and chilled raw milk and have the potential for degradation of milk products. In the present trial, there were 15 genera and including 15 species: namely *Lactococcus raffinolactis*, *Candida ceylanoides*, *Chryseobacterium indologenes*, *Raoultella ornithinolytica*, *Hafnia alvei*, *Enterobacter cloacae*, *Acinetobacter guillouiae*, *Pantoea agglomerans*, *Serratia liquefaciens*, *Enterococcus durans*, *Filobasidium uniguttulatum*, *Bacillus cereus*, *Buttiauxella brennerae*, *Carnobacterium maltaromaticum*, and *Rahnella aquatilis*. *A. guillouiae*, and *H. alvei* showed only lipolytic activity. Species belonging to *Acinetobacter* have previously been reported to be high lipolytic bacteria, but the heat-stability of their lipases has not been reported (Yuan et al., 2017). In the present trial, their lipases survived pasteurisation. *H. alvei* was one of the most common occurring contaminants in milk. *H. alvei* is found in the natural environment, particularly in soil, sewage and water, and is associated with gastroenteritis, septicaemia, and urinary infections in humans (Chen, Wei, & Chen, 2011). The production of lipase by this bacterium has not previously been reported, although all 4 isolates in the present trial produced lipase. All three *S. liquefaciens* isolates from this study produced heat-stable proteolytic enzymes, agreeing with many other researchers (Machado et al., 2015; Machado et al., 2016; Teh et al., 2011). This is also one of the well-known biofilm producers on the internal surfaces of raw milk tankers (Solimar et al., 2015; Teh et al., 2011). Their capacity to form biofilm is reported as being much higher than for *Pseudomonas* isolates due to a larger ratio of mass: cell (Cleto et al., 2012). Teh et al. (2011) reported that proteolysis by bacteria was generally higher within biofilm compared with the corresponding planktonic cultures. Therefore, the presence of *S. liquefaciens* in raw milk may have a significant effect on the quality of dairy products.

Bacillus species usually produce more than one type of protease whereas most *Pseudomonas* only produce one (Chen, Daniel, & Coolbear, 2003). Among those *Bacillus* species, *B. cereus* is a well-known psychrotrophic spore-forming bacterium that can grow at < 7°C. *B. cereus* is less common in raw milk compared to *Pseudomonas* due to their relatively long

generation time at 7°C varying from 9.4 h up to 75 h (Dufrenne et al., 1995). They showed only heat-stable proteolytic activity in this study, while Meer et al. (1991) pointed out they were able to produce both heat-stable protease and lipase. Their enzymes are produced as soon as the spores germinate following heat activation by pasteurization, which can cause a big concern in the dairy industry (De Jonghe et al., 2010). Despite this current study aiming to explore the most frequently isolated heat-stable enzyme-producing psychrotrophic bacterial communities, other bacteria with low abundance and prevalence also play a vital role in the whole complex microbial ecology of raw milk. Although some bacteria that have been isolated from this study were not able to produce protease and lipase, they are ubiquitous and have the potential for the spoilage of dairy products through acid production, the production of other enzymes not tested in this study or the effect on other bacteria in milk that do produce enzymes.

4.5 Conclusions

The combination of refrigeration and pasteurization in the dairy industry is required to produce safe and quality products. However, these processing methods have created a specific niche for the heat-stable enzyme-producing psychrotrophs. This study compared the psychrotrophic bacterial population in raw milk before and after refrigerated storage and showed that prolonged refrigerated storage of raw milk could influence the microbial diversity of raw milk. Most studies have focussed on increasing numbers of bacteria during refrigerated storage of milk, not the change in predominant bacteria. This change may influence milk quality if prolific enzyme producers predominate. A wide range of psychrotrophs was isolated from fresh and chilled raw milk followed by identification by MALDI-TOF MS. The results reinforce that extended refrigerated storage of raw milk can shift the microbiota of raw milk. Some of these psychrotrophs found in this study represent the first time these species have been reported producing heat-stable enzymes. Their enzymes not only remain active after heat-treatment but also can be active during refrigerated storage. A complete understanding of the thermal tolerance of these microbial enzymes and their potential to spoil milk and milk products in different environments is important to help develop strategies to improve the quality of milk products.

4.6 Links between Chapter 4 and Chapter 5

In Chapter 4, psychrotrophs were isolated, identified, and tested for heat stability of enzymatic activity. The six most frequent isolates showing heat-stable proteolytic activity from raw milk in Chapter 4 were selected for their growth and proteolytic activity before processing in Chapter 5.

4.7 Acknowledgement

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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Name of candidate:	Dong Zhang	
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In which Chapter is the Manuscript /Published work:	Chapter 4	
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<ul style="list-style-type: none"> The percentage of the manuscript/Published Work that was contributed by the candidate: 	80%	
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Chapter 5. Milk fat influences proteolytic enzyme activity of dairy

***Pseudomonas* species**

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Abstract

This study investigated the effect of growth conditions on proteolytic activity of six *Pseudomonas* isolates, (*Pseudomonas fragi* DZ1, *Pseudomonas koreensis* DZ138, *Pseudomonas rhodesiae* DZ351, *Pseudomonas fluorescens* DZ390, *Pseudomonas synxantha* DZ832, and *Pseudomonas lundensis* DZ845), isolated from raw milk. Proteolytic activity of all *Pseudomonas* isolates in dairy media (skim milk and whole milk) was significantly higher ($p < 0.05$) than in non-dairy media (TSB), with most activity from *Pseudomonas* grown in whole milk. The proteolytic activity of *P. lundensis* DZ845 grown in TSB with the addition of 5% (w/v) butter was higher than other dairy ingredients added to TSB and the amount of proteolytic activity increased with increasing concentrations of butter (from 0-15%). *P. rhodesiae* DZ351 showed little proteolytic activity in all TSB supplemented with dairy ingredients. Four of the six isolates produced one protease of 47-50 kDa when grown in TSB. However, all six isolates were able to produce at least one type of proteases in milk media. For *P. lundensis* DZ845, a 12% casein zymography gel confirmed that the presence of butter could induce proteolytic activity. This is the first study showing the effect of milk fat (butter) on the proteolytic activity of *Pseudomonas*. This highlights the greater vulnerability of whole milk compared to skim milk on proteolytic activity.

Keywords: Protease, dairy ingredients, butter, milk fat

5.1 Introduction

Refrigerated storage of raw milk is a common practice to minimise the spoilage of milk caused by the growth of mesophilic microorganisms. However, this could enable the growth of psychrotrophic bacteria (psychrotrophs), which can produce proteolytic enzymes, many of which are resistant to heat treatments such as ultra-high temperature (UHT) processing (Hahne et al., 2019). Proteolytic enzymes hydrolyse peptide bonds in milk proteins, resulting in the alteration of physicochemical, functional and sensory properties of milk and milk products. More specifically, proteolysis results in bitter flavours, gelation, and spoilage in milk (Chen et al., 2003; Hantsis-Zacharov & Halpern, 2007). *Pseudomonas* species are the most commonly found psychrotrophs in chilled raw milk at the time of spoilage (Ercolini et al., 2009; Raats et al., 2011). *Pseudomonas* species such as *Pseudomonas fluorescens*, *Pseudomonas fragi* and *Pseudomonas lundensis* constitute the predominant microorganisms limiting the shelf life of UHT milk (Marchand et al., 2009). Most of their proteases are alkaline metalloproteases belonging to the serralsin family (AprX), with a molecular mass of about 47-50 kDa (Matéos et al., 2015). These enzymes are typically produced in the late log or early stationary growth phase. Ercolini et al. (2009) reported that milk products develop off-flavours when the number of *Pseudomonas* spp. reaches 2.2×10^6 - 3.6×10^7 cfu/mL, most likely due to these proteases. This suggests that the quality of the final product is negatively affected as time increases since the spoilage-causing proteases produced by *Pseudomonas* correlate with the growth of the bacteria that increase with time. To maintain the quality and prolong the shelf life of the dairy products, controlling the growth of *Pseudomonas* and their proteolytic activity in raw milk before any heat treatment is of paramount importance.

There have been some studies investigating the effect of growth conditions including pH, temperature, and mineral concentrations on the proteolytic activity of *Pseudomonas* (Gügi et al., 1991; Hellio et al., 1993; Rahman et al., 2005; Zhang et al., 2015). For example, the growth rate and proteolytic activity of *P. fluorescens* grown in nutrient broth are less than when grown in skim milk (Zhang et al., 2015). The proteolytic activity of bacteria is known to require the

presence of an inducer in the culture medium (Nicodème et al., 2005). Skim milk is an efficient inducer for some *Pseudomonas* strains, but the influence of whole milk is unknown. Furthermore, the effect of individual milk components on the bacterial growth and their proteolytic activity has not been well studied.

The most common species found in raw milk are *P. fluorescens*, following by *P. fragi* and *P. lundensis*, consequently, they are the most studied species (von Neubeck et al., 2016; Zhang et al., 2015). *Pseudomonas* species *P. rhodesiae*, *P. koreensis* and *P. synxantha* have also been frequently isolated from raw milk (von Neubeck et al., 2017; Zhang et al., 2019), but there is very little information on their growth and proteolytic activity which may potentially be involved in spoilage of milk products. This research investigated the effect of dairy ingredients on the bacterial growth and proteolytic activity from six dairy isolates of *Pseudomonas* in both dairy and non-dairy media. This knowledge will enable the dairy industry to understand the role of dairy ingredients in triggering bacterial proteolytic activity and provide information to help in controlling microbial proteolytic activity in dairy product manufacture.

5.2 Materials and methods

5.2.1 Bacterial isolates

In our previous study of New Zealand raw milk samples, a large set of *Pseudomonas* species and strains were isolated and identified by MALDI-TOF MS (Zhang et al., 2019). Six dominant *Pseudomonas* isolates (*P. fragi* DZ1, *P. koreensis* DZ138, *P. rhodesiae* DZ351, *P. fluorescens* DZ390, *P. synxantha* DZ832, and *P. lundensis* DZ845) showing high heat-stable proteolytic activity by agar diffusion assay at 7°C for 7 days (see chapter 4) were selected for this current study (Chapter 4). All isolates were stored in Cryopreserved beads (Thermo Fisher Scientific, USA) at -80°C and were pre-incubated for 24 h at 20°C in tryptic soy broth before the experiments (TSB; Becton Dickinson, Cockeysville, MD, USA).

5.2.2 Preparation and inoculation of growth media

In the first experiment, all six *Pseudomonas* isolates (approximately 1.0×10^3 cfu/mL) were inoculated into non-dairy and dairy media-TSB, skim UHT milk, and whole UHT milk. The milk media were purchased from a local supermarket. In the second experiment, the two isolates showing low (*P. rhodesiae* DZ351) and high (*P. lundensis* DZ845) proteolytic activity were inoculated into five different media-TSB as the control and TSB with 5% (w/v) of the following: sodium caseinate (SC; Fonterra, New Zealand), calcium caseinate (CC; Fonterra, New Zealand), salted butter (Fonterra, New Zealand) and 90% gamma-irradiated whey protein isolate (WPI; Fonterra, New Zealand). In the third experiment, the two selected isolates were incubated in TSB enriched with butter of different concentrations: 5%, 10%, 15%, 20% and 25% (w/v). Enriched media were homogenised (Ultra Turrax T25 Basic, IKA Labortechnik, Germany) at 400 Hz for 5 min before autoclaving at 121°C for 15 min. Cultures were incubated in different media for 7 days at 7°C for microbial analyses, proteolytic activity, and zymography gels. The controls included for all conditions did not contain any inocula.

5.2.3 Microbial analyses

Tryptic soy agar (TSA; Becton Dickinson, Cockeysville, MD, USA) was used to determine the total plate count using serial 10-fold dilutions of cultures in 0.1% (w/v) peptone water (GranuCult™, Merck, Germany). Using the drop plate technique, 10 µL of each dilution was pipetted in triplicate onto the TSA plates (Herigstad, Hamilton, & Heersink, 2001). The plates were incubated at 30°C for 24 to 48 h before counting. The bacterial count was expressed in log cfu/mL.

5.2.4 Proteolysis assay

A proteolysis assay used azocasein (Sigma, USA) as the substrate, according to Teh et al. (2012) with slight modifications. A 2% (w/v) azocasein solution was prepared in 50mM of pH 7.5 phosphate buffer saline solution (Thermo Fisher Scientific, New Zealand) and 0.1% (w/v) sodium azide (Thermo Fisher Scientific, USA). After centrifugation, 0.1 mL of the supernatant

from the culture referred to in Section 5.2.2 was mixed with 0.9 mL of the 2% azocasein solution. The samples were incubated at 37°C for 24 h. The reaction was stopped with the addition of 0.8 mL of 20% (w/v) trichloroacetic acid (Thermo Fisher Scientific, USA) into 0.4 mL of the azocasein, and sample mixtures were then centrifuged at 14,100 x g for 15 min. In triplicate, 0.15 mL of the supernatant was loaded into a 96-well microtiter plate (FALCON, USA). Optical density (OD) was then read at 400 nm with a spectrometer-based absorbance microplate reader (Spectrostar Nano, BMG LABTECH, New Zealand). The OD values were baselined using the values from the controls and converted to protease units with a standard curve for proteolysis by *Streptomyces griseus* (3.5 units/mg; Sigma-Aldrich, USA). One unit of proteolytic activity was defined as equivalent to the proteolytic activity of one milligram of the Sigma-Aldrich *S.griseus* protease. The standard curves were prepared for each medium used in this study (Figs. S5.15-S5.25). From each of the three biological repeats, samples were tested in triplicate.

5.2.5 Zymographic analysis

To determine the molecular mass of the bacterial proteases produced by different *Pseudomonas* isolates in different media, a (12%) casein zymography gel method was used as described by Marchand et al. (2009) with some modifications. Cultures were grown in different media at 7°C for 7 days as previously. The cultures were centrifuged at 14,100 x g for 15 min and 1 part of the supernatant was diluted with 2 parts of zymography sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% (w/v) glycerol, 4% (w/v) sodium dodecyl sulfate (SDS), 0.01% (w/v) bromophenol blue; Bio-Rad, USA). 25 µL samples of protein solution were loaded for electrophoresis. The separating gel was made of 30% (w/v) acrylamide-bisacrylamide (Acrylamide/Bis 37.5:1, Bio-Rad, USA), 10% (w/v) ammonium persulfate (Bio-Rad, USA), 50% (w/v) glycerol, 10% (w/v) SDS tetramethylethylenediamine (TEMED; Bio-Rad, USA) and 1.5M Tris solution (pH 8.8). The substrate added into the separating gel was 0.4% (w/v) casein (Thermo Fisher Scientific, New Zealand). The stacking gel was made of 30% (w/v) acrylamide-bisacrylamide, 10% (w/v) ammonium persulfate, 10% (w/v) SDS, 1 µL TEMED and 1.5M Tris solution (pH 6.8). The samples were run at 150V for 90 min on the PowerPac power supply (Bio-

Rad, USA). After electrophoresis, the gels were washed in 2.5% (v/v) Triton X-100 (Sigma-Aldrich, USA) and then incubated for 24 h at 37°C in development Buffer Solution (50mM Tris-HCl, pH 7.5, 200mM NaCl, 5mM CaCl₂, 0.02% (v/v) Brij-35; Bio-Rad, USA). After incubation, the gels were then stained with 0.1% (w/v) Coomassie Brilliant Blue (Biochemical, England) dissolved in a mixture of 40% (v/v) methanol (AnalaR NORMAPUR, New Zealand) and 10% (v/v) acetic acid (Thermo Fisher Scientific, USA), followed by discoloration in a solution containing 40% (v/v) ethanol (LabServ, New Zealand) and 10% (v/v) acetic acid until proteolytic activity appeared as clear bands on a blue background. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, Lithuania) was used as a molecular standard. Uninoculated samples were used as negative controls.

5.2.6 Statistical analysis

The effect of medium on bacterial proteolytic activity was analysed by one-way ANOVA only considering medium as the independent variable. The means of the triplicate for each strain x incubation time combination were compared by Tukey's test ($p \leq 0.05$), using Minitab Statistical Software (Minitab Version 17, State College, Pennsylvania, USA).

5.3 Results

5.3.1 Bacterial growth and proteolytic activity in TSB, skim milk and whole milk

The growth of six *Pseudomonas* isolates at 7°C for 7 days in TSB, skim milk, and whole milk and their proteolytic activity is shown in Figs. S5.8-S5.13. The viable counts increased as expected over 7 days incubation. The isolates used in this study had similar growth curves in all three media. The initial bacterial counts ranged from 2 to 4 log cfu/mL and the final counts for all the samples were around 9-10 log cfu/mL. All the isolates reached late log phase or early stationary phase after 2 to 3 days when proteolytic activity was detected. The proteolytic activity of all *Pseudomonas* isolates in dairy media (skim milk and whole milk) was significantly higher ($p < 0.05$) than in non-dairy medium (TSB), with growth in whole milk showing the strongest proteolytic activity compared with skim milk ($p < 0.05$), indicating that the presence of dairy

ingredients, especially milk fat, may increase the proteolytic activity of *Pseudomonas*. During 7 days of refrigerated incubation, *P. lundensis* DZ845 showed the highest proteolytic activity in whole milk at 1.04 ± 0.02 units, followed by *P. fluorescens* DZ390 at 0.89 ± 0.03 units. *P. koreensis* DZ138 and *P. rhodesiae* DZ351 were weak protease-producers. The rest were considered as medium protease-producers. Within 5 days of incubation, proteolytic activity from the growth of all isolates increased. A reduction in the proteolytic activity of some isolates was observed after 6 to 7-day incubation. For example, the proteolytic activity of *P. fragi* DZ1 and *P. koreensis* DZ138 started to decrease after 5-day incubation in TSB.

5.3.2 Effect of dairy components on proteolytic activity

P. rhodesiae DZ351 and *P. lundensis* DZ845 were used for further study, as the initial screening showed a low and high level of proteolytic activity, respectively. To determine the effect of dairy components on bacterial proteolytic activity, TSB supplemented individually with five dairy ingredients was used (Figs. 5.1 and 5.2). After 7 days, butter samples produced the highest proteolysis for both isolates: 0.09 ± 0.01 units for *P. rhodesiae* DZ351 and 0.50 ± 0.03 units for *P. lundensis* DZ845. Calcium caseinate samples showed the least proteolytic activity for both isolates. For *P. rhodesiae* DZ351, the proteolytic activity in all 5 media was limited. In Fig. 5.2, dairy ingredients other than milk fat (butter) appeared to inhibit the proteolytic activity. We conclude that the addition of milk fat to the growth medium of *P. lundensis* DZ845 significantly enhances its proteolytic activity ($p < 0.05$).

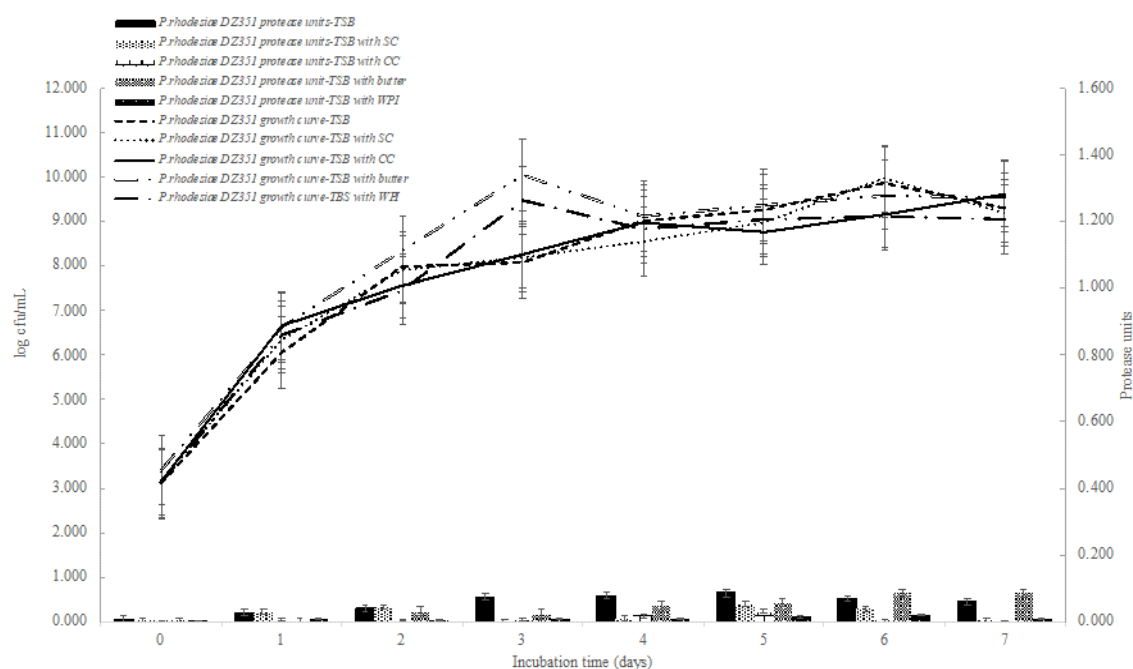


Figure 5.1 Growth curve and proteolytic activity of *P. rhodesiae* DZ351 in enriched media at 7°C for 7 days. SC-sodium caseinate, CC-calcium caseinate, and WPI-whey protein isolate. Values are the means \pm standard deviations from three independent experiments.

