UHT milk spoilage owing to lipases and proteases from psychrotrophic *Pseudomonas* species: Characteristics of enzymatic breakdown, improved assays for enzyme detection and sources of the bacteria

Philip Douglas Button
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School of Agriculture and Food Systems
The University of Melbourne

ABSTRACT

Spoilage of UHT milk is of concern to the Australian dairy industry. Bacterial lipases and proteases have been identified as a key cause of spoilage of long life dairy products. They are synthesised in raw milk by psychrotrophic *Pseudomonas* spp. during refrigerated storage and persist as active enzymes in dairy products owing to retention of activity after heat treatment. The continuing action of lipase and protease may cause spoilage of all long life dairy products, including UHT milk, butter and cheese.

The aims of this PhD project were to: I) optimise assays for lipase and protease produced by psychrotrophic *Pseudomonas* spp. and identify those which are more sensitive than those currently available, II) correlate the progress of lipolysis and proteolysis in these assays with the accumulation of products of lipolysis and proteolysis in UHT milk during storage, and relate it to common organoleptic defects in UHT milk, and, III) identify sources of psychrotrophic *Pseudomonas* spp. in raw milk and the industry practices likely to contribute to the presence of lipases and proteases from psychrotrophic *Pseudomonas* spp. in UHT milk.

The present study identified lipase and protease assays which could be used to detect limited lipolysis and proteolysis in UHT milk that may result in organoleptic defects during shelf life. These assays utilised the milk-like substrate triolein for lipase detection and the labelled substrate FITC-casein for protease detection. In these assays, a strong correlation was observed between the extent of hydrolysis of the substrate in the assay and the progress of lipolysis and proteolysis in milk. Furthermore, these assays could be used to detect levels of lipolysis or proteolysis that are likely to result in spoilage in UHT milk after three to five months of storage.

The progress of some biochemical changes in UHT milk have been followed during shelf life. The shelf life of the milk sample was limited by the development of age gelation, which occurred after viscosity increased to 120-150 mPas, although there were no appreciable off-aromas or off-flavours at this time. Age gelation was accompanied by a large decline in the κ-casein content, particularly in the days immediately prior to its development. At no time were

off-aromas or off-flavours reported. This is consistent with the absence of measurable quantities of hydrophobic peptides and free fatty acids which contribute to flavour, in the samples.

Psychrotrophic *Pseudomonas* spp. were isolated from farm, tanker and silo milk. A total of 39 PFGE Types were identified among 45 isolates which demonstrated considerable genetic diversity in *Pseudomonas* spp. in raw milk. Isolates from one of the farms and from the silo had the greatest potential for causing spoilage. This is because proportions of the strongly lipolytic and proteolytic isolates were higher and they grew to spoilage levels more quickly than isolates from other sources. Although incubation of the milk at 10 ºC led to faster *Pseudomonas* spp. growth, this growth temperature resulted in the proliferation of bacteria that were less lipolytic and proteolytic than did incubation at 4 ºC. After growing at 4 ºC, the *Pseudomonas* spp. were also more lipolytic and proteolytic than the bacteria present in the unincubated milk.

Specificity of a typical psychrotrophic *Pseudomonas* spp. protease, protease B52 from *Pseudomonas fluorescens* B52, towards α_s- and β-casein was investigated. Mass spectral analysis revealed limited specificity, with the peptides released being mostly hydrophilic. This result suggests that B52 protease is unlikely to cause bitterness from degradation of α_s- and β-casein.

The present study has improved our basic understanding of psychrotrophic *Pseudomonas* spp. lipase and protease action in UHT milk. In addition, information has been provided to industry regarding improved detection methods along with sources and practices that may contribute to increased lipase and protease contamination of UHT milk and other long-life dairy products.

DECLARATION

This is to certify that

(i) the thesis comprises only my original work towards the PhD except where indicated in the Preface,

(ii) due acknowledgement has been made in the text to all other material used,

(iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

PREFACE

Most of the work presented in this thesis was undertaken by the candidate. The exceptions were aspects of the ESI-MS/MS and MALDI-TOF MS analysis, reported in Chapters 5 and 7. This work was carried out by staff at Monash University, in the Centre for Green Chemistry's Proteomics Facility.

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Patience. This is something I know my wife, Michelle has a lot of. She has patiently waited years for my PhD to draw to a close, while making many sacrifices along the way to accommodate my PhD. Thomas, my son, has also made sacrifices. Now, he will get to know his Daddy better. You both have been wonderful – thank you heaps from the bottom of my heart for your love and support.

My parents, Doug and Julie, have seen it all, and have been there every step of the way with my education. They have done everything they can to help me achieve my dreams. Without their love and support, there is no doubt that I would not have made it this far. Thank you so much. I would also like acknowledge the rest of my close family; parents-in-law Raymond and Monica, brother-in-law Clement, sisters-in-law Emille, Jocelyn and Olivia and future brother-in-law Kevin.

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Access to information sources is vital in a major research project, and I was very fortunate to have access to one of the best at Food Science Australia, CSIRO in general and The University of Melbourne. I am extremely grateful to the efforts of the entire team of librarians at Food Science Australia in Melbourne, especially Jan Stewart and Tania Celeste, who have provided most assistance during my time there.

In the final stages of thesis preparation, I'd like to acknowledge and applaud the efforts of my wife Michelle and my parents in proofreading the entire thesis, Mum for assistance in compiling the final table of contents and Michelle and Hossein Daryaei for printing.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS OF UNITS

ºC	degrees Celsius
d	day(s)
Da	Daltons
g	g force
g	gram(s)
h	hour(s)
kDa	kilodalton(s)
kg	kilogram(s)
kHz	kilohertz
kPa	kilopascal(s)
kV	kilovolt(s)
L	litre(s)
M	molar
meq	milliequivalent(s)
mg	milligram(s)
min	minute(s)
µL	microlitre
µm	micrometre
µM	micromolar
mL	millilitre(s)
mm	millimetre
mM	millimolar
mPa	millipascal(s)
mPas	millipascal seconds
ms	milliseconds
m/z	mass to charge ratio
nL	nanolitre(s)
nm	nanometre(s)
nM	nanomolar
sec	second(s)
V	volts
v/v	volume/volume

OTHER ABBREVIATIONS

4-MU	4-methylumbelliferone

4-MUC	4-methylumbelliferyl caprylate
4-MUN	4-methylumbelliferyl nonanoate
4-MUO	4-methylumbelliferyl oleate
ADV	acid degree value
α-LA	α-lactalbumin
α_s-CN	α_s-casein
α_{s1}-CN	α_{s1}-casein
α_{s2}-CN	α_{s2}-casein
β-CN	β-casein
β-LG	β-lactoglobulin
β-NC	β-naphthyl caprylate
CCS	combined consensus (hydrophobicity) scale
cfu	colony forming unit(s)
GC/MS	gas chromatography/mass spectrometry
E.C.	enzyme classification (number)
ESI-MS/MS	electrospray ionisation mass spectrometry/mass spectrometry (ESI tandem mass spectrometry)
FAG	free amino group(s)
FFA	free fatty acid(s)
FITC	fluorescein isothiocyanate
HPLC	high performance liquid chromatography
κ-CN	κ-casein
Leu-Gly	leucine glycine (dipeptide)
MALDI-TOF MS	matrix assisted laser desorption ionisation time of flight mass spectrometry
para-κ-CN	para-κ-casein
PCA	Plate Count Agar
PF	pulsed field (types)
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEM	transmission electron microscopy

UHT	Ultra high temperature

CHAPTER 1

INTRODUCTION

Milk is one of the most widely consumed foods. However, the very nature of milk production and the composition of the product itself, make it highly susceptible to spoilage. Therefore, various forms of heat treatment have been developed, both to eliminate pathogenic bacteria and to reduce spoilage.

The bacteriological quality of heat-processed milk is largely dependent on the heat treatment applied. Bacteria, and their enzymes, differ in their susceptibility to heat, therefore the rate of spoilage and type of spoilage products appearing in milk would also vary. Bacterial quality of milk is relatively straight-forward to assess. However, assessment of enzymatic spoilage is quite different. The causes are difficult to quantify and it has thus far been impossible to establish a relationship between the progress of enzyme-catalysed reactions in the product and likely shelf life. While various assays are available for detection of lipases and proteases in milk, only few have sufficient sensitivity to detect the residues of heat-resistant lipases and proteases that may be present in UHT milk following processing. This is a problem, because UHT milk is predominately spoiled by heat-resistant lipases and proteases, often those produced by contaminating psychrotrophic *Pseudomonas* spp. A sensitive and accurate test to detect the extent of lipase and protease contamination is required. This information, in turn, could lead to a prediction of the shelf life of that product, based on the likely action of a particular enzyme.

As a major portion of milk solids are fat and protein, their degradation may lead to serious quality defects. In order to better understand the spoilage process, it is necessary to understand how typical lipases and protease act on fat and protein in UHT milk. This information could improve our appreciation of continuing enzymatic action in relation to its contribution to spoilage of UHT milk. The mechanisms of typical spoilage phenomena observed in UHT milk, such as age gelation, have not been sufficiently elucidated. Therefore, a detailed investigation into the biochemistry of UHT milk spoilage and the contribution of hydrolytic enzymes is imperative in adding to the current knowledge of age gelation and other enzyme-mediated spoilage phenomena.

Lipases and proteases are naturally present in raw milk. Those which are more important in UHT-processed milk are produced by contaminating

psychrotrophic *Pseudomonas* spp., which may enter the raw milk supply at various milk harvesting, storage and transport points. Therefore, the key to reducing the risk of hydrolytic enzyme contamination of UHT milk is to reduce the raw milk contamination of the bacterial isolates likely to contribute the problem enzymes. Reduction of contamination may involve various approaches. These can be on the farm or within the factory environment and may involve a variety of aspects related to handling and storage of the raw product. Knowledge of these particular aspects is essential to devising strategies to restrict the entry of particular isolates that are more likely to contribute enzymes that could spoil UHT milk.

There is currently a lack of understanding in a range of areas associated with the spoilage of UHT milk. These areas involve detection of the enzymes (which could lead to shelf life prediction), full characterisation of products of lipolysis and proteolysis during product storage and the contribution of handling or processing practices that may increase the likelihood of bacterial lipase and protease contamination in milk. A detailed investigation into these areas would advance basic knowledge of the microbiology and biochemistry of UHT milk spoilage and provide information that could be used by industry to reduce the risk of spoilage of UHT milk. This may lead to higher industry revenue through increased consumer confidence and additional markets for these products which in turn could increase the competitiveness of this sector of the Australian dairy industry.

CHAPTER 2

LITERATURE REVIEW

2.1 The dairy industry in Victoria and Australia

Australia's dairy industry, currently worth $3.2 billion (Australian Bureau of Statistics, 2006a), is particularly important in Victoria, where over 60% of the nation's milk is produced (Rowley, 1994; Mikkelsen, 2001). On average, milk has been the third largest agricultural income earner in Australia, after wheat and beef, over the previous three years (Australian Bureau of Statistics, 2006a). Exported dairy products are of much importance to the national economy. Although Australia accounts for 2% of world milk production (IDF, 2005), the dairy industry in Australia has a 12% share of the international dairy export market, which is third, after the European Union and New Zealand (Dairy Australia, 2006a), earning the country around $2.3 billion annually (Trewin, 2007). Overall, approximately 70% of manufactured dairy products in Australia are exported (Norwood, 2000). Asia is the biggest market for Australian dairy products; exports to Asia total approximately $1.8 billion (Dairy Australia, 2006b). More than 85% of milk powder, a long-life dairy product, manufactured in Australia is exported (Dairy Australia, 2006b) with the value of milk powder exports being $825 million over the ten months to April 2007 (Dairy Australia, 2007). UHT milk comprises 11 to 12% of all supermarket milk sales domestically over the previous three years (Dairy Australia, 2006b) with the markets in Asia being a major potential growth area. Overall, the Australian dairy industry is a major competitor internationally and contributes substantially to the national, and particularly the Victorian economy.

2.2 Milk composition

Milk is the secretion produced by the mammary gland of female mammals for the purpose of supplying nutrition and providing immune support to the young (Jenness, 1999). Apart from cows, animals from which milk is commercially utilised include buffalo, sheep and goat (Hinrichs, 2004), with these species being primarily of regional importance. Bovine milk is the most commonly available; further references will only be made to milk from *Bos taurus*. There are four major components of milk - water, lactose, protein and fat. On the average, solids comprise approximately 12.7%, of which 4.8% is

lactose, 3.7% is fat and 3.4% is protein (Fox & McSweeney, 1998). However, these figures, and the amount of protein and fat show some variability, as will be detailed later, between breeds (Auldist *et al*. 2004), and within breeds, depending on a range of factors, including hormone levels (Auldist *et al*., 2007), season (Auldist *et al*., 1998), stage of lactation (Auldist *et al*., 1998), nutrition (Mackle *et al*., 1999) and other factors, such as intervals between milkings, which, has been shown by O'Brien *et al.* (2002) to significantly influence the fat and protein concentration in milk.

2.2.1 Fat

Nearly all (approximately 99% by weight) lipids in milk are triacylglycerols, with all remaining classes of lipids (such as monoacylglycerols, diacylglycerols and phospholipids) comprising approximately 1% of the total weight of milk fat on average (Jensen *et al*., 1991). Milk fat contains approximately 69-70% saturated fatty acids, 25-27% monounsaturated fatty acids and 4-5% polyunsaturated fatty acids (Hillbrick & Augustin, 2002; Jensen, 2002). The content of fat and of particular fatty acids does vary and the factors influencing this will be discussed later.

2.2.1.1 Fatty acids

As of December 2000, there were 416 identified fatty acids in milk (Jensen, 2002), up from 400 in February 1991 (Jensen *et al*., 1991). Christie and Clapperton (1982) point out that many fatty acids do occur in any of the three positions in the triacylglycerol molecule, in widely varying proportions, and as Creamer and MacGibbon (1996) state, this can lead to many hundreds of combinations of fatty acids within triacylglycerols. Hexadecanoic (C16:0) and *cis*-9-octadecanoic (C18:1) acids are the most common fatty acids present in triacylglycerol molecules (Shi *et al*., 2001; Timmen & Patton, 1988) and as FFA (Kim *et al*., 1987).

2.2.1.2 Milk fat globules

In a review by Keenan (2001), it is stated that it has been known since the 17th century that fat is enclosed in milk fat globules. The majority of fat globules (78% on average) are between 2-6 μm in diameter (Dolby, 1957). These milk fat globules are surrounded by the milk fat globule membrane (MFGM), which contains a much lower triacylglycerol proportion compared to that of milk fat. The reported values are 36.1% (Thompson *et al.*, 1961) and 61.7% (Bracco *et al.*, 1972). Fatty acid composition has been shown to be dependent on size of the globules, with significantly ($p<0.05$) more dodecanoic (C12:0), tetradecanoic (C14:0) and hexadecanoic (C16:0) acids found in smaller globules while larger globules contained more ($p<0.05$) octadecanoic (C18:0), *cis*-9-octadecanoic (C18:1) and *cis*-9,12-octadecanoic acid (C18:2) (Briard *et al.*, 2003). There is a substantial size difference in globules isolated from cream and skim milk, with the globules from cream two to three fold larger (mean of 3.00 μm from cream and 1.41 μm from skim milk) (Timmen & Patton, 1988).

2.2.1.3 Factors responsible for variability in fat and triacylglycerol content

The fat content of milk is variable, and can be influenced by factors such as: the interval between milkings (Friggens & Rasmussen, 2001), diet (Spain *et al.*, 1995), time of year (Overman, 1945; Dolby *et al.*, 1969; Phelan *et al.*, 1982; Shi *et al.*, 2001), stage of lactation (Auldist *et al.*, 1998) and breed (Hogan *et al.*, 1990), with Jersey having the highest and Holstein the lowest fat content among the major breeds (Blanchard *et al.*, 1966; Bruhn & Franke, 1977; Cerbulis & Farrell Jr., 1975). Furthermore, there are variations in fatty acid composition in milk from individual cows of different breeds (Breckenridge & Kuksis, 1967; Zegarska *et al.* 2001), those consuming different diets (Morales *et al.*, 2000; Collomb *et al.*, 2002; Reklewska *et al.*, 2002) and between seasons (Norris *et al.*, 1973; Auldist *et al.*, 1998).

2.2.1.4 Effect of heat processing on free fatty acids in milk

The effect of heat processing on the free fatty acid content has been investigated (Kintner & Day, 1965; Withycombe & Lindsay, 1969). Kintner and Day (1965) reported an average decrease in FFA in the order of 15% after

commercial pasteurisation (conditions not specified) while in the study by Withycombe and Lindsay (1969), FFA losses were on average 13% after high temperature short time (HTST), 16% after low temperature long time (LTLT) and 18% after ultra high temperature (UHT), processing conditions. The percentage reduction was proportional across each fatty acid with the different heat treatments and neither length of the carbon chain nor saturation were associated with percentage reduction. Withycombe and Lindsay (1969) postulate that these losses may be attributed to interactions and associations with other milk components in addition to thermal decomposition.

2.2.2 Proteins

Two categories of milk proteins are recognised. These are caseins and whey proteins. The major protein fractions in milk are α_{s1}-casein (α_{s1}-CN) and β-casein (β-CN) (Bloomfield & Mead Jr., 1975; Swaisgood, 1996), which, together with α_{s2}- (α_{s2}-CN) and κ-casein (κ-CN), comprise approximately 30 g of the solids per litre of milk, or 80% of all protein, while whey proteins constitute approximately 6.5 g/L of milk (Swaisgood, 1995). As outlined above, casein is comprised of α_{s1}, α_{s2}, β and κ forms, which do not exist in isolation in their native state, but - owing to their poorly developed tertiary structure - form large colloidal complexes known as casein micelles (Swaisgood, 1992). The whey proteins are a small and structurally diverse group, of which β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and the immunoglobulins comprise the majority (Whitney, 1999). The content of individual proteins is affected by breed, stage of lactation, and cow nutrition, which is often related to the season of the year.

Genetic polymorphism of milk proteins is now well characterised, although further findings in this area are still likely. Protein heterogeneity was suspected prior to the mid-1950s, for example from the work of Ogston and Tilley (1954), who provided substantial evidence for heterogeneity of β-LG. This was confirmed by Aschaffenburg and Drewry (1955), in the first report of heterogeneity of a milk protein. Genetic variants of each milk protein only vary by the alteration of a very small number of amino acids For example, the primary structure of genetic variant F of a_{s1}-CN varies from the primary structure

of genetic variant B by a single amino acid position in position 66 (Prinzenberg *et. al*., 1998). Ng-Kwai-Hang (2003) lists a total of 44 genetic variants, among all milk proteins, with κ-CN having the most variants, 11, of any one milk protein.

The variation in the composition of milk with regard to individual caseins is of importance, because specific caseins are more likely to be involved in particular organoleptic defects. For example, κ-CN has been implicated in age gelation (along with β-LG) (Datta & Deeth, 2001) and β-CN in bitterness (Habibi-Najafi & Lee, 1996), owing to the quantity of hydrophobic amino acid residues in the primary sequence of β-CN (Ribadeau-Dumas *et al*., 1972).

2.2.2.1 Structure of casein micelles

Micelles are aggregates of casein and minerals (Ginger & Grigor, 1999). Most (95%) micelles are 30-120 nm in size (Waugh & Talbot, 1971), although some are as large as 600 nm (Donnelly *et al*., 1984). More recently, Panouillé *et al.* (2004) stated that the average micelle is 160 nm in diameter.

The casein micelles comprise α_{s1}, α_{s2}, β, κ, caseins in the ratio of approximately 3:1:3:1 (Bloomfield & Mead Jr., 1975; Swaisgood, 1996), and were originally thought to be uniformly distributed throughout the micelle (Ashoor *et al*., 1971). It is now accepted that this is not the case, with κ-CN predominating on the surface and the other caseins mostly comprising the core (Fox & McSweeney, 1998). This view is supported by the work of many authors (Sullivan *et al*., 1959; Davies & Law, 1983; Donnelly *et al*., 1984; Dalgleish *et al*., 1989) who found that the percentage of κ-CN increased with decreasing micelle size, and therefore increasing surface area relative to the overall size of the micelle. In addition, treatment of casein micelles with chymosin reduces their radius by approximately 5 nm (Holt & Roginski, 2001). Such observations imply a link between κ-CN and surface area of the micelle; in fact, results from Dalgleish *et al.* (1989) show that very little β-CN is present on the micelle surface while a substantial proportion of κ-CN is present, as suggested by Payens (1966). This is in contradiction to the observations made by

Horisberger and Vauthey (1984) of a uniform distribution of κ-CN throughout the micelle.