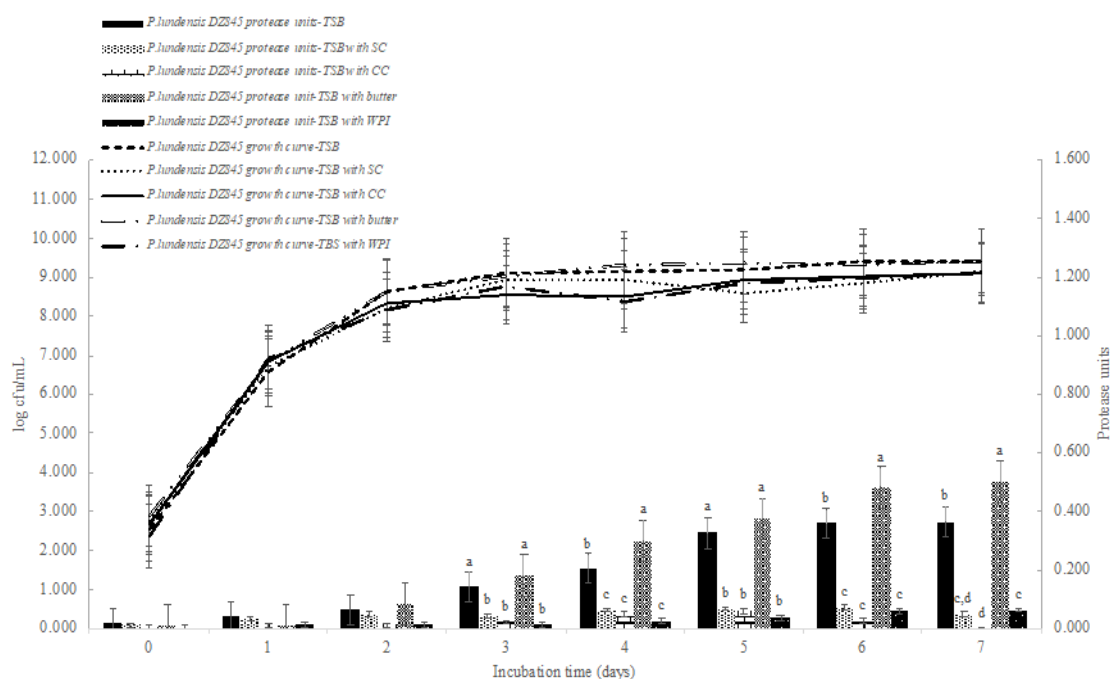


Figure 5.2 Growth curve and proteolytic activity of *P. lundensis* DZ845 in enriched medium at 7°C for 7 days. SC-sodium caseinate, CC-calcium caseinate, and WPI-whey protein isolate. Values are the means \pm standard deviations from three independent experiments. Different letters indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

5.3.3 Effect of butter concentrations on proteolytic activity

The effect of butter concentrations on proteolytic activity is shown in Fig. 5.3. *P. rhodesiae* DZ351 and *P. lundensis* DZ845 were grown for 7 days at 7°C in TSB medium supplemented with 0%, 5%, 10%, 15%, 20% and 25% (w/v) butter. *P. rhodesiae* DZ351 appeared to be unaffected by the increase in butter concentration. *P. lundensis* DZ845 on the other hand showed an increase in proteolytic activity as the butter concentration increased. For *P. lundensis* DZ845, the peak of activity was at 15% of butter, after which the proteolytic activity decreased.

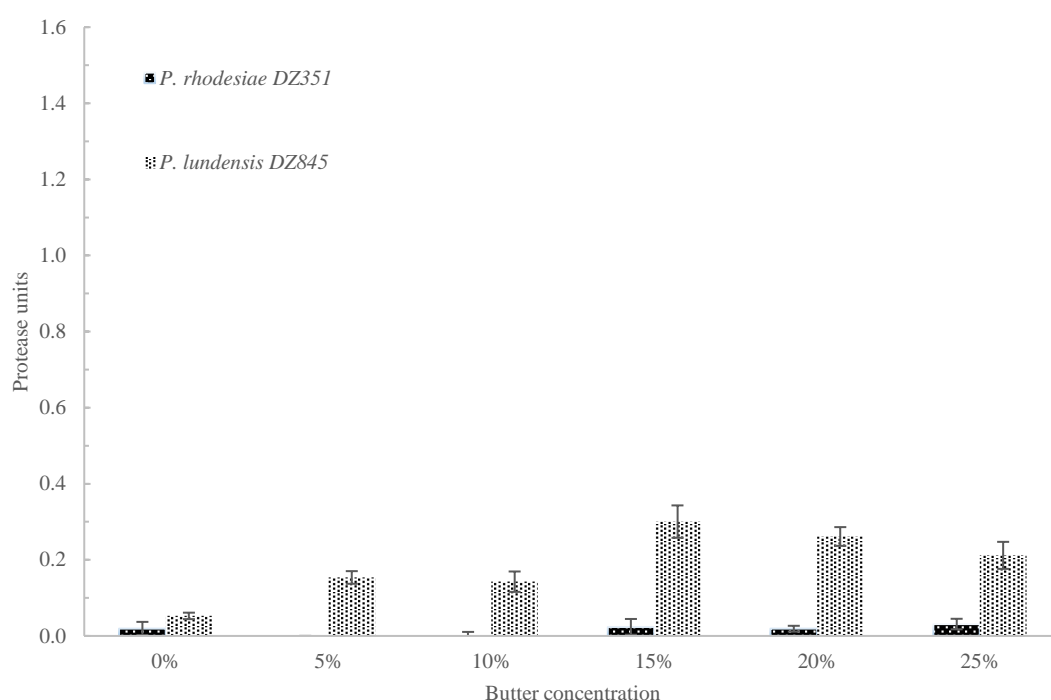


Figure 5.3 Proteolytic activity by *P. rhodesiae* DZ351 and *P. lundensis* DZ845 in TSB enriched with different butter concentrations (0-25%) after 7-day incubation at 7°C.

5.3.4 Electrophoretic protease pattern

After growth for 7 days at 7°C in TSB, skim milk, and whole milk, extracellular proteases of six isolates were separated using a zymography gel (Figs. 5.4-5.6). There was no proteolytic activity detected in the negative control samples. An example of a whole milk control sample is given in Fig. S5.14. The milk protein band was only observed in negative control samples. The protein band did not appear in the samples degraded by the proteases produced from the bacteria. Zymography gels revealed the presence of different proteolytic activity depending on the isolates

and the growth environment. *P. koreensis* DZ138 and *P. rhodisiae* DZ351 showed no proteolytic activity in TSB, which agrees with the previous proteolytic activity results. However, *P. koreensis* DZ138 and *P. rhodisiae* DZ351 were able to produce one strong band with an apparent molecular mass of 47 kDa and two distinct protease bands with apparent molecular masses of 47 and 50 kDa in milk media, respectively. For *P. fragi* DZ1 and *P. synxantha* DZ832, only one light band with a molecular mass of 50 kDa in was shown in TSB for each isolate. In contrast, the zymography revealed one strong band with an apparent molecular mass of 50 kDa in both skim milk and whole milk whereas an extra weak band with molecular mass between 130 to 180 kDa was only found in whole milk. Both *P. fluorescens* DZ390 and *P. lundensis* DZ 845 seem to produce only one protease with a molecular mass of 47 kDa in all media. However, the bands in milk samples were much stronger than in TSB. Interestingly, several smears (50-180 kDa) were observed only in milk media. These indicate stronger proteolytic activity in milk media compared with TSB. Similar results were reported by Troeberg & Nagase (2003) and Nicodème et al. (2005). This can be explained by the protein degradation of milk protein as the protease passes through the gel (Troeberg & Nagase, 2003). The phenomenon of the high molecular mass proteolysis smears does not correspond to different monomeric proteases, aggregates, or multimers.

P. lundensis DZ845 was incubated in TSB and TSB supplemented with 5% (w/v) of butter for 7 days at 7°C and the extracellular proteases were evaluated using 12% casein zymography (Fig. 5.7). *P. lundensis* DZ 845 produced only one protease with a molecular mass of 47 kDa in both media. Compared to the band in TSB, the band in TSB with butter was stronger. This suggests the presence of milk fat favours the proteolytic activity of *Pseudomonas*.

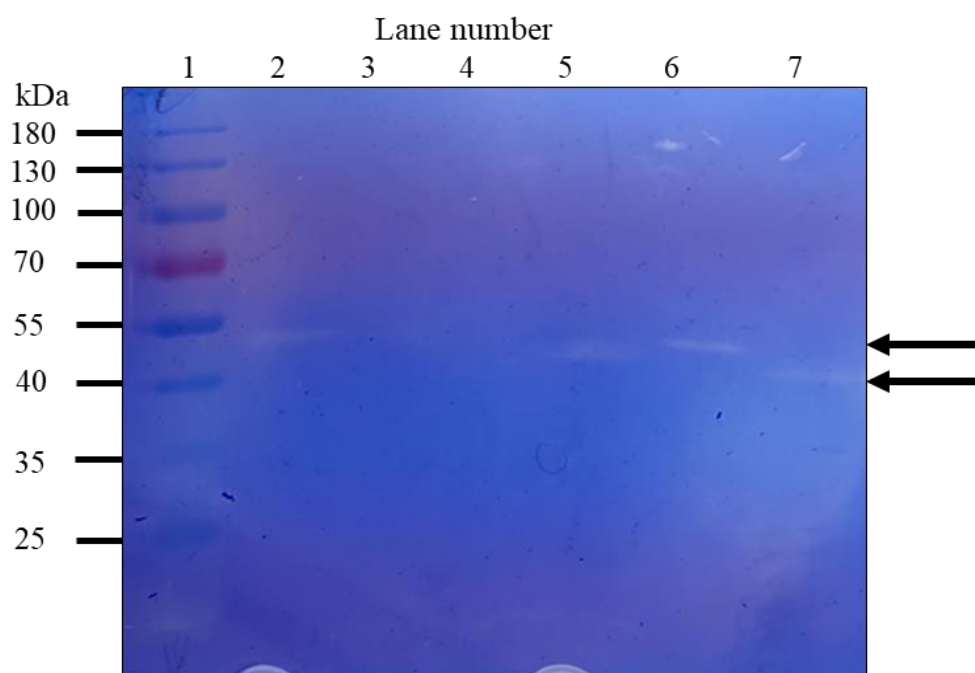


Figure 5.4 12% Casein zymography gel of cell-free culture supernatant of the 6 *Pseudomonas* isolates growing in TSB at 7°C for 7 days. Lane 1, molecular mass standard; Lane 2, *P. fragi* DZ1; Lane 3, *P. koreensis* DZ138; Lane 4, *P. rhodesiae* DZ351; Lane 5, *P. fluorescens* DZ390; Lane 6, *P. synxantha* DZ832; Lane 7, *P. lundensis* DZ845; Arrow indicates protease bands.

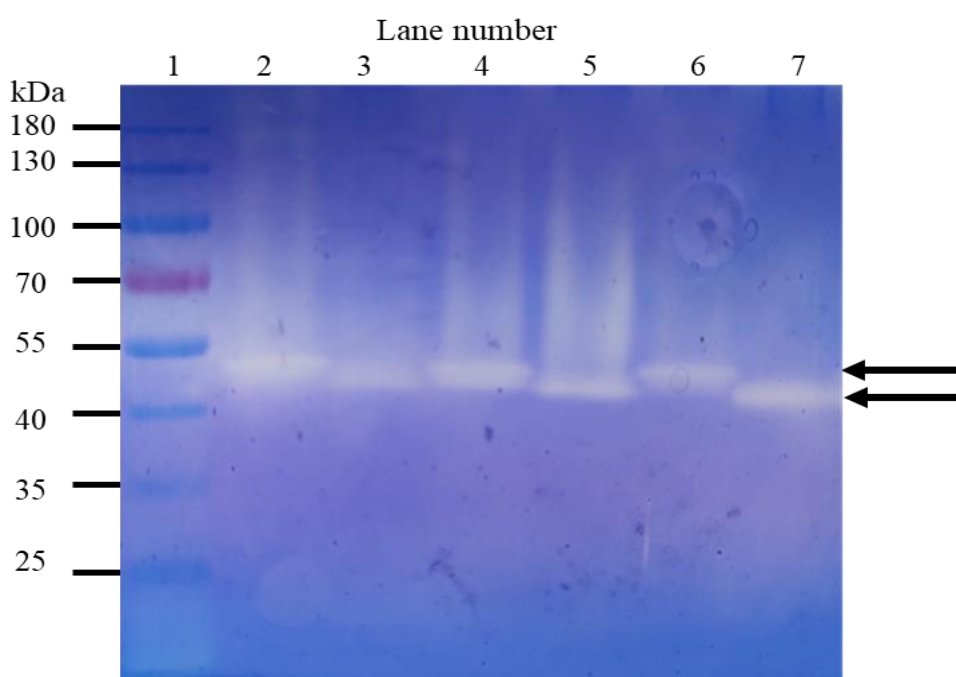


Figure 5.5 12% Casein zymography gel of cell-free culture supernatant of the 6 *Pseudomonas* isolates growing in skim milk at 7°C for 7 days. Lane 1, molecular mass standard; Lane 2, *P. fragi* DZ1; Lane 3, *P. koreensis* DZ138; Lane 4, *P. rhodesiae* DZ351; Lane 5, *P. fluorescens* DZ390; Lane 6, *P. synxantha* DZ832; Lane 7, *P. lundensis* DZ845. Arrows indicate protease bands.

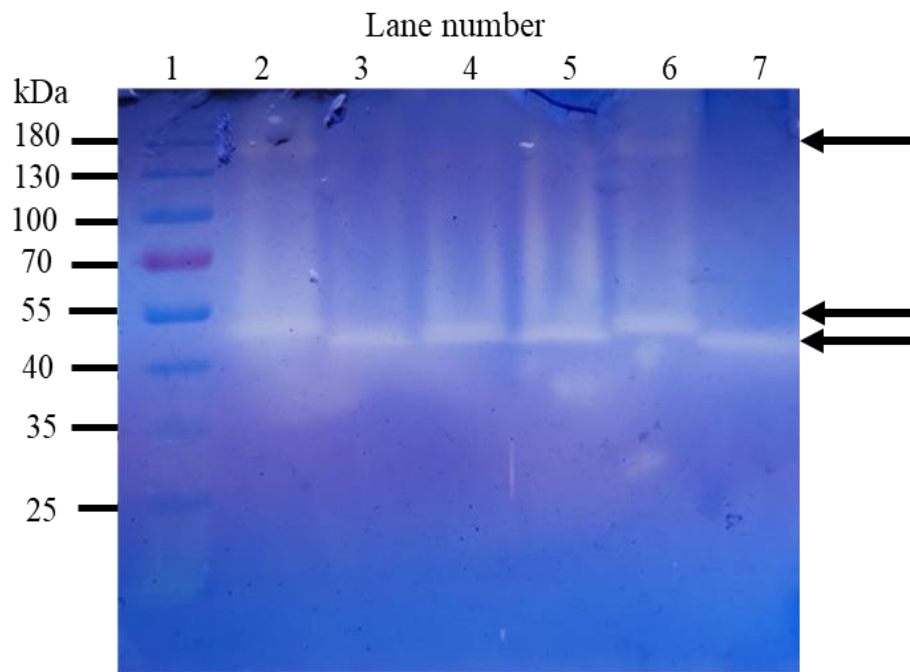


Figure 5.6 12% Casein zymography gel of cell-free culture supernatant of the 6 *Pseudomonas* isolates growing in whole milk at 7°C for 7 days. Lane 1, molecular mass standard; Lane 2, *P. fragi* DZ1; Lane 3, *P. koreensis* DZ138; Lane 4, *P. rhodesiae* DZ351; Lane 5, *P. fluorescens* DZ390; Lane 6, *P. synxantha* DZ832; Lane 7, *P. lundensis* DZ845. Arrows indicate protease bands.

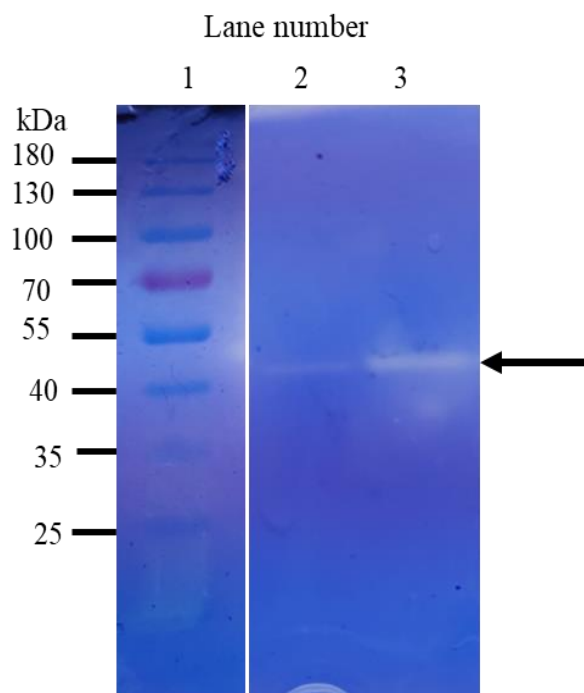


Figure 5.7 12% Casein zymography gel of cell-free culture supernatant of *P. lundensis* DZ845 growing in TSB and TSB with 5% of butter at 7°C for 7 days. Lane 1, molecular mass standard; Lane 2, TSB; Lane 3, TSB with 5% of butter. Arrow indicates protease bands.

5.4 Discussion

Pseudomonas spp. are the predominant species among the psychrotrophs in refrigerated milk due to their tendency to outgrow other bacteria present in refrigerated raw milk and are known for their production of heat-stable proteases (Raats et al., 2011; Von Neubeck et al., 2015; Xin et al., 2017). Most of these proteases are very heat-stable and even survive after UHT processing, leading to spoilage of dairy products (Samaržija, Zamberlin, & Pogačić, 2012). Although, the growth of some *Pseudomonas* (*P. fluorescens*, *P. fragi*, and *P. lundensis*) and their proteolytic activity has been extensively studied in the past (Dufour et al., 2008; Marchand et al., 2009; Nörnberg et al., 2010), the relationship between bacterial growth and proteolytic activity in different media and spoilage by other *Pseudomonas* spp. has not been well studied. The goal of this study was to evaluate the effect of culture conditions on the growth and proteolytic activity of *Pseudomonas* spp. The presence of milk fat appears to trigger the proteolytic activity of *Pseudomonas* spp., and this has not been previously reported.

All the bacterial isolates had similar growth curves in the different media tested. There was no media that appeared to provide a growth advantage over the others. With all the cultures entering the stationary phase after 2 days of cold storage, proteolytic activity increased from the start of the stationary phase, which agrees with many other studies (Chen et al., 2003; Zhang et al., 2015). This shows that proteolytic activity continues to increase once net cell growth has stopped. These results reinforce the importance of processing milk as soon as possible after milking. In this study, the maximum amount of proteolytic activity of all six isolates occurred in following order *P. lundensis* DZ845> *P. fluorescens* DZ832> *P. fragi* DZ1> *P. synxantha* DZ832> *P. rhodesiae* DZ351> *P. koreensis* DZ138. It is not possible to determine if this represents differences between the species or strains as only one isolate of each species was tested in this study. Other members of *Pseudomonas* species or strains may behave differently. The high level of the proteolytic activity of some isolates is likely to have the most effect on reducing the shelf life of dairy products. This study provides some comparisons between different isolates of

Pseudomonas spp. such as *P. rhodesiae*, *P. koreensis* and *P. synxantha* from milk that have not been well studied previously for proteolytic activity.

All the isolates showed more proteolytic activity in milk media compared with TSB. Although Griffiths. (1989) reported extracellular enzyme production by psychrotrophs growing in skim and whole milks was similar, maximum proteolytic activity appeared in whole milk rather than skim milk in this study. In this study, the bacteria growth and their proteolytic activity were carried out at 7°C for up to 7 days, to replicate a realistic, although extreme, scenario that can happen in the dairy industry while Griffith. (1989) only looked at bacterial protease production after 24 h incubation at different temperatures (2, 6, 10, and 15°C). In this present study, a high level of proteolytic activity in whole milk was observed after 3-days incubation at 7°C where the bacteria are entering the stationary phase, while incubation at 7°C for 24 h (Griffith 1989) is likely to be too early for optimal extracellular enzyme production. This could explain the report in Griffith (1989) that proteolytic activity by psychrotrophs growing in skim and whole milks was similar. Another explanation for the difference between the current study and that of Griffiths (1989) is that different bacterial strains were used. While there is a considerable amount of research showing that skim milk is more susceptible to proteolysis than whole milk (Deeth, Datta, & Wallace, 2002; López-Fandiño, Olano, Corzo, & Ramos, 1993), these studies have involved proteolytic activity in skim and whole milk following manufacture and the proteolysis may have originated from different microbial sources (eg thermophiles). The amount of proteolytic activity from *Pseudomonas* before reaching the dairy processing plant has been less well studied. Zymography confirmed the azocasein results and revealed that milk could induce *Pseudomonas* to produce different types of proteases, whereas only one protease with a mass of either 47 or 50 kDa was detected in TSB. This result supports previous work that proteolytic activity of *Pseudomonas* is predominantly from proteases with a molecular weight of 47-50 kDa (Koka & Weimer, 2000; Stuknytė et al., 2016). These proteases mostly likely include a specific caseinolytic extracellular protease, called AprX (47-50 kDa), which is the main concern in limiting the shelf life of UHT products, due to its heat-stability. AprX produced from *Pseudomonas* spp. degrades

κ -casein, α -casein, and β -casein. It causes solid and compact gels in UHT milk by specifically hydrolysing κ -casein (Zhang et al., 2019). Production of multiple proteases by some *Pseudomonas* spp. has been mentioned (Caballero et al., 2001; Fernandez et al., 1999; Nicodème et al., 2003; Rajmohan et al., 2002; Stepaniak & Fox, 1985). For example, Caballero et al. (2001) reported the aggregation of protease IV and elastase B produced by *P. aeruginosa* into complexes of 160 and 200 kDa, respectively, on casein gel zymography. Nicodème et al. (2003) reported that three strains of *P. chlororaphis* growing in mineral salt medium supplemented with 2% (v/v) skim milk showed the presence of several proteolytic bands with molecular weights of 47, 97 and 116 kDa on a 12% casein zymography gel. However, the role of milk in promoting the activity of multiple proteases has not been reported. The multiple protease bands (> 50 kDa) produced in whole milk might not be associated with AprX genes (47-50 kDa) and needs to be investigated in the future. In this study, the proteases with a molecular mass of 130-180 kDa produced by *P. fragi* DZ1 and *P. synxantha* DZ832 growing in whole milk may have a proteolytic activity that is different to the protease with a molecular mass of 47-50 kDa (La Rocca et al., 2004), and their effect on milk quality may be frequently overlooked. Further analysis of the identification of the type of proteases with different molecular mass by mass spectrometry may help in our understanding of the effect of different proteases in milk.

The hypothesis is that some dairy ingredients, especially milk fat, can induce bacterial proteolytic activity. The bacterial proteolytic activity can be influenced by many nutritional factors, such as carbon, nitrogen, and mineral sources (Adams et al., 1975; Fairbairn & Law, 1987). However, our results showed that of the individual dairy ingredients tested, only milk fat could induce high levels of proteolytic activity. A few researchers reported that bacterial lipase production or lipolytic activity can be stimulated by lipids, such as milk fat and olive oil (Aires-Barros et al., 1994; Stead, 1986), but no one mentioned bacterial proteolytic activity being stimulated by lipids. We also observed higher proteolytic activity in milk media than TSB. Milk has a higher protein and carbohydrate content than TSB, so it is not unexpected that *Pseudomonas* grown in milk should express more proteolytic activity than TSB. Also, proteins in milk are

different from those in TSB. The proteins in TSB are present in the form of small peptides. One of the components of TSB is phytone, which is an enzymatic digest of soybean meal. Milk media may enhance the production of *Pseudomonas* proteases, resulting in higher activity in the azocasein assay and zymography gel. Evidence for other milk components inducing protease production is shown by Hellio et al. (1993) where milk leucine and glutamine and a mixture of di- and tripeptides released from hydrolysis of plasma protein are inducers. However, many dairy ingredients tested in the current study (Sodium Caseinate (SC), Calcium Caseinate (CC), and Whey Protein Isolate (WPI)) were individually inhibitory to proteolytic activity. This was surprising as these protein ingredients were hypothesized to be stimulatory substrates for proteolytic activity. Calcium caseinate was the ingredient tested that produced the largest reduction in proteolytic activity after 7-day incubation. However, SC and CC are different from casein micelles and therefore may interact with the protease differently. The casein protein in calcium or sodium caseinate is not in the micelle form naturally found in milk so it does not necessarily reflect the effect of casein in milk (Southward, 2003). The reason for the inhibitory effect of WPI is something that needs investigation. One hypothesis may be that WPI contains enzymes that degrade the microbial proteases used in the present study. The same explanation could explain the observation with SC and CC.

The most surprising result is the effect of milk fat on the proteolytic activity of *P. lundensis* DZ845. Interestingly, this does not affect all *Pseudomonas* spp. with *P. rhodesiae* DZ351 appearing to be unaffected by the milk fat content. The proteolytic activity of *P. lundensis* DZ845 in whole milk is higher than in skim milk. This indicated that milk fat in whole milk is likely to influence proteolytic activity. However, other dairy ingredients may influence results (positively or negatively) (Supuran et al., 2002; Zhang et al., 2012), and therefore when milk fat was added to TSB, the result differed in terms of the degree of proteolytic activity in the presence of milk fat. In this study, we still can conclude milk fat does have a stimulatory effect on proteolytic activity. There are several possible explanations for this effect of milk fat on proteolytic activity. Firstly, fat may influence the metabolism of *Pseudomonas*, stimulating protease production.

Secondly, fat may influence the morphology of the proteases released. These proteases may be more exposed to active sites that make it easier to hydrolyse proteins. Thirdly, the fat in water emulsion in the TSB may assist with hydrophobic interactions between the proteolytic enzymes and their substrate. This is the most likely explanation resulting in an increase in the accessibility between the active sites and peptide bonds, optimizing protease activity. Moreover, autoproteolysis may occur during storage due to the partial unfolding of tertiary structures (Zhang et al., 2019). Protease is stable in the presence of organic matter, which may prevent autohydrolysis (Karadzic et al., 2004). For example, Karadzic et al. (2004) showed that a proteolytic enzyme produced by *P. aeruginosa* san-ai is stable in the presence of various organic matter, especially cutting oil (an oil that cooling and lubrication during industrial metal-working processes). Finally, the *aprX-lipA* operon is responsible for proteolytic activity and is associated with the gene *lip A* (Zhang et al., 2019). The absence of any of these genes in bacteria will lead to the loss of or relatively low proteolytic activity. It is, therefore, possible that the presence of milk fat enhances the expression of this gene resulting in increased proteolysis. Although we concluded that milk fat does increase the proteolytic activity of some *Pseudomonas* spp. the mechanisms for this are unknown. Other factors in the environment in which the protease is located may also influence the activity of the bacterial protease or enhance protease production of the microorganisms. Proteolytic activity or gene expression studies from bacteria grown in media containing individual components would help in determining the contribution of different factors that may influence proteolytic activity in a complex environment such as milk. Future work may also examine the effect of different dairy components as well as non-dairy hydrophobic compounds (e.g. vegetable oil and animal oil) to determine the effect of dairy ingredients and hydrophobicity on proteolytic activity.

5.5 Conclusions

Dairy ingredients influence the proteolytic activity of some of the dairy *Pseudomonas* isolates. Proteolytic activity from *Pseudomonas* isolates can be enhanced in the presence of milk fat and other milk components may inhibit proteolysis. Whole milk products are more likely to be

influenced by bacterial protease compared with skim milk products. The mechanism for this effect is unknown.

5.6 Links between Chapter 5 and Chapter 6

In Chapter 5, the growth of six *Pseudomonas* and their protease activity in raw milk before any heat treatment was studied. However, the heat stability of their protease was not studied in this Chapter whereas it was studied in Chapter 6. Chapter 6 continues to give more practical information to the industry. The effect of their numbers on the quality of final product quality was studied in Chapter 6.

5.7 Acknowledgement

This work was financially supported by Tetra Pak, New Zealand.

5.8 Supplementary materials

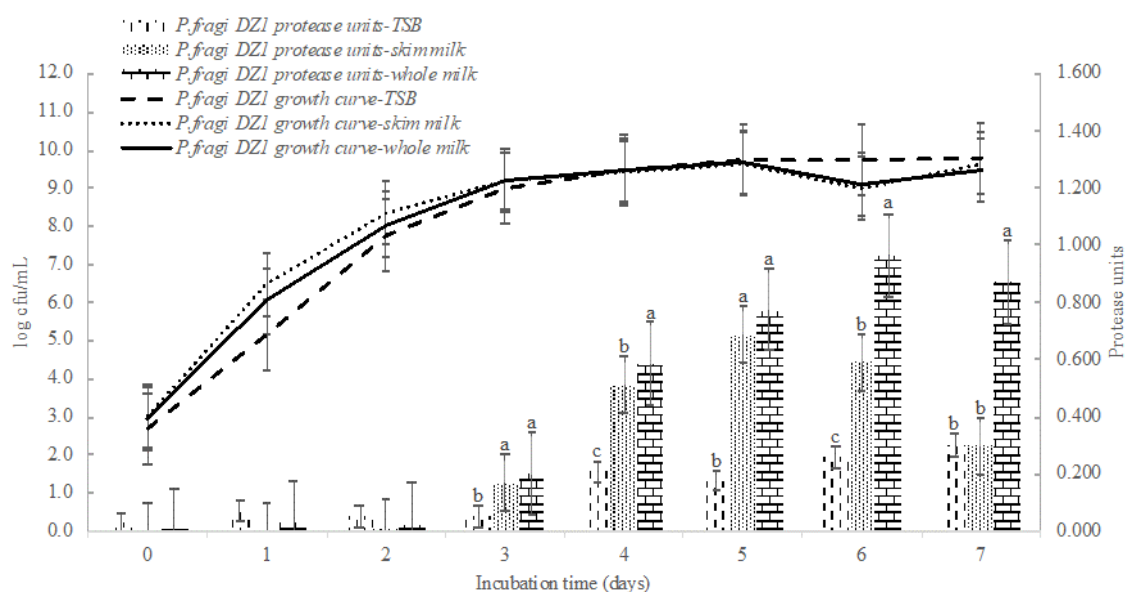


Figure S5.8 Growth curve and proteolytic activity of *P. fragi* DZ1 in TSB, skim milk and whole milk at 7°C for 7 days. Values are the means \pm standard deviations from three independent experiments. Different letters indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

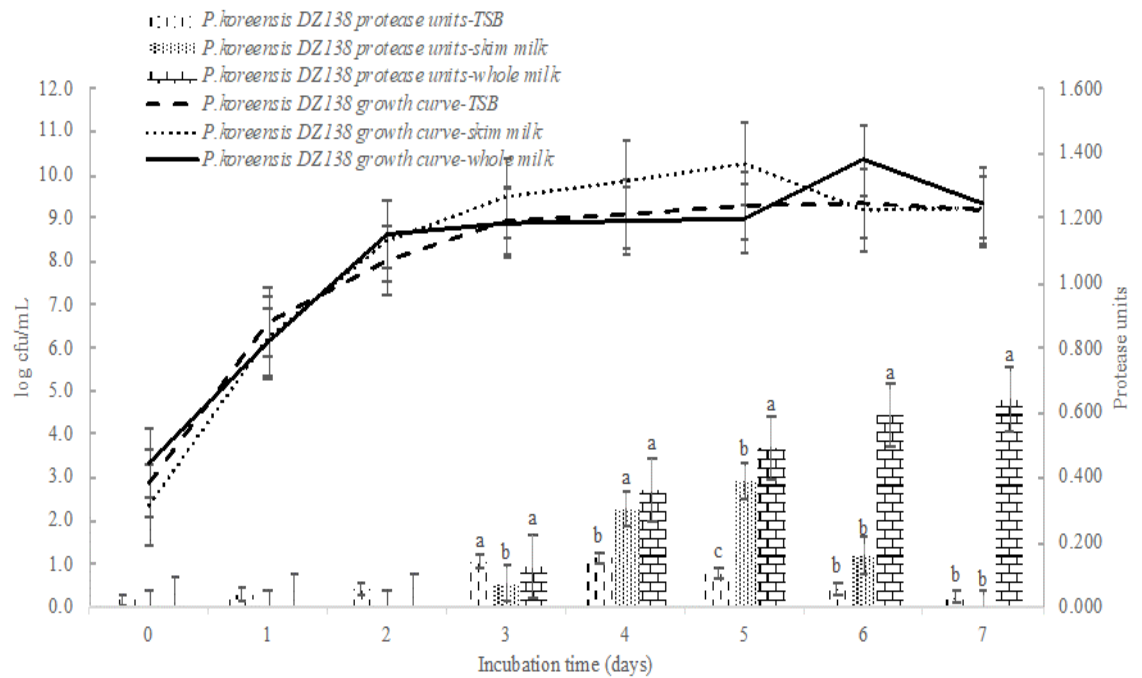


Figure S5.9 Growth curve and proteolytic activity of *P. koreensis* DZ138 in TSB, skim milk and whole milk at 7°C for 7 days. Values are the means \pm standard deviations from three independent experiments. Different letters indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

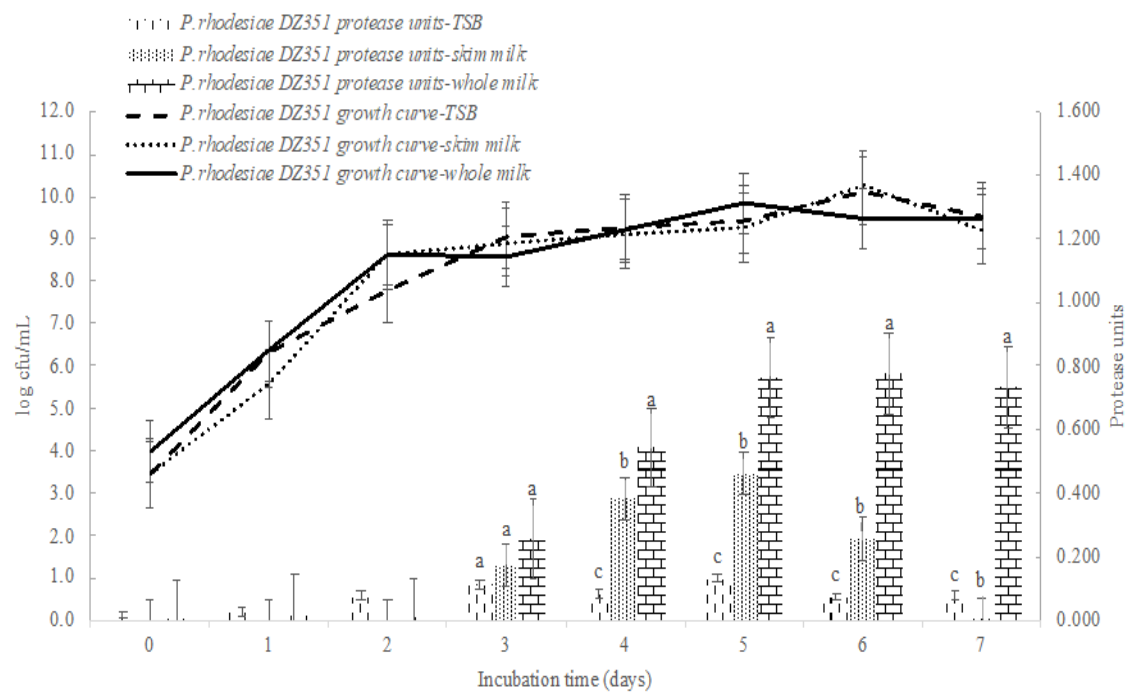


Figure S5.10 Growth curve and proteolytic activity of *P. rhodesiae* DZ351 in TSB, skim milk and whole milk at 7°C for 7 days. Values are the means \pm standard deviations from three independent experiments. Different letters indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

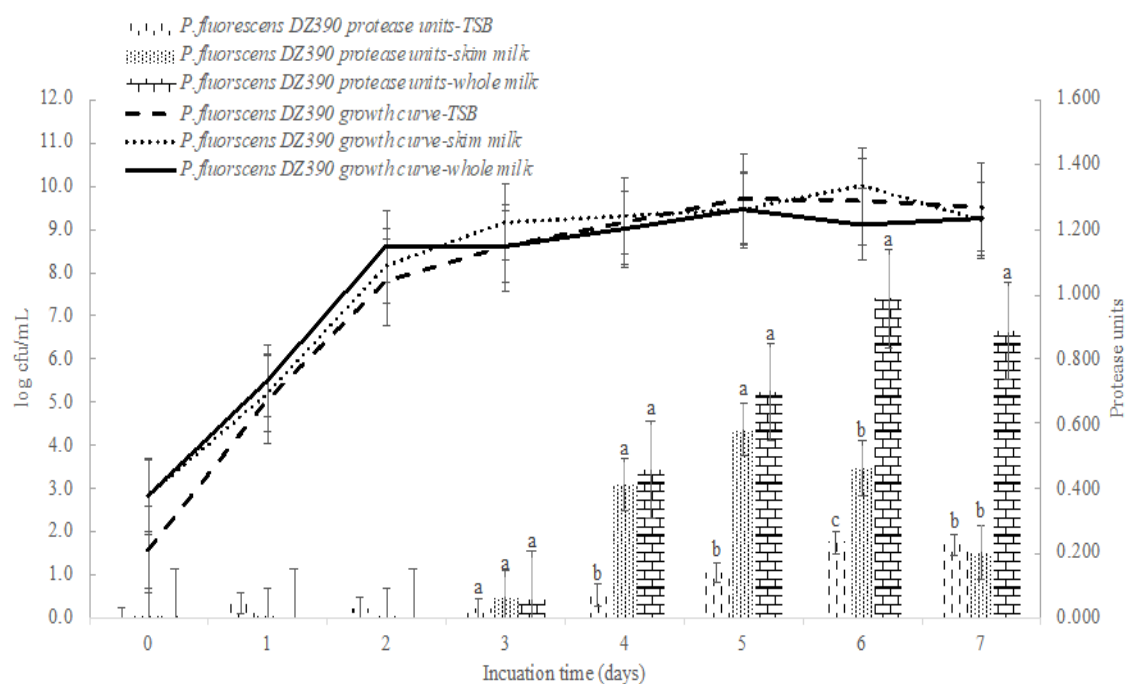


Figure S5.11 Growth curve and proteolytic activity of *P. fluorescens* DZ390 in TSB, skim milk and whole milk at 7°C for 7 days. Values are the means \pm standard deviations from three independent experiments. Different letters indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

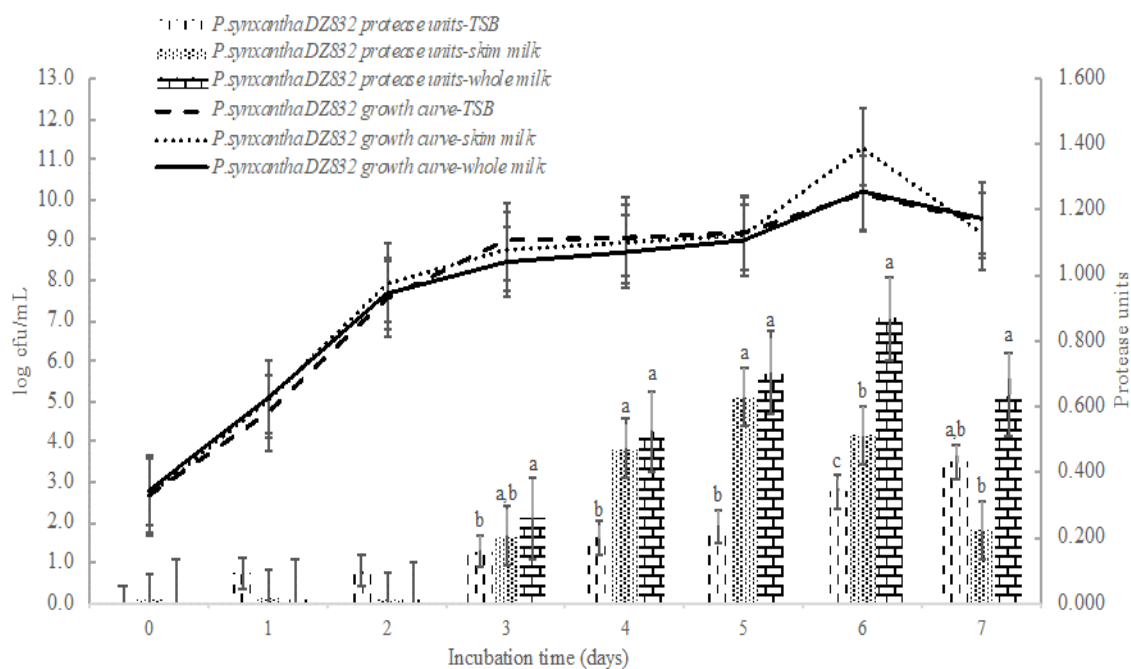


Figure S5.12 Growth curve and proteolytic activity of *P. synxantha* DZ832 in TSB, skim milk and whole milk at 7°C for 7 days. Values are the means \pm standard deviations from three independent experiments. Different letters indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

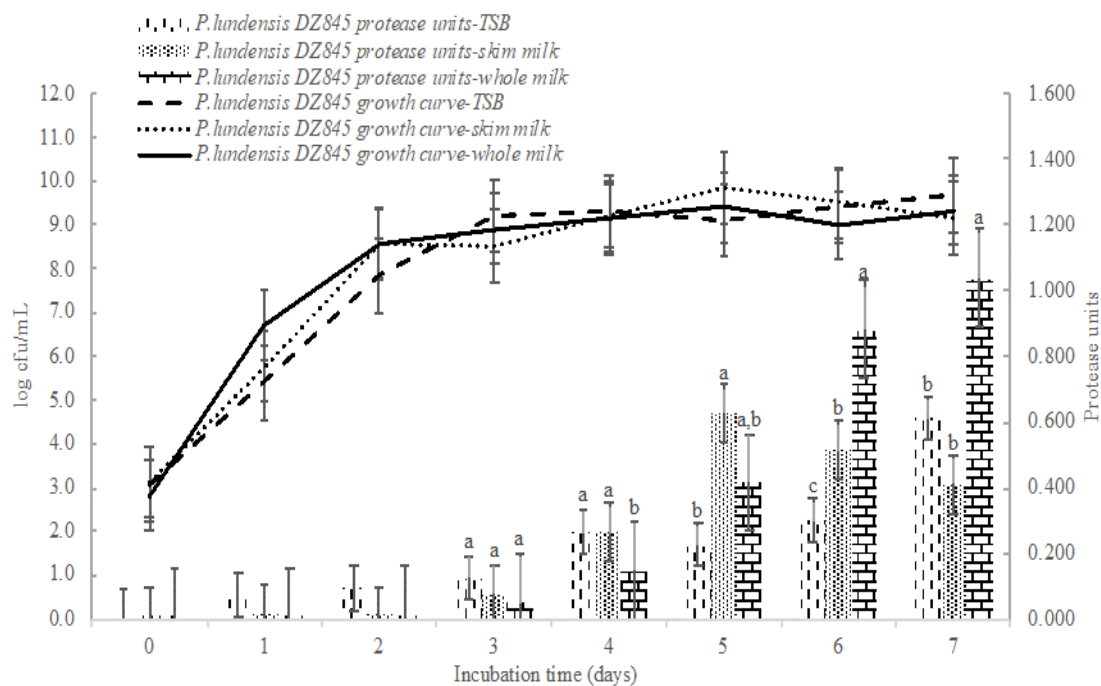


Figure S5.13 Growth curve and proteolytic activity of *P. lundensis* DZ845 in TSB, skim milk and whole milk at 7°C for 7 days. Values are the means \pm standard deviations from three independent experiments. Different letters indicate statistically significant differences, $P \leq 0.05$ (Tukey's test).

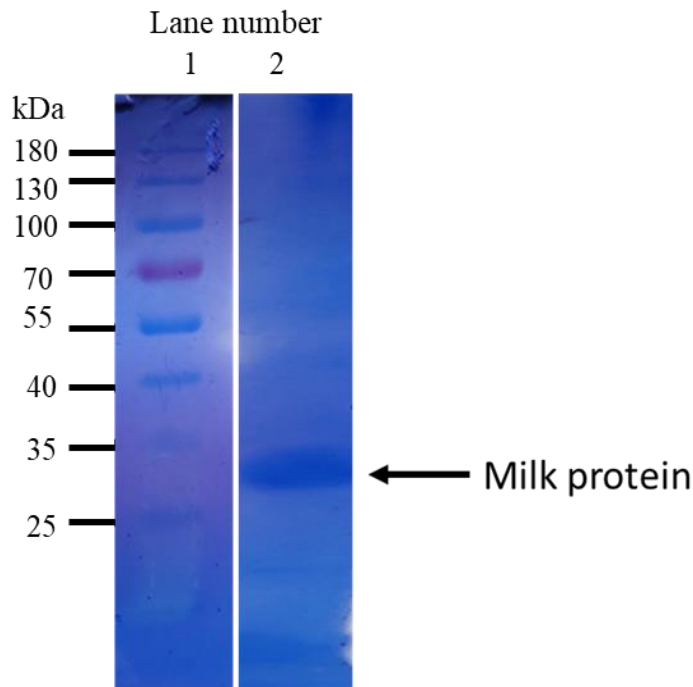


Figure S5.14 12% Casein zymography gel of cell-free culture supernatant of uninoculated milk sample. Lane 1, molecular mass standard; Lane 2, whole UHT milk. Arrow indicates protein band.