The concept of a sub-unit structure for casein micelles was first suggested by Moor (1967). In this model, micelles consist of sub-micelles of approximately 15 nm in diameter held together by calcium phosphate bridges (Walstra, 1999) with this sub-unit structure giving a raspberry-like appearance. The description of the sub-unit model by Phadungath (2005) includes mention of exposed hydrophilic domains of κ-CN, which could be the protein chains that were observed by McMahon and McManus (1998). This model has largely been dismissed (Horne, 2006) with various other models having been suggested. Transmission electron microscopy (TEM) and scanning electron microscopy have produced variable results (McMahon & McManus, 1998) with TEM images in particular seemingly adding little to the debate on micelle structure. However, recent findings by a group utilising a field emission scanning electron microscope (Dalgleish *et al.*, 2004), indicate the absence of sub-micelles. Instead, Dalgleish *et al.* (2004) explains how caseins are organised into tubular structures within the micelle.

Criticism of the sub-micelle model by Holt (1994) which led to the development of the "tangled web" model, includes a lack of openness and some disorder. Calcium phosphate nanoclusters are the cornerstone of this hypothesis which involves attachment of both α_s- and β-CN, present in equal proportions. A major difference between the sub-micelle model and the model proposed by Holt (1992) is related to the type of bonds that hold the micelle together (Horne, 2006). In Holt's model, a fairly uniform matrix of polypeptide chains is held together in calcium phosphate nanoclusters, with the outer parts of the micelle consisting of κ-CN and hydrophilic domains of the remaining caseins (Holt & Roginski, 2001).

Work by Dalgleish *et al.* (2004) indicated, using FESEM micrographs, that many protrusions extend from the micelle surface, greatly increasing the surface area of the micelle over what was previously thought. They suggest that the concentration of micelle κ-CN would not be sufficient to cover the entire surface area, and that it is only present at the tips of the protrusions. It has

been proposed that 33% of the surface of the micelle is comprised of κ-CN (Dalgleish, 1998), which implies that other caseins are also present on the surface, a suggestion consistent with Holt's model.

The main function of κ-CN in milk is thought to be the prevention of micelle aggregation (Creamer *et al.*, 1998) and as early as the 1950s, the key role played by κ-CN in micellar stabilisation was recognised (Waugh & von Hippel, 1956). Casein micelles are negatively charged at the pH of raw milk, therefore repel each other electrostatically. In addition, they are surrounded by water dipoles ("hydration water"). With the removal of the hydrophilic f106-169 of κ-CN by chymosin or with neutralisation of negative charges on casein micelles, which occurs at pH 4.6, micelles can no longer stay apart and form a casein coagulum.

The structure of micelles is still being debated (Horne, 2006).

2.2.2.2 Variability in the content of individual proteins

Variation in β-LG content has been observed between breeds (Cerbulis & Farrell Jr., 1975), while variation in the content of individual caseins can vary with stage of lactation (Ostersen *et al.*, 1997). This variability in individual proteins could suggest the likelihood of a particular milk to undergo a specific organoleptic defect. For example, if a particular milk was richer in β-CN, then in theory, there could be an increased likelihood for that milk to become bitter, if hydrolysis of β-CN took place.

2.2.2.3 Effect of heat on proteins

There is a marked difference in heat sensitivity between the caseins and whey proteins. Caseins are remarkably resistant to heat (Fox & McSweeney, 1998). The most heat-sensitive milk protein is β–LG (Fox & McSweeney, 1998). The most heat-tolerant whey protein is α-LA (de Wit & Klarenbeek, 1984), which has a relatively low denaturation temperature but a high degree of renaturation (Rüegg *et al.*, 1977). Hansen and Melo (1977) reported that after UHT processing 97% of the original casein remained soluble compared to whey proteins, where only 44% of α-LA and 32% of β-lactoglobulin remained soluble.

Maximum denaturation of many whey proteins occurs at approximately 100 ºC, with further heating having little effect on additional denaturation (Law *et al.*, 1994). During heat treatment of milk, interactions occur between whey proteins and the casein micelles, primarily dependent on complex formation between β- and κ-CN (Hill, 1989) through disulphide bonds. Formation of these complexes is both temperature (above 70 ºC) and pH (above 6.7) dependent (Anema *et al.*, 2004).

2.2.3 Indigenous hydrolytic enzymes of importance in dairy technology

There are approximately 70 known indigenous enzymes in milk at present (Fox and Kelly, 2006), with 30 known in the late 1980s (Grufferty & Fox, 1988). Two of the most important in dairy technology are lipoprotein lipase, and the indigenous milk protease, plasmin.

2.2.3.1 Lipoprotein lipase

There was much debate over the presence of an indigenous lipase in bovine milk early in the 20th century (Palmer, 1922; Rice & Markley, 1922). Subsequently, the existence of multiple indigenous lipases was apparently demonstrated (Albrecht & Jaynes, 1955; Tarassuk & Frankel, 1957), before the single indigenous lipase was first purified by Egelrud and Olivecrona (1972). It is a dimer of 96.9 kDa (Iverius *et al.*, 1976), much of which is associated with the casein micelles (Downey & Andrews, 1966). Optimal activity is observed at pH 9.0 and 37 ºC (Frankel & Tarassuk, 1956). The optimal pH appears linked to inactivation of the enzyme at higher pH values, which results in decreased activity with increasing pH above 9.0 (Bengtsson & Olivecrona, 1982). Characteristically, the enzyme does not discriminate between saturated and polyunsaturated long-chain fatty acids as well as medium-chain fatty acids in the triacylglycerol molecule (Morley & Kuksis, 1977), although there is positional specificity within the molecule, typically for the *sn*-1 position (Morley & Kuksis, 1972). Ouattara *et al.* (2004) demonstrated that *cis*-9-octadecanoic acid (C18:1) was clearly the fatty acid that increased in concentration most during raw milk incubation, with hexadecanoic acid (C16:0) second. This would be

due to the fact that the main fatty acids in milk, which are hexadecanoic (C16:0) and *cis*-9-octadecanoic (C18:1), are primarily located in the *sn*-1 position, which is the position that lipoprotein lipase preferentially hydrolyses (Morley & Kuksis, 1972). Spontaneous lipolysis can occur in raw milk as a result of lipoprotein lipase action (Kitchen & Cranston, 1969; Olivecrona *et al.*, 2003) and due to the high turn-over rate of the enzyme, milk could develop lipolysed flavour in 10-20 sec (Driessen, 1989). However, spontaneous lipolysis is usually prevented by the integrity of the milk fat globule membrane (Sundheim & Bengtsson-Olivecrona, 1987). Susceptibility of a given milk sample to spontaneous lipolysis is linked to various factors (Cartier & Chilliard, 1990), which include fat concentration of the milk (Deeth & Fitz-Gerald, 1977) and diet of the cow (Stobbs *et al.*, 1973). In addition, external factors can also contribute considerably. For example, activation of spontaneous lipolysis by agitation has been known for a long time (Krukovsky & Sharp, 1938). The degree of activation can be influenced by a range of factors including temperature (Krukovsky & Herrington, 1939; Kitchen & Aston, 1970; Fitz-Gerald, 1974; Sundheim & Bengtsson-Olivecrona, 1987), formation of foam during agitation (Tarassuk & Frankel, 1955) along with speed (Deeth & Fitz-Gerald, 1977) and duration (Deeth & Fitz-Gerald, 1978) of agitation. The minimum pasteurisation standard in Australia consists of a 15 second heat treatment at 72 ºC (FSANZ, 2006). Shipe and Senyk (1981) found that residual activity remained following pasteurisation conditions (72 ºC/16 sec) similar to the minimum standards in Australia, with complete inactivation of the enzyme taking place after pasteurisation at 77 ºC for 16 sec, therefore lipoprotein lipase can be considered a reasonably heat-labile enzyme (Farkye & Imafidon, 1995). As a consequence, this enzyme is mostly of importance in raw milk.

2.2.3.2 Plasmin

There are several proteases indigenous to milk, of which the major one is plasmin, an alkaline serine proteinase (Fox & Kelly, 2006). In addition to

plasmin, the plasmin system involves plasminogen (the plasmin precursor), plasminogen activators as well as inhibitors of plasminogen activators and plasmin (Kelly & McSweeney, 2003). Purification of plasmin was first reported by Dulley (1972) who also established temperature (37 ºC) and pH (6.5-9.0) optima. In addition, Dulley (1972) also estimated the molecular weight of the enzyme, at approximately 100 kDa. Also published in that year (Kaminogawa *et al.*, 1972), was a comparative study of "plasmin" with "bovine milk protease", where a much lower molecular weight, 48 kDa, was reported. Clearly, there was some confusion during that period as to the nature, and the number of major proteases indigenous to milk, because in Kaminogawa *et al.* (1972), the name plasmin seems, at times, synonymous with plasminogen. However, Eigel *et al.* (1979) stated that plasmin was identical to the bovine alkaline protease and that plasminogen was a precursor for plasmin. The level of both plasmin and plasminogen have been found to vary with stage of lactation, with a higher level in late lactation milk (Korycka-Dahl *et al.*, 1982; Richardson, 1983), while plasmin activity also varies between breeds (Schaar, 1985). In contrast to lipoprotein lipase, plasmin is highly heat-stable. Pasteurisation (time/temperature unknown) had little effect on plasmin activity (Harper *et al.*, 1960) while Metwalli *et al.* (1998) observed approximately 22% retention of activity, following a heating slightly in excess (74 ºC) of the minimum pasteurisation temperature (72 ºC) for around 10min, and Driessen (1989) reported a decimal reduction time (D value) of 35 min 42 sec at 73 ºC and of 7 sec at 142 ºC. Action of plasmin on α_s-CN and κ-CN has been shown to be similar, while hydrolysis of β-CN was considerably lower (Yamauchi & Kaminogawa, 1972). This is, however, in contradiction to the findings of Andrews (1983) who observed action on α_{s1}-CN and β-CN to be similar and to the observation of Reimerdes (1981) that β-CN is the preferred substrate when testing for the alkaline serine protease in milk.