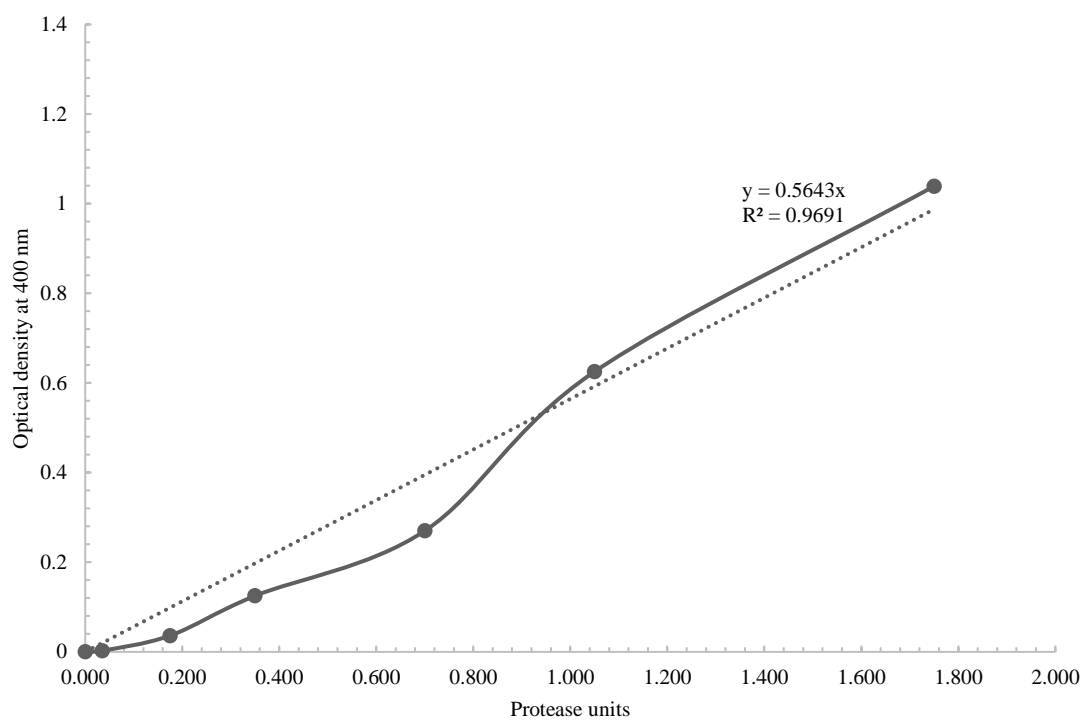


Figure S5.15 Standard curve of the proteolysis by *S. griseus* in TSB.

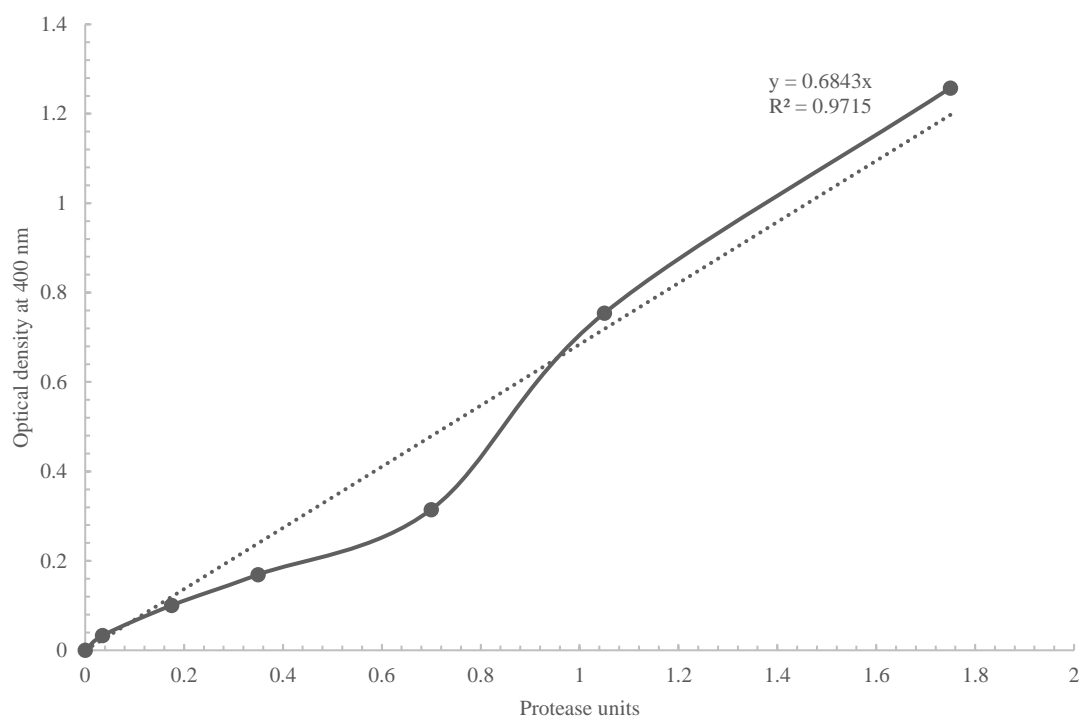


Figure S5.16 Standard curve of the proteolysis by *S. griseus* in skim milk.

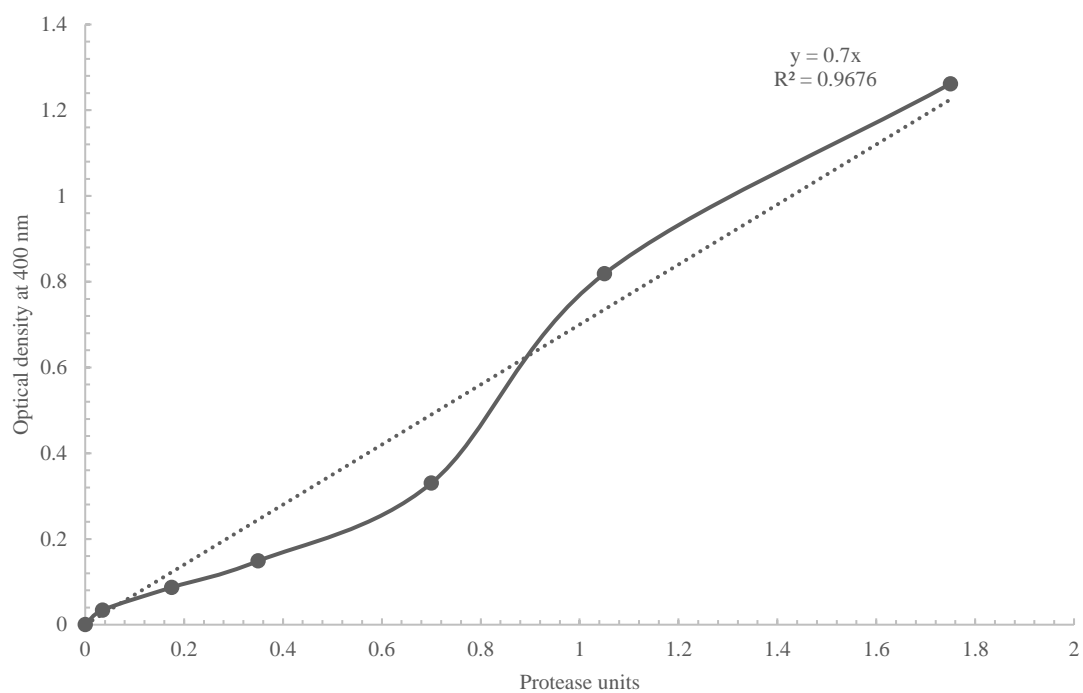


Figure S5.17 Standard curve of the proteolysis by *S. griseus* in whole milk.

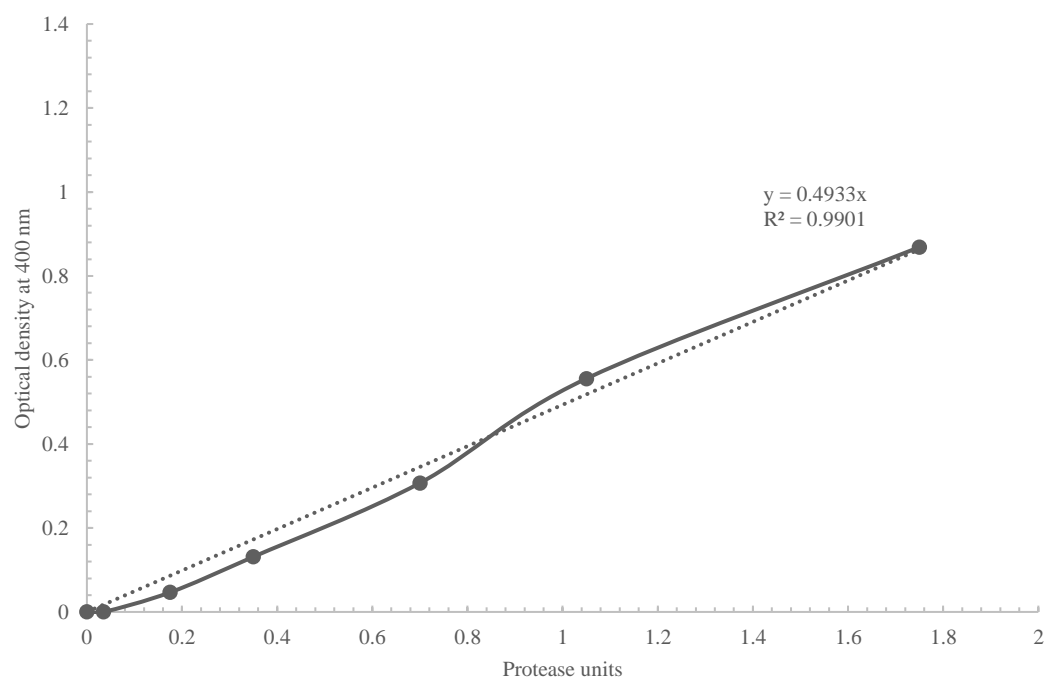


Figure S5.18 Standard curve of the proteolysis by *S. griseus* in 5% SC.

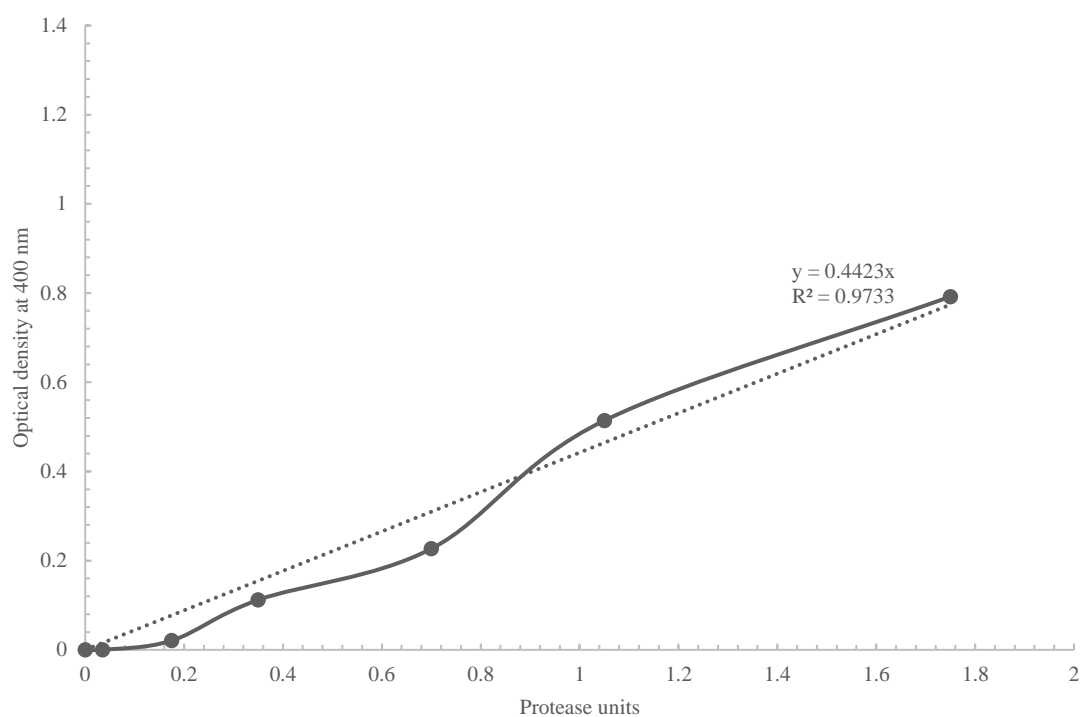


Figure S5.19 Standard curve of the proteolysis by *S. griseus* in 5% CC.

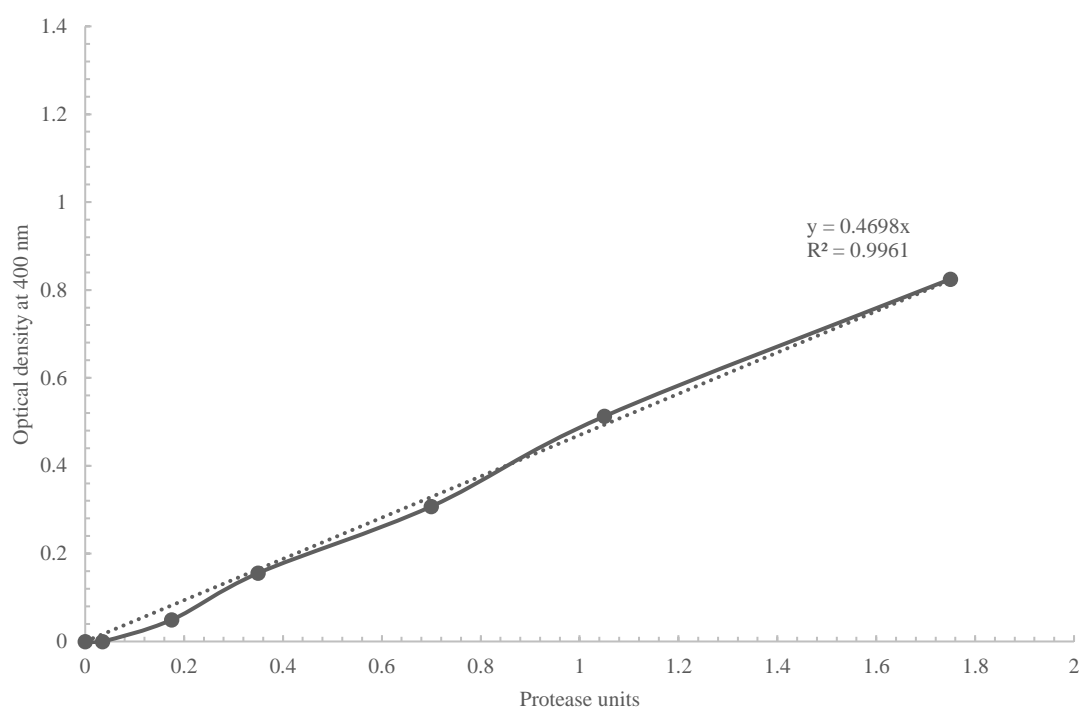


Figure S5.20 Standard curve of the proteolysis by *S. griseus* in 5% WPI.

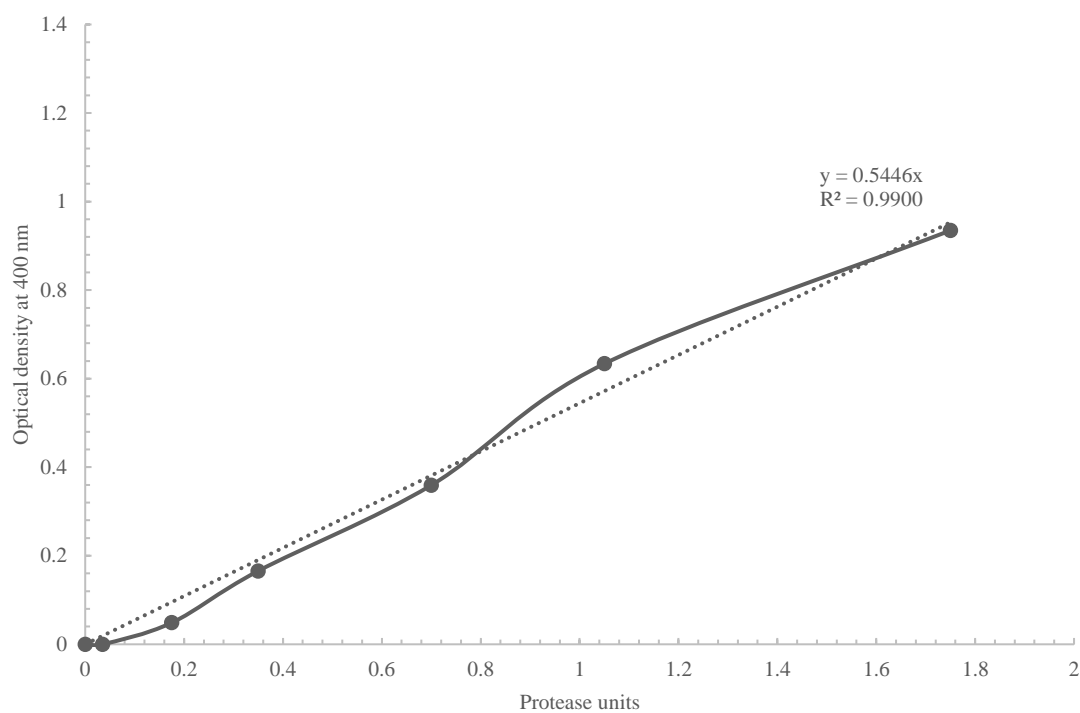


Figure S5.21 Standard curve of the proteolysis by *S. griseus* in 5% butter.

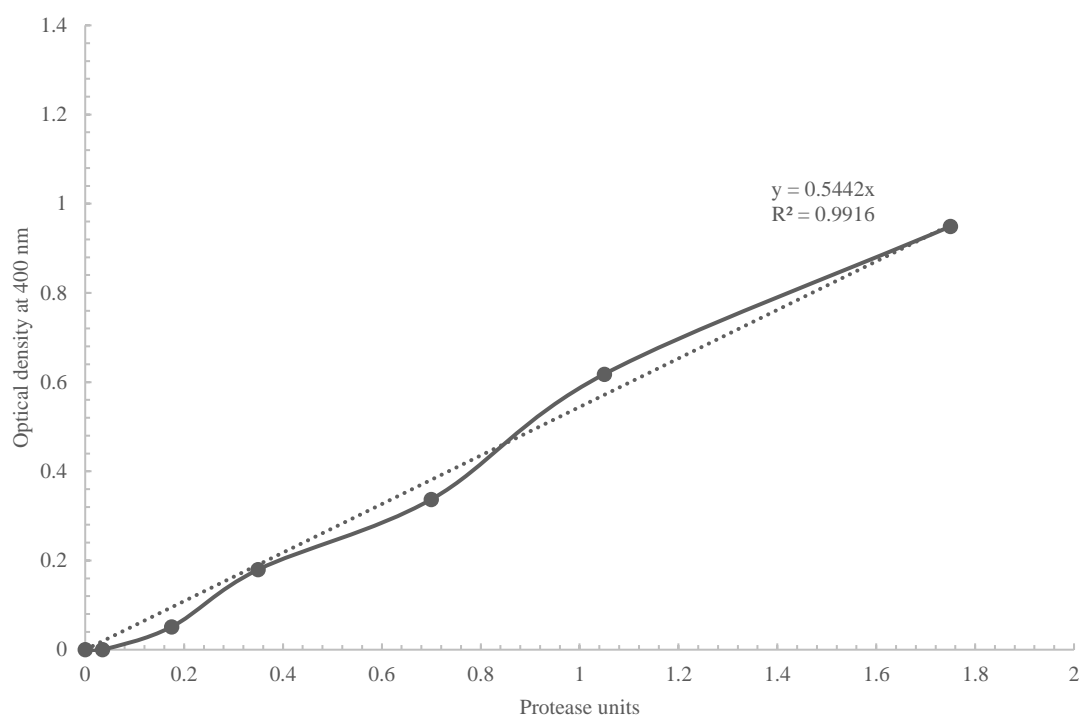


Figure S5.22 Standard curve of the proteolysis by *S. griseus* in 10% butter.

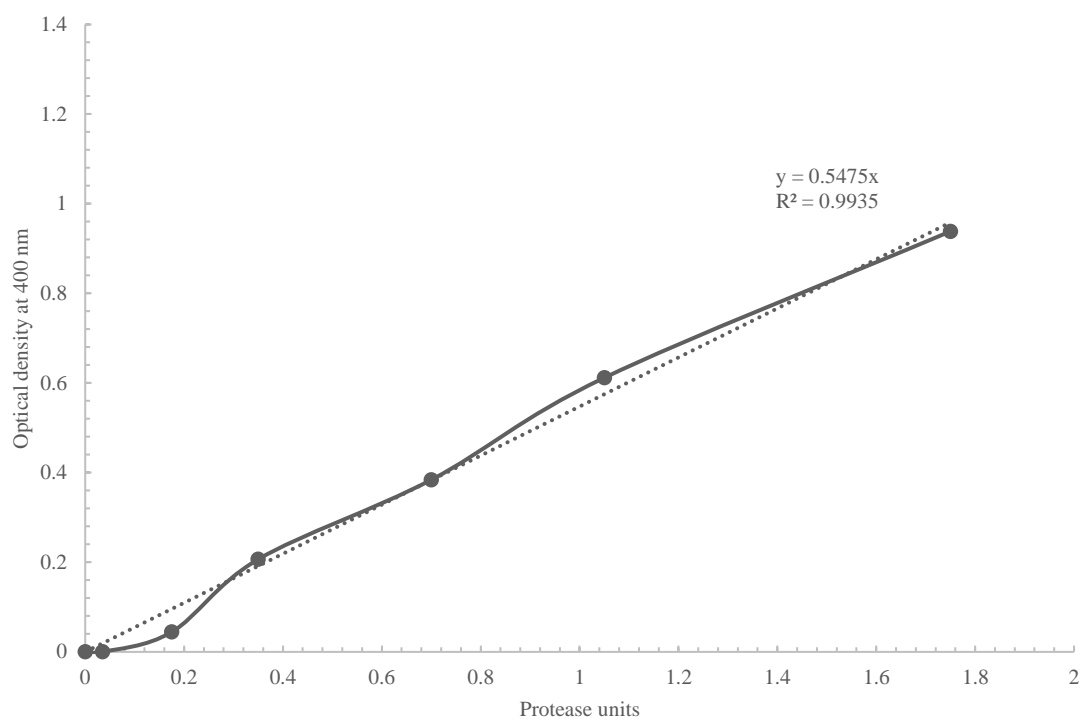


Figure S5.23 Standard curve of the proteolysis by *S. griseus* in 15% butter.