2.3 Ultra high temperature processing of milk

In the early 1950s, experimental UHT processing was underway in the United States (Tobias, 1955), while in Switzerland, UHT-type processing was

introduced commercially in 1953 (Zadow, 1993a; 1998). Once the problem of aseptic filling had been addressed (Zadow, 1975), commercial UHT processing commenced in Australia around 1964 (Zadow, 1993a; 1998). This followed research and development around the world on aseptic processing since around the 1870s (Hostettler, 1972a). Two variations of UHT treatment are in use: direct and indirect. A detailed description of these two techniques is provided by Datta *et al.* (2002) who also cover effects on milk while Jelen (1982) discusses both direct and indirect techniques from various perspectives of industrial relevance. The basis of the direct heating system is the direct mixing of steam with milk. This can be achieved in one of two ways: either steam injection, where steam is injected into the milk or steam infusion, in which milk enters an infusion chamber containing superheated steam. Indirect UHT treatment occurs when milk is heated by contact with steam or superheated water across the metal surface of a heat exchanger. The major difference between the two forms of UHT treatment is the temperature profile during treatment. Direct UHT treatment is less severe because an almost instantaneous rise and fall in temperature takes place, whereas the increase and decrease in temperature in milk heated indirectly is considerably more gradual, therefore temperatures are elevated for a longer period. Severity of indirect heating is reflected by the degree of denaturation of whey proteins, with more than twice the amount of β-LG denatured in UHT milk processed indirectly compared to that directly treated (Zadow, 1969).

A myriad of nutritional and organoleptic changes have been observed in milk following UHT processing, some of which are unique to this product (Niroumand, 1977). Changes to proteins induced by ultra high temperature (UHT) processing can be detrimental to stability of UHT milk products. This is primarily due to formation of a complex between β-LG and the micelle surface proteins (predominately with κ-CN), in addition to interaction of β-LG with α-LA, which all occur at even moderate temperatures (Fairise *et al.*, 1999), even as low as 80 ºC (Cho *et al.*, 2003). Pasteurisation is the most widely used heat processing method for raw milk, leading to reduction in numbers of spoilage bacteria and elimination of pathogenic bacteria. Higher temperatures (between

135 and 150 °C) can be applied for a (usually) shorter duration (between two and 20 sec) (Ahrné, 1988), for more effective control of bacterial contaminants. This form of heat processing is ultra high temperature (UHT) treatment which provides a very long shelf life, compared to pasteurised milk, even when stored at ambient temperatures. Such processing conditions give "commercial sterility". This aspect is discussed by Luck (1975) and Zadow (1980) and implies that, theoretically, all microorganisms have been eliminated, but in practice this is not attainable and therefore commercial sterility is a measure of the failure rate (Dentener, 1986). Dentener (1986) also explains that processors usually strive for failure rate of no more than one in 5000.

2.4 Spoilage of UHT milk

Spoilage encompasses a range of causes: biological, chemical or physical. A food product is spoiled at the end of shelf life, therefore it is useful to consider what constitutes "shelf life". A definition of shelf life relevant to milk, has been suggested by Smithwell and Kailasapathy (1995) as: "the period between packaging and when milk becomes unacceptable to consumers". As a result of the high heat applied in UHT processing of raw milk, microbial load of UHT milk is insignificant. Spoilage due to enzyme activity is more important. However, microbial contamination does occur, albeit infrequently, as absolute sterility in a UHT product cannot be guaranteed.

2.4.1 Spoilage due to the presence of vegetative bacterial cells or spores

Mesophilic, psychrotrophic and psychrophilic microorganisms are usually eliminated during UHT treatment; heat resistance enabling survival of these microorganisms is extremely unusual. However, spoilage is possible through the presence of mesophilic organisms in the finished product. For example, there is an isolated report of UHT milk spoilage due to growth of *Enterobacter* sp. and *Micrococcus* sp., which demonstrated remarkably high levels of heat resistance (Antoine & Donawa, 1990). It is possible that in this particular case time/temperature combination typical of the UHT process was not achieved. In some countries, contamination of UHT milk does appear to be a major problem.

For example, in Egypt, some samples of UHT milk had a total bacterial count of up to 10^5 cfu/mL (El-Samragy *et al.*, 1992). Due to the heat treatment conditions typical of UHT processing, thermophilic and thermoduric microflora or their spores are most often responsible for contamination of UHT milk (Foschino *et al.*, 1990; Lewis, 1999). Although these bacteria are present in freshly drawn raw milk in low numbers (Flint *et al.*, 2001), they may be present in long-life dairy products (Westhoff & Dougherty, 1981), and are very important owing to their potential involvement in spoilage of those products, which has been recognised for a long time (Grinstead & Clegg, 1955). *Bacillus sporothermodurans* has been one of the most frequently implicated (Klijn *et al.*, 1997). As a result of the extreme heat resistance of its spores (Huemer *et al.*, 1998), which may lead to the possibility of spore survival during UHT processing and subsequent germination in UHT milk (Pettersson *et al.*, 1996), it can be regarded as one of the most important. Also, high levels of *Geobacillus stearothermophilus* vegetative cells may cause flat sour spoilage of low-acid foods, including milk (Ito, 1981), especially evaporated milk (Kalogridou-Vassiliadou *et al.*, 1989). It is not unreasonable to expect one in 100 000 1 L packs of UHT milk to be contaminated with thermophilic or thermoduric bacteria, which is considered acceptable (Luck, 1975), although a total count of 10^2 cfu/mL after 15 d at 30 ºC also seems to be acceptable for UHT milk (Lewis, 1999), which would appear odd as this does seem significant contamination. Although many factors can be influential, the single most important factor in ensuring commercial sterility is a strict maintenance schedule (Farahnik, 1982).

2.4.2 Spoilage due to the action of heat-stable hydrolytic bacterial enzymes

UHT treatment is effective in achieving commercial sterility and a long shelf life at room temperature, but the high temperatures used cannot inactivate some hydrolytic enzymes, particularly lipases and proteases. These enzymes can be either those native to milk (such as the native milk protease, plasmin), or produced by microorganisms. As a result of their high heat stability, these lipases and proteases can retain activity, sometimes at a substantial level, following UHT treatment. During long term storage of the product, a range of

defects arising from proteolytic and lipolytic activity can be seen (Rowe & Gilmour, 1985).

Heat treatment to completely inactivate hydrolytic enzymes is extreme. For example, it is not unusual for the D values for inactivation of lipases and proteases of *P. fluorescens* to be approximately two to three minutes at 140 ºC (Andersson *et al.*, 1979; Mitchell *et al.*, 1986), the typical temperature of UHT processing, but for a much shorter time. Temperatures and/or times to inactivate these enzymes would adversely affect the quality of the product and therefore cannot be applied (Renner, 1988). Therefore, UHT milk is quite vulnerable to enzymatic spoilage (Craven *et al.*, 2001), and in fact, enzymatic spoilage is regarded as the most important form of spoilage in UHT products (Renner, 1988; Stepaniak, 1991), as a consequence of the heat stability of the enzymes produced by the contaminating bacteria in the raw product. A possible alternative to traditional UHT treatment, which has resulted in a substantial inactivation of hydrolytic enzymes, is standard UHT treatment followed by 5 min at between 57 and 65 °C (Bucky *et al.*, 1987a; Bucky *et al.*, 1988) or 60 min at 55 °C (West *et al.*, 1978); however, such treatment has little effect on the onset of age gelation (Kocak & Zadow, 1985b). These additional heat treatments are costly, and double heat processing of milk is not allowed in some countries. Furthermore, due to variation in degree of inactivation between isolates, the additional heat treatments may have little practical value (Griffiths *et al.*, 1981). In addition, combinations of storage temperature, pH, water activity and heat treatment devised by Braun *et al.* (1999), will inhibit the activity of lipases and proteinases produced by *P. fluorescens*. In addition, a combination of heat treatment (lower than that of standard UHT) with nisin has been found to be very effective in spoilage control while eliminating any undesirable heat effects (Lewis, 1999).

Bacterial proteinases in general have been found to play a leading role in milk spoilage (Guinot-Thomas *et al.*, 1995a); in particular, in the spoilage of UHT milk (Oakenfull *et al.*, 1997). Age gelation is one important spoilage defect often attributed to proteolytic activity, but a range of other defects do occur, including bitterness and sedimentation (Datta *et al.*, 2002). In addition,

accumulation of deposits when aged skim milk is heated has been suggested to be caused by the action of proteolytic enzymes (Jeumink, 1991).

Action of lipase results in various off-flavours in UHT milk, widely known and generally described as rancidity. Such terminology is ambiguous as rancidity can also refer to the spoilage defect resulting from lipid oxidation and therefore “lipolysed flavour” is the descriptive term given to the off-flavours resulting from enzymatic lipolysis (Shipe *et al.*, 1978).

2.4.3 Age gelation

Age gelation is widely recognised as the major factor limiting the shelf life of UHT milk. Several hypotheses still seek to provide an explanation of the mechanism of age gelation, which is not yet fully understood. Some (Andrews *et al.*, 1977; Manji & Kakuda, 1988; McKenna & Singh, 1991; Venkatachalam *et al.*, 1993) have concluded that gelation is a two-stage process and has both an enzymatic and a physicochemical basis. However, the most convincing idea to have been presented is the enzymatic theory proposed by McMahon (1996) in which three major steps have been postulated - starting with the casein micelle with κ-CN on its surface: I) covalent bonding of β-LG to κ-CN on the surface of the micelle to form beta-kappa (βκ) complexes, II) dissociation of the βκ complex from the micelle, by proteolysis, III) cross-linking of the βκ complex to form aggregations and finally a gel.

The type of genetic variant of β-LG present appears to play a role in this process as they have different heat sensitivities (McKenzie *et al.*, 1971). Furthermore, while there is widespread acknowledgement of the possible role of proteases from *Pseudomonas* spp. in age gelation, the contribution from proteases of other bacteria present in raw milk should not be discounted (Keogh & Pettingill, 1982).