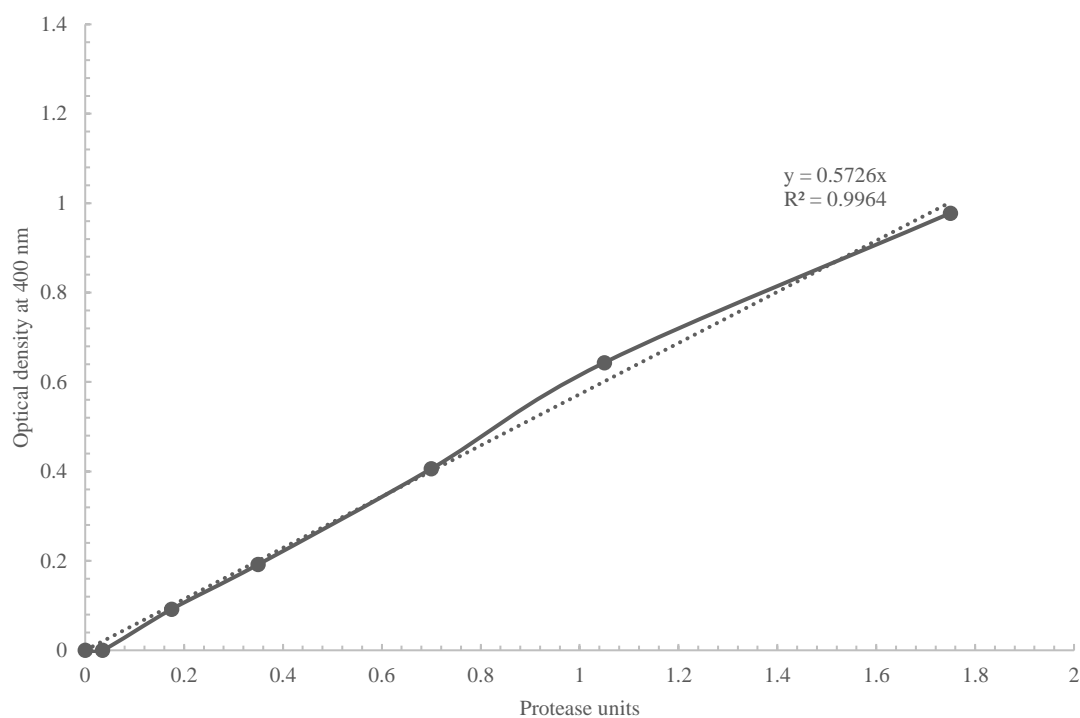


Figure S5.24 Standard curve of the proteolysis by *S. griseus* in 20% butter.

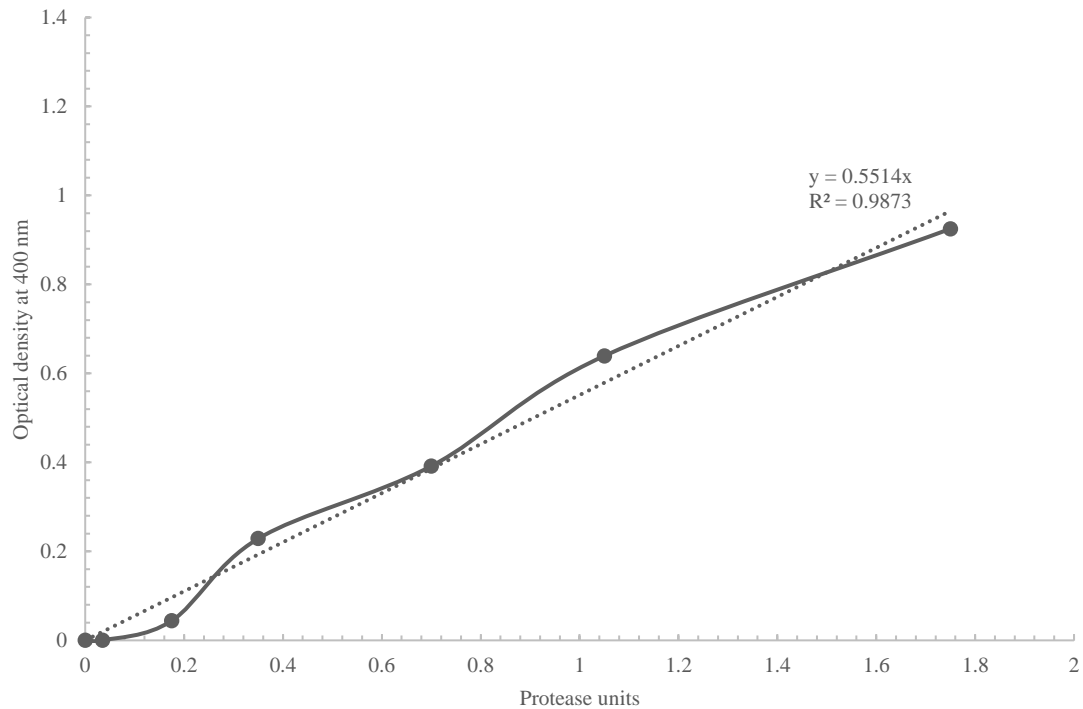


Figure S5.25 Standard curve of the proteolysis by *S. griseus* in 25% butter.

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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Name of candidate:	Dong Zhang	
Name/title of Primary Supervisor:	Steve Flint	
Name of Research Output and full reference:		
Zhang, D., Palmer, J., Teh, T. H., Calinisan, M. M. A., & Flint, S. (2020). "Milk fat influences proteolytic enzyme activity of dairy <i>Pseudomonas</i> species." <i>International Journal of Food Microbiology</i> , 320.		
In which Chapter is the Manuscript /Published work:	Chapter 5	
Please indicate:		
<ul style="list-style-type: none"> The percentage of the manuscript/Published Work that was contributed by the candidate: 	80%	
and		
<ul style="list-style-type: none"> Describe the contribution that the candidate has made to the Manuscript/Published Work: 		
The candidate carried out the laboratory work and data analysis (the proteolysis assay was carried out with the help of Singaporean intern, Miranda Maru Angeli Calinisan) and prepared the manuscript with input in guidance of direction and editorial help from the co-authors and supervisors.		
For manuscripts intended for publication please indicate target journal:		
International Journal of Food Microbiology (published manuscript)		
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Chapter 6. The relationship between numbers of proteolytic *Pseudomonas* bacteria in milk used to manufacture UHT milk and the effect on product quality

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Abstract

Gelation and bitterness in UHT milk during its shelf life are mainly determined by the numbers and types of bacterial proteases present. The stability of UHT milk is of increasing concern because of the possibility of prolonged shipping times and high storage temperatures for UHT milk products. In this study, UHT milk was made from milk intentionally contaminated with two single isolates of *Pseudomonas* (*P. rhodesiae* DZ351 and *P. synxantha* DZ832), and one cocktail of isolates of *Pseudomonas* (*P. fragi* DZ1, *P. koreensis* DZ138, *P. rhodesiae* DZ351, *P. fluorescens* DZ390, *P. synxantha* DZ832 and *P. lundensis* DZ845) inoculated at different levels (4, 5, 6 and 7 log cfu/mL). The milk was stored at 20, 30, and 55°C for up to 9 months and the physical and chemical properties were studied monthly. The milk inoculated with 7 log cfu/mL before UHT treatment formed a compact gel immediately after manufacture and κ -casein, α -casein, and β -casein were hydrolyzed. The milk inoculated with *P. rhodesiae* DZ351 had less hydrolysis of casein compared with the milk that was inoculated with *P. synxantha* DZ832 and the cocktail of isolates. The mixed isolates formed new peptides in gelled samples. After 4 months of storage, the milk containing 6 log cfu/mL before UHT started to gel and had more sediment than the milk that contained 4 and 5 log cfu/mL. After 7 months of storage, all the milk samples had gelled. HPLC analysis provided a more accurate indication of milk quality, well before the onset of gelation. This enables the prediction of sensory flaws that will precede any gelation. This information will help develop industry guidelines to select the milk for UHT processing and recommend storage conditions to delay the spoilage of UHT milk.

Keywords: UHT milk, *Pseudomonas*, gelation, bitterness

6.1 Introduction

A typical ultra-high temperature (UHT) process is 135-150°C for 1-10 s, producing a commercially sterile product with a 6-9 month shelf life without refrigeration (Sunds et al., 2018). UHT treatment eliminates pathogenic bacteria, minimises spoilage bacteria, and deactivates enzymes (Datta & Deeth, 2003). The worldwide demand for UHT milk is increasing due to the convenience of a shelf-stable ambient stored product (Malmgren et al., 2017). However, physicochemical and enzymatic changes during transportation and storage can cause the deterioration of the product, including undesirable flavours, sedimentation, and gelation of UHT products, all of which can be detected by consumers. Proteolysis of milk casein by the enzymes originating from psychrotrophic bacteria (psychrotrophs) are suspected as the major contributors to inferior UHT milk quality (Matéos et al., 2015). However, details on the numbers and types of psychrotrophs in the milk before UHT treatment influencing product quality are lacking.

Psychrotrophs grow in refrigerated milk after milking. Most of these bacteria secrete heat-stable extracellular proteases that remain active after UHT treatment, leading to a bitter taste and gelation of final UHT products (Hantsis-Zacharov & Halpern, 2007). Under good hygienic conditions, the population of psychrotrophs in fresh raw milk drawn from a healthy cow is less than 10^2 cfu/mL, accounting for 10% of the total microbiota (Decimo et al., 2014). However, raw milk is usually stored and transported under refrigeration for a few days before processing, which can favour the growth of psychrotrophs. Raw milk may be kept refrigerated for up to 5 days from milking until the start of processing at the dairy manufacturing plant (Vithanage et al., 2016). The levels of psychrotrophs will increase over this time but the numbers and types of psychrotrophs that need to be reached before there is a critical effect on UHT milk quality have not been accurately determined. According to Law et al. (1977), UHT milk made from raw milk with a level of psychrotrophs exceeding 5.0×10^6 and 8.0×10^6 cfu/mL, has a limited shelf-life of less than 63 and 12 days, respectively. Sørhaug & Stepaniak (1997) reported that a psychrotrophic count of 3.2×10^5 cfu/mL in raw milk causes UHT milk gelation after 20 weeks of storage, while counts between 7.9×10^6 cfu/mL and 1.6×10^7 cfu/mL will cause the same outcome within 10 weeks

and 2 weeks, respectively. Raw milk containing more than 1.0×10^6 cfu/mL of psychrotrophs is not suitable for UHT milk manufacture and this is supported by many researchers (Law et al., 1977; Oliveira et al., 2015; Sørhaug & Stepaniak, 1997). However, the data supporting this are fragmented, and there are questions around the effect of lower numbers of psychrotrophs on shelf life. There is no clear relationship between the numbers and types of bacteria and the actual predicted shelf life. The limit of psychrotrophic counts that will ensure the manufacture of UHT milk with a 9-month shelf life is not known. Moderate levels of those psychrotrophs in raw milk (e.g. 1.0×10^6 cfu/mL) may be still beneficial for cheese making owing to the development of desirable flavours and aromas; however, this milk may not be suitable for long-life UHT milk (Barbano, Ma, & Santos, 2006; Champagne et al., 1994). Thus, to understand psychrotrophic bacterial quality of raw milk before UHT processing and the effect on final UHT milk quality, some data are needed to establish a guideline for UHT milk manufacture.

The *Pseudomonas* genus is the predominant genus in raw milk (Zhang et al., 2019). Most species can produce one type of alkaline metalloprotease belonging to the serralyisin family with an apparent molecular size of about 47-50 kDa (Marchand et al., 2009). *P. fluorescens*, *P. fragi*, and *P. lundensis* are the most commonly reported species in raw milk (Mallet et al., 2012; Meng et al., 2017), but *P. rhodesiae*, *P. koreensis* and *P. synxantha* are also frequently implicated in the spoilage of dairy products although they have been less studied (Munsch-Alatossava & Alatossava, 2006). The proteases with different molecular mass produced by different *Pseudomonas* species have diverse ways of degrading milk proteins, resulting in diverse effects on shelf life and flavour of UHT milk (Stoeckel et al., 2016).

This study aimed to find out the maximum level of proteolytic psychrotrophic bacterial contamination in raw milk that can be used for long shelf life (9 months) UHT milk products and evaluate the stability of UHT milk contaminated with single and mixed isolates under different storage conditions.

6.2 Materials and methods

6.2.1 Isolates selection and protease heat stability

Six dairy *Pseudomonas* isolates (*P. fragi* DZ1, *P. koreensis* DZ138, *P. rhodesiae* DZ351, *P. fluorescens* DZ390, *P. synxantha* DZ832, and *P. lundensis* DZ845) were selected based on the frequency of isolation of proteolytic isolates from the psychrotrophic bacterial populations from a previous study (Chapter 3 and 4). The cells were revived from Cryopreserved beads (Thermo Fisher Scientific, USA) at -80°C and pre-incubated for 24 h at 20°C in tryptic soy broth (TSB; Becton Dickinson, Cockeysville, MD, USA) before use in the experiments. A mixed culture of all 6 isolates was reconstituted with equal parts of the six single cultures. All seven *Pseudomonas* cultures (approximately 1.0×10^3 cfu/mL) were inoculated into whole UHT milk and incubated for 7 days at 7°C (condition used for psychrotrophs testing) to provide the best opportunity to screen for protease production. Uninoculated UHT whole milk was used as controls. After 7 days, sodium azide (Thermo Fisher Scientific, USA) was added to the samples to a final concentration of 0.01% (w/v) to prevent further growth of bacteria, and milk samples were centrifuged (14, 100 x g, 4°C, 5 min). 100 µL of supernatant from each sample was heat-treated in an oil bath (141°C, 10 s) in sealed glass capillary tubes and then iced for 5 min following treatment. Then, the ends of the capillary tubes were removed with a pair of sterile scissors and the heat-treated supernatants collected. As described in Chapter 5, the azocasein assay was used to quantify the proteolytic activities of different cultures with and without heat treatment to observe the effect of heat treatment on the activity of the proteases. Tests were done in triplicate.

6.2.2 UHT milk preparation

Raw milk was aseptically collected from Massey University No. 4 Dairy Farm (Palmerston North, New Zealand), which consisted of 614 Friesian-Jersey cross-breed cows. The milk samples were then transferred to the Massey Pilot Plant under refrigeration and processed within 60 min to avoid bacterial growth that occurs during transportation and storage and thus limit bacterial proteases that may interfere with the results. After preheating to 50°C for 20 s, the

milk was homogenized at 200/50 bar. The milk was immediately UHT treated at 140°C for 10 s using an indirect (plate heat exchanger) pilot-scale heating system, followed by vacuum cooling to 20°C. The UHT milk was aseptically packed in 4 sterile storage containers (50 L).

6.2.3 Preparation of inoculum

Two single isolates showing heat-stable proteolytic activity, *P. rhodesiae* DZ351 and *P. synxantha* DZ832, and one cocktail of all six isolates were used in this study. To prepare the inoculum for testing, the cells were revived from Cryopreserved beads at -80°C and pre-incubated for 24 h at 20°C in TSB before commencing the experiments. The final cell count was $\sim 10^8$ cfu/mL for all the three isolates. For each isolate, an appropriate dilution of the culture was aseptically inoculated into UHT milk to provide an initial concentration of 10^3 cfu/mL. More specifically, 90 μ L of the overnight culture was inoculated in 100 mL of UHT milk. A mixed culture sample was prepared by mixing all six strains (15 μ L of each overnight culture in 100 mL of UHT milk). UHT milk without inoculum was used as a negative control. All milk samples were kept in 16 sterile sealed containers at 7°C for up to 4 days. After each day of storage, 4 containers of inoculated milk were UHT treated again using the same conditions as described previously and aseptically packed in 100 mL sterile containers. All the products were kept at 20, 30, and 55°C for future analysis.

6.2.4 Microbial analysis

Analysis of the total microbial plate count of the milk following the first UHT treatment was done each day following inoculation during refrigerated storage before the 2nd UHT treatment. The drop plate method (the minimum numbers that can be detected by the drop plate method is 10^2 cfu/mL) using tryptic soy agar (TSA; Becton Dickinson, Cockeysville, MD, USA) was used (Herigstad, Hamilton, & Heersink, 2001), and samples were tested in triplicate. Agar plates were incubated at 30°C for 24 to 48 h before counting. The sterility of the uninoculated UHT milk samples was checked initially and then monthly after storage at 20, 30 and 55°C by streaking 0.1 mL samples on TSA, and incubating the plates under either anaerobic or aerobic conditions

at 30 °C for 72 h to ensure sterility of the milk before inoculation and during storage. The psychrotrophic and mesophilic bacterial counts of the fresh raw milk were analysed on TSA plates at 7°C for 7 days and 30°C for 48 h, respectively.

6.2.5 Gelation, pH, and sedimentation

During storage, gelation was visually assessed monthly. Only samples that had completely gelled were referred to as gelled.

The milk samples were equilibrated at room temperature for 1 h before pH measurement using a digital pH meter (Orion Research Inc., Boston, MA, USA). Before use, the pH meter was standardized with standard buffer solutions of pH 4, 7, and 10. All the samples were analysed monthly.

A sedimentation test followed the method described by Boumpa et al. (2008) with some modifications. The amount of sediment was determined by transferring 10 g of milk samples to a 15 mL centrifuge tube. The tube was then centrifuged for 10 min at 14, 100 x g, after which the supernatant was decanted and the amount of the settled substance was determined by weighing the tube containing the sediment. The wet sediment was transferred from the tube into an aluminium dish with the help of 2 mL distilled water, and the dry mass of sediment was determined after drying for 3 h at 115°C. The results (in g/100g) were based on analyses of triplicate samples. All the samples were analysed monthly.

6.2.6 Analysis of protein changes by HPLC

The changes in milk casein proteins were analysed monthly by reversed-phase high-performance liquid chromatography (HPLC; Shimadzu, Tokyo, Japan) according to Bobe et al. (1998) and Li et al. (2019) with some modifications. 500 µL of milk samples were added to a solution containing 0.1M BisTris buffer (pH 6.8; Sigma, St. Louis. MO, USA), 6M Guanidine hydrochloride (GdnHCl; Sigma, St. Louis. MO, USA), 5.37 mM sodium citrate (Thermo Fisher, Scoresby VIC, Australia), and 19.5 mM Dithiothreitol (DTT, pH 7; Sigma, St. Louis. MO, USA) in a 1:1 ratio (v:v) at room temperature. The mixture was shaken for 30 s and incubated for 1 h at

room temperature, and centrifuged for 10 min at 14, 100 x g. The fat layer was removed with a pipette tip. The remaining supernatant was diluted 1:3 (v:v) with a solution containing 4.5 M GdnHCl and solvent A, which consisted of acetonitrile (Sigma, Shanghai, China), water and trifluoroacetic acid (Sigma, St. Louis, MO, USA) in a ratio 100:900:1 (v:v:v; pH 2). The samples were then filtered through a 0.2 µm RC filter (Sartorius, Gottingen, Germany). Chromatographic conditions and protein identification were carried out on a reversed-phase C18 column (Aeris Widepore 3.6 µm XB-C18 RP; Phenomenex, Torrance, CA). For identification of κ-casein (κ-CN), α-casein (α-CN) and β-casein (β-CN) peaks, purified bovine milk proteins (purity for κ-CN ≥ 70%, α-CN ≥ 70% and β-CN ≥ 98%; Sigma, St. Louis, MO, USA) were prepared (Figs. S6.7-6.9). The degree of proteolysis of UHT milk was defined as the peak area ratio of κ-CN, α-CN, and β-CN in protein compared with the peak area of the control UHT milk (without inoculation after 1st UHT process). The effect of storage time, temperature and isolate variations on milk quality was compared as the difference between the total proteolysis of κ-CN, α-CN, and β-CN. All the samples were tested once, as all the samples were dispatched from the same container and a preliminary test on fresh raw milk showed the whole system produced consistent results (Fig S6.10).

6.2.7 Statistical analysis

Excel (Microsoft, USA) was used for the analysis of variance using Tukey's with a critical probability of $p \leq 0.05$.

6.3 Results

6.3.1 Heat stability test

This study aimed to understand if these bacterial proteases remained active after UHT process. The heat stability to UHT treatment of the proteases produced by the 6 single and 1 mixed *Pseudomonas* isolates was quantified by proteolytic activity in the azocasein assay. Optical density was baselined using values from controls and converted to protease units based on standard curves established in a previous study (Chapter 5). The results are provided in the

supplementary material (Table S6.5). The heat stability of different *Pseudomonas* proteases was defined as the value ratio of “with heat treatment” to “without heat treatment” (Table 6.1). Proteases produced by *P. koreensis* DZ138, *P. rhodesiae* DZ351, *P. synxantha* DZ832 and mixed isolates remained 100% active after UHT treatment whereas *P. fluorescens* DZ390, *P. fragi* DZ1 and *P. lundensis* DZ845 retained 94.81%, 80.85% and 55.32% of the initial activity, respectively. *P. rhodesiae* and *P. synxantha* were the two common species isolated from raw milk (Chapter 4) but have rarely been studied. Thus, isolates *P. rhodesiae* DZ351 and *P. synxantha* DZ832 showing heat-stable proteolytic activity were selected for a UHT shelf life study.

Table 6.1 Heat stability of the protease produced by the 6 single and 1 mixed isolate.

Heat stability	Isolates						
	<i>P. fragi</i>	<i>P. koreensis</i>	<i>P. rhodesiae</i>	<i>P. fluorescens</i>	<i>P. synxantha</i>	<i>P. lundensis</i>	Mixed culture
(141°C, 10 s)	DZ1	DZ138	DZ351	DZ390	DZ832	DZ845	of 6 isolates
	80.58%	100%	100%	94.81%	100%	55.32%	100%

6.3.2 Microbial analysis

Table 6.2 shows the growth of the three inocula (1) *P. rhodesiae* DZ351, (2) *P. synxantha* DZ832, and (3) a mixture of all six isolates at 7°C for 4 days in fresh UHT milk. The growth of the three inocula was similar. The cell counts from an initial inoculum of 3-4 log cfu/mL increased about 1 log cfu/mL/day at 7°C, reaching 7-8 log cfu/mL after 4-days incubation. The mesophilic bacterial counts of fresh raw milk from the farm was 3.87 ± 0.06 log cfu/mL. There was no detectable psychrotrophic bacteria count in the fresh raw milk, indicating the microbial quality of the milk samples was very high. This milk was taken immediately after milking (analysis within 4 h). The UHT milk was sterile, confirmed by no evidence of bacterial growth during the shelf life.

Table 6.2 The population of *Pseudomonas* spp. in raw milk before the 2nd UHT processing.

Microbial analysis (log cfu/mL)	0-day storage	1-day storage	2-day storage	3-day storage	4-day storage
UHT milk inoculated with <i>P. rhodesiae</i> DZ 351	3.82±0.02	4.56±0.14	5.12±0.03	6.71±0.08	7.98±0.21
UHT milk inoculated with <i>P. synxantha</i> DZ 832	3.85±0.04	4.55±0.07	5.87±0.11	6.64±0.02	7.32±0.07
UHT milk inoculated with mixed isolates	3.83±0.07	4.28±0.14	5.32±0.32	6.32±0.32	7.45±0.13

6.3.3 Gelation, pH, and sedimentation

The time taken for the gelation of the UHT milk samples during storage is given in Table 6.3. This was observed visually as a coagulation of the milk. The UHT milk made with raw milk containing 7 log cfu/mL of *Pseudomonas* spp. all formed gelation at less than 1-month storage at 20 and 30°C. UHT milk contaminated with *P. synxantha* DZ832 and mixed isolates showed stronger signs (more gelation) of deterioration than the samples contaminated with *P. rhodesiae* DZ351. The samples stored at 30°C showed faster gelation than the samples at 20°C. An example of gelled samples is shown in Fig. 6.1.