While there is generally only one enzyme-based hypothesis to explain age gelation, a number of non-enzymatic mechanisms of gelation have been put forward, some of which are detailed below:

Heat induced complex formation

(Hostettler, 1972b)

The basis of this theory is that the nature and extent of heat treatment is critical to determining the degree of complex formation between whey proteins and caseins. With the application of severe heat treatment to milk (such as retort sterilisation - autoclaving), advanced and irreversible interactions occur between the whey proteins and caseins. Covalent bonding, primarily through disulphide bridges, is the likely basis of these interactions. This protects casein micelles from direct association which could lead to aggregation and other structural changes. The use of milder heat treatment results in formation of a reversible complex, and as a result, the micelles have the opportunity to interact directly. This is particularly so because UHT treatment does not transform the micelles, thus allowing them to remain in an intermediate state of instability.

Evidence against this idea is that age gelation was still observed in milk heat treated by the retort method.

Chemical modification of micelles

(Andrews & Cheeseman, 1971; Andrews & Cheeseman, 1972; Andrews, 1975)

During prolonged storage of UHT milk Maillard-type reactions can lead to polymerisation of caseins and whey proteins. The changes to the casein component associated with the Maillard-type reactions in model systems have been found to be similar to those that occur during prolonged storage of UHT milk. The rate of Maillard-type reactions increases with with a rise in temperature, but as age gelation was not observed above 35 °C, this theory is less convincing. Lactose interactions block ε-NH_2 groups in lysine residues, thereby preventing gelation. However, substitution of lactose with sorbitol has been found to result in gelation. All these modifications are presumed to alter the charge on casein micelles.

Free energy change

(Graf & Bauer, 1976)

In this hypothesis, micelles are said to be in a metastable state with a high surface potential. There is a progressive, spontaneous transformation from the unstable high free energy potential state to the low potential state, which is thermodynamically stable. This transformation in potential occurs by

random potential jumps, and it is the difference in the surface potential that promotes aggregation. Intense heat treatment or additives, particularly sodium hexametaphosphate, can decrease changes in surface potential, therefore reducing the likelihood of aggregation.

Plastein-induced mechanism

(Pande & Mathur, 1994)

As early as the start of the 20th century, the term "plastein" was introduced. It referred to a gel-like product formed over time in an enzymatic protein hydrolysate and it was based on the observations of Danilewski, Okunew and Sawjalow (Sawjalow, 1901). In the early 1920s, a study was carried out by Beard (1927) who confirmed that plastein was a protein, by its nutritional value in feeding experiments with mice. Wasteneys and Borsook (1930) stated that the coagulum formed in the plastein reaction was indeed a protein and Alcock (1936) concluded that the reaction involved proteases. Horowitz and Haurowitz (1959) stated that plastein formation was often considered to be the reversal of proteolysis. This did seem likely, based on the final product of the plastein reaction. Goepfert *et al.* (1999) carried out an investigation into the mechanism of synthesis of a peptide reported by Lorenzen *et al.* (1997) to have occurred during proteolysis, and established that a new peptide bond was formed through transpeptidation. Although appearing conclusive, one puzzling observation remains, namely that made by Horowitz and Haurowitz (1959). In their study, plastein formation was not accompanied by a decrease in FAG content, which would suggest that new peptide bonds were not formed. However, the method of FAG determination may not have been sensitive enough to reflect small changes in FAG content.

Pande and Mathur (1994) postulate a three-stage process for gelation in UHT milk, involving the plastein reaction. Firstly, proteolysis results in degradation of casein. The second stage is also enzymatic, with plastein products synthesised through peptide bond formation from the peptides produced in first-stage proteolysis. In the third stage, the hydrophobic peptides formed in the plastein reaction aggregate, ultimately leading to age gelation. A

key aspect, which must be considered are the conditions which are prerequisites for plastein formation. Watanabe and Arai (1988) have stated that three factors are essential for plastein formation: I) the peptides must be of low molecular weight, II) the substrate concentration in the range of 20 to 40% (w/v) and III) a pH between four and seven. Therefore, a plastein-development mechanism for age gelation appears quite unlikely based on the conditions found in UHT milk, where peptides are present at a far lower concentration than that required for plastein synthesis.

2.5 Importance of psychrotrophic bacteria in the spoilage of dairy products

Prior to introduction of refrigerated bulk milk collection, temperature control of stored raw milk was not tightly regulated, with milk held at ambient temperatures for varying periods. Consequently, spoilage by lactic acid bacteria was common (Cox, 1993). The introduction of bulk refrigerated raw milk storage, which on Australian dairy farms took place in the early 1960s (Tarrant, 1961), has virtually eliminated spoilage by lactic acid bacteria (Law, 1979) but has also provided conditions selective for the growth of psychrotrophic bacteria. Originally, these bacteria were erroneously classified as psychrophilic (Ingraham & Stokes, 1959). They can be defined as those being able to grow at temperatures below 8 ºC regardless of their optimal growth temperatures (Cousin, 1982). Many psychrotrophic bacteria produce heat-resistant hydrolytic enzymes which are capable of degrading fat and protein, the major solid components of milk. Owing to the conditions which select for the growth of psychrotrophic bacteria becoming widespread, enzyme-related spoilage of dairy products has increased in prevalence and importance, particularly with the advent of long-life dairy products, especially UHT milk.

2.6 Characteristics of lipases and proteases isolated from *Pseudomonas* spp.

Much research involving purification and characterisation of lipases and proteases was carried out during the 1980s; the reviews in a monograph edited by McKellar (1989) are excellent sources of information. One important shared characteristic of both lipases and proteases is the phase of culture growth

during which they are produced. Production of both proteolytic and lipolytic enzymes in milk has been observed only once the culture has reached late exponential or stationary phase (Griffiths, 1989; Jaspe & Sanjose, 1999; Stevenson *et al*, 2003). Increased production of both lipase and protease takes place at a lower temperature, often 15 to 20 ºC (McKellar, 1982; Griffiths, 1989), which is below the temperature recognised for optimal growth, typically 25 to 30 ºC, as these bacteria occupy an environmental niche. While details will be provided in the sections that follow, it is interesting to consider the suggestion of Villafafila *et al.* (1993), who speculate that the increased rate of lipase and protease synthesis compensates for their decreased activity at temperatures below the optimal temperature for growth.

2.6.1 Lipases

Bacterial lipases are responsible for much of the lipolysis in heat-processed (UHT) milk, whereas lipoprotein lipase is responsible for most of the lipolysis in raw milk. This can mostly be attributed to the heat stability of bacterial lipases and the heat sensitivity of lipoprotein lipase.

2.6.1.1 Location and number of lipases produced

Pseudomonas spp. are widely known to produce one lipase (Mencher & Alford, 1967; Bozoglu *et al*., 1984), with production of multiple lipases rare. However, Fox and Stepaniak (1983) reported the production of three lipases by *P. fluorescens* AFT36, while *P. fluorescens* C9 produced two lipases (Beven *et al*., 2001). There are descriptions in the literature of intracellular lipase production, such as by *P. fragi* (Schuepp *et al*., 1997) and *P. fluorescens* (Beven *et al*., 2001), which has also been reported to have produced an extracellular lipase.

2.6.1.2 Molecular mass

The typical size of lipases is in the range of 25-50 kDa (Deeth & Fitz-Gerald, 2006). Some exceptions have been reported, such as 16 kDa (Dring &

Fox, 1983), 55 kDa (Bozoglu *et al*., 1984), 67 kDa (Kojima & Shimizu, 2003) and 155 kDa (Bucky *et al*., 1987b), all from different isolates of *P. fluorescens*.

2.6.1.3 Heat stability

A high degree of heat stability of lipases produced by *P. fluorescens* has been reported. After HTST pasteurisation, loss of activity was minimal (Nashif & Nelson, 1953), and is reported to be as low as 1.5% for *P. fluorescens* (Kalogridou-Vassiliadou, 1984), while 20% loss of activity was seen with heat treatment in excess of normal UHT treatment (Adams & Brawley, 1981). Maximum loss (99%) of activity of the lipase from *P. fluorescens* NC1 was reported following low temperature, long time (LTLT) pasteurisation of 63 ºC for 30 min, with minimum loss (41%) after UHT treatment at 150 ºC for 1 sec (Abad *et al*., 1993). From many reports of activity loss of lipases, this appears a typical situation. Some lipases from *Pseudomonas* spp. are termed "cold-active" and are characterised by their heat sensitivity (Alquati *et al*., 2002), in contrast to the lipases of most *Pseudomonas* spp. A substantial loss of activity at relatively mild temperatures (50-60 ºC), has been reported (Schokker & van Boekel, 1998), although this inactivation is not consistent across isolates (Griffiths *et al*., 1981; Fitz-Gerald *et al*., 1982).

2.6.1.4 Optimal temperature

While lipases from *Pseudomonas* spp. exhibit maximum activity between 35 °C and 45 °C (Mencher & Alford, 1967; Kumura *et al*., 1993), 37 °C is the optimal temperature for activity of lipases from pseudomonads isolated from milk (Cousin, 1982; Fox *et al*., 1989). Exceptions to the range mentioned above have been noted. In early work on *P. fragi* by Lu and Liska (1969), 54 °C was the optimal temperature while Wahab *et al.* (1999) has reported 80 °C as the optimal temperature for the lipase from *P fragi* CRDA 037. Reports of pseudomonad lipases or protease that are optimally active below 35 °C are rare. Therefore, the reports of the heat-stable lipase from *P. fragi* IFO 3458 (optimal temperature 29 °C) (Alquati *et al*., 2002) and *P. fluorescens* AFT29 (optimal temperature of 22 °C) (Dring and Fox, 1983) are of special note.

2.6.1.5 Optimal pH

The pH range of bacterial lipases is wide, with most falling between pH 6.5-8.5 (Mencher & Alford, 1967; Dring & Fox, 1983; Pabai *et al.*, 1995; Abdou & Ohashi, 1996). With any single isolate, the optimal pH is usually within a small range. An exception, for *P. fragi*, was reported by Lawrence *et al.* (1967) and Wahab *et al.* (1999), where optimal activity was seen between pH 8.5 and 10.0. This optimal pH range has also been reported for *P. fluorescens* (Kojima *et al.*, 1994).