Table 6.3 Relationship between inoculum type, inoculum number, storage temperature, and time (up to 9 months) for visible gelation UHT milk.

Temperature (°C)	<i>P. rhodesiae</i> DZ351			<i>P. synxantha</i> DZ832			Mixed isolates		
	4 log	5 log	6 log	4 log	5 log	6 log	4 log	5 log	6 log
	cfu/mL	cfu/mL	cfu/mL	cfu/mL	cfu/mL	cfu/mL	cfu/mL	cfu/mL	cfu/mL
20	7	6	5	6	5	5	6	6	5
30	5	4	4	4	4	3	4	4	3

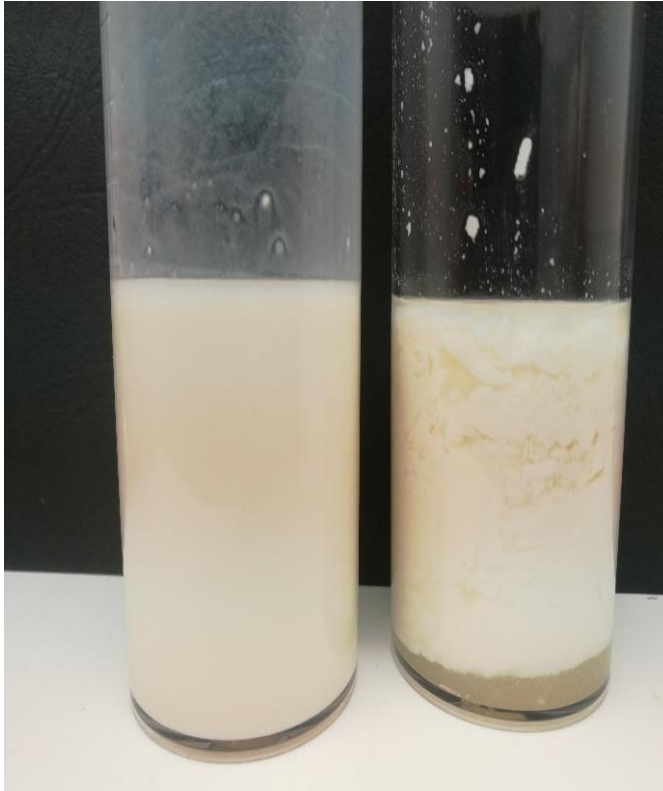


Figure 6.1 An example of the control sample (left) and gelled milk sample (right).

The pH during the storage of all milk samples decreased over time. The initial pH of all milk samples was above 6.6 and reduced to 6.0. The gelled samples had a significantly lower pH than the non-gelled samples ($p < 0.05$). The details are provided in the supplemental materials (Table S6.7).

The onset of sedimentation is the early stage of gelation. The sediment levels in UHT milk that had not gelled 1 month after manufacture was about 1 g/100g, which is equal to the control milk, whereas for the gelled samples this was about 10 g/100g. Small increases in sediment were observed in all milk samples before gelling. The higher the levels of contamination in the milk before the 2nd UHT treatment and the higher the storage temperature of UHT milk, the faster and stronger the sediment formation. The sediment levels in gelled samples were significantly higher than in the other milk samples ($p < 0.05$). The data are shown in the supplemental documents (Table S6.8).

After 1-month storage at 55°C, the UHT milk became brownish. The samples were submitted for HPLC analysis for casein protein changes. There were no casein proteins detected

by HPLC, but the milk had not gelled. One of the possible reasons is that casein proteins were completely degraded after 1-month storage at 55°C. Thus, it was not tested further. The pH was about 5.5, which is significantly lower than other samples ($p < 0.05$).

6.3.4 Analysis of protein changes by HPLC

To describe how the level of microbial contamination in raw milk and storage temperature affected the shelf life of UHT milk, the ratios of κ -CN, α -CN, and β -CN between UHT milk samples made with raw milk contaminated with *P. rhodesiae* DZ351 and uninoculated milk is shown in Figs. 6.2-6.4 and the hydrolysis of the caseins by *P. synxantha* DZ832 and mixed isolates are provided in the supplemental materials (Table S6.6). The level of κ -CN was largely reduced in all inoculated milk samples after 2-month storage, indicating that κ -CN is very vulnerable to *Pseudomonas* spp. proteases. Over time, the levels of α -CN and β -CN also decreased. In comparison with κ -CN and α -CN, the degradation of β -CN was relatively slow. Fig 6.5 is an example of the protein profile of gelled and non-gelled milk samples. Fig 6.6. shows the mixed inoculum hydrolyzed milk proteins differently compared with the single cultures, forming some new peptides.

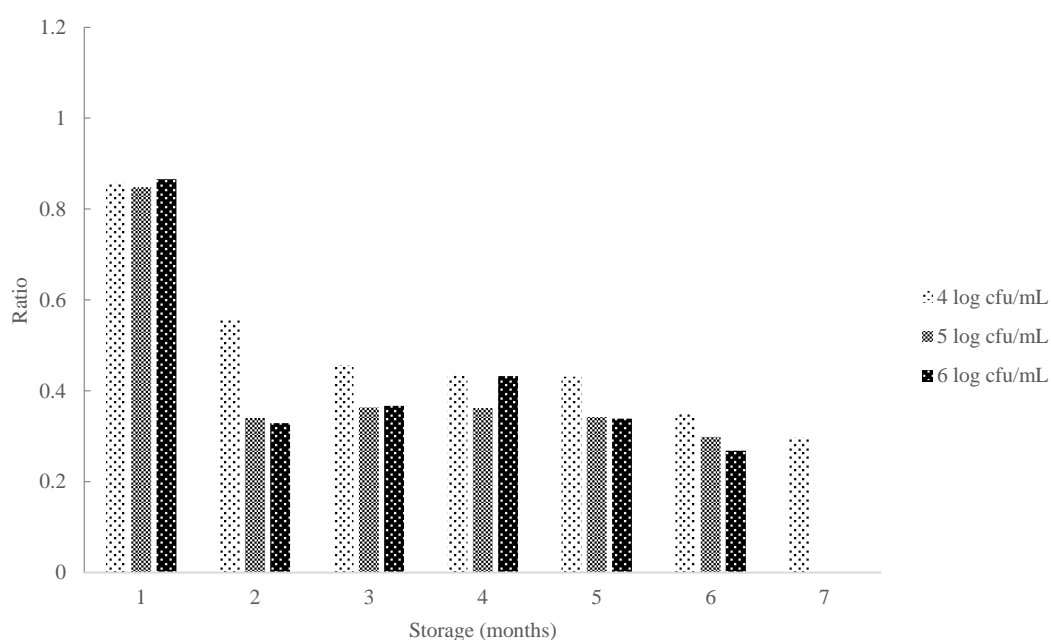


Figure 6.2 The ratio of κ -CN compared with the start of the trial (control) for *P. rhodesiae* DZ351 inoculated UHT milk stored at 20°C for up to 9 months.

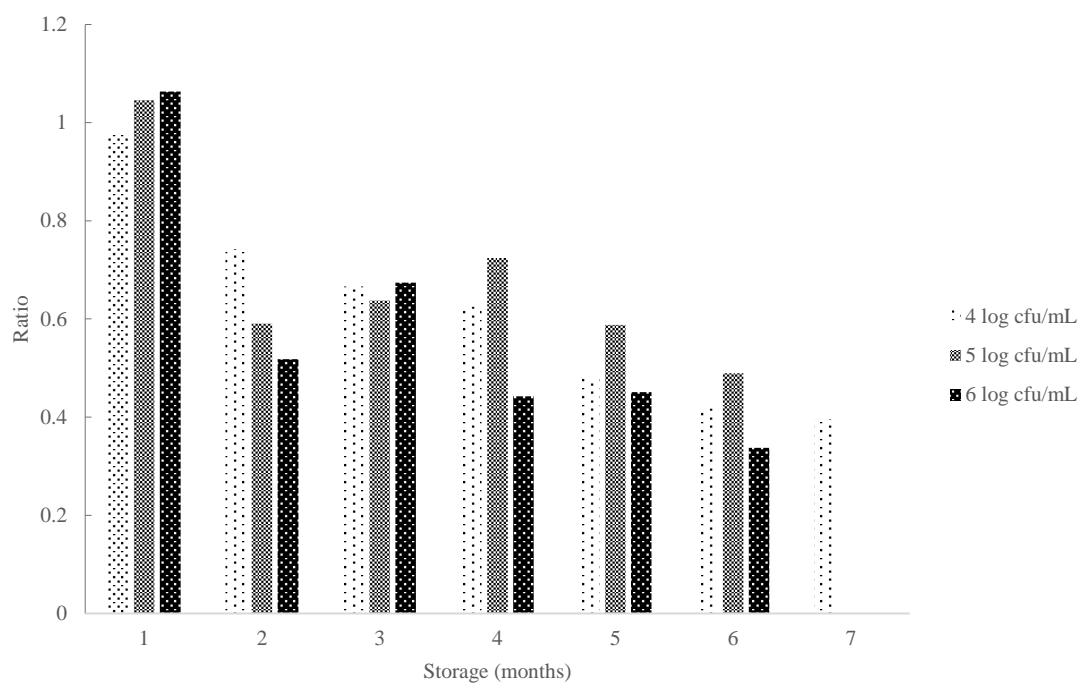


Figure 6.3 The ratio of α -CN compared with the start of the trial (control) for *P. rhodesiae* DZ351 inoculated UHT milk stored at 20°C for up to 9 months.

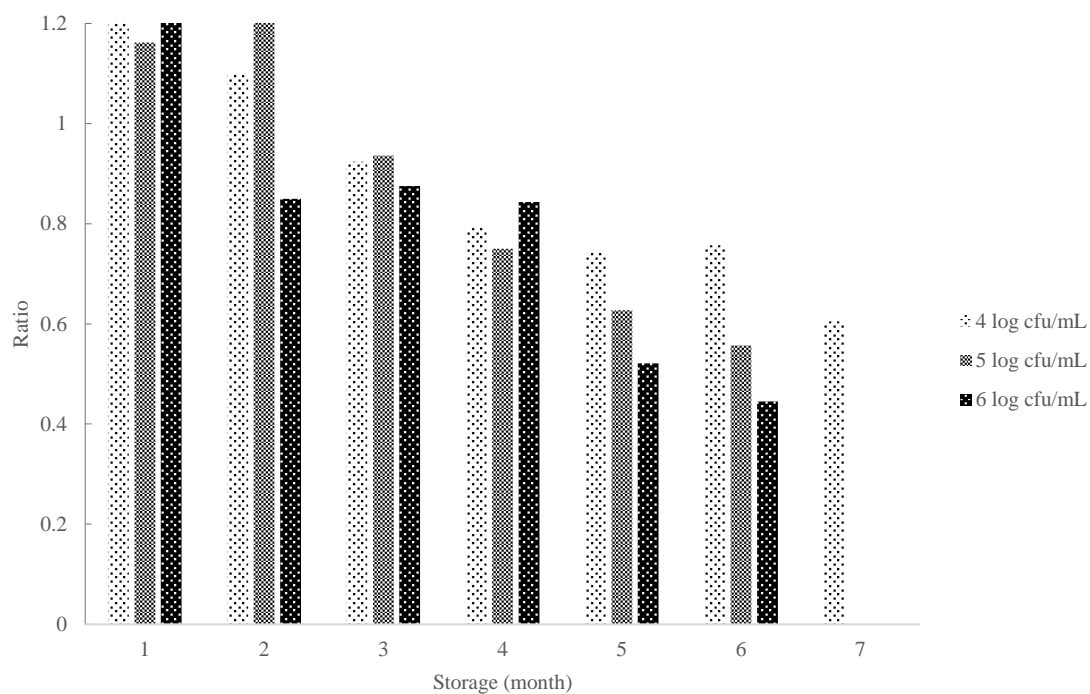


Figure 6.4 The ratio of β -CN compared with the start of the trial (control) for *P. rhodesiae* DZ351 inoculated UHT milk stored at 20°C for up to 9 months.

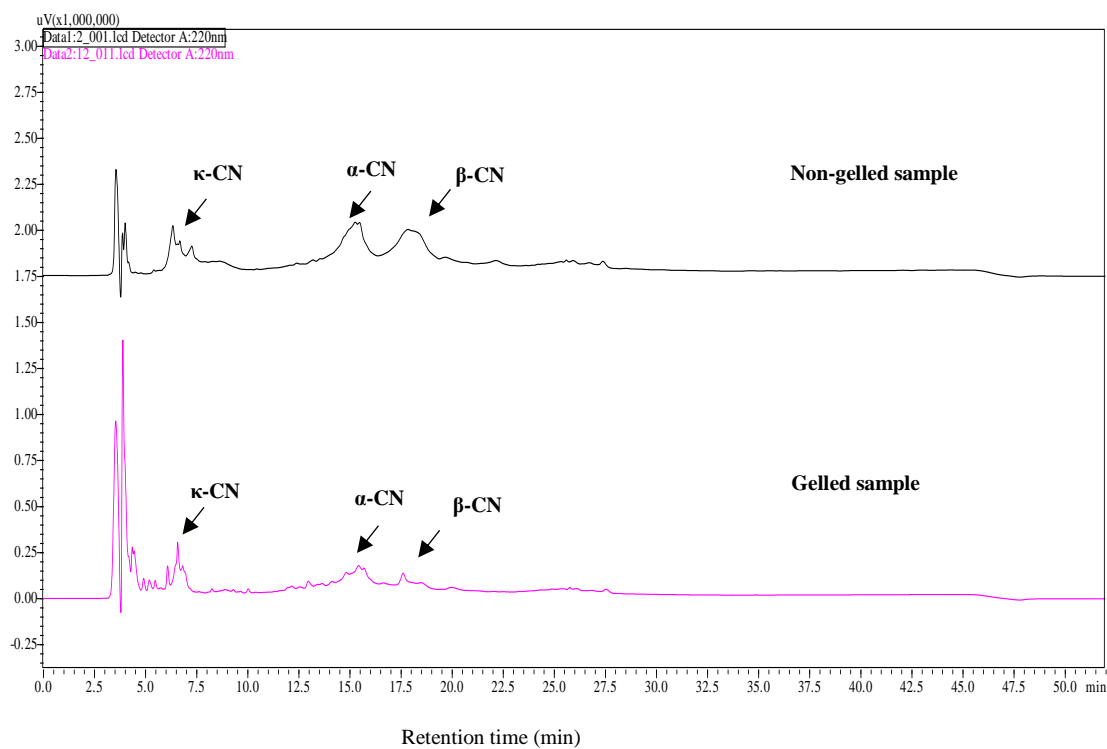


Figure 6.5 Protein and peptide composition of milk samples analyzed by HPLC before and after gelation. UHT milk contaminated with 4 log cfu/mL of *P. rhodesiae* DZ351 after 1-month storage at 20°C (black line) and UHT milk contaminated with 7 log cfu/mL of *P. rhodesiae* DZ351 after 1-month storage at 20°C.

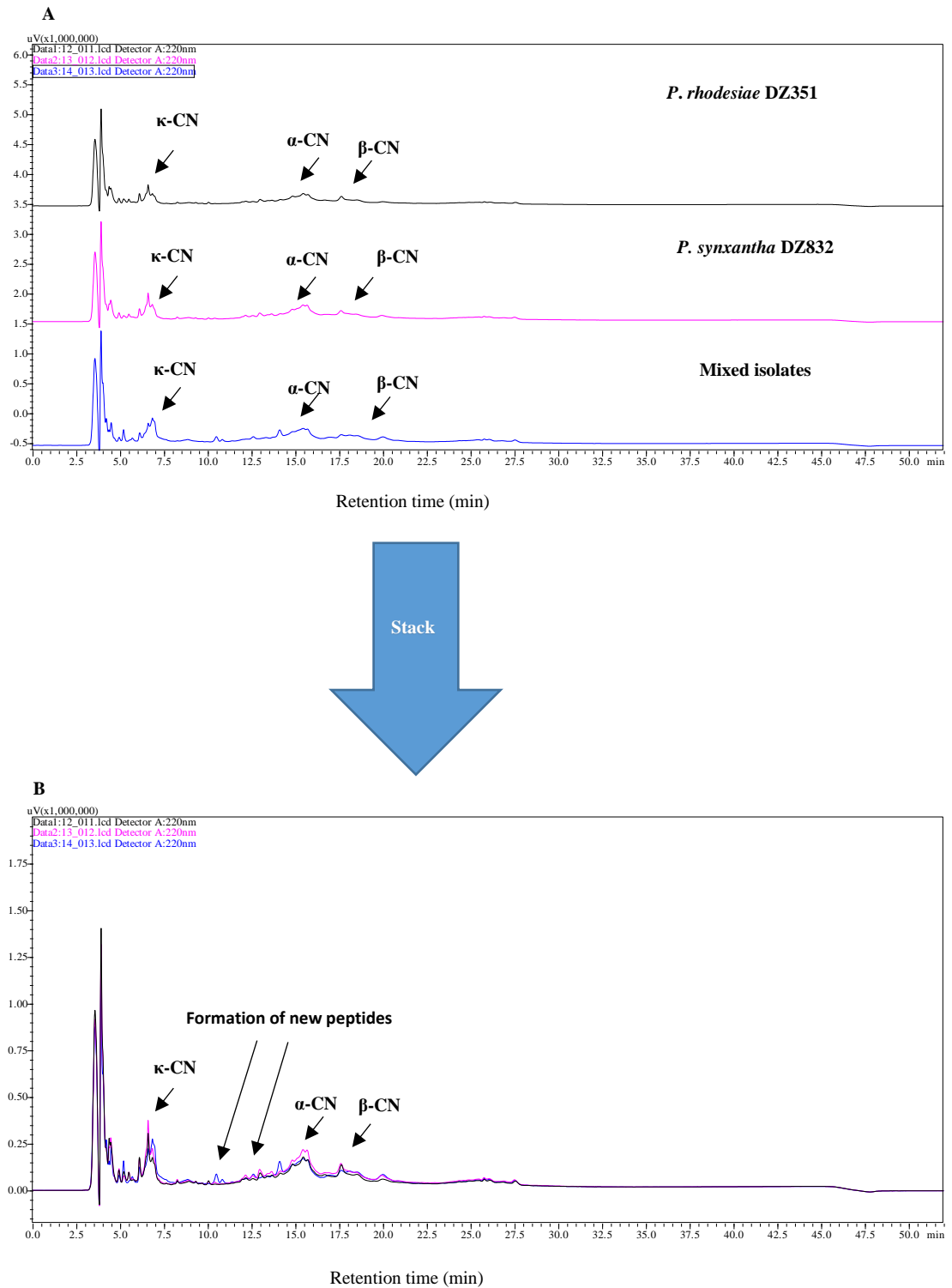


Figure 6.6 A: Protein and peptide composition of gelled milk hydrolyzed by different isolates analyzed by HPLC. UHT milk contaminated with 7 log cfu/mL of *P. rhodesiae* DZ351 (black line), *P. synxantha* DZ832 (pink line), and mixed isolates (blue line) after 1-month storage at 20°C, respectively. B: Combined version.

6.4 Discussion

According to Champagne et al. (1994), raw milk containing more than 10^6 cfu/mL of psychrotrophs is not recommended for UHT milk production. However, the spoilage threshold of psychrotrophic bacterial contamination in raw milk that can be used for UHT manufacture is still

unknown. The goal of this study is to predict the correlation between the microbial quality of raw milk and the onset of spoilage of UHT milk.

Table 6.4 A summary of proteolytic psychrotrophic bacteria numbers and the shelf life (up to 9 months) of UHT milk based on proteolytic activity at different temperatures.

Raw milk quality (numbers)	<i>P. rhodesiae</i> DZ351		<i>P. synxantha</i> DZ832		Mixed isolates	
	20°C	30°C	20°C	30°C	20°C	30°C
⁴ ~10 ⁴ cfu/mL	7	5	6	4	6	4
⁵ ~10 ⁵ cfu/mL	6	4	5	4	5	4
⁶ ~10 ⁶ cfu/mL	5	4	6	3	6	3
⁷ ~10 ⁷ cfu/mL	1	1	1	1	1	1

A summary of the level of psychrotrophic bacterial contamination and shelf life of UHT milk based on protease activity at different temperatures is shown in Table. 6.4. The initial populations and types of psychrotrophs in raw milk are strongly related to the spoilage of UHT milk throughout the shelf life. In this study, raw milk exposed to different levels and types of *Pseudomonas* spp. were used to make UHT milk. *Pseudomonas* spp. is one of the most well-known and predominant psychrotrophs present in raw milk. Spoilage of UHT milk, manifest by sediment formation, gelation, and bitterness, caused by heat-stable proteases of *Pseudomonas* spp. is described and reported by many researchers (Matéos et al., 2015; Stoeckel et al., 2016). The mixed inoculum (a mixture of 6 isolates) was used to mimic a more complex microbial composition in raw milk (Vithanage et al., 2016). All the *Pseudomonas* isolates used in this trial showed heat-stable proteolytic activity after UHT treatment and were able to grow in raw milk at 7°C with approximately 1 log cfu/mL increase per day. This suggests that the refrigerated storage of raw milk favours the growth of protease-producing bacteria and the production of their heat-stable protease under refrigeration can potentially affect the quality of UHT milk during its shelf life. High bacterial counts result in more rapid proteolytic activity. The results showed UHT milk manufactured using raw milk containing more than 10⁶ cfu/mL of psychrotrophs has a shorter shelf life, which agrees with other researchers (Lin et al., 2016; Paludetti et al., 2018). This study also observed that the onset of spoilage of UHT milk occurred when the bacterial population was low (< 6 log cfu/mL). Deterioration of UHT milk made with low numbers of *Pseudomonas* spp.

occurred within 7 months, which is shorter than the shelf life expected for good UHT milk (9 months) (Sunds et al., 2018). Gelation caused by the bacterial proteases is usually attributed to the hydrolysis of κ -CN, forming “para- κ -casein-like” micelles and a “glyco-macro-peptide” (Anema, 2019; Zhang, Bijl, & Hettinga, 2018). During storage, the κ -CN started to deplete after 1-month of storage, which is earlier than other caseins. It is the hydrolysis of the κ -CN that manifests itself into very unstable casein micelles, which may give the impression of preferential proteolysis of κ -CN (Datta & Deeth, 2001). Gelation is the final step during the spoilage of UHT milk. Sediment formation, pH decline, and hydrolysis of milk protein occurred before gelation. The sediment occurred as a precipitate that in some cases, turned into a gel. Sedimentation increased when the pH was low. This has also been reported by Gaur et al. (2018). This pH decline may lead to an unstable colloid of the casein micelles, resulting in slow aggregation of the κ -casein-depleted micelles, thereby causing sediment formation (Anema, 2019). The drop in pH of the products was observed during the shelf life. Anema et al (2017) also reported that the pH of reconstituted UHT skim milk continued to decline during 10 months storage due to proteolysis. The pH was 0.2 pH units lower than the initial pH but not significant. The reason for the decline of pH will be investigated in future work. κ -CN hydrolysis is associated with physical changes in UHT milk while other caseins, in particular α -CN and β -CN, which are associated with undesirable bitter flavours in UHT milk, have also been reported to be hydrolysed, but not always at the same rate as κ -CN (Anema, 2019; Baglinière et al., 2013; Swamylingappa et al., 2003; Zhang & Lv, 2014). The preferential proteolysis of κ -CN in this study agrees with many other researchers (Adams, Barach, & Speck, 1976; Datta & Deeth, 2001; Zhang, Bijl, & Hettinga, 2018). Zhang, Bijl, & Hettinga. (2018) state that this is probably because the main protease produced by *Pseudomonas* species (metalloproteases AprX) can easily access and breakdown κ -CN.