2.6.1.6 Factors influencing production

During the course of culture growth, lipase production can vary. Fluctuations in lipase production in milk during prolonged incubation (up to 60 d) of *P. fluorescens* have been reported (Stead, 1987). This involved lipase production, proteolytic degradation of lipase and an increase in production once again (Stead, 1987).

The optimal temperature for lipase synthesis has been reported at around 15-22 ºC for many isolates (Alford & Elliott, 1961; Lawrence *et al.* 1967; Juffs, 1976; Sztajer & Maliszewska, 1988; Griffiths, 1989; Merieau *et al.*, 1993; Burini *et al.*, 1994; Makhzoum *et al.*, 1995), although some isolates produce lipase optimally below 10 ºC (Andersson, 1980; Bucky *et al.*, 1986) while others at a temperature as high as 25 ºC (Harris *et al.*, 1990).

From the work of Alford and Elliott (1961) as well as Malik *et al.* (1985), pH values just above 7.0 are optimal for lipase production.

A comprehensive investigation by Makhzoum *et al.* (1995) demonstrated the effect of various amino acids, nitrogen sources, carbon sources, organic acids, sugars, triacylglycerols, free fatty acids, phosphate, metal ions and antibiotics on lipase production. Iron(III) chloride for example, delays the commencement of lipase production by *P. fluorescens* in raw milk (Fernandez *et al.*, 1992), while the pyoverdine-based iron acquisition system appears to be closely linked with lipase production in this species (Fernandez *et al.*, 1988), by stimulating production (McKellar *et al.*, 1987). Furthermore, various compounds

normally regarded as preservatives have a demonstrated inhibitory effect on lipase production while not necessarily inhibitory to growth (Andersson *et al.*, 1980). Matselis & Roussis (1992) reported much higher production in skim milk compared with synthetic media (nutrient broth). In contrast, Bucky *et al.* (1987b) noted the reverse situation when using half strength peptone water as the synthetic medium.

Aeration can have a major effect, whether due to agitation of cultures or flushing with air. For example, Myhara and Skura (1990) report that the percentage of oxygen present in cultures of *P. fragi* was the most significant growth parameter influencing production of lipase. There are several reports (Dring & Fox, 1983; Birkeland *et al.*, 1985; Malik *et al.*, 1985; Bucky *et al.*, 1986) describing how static incubation restricts lipase production compared to shaken cultures available while Jaspe *et al.* (2000) describes the same effect with air-flushing.

Atmospheric parameters also play a role in hydrolytic enzyme production, as demonstrated by Rowe (1988). Addition of carbon dioxide to the culture delayed the commencement of lipase production and resulted in the final production of lipase approximately 50% below the control culture.

2.6.1.7 Specificity

Lipases from many bacteria preferentially hydrolyse fatty acids at a particular position on the triacylglycerol molecule (Alford *et al.*, 1964). These positions are often the *sn*-1 and *sn*-3 positions (Rashid *et al.*, 2001), and because positional specificities exist, for example, butanoic acid (C4:0) is found only in the *sn*-3 position (Christie & Clapperton, 1982), this has implications for the pattern of fatty acid release and sensory perceptions of that product. Lipolytic activity of *P. fragi* most often results in release of butanoic (C4:0) and hexanoic (C6:0) acids (Shipe *et al.*, 1978), with the predominance of butanoic acid (C4:0) in the *sn*-3 position (Christie & Clapperton, 1982), such a result is understandable. Triacylglycerols containing either of these two fatty acids only were the preferred substrate for *P. fluorescens* HU380 (Kojima & Shimizu, 2003).

2.6.2 Esterases

Esterase production in food-spoilage *Pseudomonas* spp. has been observed for nearly 40 years, since the work of Reddy *et al.* (1970). These enzymes, from pseudomonads, appear to be optimally active in conditions similar to lipases (Khalameyzer *et al.*, 1999; Suzuki *et al.*, 2003). However, they are distinguished from lipases by their substrate preference. Esterases demonstrate a preference for hydrolysis of ester bonds of shorter chain fatty acids whereas lipases possess a much wider substrate range (Fojan *et al.,* 2000). Suzuki *et al.* (2003) carried out an investigation of an esterase from a psychrotrophic pseudomonad. In this study, activity was seen towards a wide range of chain length of fatty acids, although activity peaked with *p*-nitrophenyl butyrate as substrate, with a considerable activity observed. The substrate preference of esterases was employed by Brand *et al.* (2000) who developed a lipase assay that possessed a ten-fold increase in sensitivity for detection of lipase over esterase. Owing to this substrate preference for short-chain fatty acids, and the observation by Al-Shabibi *et al.* (1964) that these fatty acids contribute little to lipolysed flavour in milk, it is unlikely that esterases make a perceptible contribution to lipolytic spoilage of dairy products.

2.6.3 Proteases

P. fluorescens is widely acknowledged as the most important proteolytic psychrotroph in dairy products, although *P. fragi* and other species do produce protease(s). However, when compared to *P. fluorescens*, *P. fragi* is much less proteolytic (Alanis *et al.*, 1999).

2.6.3.1 Location and number of proteases produced

As with production of lipase, many authors have reported pseudomonads producing one protease, for example Mayerhofer *et al.* (1973), Noreau and Drapeau (1979) and Richardson (1981). Nevertheless, encountering isolates capable of secreting multiple proteases is not uncommon. For example, some isolates of *P. fluorescens* can produce three (Petersen &

Gunderson, 1960; Stepaniak *et al.*, 1987) and other isolates, five (Leinmuller & Christophersen, 1982; Stepaniak & Fox, 1985) proteases.

2.6.3.2 Molecular mass

The majority of proteases are in the size range of 40-50 kDa (Yan *et al.*, 1985; Gobbetti *et al.*, 1995; Schokker & van Boekel, 1998), with few exceptions reported. Possibly one of the smaller proteases described was 33 kDa in size, produced by *P. fluorescens* (Azcona *et al.*, 1988; Kojima *et al.*, 1994). Proteases composed of subunits have been observed. Yasuda *et al.* (1992) reported a pseudomonad protease of 60 kDa, comprised of four subunits of identical size.

2.6.3.3 Heat stability

In any given isolate, proteases are generally more heat-stable than lipases (Braun & Fehlhaber, 2002). A particular feature of industry relevance is the heat stability of pseudomonad proteases. In a comparison between different genera, proteases of *Pseudomonas* spp. often possess a higher heat stability compared to proteases of other genera (Khurana *et al.*, 1989). Similar to lipases, some proteases are heat sensitive at relatively mild temperatures, such as 55 °C (Baral *et al.*, 1995).

2.6.3.4 Optimal temperature

The optimal temperature for activity of pseudomonad proteases is generally between 35 and 45 °C (Fortina *et al.*, 1989; Koka & Weimer, 2000b; Kojima & Shimizu, 2003), although those produced by *Pseudomonas* spp. isolates originating from milk have an optimal temperature for activity of 37 °C (Cousin, 1982; Fox *et al.*, 1989). Two of the most notable exceptions to this were optimal temperatures of 60 °C (Yasuda *et al.*, 1992) and 65 °C (Malik & Mathur, 1984). Other less extreme exceptions, slightly beyond the range given above, are more likely to be at higher temperatures. For example, the temperature of optimal activity for the *P. fluorescens* B52 protease is between

45 and 50 °C (Richardson, 1981), and for *P. fluorescens* 22F, at 49 °C (Schokker & van Boekel, 1998).

2.6.3.5 Optimal pH

Proteases tend to possess most activity at a slightly alkaline pH (Richardson & Te Whaiti, 1978; Fortina *et al.*, 1989; Kojima & Shimizu, 2003), with few reports of optimal activity outside of the range 7.0 to 8.5. However, notable exceptions do exist, such as the optimal pH of 5.0 for *P. fluorescens* RO98 (Koka & Weimer, 2000b) and 9.8 for *P. fluorescens* 22F (Schokker & van Boekel, 1998). In addition, the protease from *P. fluorescens* P1 demonstrated optimal activity between pH 6.0 and 8.0 (Stepaniak *et al.*, 1987), which is an unusually wide range.

2.6.3.6 Factors influencing production

A myriad of factors can affect production of proteases, in a similar fashion to production of lipases. The optimal temperature for protease production is essentially the same as for lipase production. Many isolates have been reported to produce most protease at 15 to 22 ºC (Mayerhoffer *et al.*, 1973; Juffs, 1976; McKellar, 1982; Malik *et al.*, 1985; Fairbairn & Law, 1987; Nicodeme *et al.*, 2005). However, temperatures as high as 27 ºC (Al-Saleh & Zahran, 1997) or as low as 0 ºC (Peterson & Gunderson, 1960) have also been reported for optimal protease production.

In addition, another commonly shared feature is stimulation of their production by specific metal ions, in particular Ca^{2+} (Alquati *et al.*, 2002; Liao & McCallus, 1998; Rashid *et al.*, 2001). Iron(III) chloride has delayed the commencement of production of protease by *P. fluorescens* in raw milk (Fernandez *et al.*, 1992) while the pyoverdine-based iron acquisition system stimulates production of protease (McKellar *et al.*, 1987).

Chemical composition of the growth media also affects protease production. Juffs (1976) observed that presence of glucose or sodium lactate had various effects on protease production in synthetic media while Rowe and Gilmour (1983) observed a strong synergism between whey proteins and

casein, in the increase of protease production, when added to a minimal medium. Furthermore, Matselis and Roussis (1992) found much higher production of protease in skim milk compared with synthetic media (nutrient broth).

Fairbairn and Law (1987) observed both stimulation and inhibition of protease production with aeration, at different phases of growth. With aeration, increased protease production was seen initially. The production increased rapidly before a reduction to a level which remained constant. However, in the static-incubated cultures, a slow gradual increase in protease production took place, with the maximum level higher in the static-incubated culture than the maximum in the aerated culture. Related to aeration, addition of carbon dioxide to a simulated milk medium delayed production of protease by *P. fluorescens* B52, although the original maximum level of production was achieved eventually (Rowe, 1988).