In this study, the *Pseudomonas* spp. showed variations in the proteolysis of UHT milk. *P. synxantha* DZ832 and the mixed isolates showed a stronger proteolysis of UHT milk than *P. rhodesiae* DZ351, which is also in agreement with the proteolytic activity shown in Table S6.5. Most *Pseudomonas* can produce only one type of protease, a neutral zinc metalloproteinase with

molecular weights ranging from 47 to 50 kDa (Marchand et al., 2009). Stoeckel et al. (2016) reported that the proteases produced by different *Pseudomonas* species have different effects on protein destabilization and flavour defects of UHT milk. Also, the production of proteases by dairy *Pseudomonas* varies from species to species. *P. synxantha* DZ832 might be a strong producer while *P. rhodesiae* DZ351 is not. For example, *P. chlororaphis* CIP 103295 was reported to have higher protease production compared with *P. fluorescens* CIP 69.13 and *P. chlororaphis* CIP 75.23 when incubated at 30°C (Nicodème et al., 2005). Although this did not refer to protease production at 7°C, it is suggested that the protease production of different *Pseudomonas* spp. might vary.

In New Zealand, UHT milk is exported to some equatorial countries (e.g. Singapore) by ship without a cold chain. From our discussion with some dairy companies, the temperature can reach to 55°C when the products are distributed in equatorial regions. Therefore, different storage conditions were studied to give the dairy industry some fundamental information to keep the products in good condition before reaching the market. The storage of UHT milk at higher temperatures, especially at 30°C, appears to increase the propensity of the milk to spoilage. This may be because the bacterial proteolytic activity is higher in 30°C than 20°C. A similar study was reported by Nicodème et al. (2005). This demonstrates that proteolysis, if present, can be accelerated by increasing the storage temperature. The brownish colouration of UHT milk at 55°C is thought to be caused by polymerization of casein and whey proteins by Maillard reactions (Renner, 1988).

6.5 Conclusions

UHT processing can kill all the vegetative cells of *Pseudomonas*, but the protease produced by these cells during the cold chain may remain active in UHT milk, resulting in the spoilage of final products. The UHT milk made with raw milk containing less than 10⁶ cfu/mL of protease-producing *Pseudomonas* (commonly regarded as the maximum level to produce quality UHT milk) can reduce the shelf life of UHT milk. This suggests a revision of the guidelines for the dairy industry in terms of the microbial load in raw milk used for UHT milk manufacture would

be useful. As the psychrotrophs can grow at refrigeration temperatures, issues may arise when the raw milk is stored for long periods before UHT processing, as these conditions will promote microbial growth and the production of proteases that are responsible for the deterioration of final products. This reinforces the need for rapidly processing raw milk. The present study provides the dairy industry with some fundamental information to enable the selection of raw milk for UHT manufacture.

6.6 Acknowledgement

This work was financially supported by Tetra Pak, New Zealand.

6.7 Supplementary materials

Table S6.5 Average protease units measurements of *Pseudomonas* isolates before and after UHT treatment in whole milk (mean and standard deviation)

Isolates	UHT Whole milk (protease units)	
	Before (protease units)	After (protease units)
<i>P. fragi</i> DZ1	0.623 ± 0.016	0.502 ± 0.046
<i>P. koreensis</i> DZ138	0.245 ± 0.018	0.255 ± 0.010
<i>P. rhodesiae</i> DZ351	0.189 ± 0.026	0.259 ± 0.043
<i>P. fluorescens</i> DZ390	0.347 ± 0.012	0.329 ± 0.010
<i>P. synxantha</i> DZ832	0.416 ± 0.032	0.470 ± 0.008
<i>P. lundensis</i> DZ845	0.311 ± 0.055	0.172 ± 0.021
Mixed isolates	0.648 ± 0.133	0.681 ± 0.011

Represents P < 0.05

Represents P > 0.05 or increase in reading after heat treatment

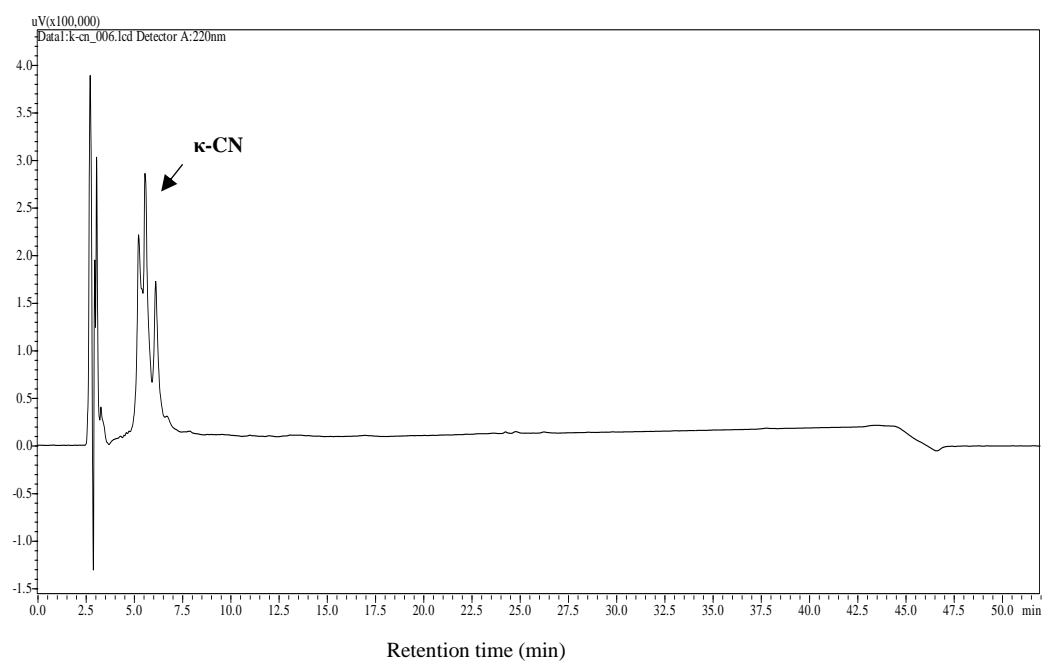


Figure S6.7 Identity of peaks of κ -CN standard by HPLC.

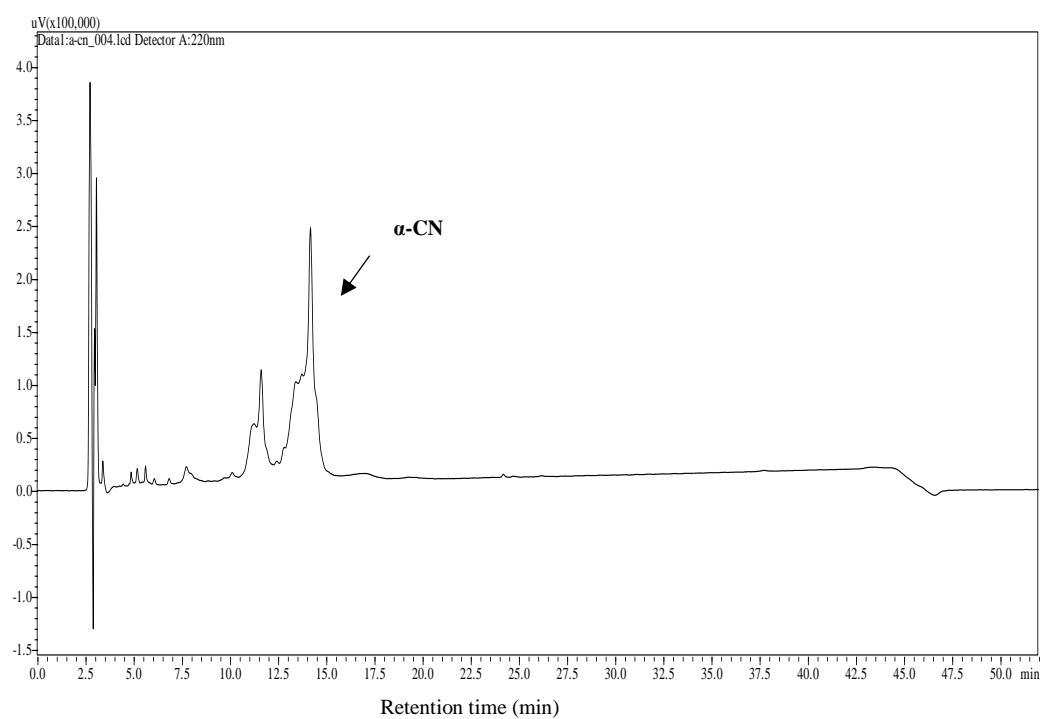


Figure S6.8 Identity of peaks of α -CN standard by HPLC.

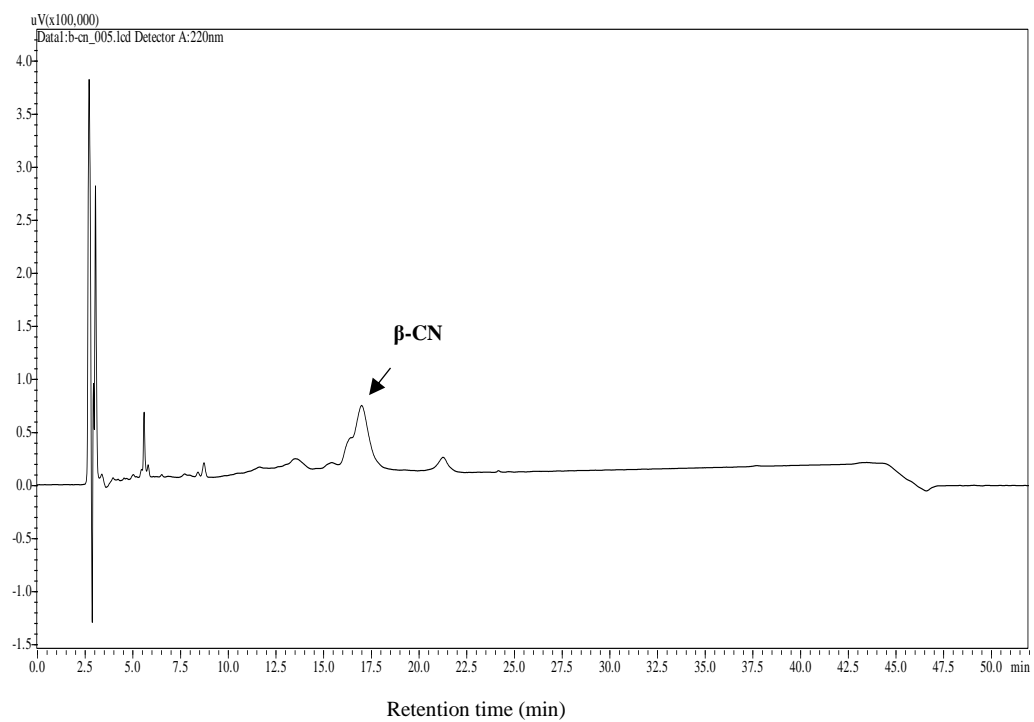


Figure S6.9 Identity of peaks of β -casein standard by HPLC.

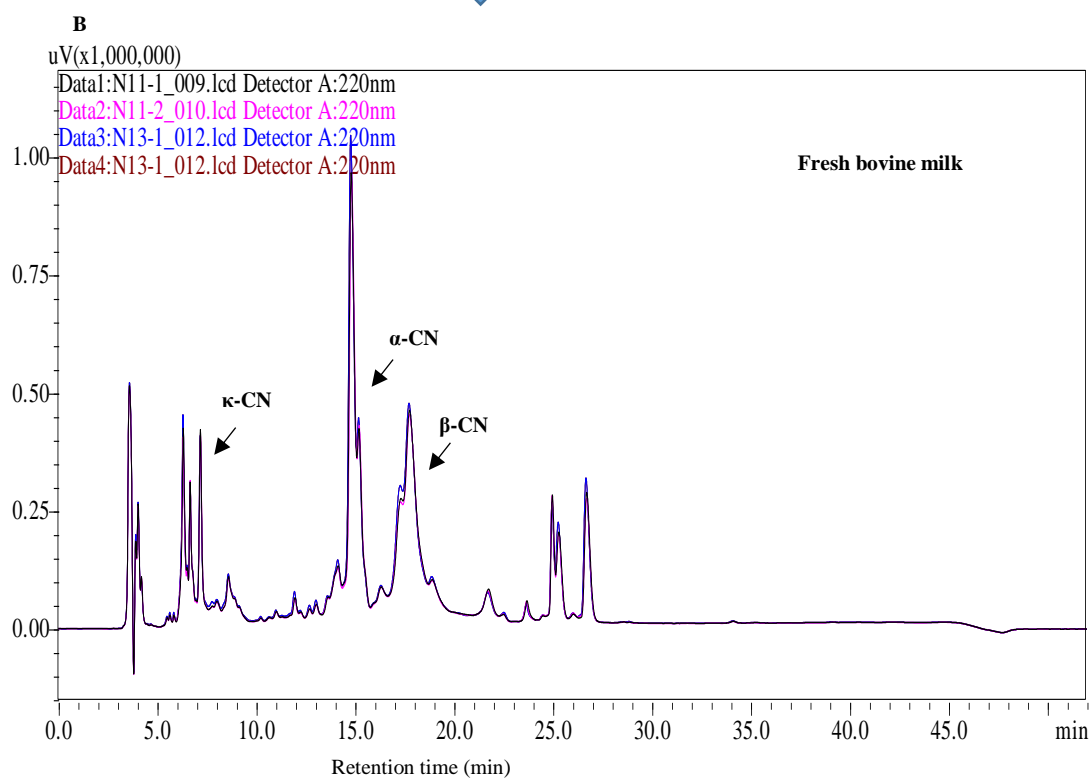
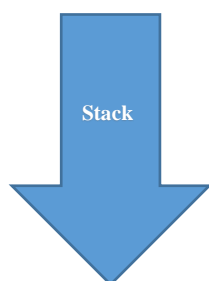
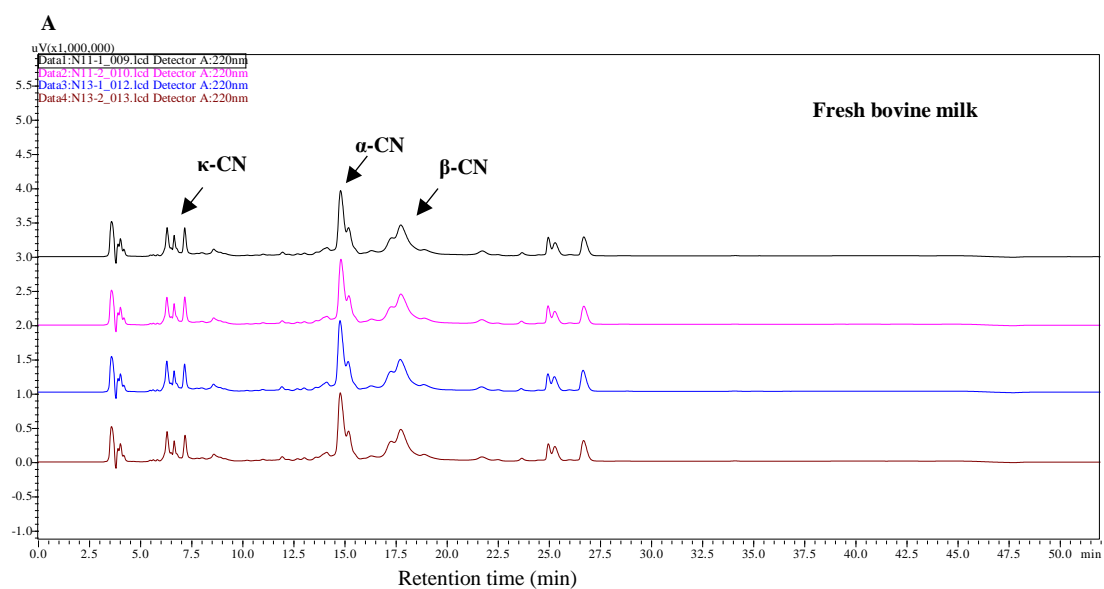


Figure S6.10 A: Separations of fresh bovine milk proteins by HPLC were done in quadruplicate. B: Combined samples.

Table S6.6 UHT milk with *P. rhodesiae* DZ351 (A), *P. synxantha* DZ832 (B) and mixed isolates (C) ratio of κ -CN, α -CN, and β -CN in total of control milk stored at 20°C and 30°C up to 9 months.

A

<i>P. rhodesiae</i> DZ351																								
Month	20°C												30°C											
	4 log cfu/mL			5 log cfu/mL			6 log cfu/mL			7 log cfu/mL			4 log cfu/mL			5 log cfu/mL			6 log cfu/mL			7 log cfu/mL		
	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN
1	0.86	0.97	1.22	0.85	1.05	1.16	0.87	1.06	1.21	0.38	0.38	0.35	0.82	0.92	1.15	0.91	1.13	1.20	0.93	1.12	1.14	0.38	0.41	0.31
2	0.56	0.74	1.10	0.34	0.59	1.32	0.33	0.52	0.85				0.43	0.75	0.94	0.42	0.94	0.90	0.44	0.83	0.82			
3	0.46	0.67	0.92	0.36	0.64	0.94	0.37	0.67	0.88				0.45	0.61	0.82	0.41	0.77	0.78	0.39	0.72	0.78			
4	0.43	0.63	0.79	0.36	0.72	0.75	0.43	0.44	0.84				0.35	0.50	0.63	0.29	0.47	0.54	0.28	0.39	0.48			
5	0.43	0.48	0.74	0.34	0.59	0.63	0.34	0.45	0.52				0.25	0.39	0.57	0.27	0.25	0.43	0.25	0.25	0.41			
6	0.35	0.42	0.76	0.30	0.49	0.56	0.27	0.34	0.44															
7	0.30	0.40	0.60																					

B

<i>P. synxantha</i> DZ832																								
Month	20°C												30°C											
	4 log cfu/mL			5 log cfu/mL			6 log cfu/mL			7 log cfu/mL			4 log cfu/mL			5 log cfu/mL			6 log cfu/mL			7 log cfu/mL		
	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN
1	0.86	1.03	1.11	0.89	1.16	1.30	0.90	1.20	1.32	0.53	0.54	0.22	0.84	0.96	1.09	0.82	0.98	1.03	0.84	1.02	1.15	0.51	0.57	0.18
2	0.40	0.54	1.17	0.38	0.77	1.25	0.39	0.61	1.02				0.36	0.61	0.85	0.34	0.68	0.95	0.43	0.81	0.87			
3	0.38	0.64	0.81	0.38	0.72	0.93	0.39	0.75	0.91				0.38	0.74	0.87	0.38	0.79	0.85	0.42	0.61	0.43			
4	0.35	0.62	0.81	0.36	0.72	0.90	0.44	0.50	0.87				0.35	0.31	0.43	0.28	0.45	0.58	0.31	0.29	0.35			
5	0.33	0.50	0.71	0.32	0.54	0.66	0.38	0.41	0.56				0.24	0.49	0.49	0.25	0.37	0.47	0.23	0.25	0.32			
6	0.32	0.34	0.56	0.26	0.36	0.44	0.26	0.40	0.45															
7	0.31	0.16	0.38																					

C

Mixed isolates																								
Month	20°C												30°C											
	4 log cfu/mL			5 log cfu/mL			6 log cfu/mL			7 log cfu/mL			4 log cfu/mL			5 log cfu/mL			6 log cfu/mL			7 log cfu/mL		
	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN	k-CN	a-CN	B-CN
1	0.88	1.10	1.20	0.88	1.17	1.31	0.90	1.18	1.31	0.35	0.28	0.17	1.01	1.12	1.18	0.87	1.18	1.27	0.95	1.21	0.98	0.51	0.39	0.22
2	0.30	0.44	0.93	0.30	0.55	0.93	0.37	0.65	0.97				0.42	0.66	0.78	0.40	0.66	0.86	0.41	0.73	0.87			
3	0.37	0.56	0.74	0.34	0.55	0.73	0.37	0.61	0.75				0.40	0.69	0.86	0.41	0.86	0.92	0.41	0.57	0.60			
4	0.34	0.54	0.71	0.25	0.40	0.53	0.43	0.48	0.79				0.38	0.50	0.53	0.27	0.36	0.49	0.29	0.32	0.49			
5	0.31	0.49	0.71	0.36	0.60	0.75	0.24	0.34	0.46				0.30	0.29	0.55	0.26	0.48	0.60	0.24	0.26	0.37			
6	0.34	0.38	0.51	0.35	0.41	0.44	0.35	0.45	0.40															
7																								

Table S6.7 pH changes of UHT milk with *P. rhodesiae* DZ351 (A), *P. synxantha* DZ832 (B) and mixed isolates (C) stored at 20°C and 30°C up to 9 months. (Mean and standard deviation).