2.6.3.7 Specificity

A number of researchers have found κ-CN to be more often (but not universally) preferentially hydrolysed by *P. fluorescens* proteinase compared to other caseins (reviewed by Sorhaug & Stepaniak, 1991). Further specificity can be established, down to the level of a particular region within the protein, as Recio *et al.* (2000) found in a study on proteolysis of κ-CN. Little (Mitchell & Marshall, 1989), or no (Mitchell & Ewings, 1985) whey proteins are hydrolysed by *Pseudomonas* spp. proteinases. This is true for many, but not all isolates (Adams *et al.*, 1976). This is because wide variations in their ability to hydrolyse the whey proteins are possible (Gebre-Egaziabher *et al.*, 1980).

2.7 Existing lipase and protease assays

Lipase and protease assays can be used to either quantify enzyme activity or progress of hydrolysis and the distinction between measuring activity and determining the progress of hydrolysis should be defined and made clear. Many researchers investigating this topic have referred erroneously to “enzyme activity” while reporting progress or degree of hydrolysis by showing

accumulation of enzymatic breakdown products. This is incorrect terminology because the term 'activity' refers to enzyme kinetics. However, the rate at which products of enzyme action accumulate is related to enzyme activity. The complete procedure for measurement of either activity or progress of lipolysis involves two phases. First, the enzyme preparation is incubated under appropriate conditions, selected for the optimal activity of the enzymes known or anticipated to be present in the sample. Second, an assay is needed that can be used to determine the products of the enzyme's action. Many assays have been developed but none appear to be appropriate for use in accurately predicting the shelf life of the dairy products under test. For an enzyme assay to be useful for these purposes, it must provide specific substrates for the enzymes which spoil dairy products and must have a high sensitivity in order to detect low concentrations of products of hydrolysis. This project will address this shortcoming in current assay methods (i.e. lack of sensitivity) for detection of bacterial hydrolytic enzymes in dairy products.

2.7.1 Lipase assays

A multitude of chromogenic and fluorogenic substrates for lipase detection have been developed, but few are used commercially. The principle of the use of each type differs in the form of detection used. Detection of each makes use of a spectrophotometer, although of a different type - a UV/Vis spectrophotometer if the substrate is chromogenic or a fluorescence spectrophotometer when a fluorogenic substrate is used. Development of colourimetric and fluorometric lipase assays started during the late 1950s and early 1960s. However, it wasn't until the early to mid 1980s that these assays were developed for dairy applications, usually for lipases produced by psychrotrophic bacteria.

2.7.1.1 Chromogenic substrates

Colourimetric assays do not offer the sensitivity that is typical of fluorometric assays (Stauffer, 1989), but they do possess a range of benefits, and are possibly more widely used. Speed and ease of technical procedures is

an advantage. For some chromogenic substrates, such as *p*-nitrophenyl esters, the equipment and reagents are easily available and fairly inexpensive (Thomson *et al.*, 1999). The copper-based methods of lipase detection were the first colourimetric lipase detection techniques developed. Improvements in sensitivity of the copper soap method were obtained by replacing diethyldithiocarbamate, used in the method of Duncombe (1963) in the assay mixture with 1,5-diphenylcarbohydrazide (Mahadevan *et al.*, 1969) and increasing the copper and sodium chloride concentrations (Nixon & Chan, 1979).

2.7.1.1.1 β-naphthyl esters

The first lipase assay to employ β-naphthyl esters appears to have been developed by Nachlas and Blackburn (1958), who used various esters of β-naphthol as substrate to investigate a lipase present in human urine. However, in earlier work, α-naphthyl acetate was used by Greenbank and Wright (1950) to determine lipase (presumably lipoprotein lipase) activity in milk. Not until the work of McKellar (1986) did a colourimetric determination of lipase with a dairy emphasis appear. In this paper, McKellar (1986) describes the use of various β-naphthol esters as substrate for lipase of *P. fluorescens* B52, a widely studied raw milk isolate. The principle of this method, as detailed by McKellar (1986) and Versaw *et al.* (1989), is the release of β-naphthol as a result of lipolysis. The β-naphthol reacts with a diazonium salt resulting in an azo dye, the concentration of which is measured at 540nm. It has been found that the most appropriate β-naphthyl esters for detection of lipase activity are β-naphthyl laurate (Morgan *et al.*, 1967) and β-naphthyl oleate (Barrowman & Borgstrom, 1968), as both these studies found that lipases were highly active against these substrates but esterases did not show any activity. A colour change to an "intense red" through the inclusion of Fast Violet B as indicator is the basis of a method employing an α-naphthyl ester, α-naphthyl palmitate (Whitaker, 1973). The action of lipase on this substrate liberates α-naphthol, which results in the red colour when it forms a complex with the indicator. As would be expected,

the short chain fatty acid ester of β-naphthyl, β-naphthyl butyrate, has been used in an esterase characterisation study (Smacchi *et al.*, 2000).

2.7.1.1.2 *p*-nitrophenyl esters

Use of *p*-nitrophenyl esters as lipase substrates is widespread, and these substrates can possess a high level of specificity for particular enzymes. As an example, *p*-nitrophenyl laurate is hydrolysed by lipase only while *p*-nitrophenyl acetate is hydrolysed by esterase only (Barrowman & Borgstrom, 1968). The use of *p*-nitrophenyl acetate as an esterase-only substrate has been developed further in the work of Erlanson (1970). This followed early work, in which *p*-nitrophenyl acetate was described as an esterase substrate (Huggins & Lapides, 1947). Further supporting the use of short chain esters as an esterase-only substrate, is the work of Kilcawley *et al.* (2002) who determined esterase activity with *p*-nitrophenyl butyrate as substrate. It has been suggested that a specific region (fragment 336-449) of porcine pancreatic lipase can hydrolyse *p*-nitrophenyl acetate (De Caro *et al.*, 1986). These important findings need to be considered when selecting appropriate substrates. Many studies on human milk bile salt activated lipase use the esterase substrate, *p*-nitrophenyl acetate, for detection (Wang, 1981; Wang & Johnson, 1983). This substrate is clearly unsuitable, if “lipase-only” activity is to be detected. Lipase from *Pseudomonas fluorescens* P38 has been detected in an assay containing *p*-nitrophenyl caprylate as substrate (Owusu *et al.*, 1992). Furthermore, this substrate has been used in heptane, in which *p*-nitrophenyl palmitate is soluble (Pencreac’h & Baratti, 2001). However, these workers found that close to a 50-fold reduction in activity of *P. fluorescens* lipase was observed in heptane compared to water. In a survey of a range of different lipase substrates, the assay with *p*-nitrophenyl palmitate as substrate was found to be the most sensitive (Vorderwulbecke *et al.*, 1992). An investigation into nine *p*-nitrophenyl esters as the substrate for *P. fluorescens* lipase was conducted by Craven *et al.* (1997). The findings of that study indicated that *p*-nitrophenyl caprylate was the best substrate. In addition, *p*-nitrophenyl acetate was hydrolysed, albeit to a limited extent. This need not indicate the activity of

lipase against such a short chain fatty acid, rather that the enzyme preparation used, being crude, could contain some esterase. A problem with the use of *p*-nitrophenyl esters is the turbidity which often develops, interfering with spectrophotometric measurement. This can be overcome by the laborious techniques of filtering or centrifuging. In addition, Blake and Weimer (2000) have described the use of a reflectance colorimetric method to address this problem. The use of Clarifying Reagent has been found to be suitable for the purpose of stopping the enzymatic reaction and improving clarity in the assay mixture (Humbert *et al.*, 1997). More recently, it has been found that addition of Triton X-100 can also eliminate turbidity (Gupta *et al.*, 2002). In a comparison-based study of assay comprising *p*-nitrophenyl caprylate as substrate, the Humbert *et al.* (1997) method was the technique of choice as sensitivity, precision and accuracy were all good (Bendicho *et al.*, 2001).

2.7.1.1.3 Other chromogenic substrates

Other much less encountered colourimetric tests, at least from the perspective of potential dairy applications, have been developed.

The indoxyl acetate test appears, from the literature, to be more of an identification test in clinical microbiology than for research or in industry testing for lipase. Very few papers mention use of the technique, except in the purely clinical microbiology area. Slack (1987) describes methodology for this test which involves a filter paper impregnated with the substrate, following condensation. Gehauf and Goldenson (1957) describe how the substrate can be formed from indole and that products of the reaction are the intensely green fluorescent indigo white and subsequently the non-fluorescent indigo blue.

Trinitrobenzenesulphonic acid (TNBS) is often used as a basis for detection of free primary amino groups (Rollema *et al.*, 1989), the result of proteolytic activity. However, a derivative of this reagent has been used for the colourimetric determination of FFA (Goldberg *et al.*, 1978). Goldberg *et al.* (1978) describe the principle of the method as follows: trinitrophenylaminolauric acid is formed from TNBS and ω-aminolauric acid, which is then reacted with glycerol to form mono-, di- and tri-acylglycerol esters of this acid. These esters

are used as substrates, the hydrolysis of which leads to the isolation of chromogenically-labelled FFA after a solvent extraction step. The absorbance is measured at 330 nm.

Thioesters and their ^{14}C-labelled oxyester analogues have been reported as substrates for lipases (E.C. 3.1.1.3) and phospholipases (E.C. 3.1.1.4 and E.C. 3.1.1.5) (Aarsman & van den Bosch, 1979). A review published a few years after this work described such assays as “fast, specific, continuous and convenient”, with the use of thiol capture reagents being the major drawback, as they inhibit enzyme activity (Farooqui *et al.*, 1984).

Additional chromogenic esters that have been used include esters of 5-(4-hydroxy-3,5-dimethoxyohenylmethyl-ene)-2-thioxothiazoline-3-acetic acid (SRA) (Miles *et al.*, 1992) and 2,4-dinitrophenyl butyrate (Mosmuller *et al.*, 1992). In particular, the decanoate ester of SRA was found to be a good lipase substrate (Miles *et al.*, 1992). The principle of the SRA-based technique, as described by Miles *et al.* (1992) is a change in colour of the solution from pale yellow to red, when the esters are hydrolysed, however when 2,4-dinitrophenyl butyrate is subjected to lipase, hydrolysis results in release of 2,4-dinitrophenol, the absorbance of which is measured at 360nm (Mosmuller *et al.*, 1992).