A

Month	<i>P. rhodesiae</i> DZ351							
	20°C				30°C			
	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL
1	6.68 ± 0.02	6.78 ± 0.07	6.73 ± 0.06	5.96 ± 0.06	6.68 ± 0.01	6.77 ± 0.01	6.77 ± 0.01	5.83 ± 0.05
2	6.60 ± 0.01	6.63 ± 0.03	6.57 ± 0.03		6.61 ± 0.01	6.58 ± 0.06	6.55 ± 0.01	
3	6.41 ± 0.02	6.55 ± 0.01	6.60 ± 0.05		6.33 ± 0.03	6.21 ± 0.01	6.42 ± 0.02	
4	6.39 ± 0.02	6.37 ± 0.03	6.32 ± 0.01		6.11 ± 0.01	5.90 ± 0.01	5.98 ± 0.02	
5	6.33 ± 0.01	6.24 ± 0.04	5.95 ± 0.05		5.96 ± 0.05			
6	6.22 ± 0.01	5.98 ± 0.07						
7	5.83 ± 0.04							

B

Month	<i>P. synxantha</i> DZ832							
	20°C				30°C			
	4 log cfu cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL
1	6.70 ± 0.01	6.68 ± 0.02	6.81 ± 0.01	5.86 ± 0.05	6.79 ± 0.01	6.79 ± 0.01	6.76 ± 0.1	5.98 ± 0.01
2	6.73 ± 0.04	6.61 ± 0.01	6.55 ± 0.05		6.43 ± 0.01	6.55 ± 0.01	6.32 ± 0.02	
3	6.50 ± 0.01	6.59 ± 0.02	6.51 ± 0.02		6.35 ± 0.01	6.23 ± 0.01	5.74 ± 0.04	
4	6.34 ± 0.01	6.45 ± 0.01	6.32 ± 0.01		5.93 ± 0.06	5.90 ± 0.01		
5	6.18 ± 0.05	6.11 ± 0.02	5.89 ± 0.01					
6	5.74 ± 0.06	5.81 ± 0.01						
7								

C

Month	Mixed isolates							
	20°C				30°C			
	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL
1	6.80 ± 0.01	6.82 ± 0.01	6.78 ± 0.01	5.79 ± 0.09	6.75 ± 0.05	6.73 ± 0.06	6.74 ± 0.06	5.73 ± 0.04
2	6.78 ± 0.01	6.69 ± 0.03	6.65 ± 0.01		6.62 ± 0.03	6.64 ± 0.03	6.21 ± 0.01	
3	6.55 ± 0.01	6.43 ± 0.00	6.42 ± 0.03		6.40 ± 0.07	6.23 ± 0.02	5.82 ± 0.03	
4	6.23 ± 0.01	6.25 ± 0.02	6.35 ± 0.04		5.93 ± 0.05	5.87 ± 0.06		
5	6.23 ± 0.01	6.11 ± 0.00	5.99 ± 0.02					
6	5.75 ± 0.05	5.91 ± 0.02						
7								

Table S6.8 Sedimentation changes of UHT milk with *P. rhodesiae* DZ351 (A), *P. synxantha* DZ832 (B) and mixed isolates (C) stored at 20°C and 30°C up to 9 months (100%). (Mean and standard deviation).

A

Month	<i>P. rhodesiae</i> DZ351							
	20°C				30°C			
	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL
1	0.85 ± 0.03	0.91 ± 0.04	0.90 ± 0.08	8.00 ± 0.18	1.24 ± 0.07	0.97 ± 0.07	1.20 ± 0.21	8.46 ± 0.28
2	1.41 ± 0.16	1.28 ± 0.23	1.36 ± 0.39		1.21 ± 0.02	1.78 ± 0.29	2.20 ± 0.10	
3	2.41 ± 0.40	1.48 ± 0.29	2.27 ± 0.21		3.38 ± 0.17	3.70 ± 0.20	2.94 ± 0.04	
4	2.88 ± 0.11	2.92 ± 0.06	4.44 ± 0.39		3.71 ± 0.23	9.18 ± 0.37	10.46 ± 0.82	
5	2.96 ± 0.16	4.06 ± 0.22	9.43 ± 0.59		10.88 ± 0.39			
6	3.50 ± 0.05	9.80 ± 0.26						
7	9.13 ± 0.32							

B

Month	<i>P. synxantha</i> DZ832							
	20°C				30°C			
	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL
1	1.07 ± 0.14	1.19 ± 0.16	1.19 ± 0.16	8.39 ± 0.07	1.30 ± 0.13	1.22 ± 0.23	1.92 ± 0.14	7.88 ± 0.07
2	1.23 ± 0.12	1.45 ± 0.22	2.85 ± 0.08		2.74 ± 0.27	1.49 ± 0.25	2.66 ± 0.50	
3	1.71 ± 0.32	1.77 ± 0.12	3.18 ± 0.33		3.26 ± 0.33	3.30 ± 0.12	9.79 ± 1.02	
4	2.33 ± 0.48	2.66 ± 0.49	3.47 ± 0.34		10.47 ± 0.51	10.92 ± 0.83		
5	4.20 ± 0.21	3.58 ± 0.42	10.13 ± 0.22					
6	10.09 ± 0.19	12.87 ± 0.49						
7								

C

Month	Mixed isolates							
	20°C				30°C			
	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL	4 log cfu/mL	5 log cfu/mL	6 log cfu/mL	7 log cfu/mL
1	0.86 ± 0.06	0.99 ± 0.10	1.17 ± 0.17	8.17 ± 0.25	1.58 ± 0.43	1.58 ± 0.28	1.97 ± 0.07	8.12 ± 0.20
2	1.23 ± 0.02	1.37 ± 0.37	1.56 ± 0.47		2.56 ± 0.50	2.71 ± 0.26	3.68 ± 0.27	
3	2.92 ± 0.03	2.50 ± 0.32	2.67 ± 0.33		3.47 ± 0.49	3.29 ± 0.52	12.07 ± 0.21	
4	3.52 ± 0.05	3.30 ± 0.57	4.15 ± 0.28		9.93 ± 0.23	11.46 ± 0.54		
5	3.86 ± 0.07	3.66 ± 0.55	9.09 ± 0.56					
6	9.46 ± 0.45	11.01 ± 0.91						
7								

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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Name of candidate:	Dong Zhang	
Name/title of Primary Supervisor:	Steve Flint	
Name of Research Output and full reference:		
hang, D., Li, S. Q., Palmer, J., Teh, T. H., Leow, S., & Flint, S. (2020). "The relationship between numbers of <i>Pseudomonas</i> bacteria in milk used to manufacture UHT milk and the effect on product quality." <i>International Dairy Journal</i> , 105.		
In which Chapter is the Manuscript /Published work:	Chapter 6	
Please indicate:		
<ul style="list-style-type: none"> The percentage of the manuscript/Published Work that was contributed by the candidate: 	80%	
and		
<ul style="list-style-type: none"> Describe the contribution that the candidate has made to the Manuscript/Published Work: 		
The candidate carried out the laboratory work and data analysis (the protease heat stability assay and analysis of protein changes by HPLC were carried out with the help of Singaporean intern, Sharon Leow and Siqi Li, a Ph.D. student from Riddet Institute, separately) and prepared the manuscript with input in guidance of direction and editorial help from the co-authors and supervisors.		
For manuscripts intended for publication please indicate target journal:		
International Dairy Journal (published manuscript)		
Candidate's Signature:		
Date:	15/05/2020	
Primary Supervisor's Signature:		
Date:	15/05/2020	

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

Chapter 7. Summarising discussion and conclusions

7.1 Introduction

The microbiological quality of incoming raw milk is of utmost importance for high-quality dairy products. Poor milk quality damages the quality of final products, in particular for products with an extended shelf life (Chambers, 2005). Refrigeration and heat treatment are used to increase the quality and shelf life of dairy products (Machado et al., 2016). However, the presence of psychrotrophs in raw milk is a threat to product quality despite refrigeration and heat treatment because they can rapidly multiply under refrigeration and produce heat-stable proteases and lipases. These proteases and lipases are responsible for the hydrolysis of milk proteins and fats, resulting in gelation, sedimentation, off smell, and fat separation of UHT milk (Barbano, Ma, & Santos, 2006).

The problem with psychrotrophic bacterial contamination of raw milk and product quality is well reported but these reports are based on the culturable bacterial population and the relationship between bacterial numbers and types relating to product quality has not been quantified. The use of metagenomics has only recently become available as a tool for the investigation of comprehensive microbial load of raw milk, enabling both culturable and non-culturable populations to be investigated. There is lack of knowledge around the kinetics of psychrotrophic bacterial growth and proteolytic activity prior to processing, enabling the prediction of the shelf life of products such as UHT milk. This study used both culture-independent and culture-dependent methods to investigate the whole psychrotrophic bacterial population of raw milk. Also, the growth and proteolytic activity of selected psychrotrophs in different environments before any dairy processing was studied. Finally, the levels and types of psychrotrophic bacterial contamination in raw milk were studied to enable the prediction of the shelf life of UHT.

7.2 Highlights or key findings

- The combination of culture-independent and culture-dependent approaches provided a better understanding of whole psychrotrophic bacterial population (including both culturable and non-culturable) of New Zealand raw milk across seasons and regions, which is very important to the New Zealand dairy industry.
- This is the first report to compare both advantages and disadvantages of HTS and MALDI TOF MS, showing that it is a novel and complementary way to investigate the microbial quality of raw milk.
- The non-culturable microorganisms, such as *P. Psychrophila*, existed in raw milk sampled in this study. These were not isolated and identified by culture-dependent methods but can potentially affect the quality of the final product, which could be overlooked in the dairy industry previously using only culture-dependent methods. Extended refrigeration can shift the microbial diversity of raw milk at both the genus and species level. The changes in the microbial composition of raw milk under refrigerated storage results in the predominance of specific bacteria that can potentially affect the quality of final milk products.
- Some of the isolates were first isolated and identified from raw milk.
- Some of the isolates found in this study were reported for the first time as species producing heat-stable enzymes under pasteurization temperature (72°C, 15 s).
- The maximum proteolytic activity of different *Pseudomonas* isolates varies with six dairy isolates of *Pseudomonas*, showing the following order of proteolytic activity: *P. lundensis* DZ845> *P. fluorescens* DZ390> *P. fragi* DZ1> *P. synxantha* DZ832> *P. rhodesiae* DZ351> *P. koreensis* DZ138.
- *Pseudomonas* showed more proteolytic activity in a milk medium than TSB. More than one protease is produced in milk media but only one protease band was observed in TSB.

- Milk fat induces the activity or production of protease from some strains of *Pseudomonas* while other dairy ingredients (calcium caseinate, sodium caseinate, and whey protein isolate) inhibited protease activity.
- The UHT milk made from raw milk contaminated with 4 log cfu/mL of proteolytic psychrotrophs only had 6 and 4-month shelf life at 20°C and 30°C, respectively.
- The UHT milk made from raw milk contaminated with different proteolytic psychrotrophs resulted in different shelf life. One key contribution of this project to the dairy industry was the threshold of the maximum level of psychrotrophic bacterial contamination in raw milk that can be used for long shelf life (9 months) UHT milk products was reduced to 4 log cfu/mL, compared with the formerly established guideline of 6 log cfu/mL.

7.3 Summary of studies

7.3.1 Investigation of psychrotrophic bacterial composition of raw milk using culture-independent and culture-dependent methods.

Psychrotrophic bacterial diversity of raw cow's milk across four seasons and six regions was investigated using culture-independent (HTS) and culture-dependent (MALDI-TOF MS) approaches. Both approaches showed that the *Pseudomonas* genus predominated, representing 78.67% of the total microbiota by HTS and 93.68% by MALDI-TOF MS. HTS identified more than 11 genera whereas MALDI-TOF MS identified 9 genera. Of the remaining genera, HTS revealed that *Acinetobacter*, *Lactococcus*, *Serratia*, and *Kluyvera* were the only four genera that were higher than 1% of the total microbiota, whereas all other remaining genera represented a small proportion. The proportion of each of the 8 remaining genera identified by MALDI-TOF MS was 0.79%. At the species level, the abundance of predominant species varied between these two identification methods. HTS showed that *P. psychrophila* (39.02%) and *P. fluorescens* (37.53%) were predominant species, while *P. lundensis* (24.41%) and *P. fragi* (20.47%) appeared to be the predominant species by MALDI TOF MS. A total of 28.33% of *Pseudomonas* were not able to be identified to the species level by MALDI-TOF MS. HTS is a more accurate method to

determine the total microbiota of raw milk as it does not rely on cultivation needed for MALDI-TOF MS. HTS is relatively costly compared with MALDI-TOF MS and will also detect dead bacteria which may not be important. However, HTS will also detect non-culturable bacteria that may be a concern in raw milk quality even if they cannot be cultured in the laboratory. MALDI-TOF MS is limited by the extent of the database, however, it is a relatively cheap and rapid method for identifying culturable psychrotrophs. A seasonal and regional variation in the psychrotropic bacterial composition in raw milk was observed by HTS. *P. psychrophila* and *P. fluorescens* were the two predominant species across all seasons and regions. The abundance of the second dominant species varied with seasons and with regions.

This is the first report to evaluate the psychrotrophic bacterial quality of refrigerated raw milk in New Zealand across four seasons and six regions using HTS and MALDI-TOF MS. The combination of the two identification methods can provide a more accurate and comprehensive insight into the psychrotropic bacteria present in raw milk through a one-year dairy season, which may assist the dairy industry in selecting the best quality milk prior to the processing. This information is important to the New Zealand dairy industry as the cow's feed in New Zealand is mainly pasture, which will influence the microbial ecology of raw milk. This is likely to differ from countries that rely on non-pasture feeds. The dairy industry can use MALDI TOF to look at the microflora of raw milk and quickly get an idea of whether they do have these strong enzyme-producers in their milk. Although the combination of HTS and MALDI-TOF is not able to give a real-time result, it can be used to monitor the quality of incoming raw milk and monitor trends over seasons and between regions. The quality of dairy products is dependent on the quality of raw milk from the farm and maintain that quality during transportation and storage. Knowing the natural variation in quality will assist, in making decisions on prioritising the types of products being manufactured.

7.3.2 Selection of heat-stable enzyme-producing bacteria from raw milk

One of the aims of this chapter is to select the predominate proteolytic isolates for further product quality assay. The numbers and types of psychrotrophs in raw milk with and without refrigerated enrichment were compared to understand the effect of extended refrigeration on the changes in raw milk microbial ecology. Two-hundred and six isolates from both fresh and enriched raw milk were identified by MALDI-TOF MS and assessed for proteolytic and lipolytic activity at 7°C and the heat stability of these enzymes during pasteurization was measured. The values of psychrotrophic bacterial counts (PBC) and mesophilic bacterial counts (MBC) before chilled enrichment ranged from 2.36 ± 0.10 to 3.87 ± 0.39 log cfu/mL and 3.42 ± 0.10 to 4.94 ± 0.16 log cfu/mL respectively, with a PBC to MBC ratio of 1: 10. After 5-days chilled enrichment, the mean for PBC and MBC was a ratio of 1:1 with 7.78 ± 0.16 and 7.60 ± 0.20 log cfu/mL, respectively, showing the selective pressure of refrigeration. In fresh raw milk, 119 isolates were identified, representing 12 different genera and 23 species, whereas 127 isolates were identified in chilled raw milk, representing 9 genera, and 20 species, indicating that the psychrotrophic bacterial composition of chilled enriched milk is less diverse compared with fresh raw milk. *P. fragi* (10.92%) was the predominant species in fresh raw milk, followed by *P. lundensis* (6.72%) and *P. fluorescens* (6.72%). *P. lundensis* was the predominant species (24.41%) in chilled raw milk, followed by *P. fragi* (20.47%) and *P. fluorescens* (5.51%). Among the 246 isolates, 110 isolates showed proteolytic activity at 7°C and 94 of these isolates were able to produce heat-stable proteases. 91 of these isolates were able to produce lipases and 81 of them were heat stable. The heat stability of enzymatic activity of some other isolates, such as *Acinetobacter* and *Hafnia* have not reported previously.

Although several researchers have produced similar reports on the psychrotrophic bacterial changes in raw milk, the microbial ecology at both genus and species levels has been less well studied. Moreover, the identification method (MALDI-TOF) used in this project has quite a few advantages over methods used in previous reports (e.g. API test). The main advantages are the speed and simplicity of testing using MALDI-TOF, as well as accuracy if a comprehensive

database is used. That's the reason that some of the psychrotrophs were first isolated and identified in this report. The role of these psychrotrophs appears to have been overlooked previously and their effects on the final dairy product quality need to be investigated in more detail in the future. This study also reinforced that the prolonged refrigeration of raw milk can change the microbial composition of raw milk, supporting the importance of processing raw milk as soon as possible after milking. Further investigation of the predominant isolates producing heat-stable enzymes will help understand the potential for spoilage of milk products.

7.3.3 The effect of dairy components on the growth and proteolytic activity of selected *Pseudomonas* before any dairy processing

This study investigated the effect of the environment on growth and proteolytic activity of six dairy *Pseudomonas* isolates at 7°C, following raw milk handling on the farm, during transportation, and in the processing plant. Some scientific papers have been published on the proteolysis of whole UHT milk and skim milk during shelf life, showing that skim milk is more susceptible to bacterial protease activity, irrespective of the source. However, this study aimed to understand the proteolytic activity directly from psychrotrophs in raw milk in UHTs product during storage. This information is important for the dairy industry to control and maintain the quality of the raw materials used for dairy product manufacture. Six dominant *Pseudomonas* showing heat-stable protease activity from Chapter 4 were grown in TSB, skim milk, and whole milk at 7°C for 7 days. The results showed the growth curves of all six isolates were similar in dairy and non-dairy media at 7°C for 7 days. However, the proteolytic activity of all isolates, determined by azocasein analysis, was significantly higher in milk media than TSB ($p < 0.05$), especially whole milk. Zymography gels supported the azocasein result in that *Pseudomonas* produced more proteolytic activity in milk medium than TSB. In milk media, more than one protease band was detected but only one protease band was observed in TSB. The nature of the multiple bands and their impact on the high levels of proteolytic activity needs to be further investigated. The maximum proteolytic activity of six dairy isolates of *Pseudomonas* was reported for the first time in this study, in the following order, *P. lundensis* DZ845> *P. fluorescens* DZ832>

P. fragi DZ1> *P. synxantha* DZ832> *P. rhodesiae* DZ351> *P. koreensis* DZ138. The results also gave some fundamental information for further study on the effect of each individual *Pseudomonas* and specific proteases on the quality of the product.

To determine the effect of dairy components on bacterial proteolytic activity, *P. rhodesiae* DZ351 (weak proteolytic activity) and *P. lundensis* DZ845 (strong proteolytic activity) were selected and inoculated in TSB supplemented individually with five dairy ingredients (sodium caseinate, calcium caseinate, salted butter, and 90% gamma-irradiated whey protein isolate). After 7 days, the medium containing butter produced the highest proteolysis for both strains: 0.09 ± 0.01 units for *P. rhodesiae* DZ351 and 0.50 ± 0.03 units for *P. lundensis* DZ845. Also, *P. lundensis* DZ845 showed an increase in proteolytic activity as the butter concentration increased (0% -15%). Zymography showed that *P. lundensis* DZ 845 produced one stronger protease with a molecular mass of 47 kDa in TSB with butter compared with TSB without butter. The conclusion is that the addition of milk fat to the growth medium of *P. lundensis* DZ845 significantly enhances its proteolytic activity ($p < 0.05$) but the mechanisms are something for future investigations. The information from this Chapter, provides some new understanding of the proteolytic psychrotrophs before processing, and how dairy components influence proteolytic activity, which can help and guide the industry to plan to maintain the quality of raw milk before processing.

7.3.4 The effect of the level of proteolytic *Pseudomonas* bacterial contamination in raw milk on the shelf life of UHT milk

UHT milk was made from sterile milk intentionally contaminated with two single *Pseudomonas* isolates (*P. rhodesiae* DZ351 and *P. synxantha* DZ832) and a mixture of six *Pseudomonas* (*P. fragi* DZ1, *P. koreensis* DZ138, *P. rhodesiae* DZ351, *P. fluorescens* DZ390, *P. synxantha* DZ832, and *P. lundensis* DZ845) showing heat stable proteolytic activity, and inoculated at different levels (4, 5, 6, and 7 log cfu/mL). The samples were stored at 20, 30, and 55°C for up to 9 months and the degradation of casein protein, gelation, sedimentation, and pH were analyzed monthly. The UHT milk made from raw milk contaminated with 4, 5, 6, and 7 log

cfu/mL of *Pseudomonas* had less than 7, 6, 5 and 1 months' shelf life at 30°C, respectively. The onset of hydrolysis of κ -CN occurred earlier than α -CN and β -CN. *P. synxantha* DZ832 and the mixed culture showed a stronger proteolysis of UHT milk than *P. rhodesiae* DZ351. The UHT milk stored at 30°C had a shorter shelf life compared to UHT milk stored at 20°C. This can be explained by the proteolytic activity of *Pseudomonas* being higher at 30°C than 20°C. UHT milk stored at 55°C for one month became brownish, indicating that UHT milk storage at that temperature is not practical. This Chapter recommended a threshold for the maximum level of psychrotrophic bacterial contamination in raw milk 4 log CFU/mL that can be used to ensure 9 months shelf life for UHT milk. Of course, milk contains a complex microbial ecology that cannot be 100% replicated. However, work with several strains of *Pseudomonas* in this study, gives some idea of the activity of a mixed microbial population. Knowing the specific bacterial composition of raw milk is very important for selecting the top-grade raw material. Raw milk containing 4 log cfu/ml of psychrotrophs (with majority proteolytic microbes) used for UHT milk has a high risk of quality issues. This study provides the dairy industry with fundamental information and guidelines for selecting the raw milk to meet the criteria for UHT milk processing to enable quality products after typical storage conditions in the supply chain.

7.4 Future work

The effect of raw milk quality on dairy product quality has been studied by many researchers for many years. However, the results from the present study show that there is still much more to learn. Based on the findings in this thesis, it is recommended that future work include the following:

- Identify the effect of the growth environment on protease production of *Pseudomonas* isolates
- Identify the effect of different fat (e.g. anhydrous milk fat, vegetable fat, lard, and unsalted fat) on the proteolytic activity of *Pseudomonas*
- Identify the types of proteases from the zymography gels using mass spectrometry

- Identify the regulatory mechanisms that trigger *Pseudomonas* to show more proteolytic activity in the presence of milk fat by gene expression
- Identify the effect of the environment on the growth and lipolytic activity/lipase production of the selected *Pseudomonas*
- Identify the enzymatic activity or the enzyme production of *Pseudomonas* within a biofilm
- Examine the heat stability of psychrotrophic microbial lipases under UHT conditions
- Investigate the lipolysis of UHT milk during storage
- Develop an accurate and rapid method to determine the quality of incoming raw milk before UHT processing
- Identify the effect of raw milk quality on the shelf life of milk powder
- Investigate the effect of other isolated spoilage enzyme-producing bacteria (e.g. *B. cereus* and *A. guillouiae*) on the quality of dairy products

7.5 References

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