An assay medium that is optically clear is an ideal situation, and would be considered essential for continuous measurement. Such an assay medium has been developed by Renard *et al.* (1987) through the use of 1-mercapto-2,3-propanediol and 2-mercaptoethanol in hexamethylphosphoric triamide (HMPA).

A slightly different approach to colourimetric determination was investigated by Rawyler and Siegenthaler (1989). These workers made use of the metachromatic properties of safranine, included in the assay mixture. The association of lipase and substrate led to a net negative charge and the lipid/water interface they describe, which in turn, resulted in an absorbance change in safranine.

2.7.1.2 Fluorogenic substrates

A number of clear advantages are offered by the use of an assay with a fluorogenic substrate compared to other methods. Thomson *et al.* (1999) lists

these advantages, which are sensitivity and ease of preparation of the substrate and its emulsion. In addition, the end products can be analysed without extensive treatment, common for some methods. A disadvantage could be the use of detergents in the assay mixture, if required, which may have a deleterious effect on lipase activity.

2.7.1.2.1 Early work: fluorescein, riboflavin esters

One of the very earliest fluorogenic substrates were fatty acid esters of riboflavin. These were investigated as a substrate for pancreatic lipase with an indication of FFA by yellow fluorescent spots on paper chromatography (Yagi *et al*., 1961). Not long after that report, esters of fluorescein were used as lipase substrates, which importantly, were not hydrolysed by esterases (Guilbault & Kramer, 1966; Fleisher & Schwartz, 1971).

2.7.1.2.2 4-methylumbelliferone esters

The first reports on the use of 4-methylumbelliferone (4-MU) esters for detection of lipase activity were in the late 1960s, by Jacks and Kircher (1967) and Guilbault *et al.* (1968). In these studies, animal and plant lipases were investigated. Following this, Deeth (1978) described an assay employing 4-methylumbelliferyl heptanoate for an esterase native to milk. Within five years, Fitz-Gerald and Deeth (1983) and Stead (1983) published work detailing first use of a fluorogenic substrate for detection of true lipase from pseudomonads of dairy origin. These papers, containing details of detection of lipase from psychrotrophic dairy bacteria, described the use of various 4-MU esters as lipase substrate. Fitz-Gerald and Deeth (1983) used two 4-MU esters, 4-methylumbelliferone oleate (4-MUO) and 4-methylumbelliferone heptanoate (4-MUH) while Stead (1983) investigated 4-MUO. The principle of this method relies on lipolytic activity against the non-fluorescent 4-MU ester, which results in release of the fluorescent 4-MU (Stead, 1983). In an extension to these studies, Stead (1984a) compared the detection of lipase from various *P. fluorescens* and *P. fragi* isolates using 4-MUO and another 4-MU ester, 4-methylumbelliferone nonanoate (4-MUN). Improvements to the 4-MUO-based

lipase assay have been reported by Vercet *et al.* (1997) and Tsuzuki *et al.* (2001), who altered various assay conditions.

2.7.1.2.3 Umbelliferyl esters

While 4-MU esters have been used for many years, the first report of the use of an umbelliferyl (UM) ester, umbelliferyl oleate (UMO), was published in the early 1990s by de Laborde de Monpezat *et al.* (1990). Their work involved hydrolysis of UMO by two fungal lipases.

2.7.1.2.4 Other fluorogenic substrates

The fluorescent carboxyl group labelling reagent 9-bromomethylacridine (9BMA) has been used in fluorometric detection of lipase (Tsuzuki *et al.*, 2002). After the enzymatic reaction has been terminated, the FFA are fluorometrically labelled through addition of 9BMA to the solution. Tsuzuki *et al.* (2002) reported an increase of sensitivity of 1000-fold for this technique over titrimetric methods.

Many additional fluorometric methods have been devised, but are not widely used as their full potential, in many cases, has not been investigated. Therefore, most of these have thus far been limited to specific research applications. Celestino *et al.* (1996) detail a method to determine pyrenyl butyric acid using the fluorescent triacylglycerol, 1(3)-pyrenylbutanoyl-2,3(1,2)-dipalmitoyl-sn-glycerol (1,2-DPPBA) as substrate. The emission wavelength for detection was 396 nm while the excitation wavelength was 340 nm. For continuous measurement of lipase activity, use of pyrenemethyl laurate, hydrolysed by lipase to form pyrenemethanol has been reported (Negre *et al.*, 1989). Another, described by Chemnitius *et al.* (1992), is formation of resorufin from a 1,2-*o*-diauryl-rac-glycero-3-glutaricresorufin (DGGR) ester. Common components of fluorometric lipase assay mixtures are detergents. They are required to “create a substrate interface suitable for enzyme activity” but can also have deleterious effects against the assay system (Hendrickson, 1994). One particular method uses no detergent and has the added benefit of requiring only a low concentration of substrate (Wilton, 1991). The principle involves long

chain fatty acid released following lipolysis which leads to displacement of the highly fluorescent protein-binding fatty acid, 11-(dansylamino)undecanoic acid.

Methods which employed a change in dye properties are those based on the fluorescent dye rhodamine 6G. One of the earliest reports of such a method was by Anderson and McCarthy (1972), who described a method which detects FFA from hydrolysed galactolipids partially purified from membranes of spinach subchloroplast particles. An improvement of the rhodamine 6G assay method appeared in the same year; the authors suggested the use of *n*-hexane rather than benzene in the solvent mixture which was added to the enzyme reaction mixture (Hirayama & Matsuda, 1972). A plate-based rhodamine assay technique has been developed, utilising rhodamine B. In this method, olive oil is substrate and cultures are grown or lipase preparations applied to the plate before exposure of the plate to UV light at 350 nm (Kouker & Jaeger, 1987). A further improvement of the rhodamine plate assay is the miniaturisation of the assay. Jette and Ziomek (1994) have conducted this assay in 96-well microtitre plates, with triolein as substrate and excitation at 485 nm and emission at 535 nm.

2.7.1.3 Applications in dairy research

In dairy research, the purpose of lipase assays utilising a chromogenic or fluorogenic substrate is most often to quantify enzymatic breakdown products which provide an indication of potential hydrolytic spoilage in milk and dairy products. This was the topic of two papers, where the quality, in terms of lipase present, in milk powder (Celestino *et al.*, 1997a) and UHT milk (Celestino *et al.*, 1997b) were assessed with 1,2-DPPBA as substrate. Research into the lipolytic deterioration of stored UHT milk employed a modified copper soap method (Christen *et al.*, 1986). Duration of UHT milk storage was four weeks.

Through the use of chromogenic or fluorogenic substrates, much information has been obtained about spoilage. Research conducted by Stevenson *et al.* (2003) has resulted in confirming when lipases are produced (late log/early stationary phase), in addition to providing an insight into the relationship between growth kinetics and lipase production, for example, *P.*

fluorescens and *P. fragi* studied had similar levels of lipase production and commenced lipase production at similar times. Therefore, they would be expected to have similar spoilage potential. Whole milk would be expected to spoil sooner than skim milk due to lipase action. Not only because it contains more fat, but because *Pseudomonas* spp. growing in whole milk produced significantly more lipase than those growing in skim milk, as reported by Deeth *et al.* (2002). Possibly important in a spoilage outcome, was a finding by Rajmohan *et al.* (2002) that in a particular strain of *P. fluorescens* cultured in skim milk, a rise in lipase activity corresponded to a fall in protease activity.

In addition, some research was focused on characterisation of the enzymes, primarily with reference to kinetics. For example, in a comparison study, *p*-nitrophenyl palmitate was the substrate of choice in determining the activity of a range of lipases (Kilcawley *et al.*, 2002). In an initial characterisation of a newly-isolated lipase from *Pseudomonas tolaasii* the effect of various conditions on lipase activity was determined, with β-naphthyl caprylate (β-NC) as substrate (Baral & Fox, 1997). A similar initial characterisation study was carried out by Beven *et al.* (2001) on a lipase isolated from *P. fluorescens*. These workers chose 4-MUO as substrate, as did Abad *et al.* (1993) in another such study on a newly isolated lipase from *P. fluorescens* NC1. Jaspe and Sanjose (1999) used β-NC to determine the presence of lipase in milk cultures of *P. fluorescens*. In a study of similar design, Fernandez *et al.* (1992) employed the use of a lipase assay based on the same substrate to determine the presence of lipase in milk cultures, with variable iron content, of *P. fluorescens*. Furthermore, validation and comparisons between assay techniques can be an additional application, as in Bendicho *et al.* (2001). One drawback of the use of chromogenic or fluorogenic substrates has been raised by Stead (1983), namely the interference by fat (cream) present in the sample.

2.7.2 Protease assays

Early interest in proteolytic enzymes was mostly in the medical physiology area. Hence, an initial substrate used in assays for proteolytic

activity was often haemoglobin, a commonly obtained animal protein. Casein was the second most commonly used substrate (Stauffer, 1989). In addition to these commonly available and widely used protein substrates, a variety of synthetic (usually labelled) protein derivatives have been developed as protease substrates. A disadvantage of using chromogenic or fluorogenic substrates is that milk proteins can interfere with the assay (Bastian & Brown, 1996). End products of proteolytic activity, in which a protein substrate has been used, are usually detected by a colourimetric (such as the TNBS assay) or fluorometric method (such as the fluorescamine assay). Fluorometric detection is more sensitive than colourimetric (Stauffer, 1989) and while colourimetric methods are quick to perform, they may not reflect accurately a protein's proteolytic potential (Mottar & Driessen, 1987). Detection of end products in a protease assay comprising synthetic substrates will be specific to the labels of detection. This will often be colourimetric of fluorometric determination at wavelength(s) most suitable for the label used. An approach described by Berman *et al.* (1992) to ascertain optimal protease substrate appears to have potential. The procedure involved "the ability to synthesize defined mixtures of amino acids" at subsites of the peptide substrates and assignment of a structure to each component (Berman *et al.*, 1992) and protease screening of these peptide libraries (Singh *et al.*, 1995). Immunological-based assays (such as those described by Clements *et al.*, 1990 and Jabbar & Joishy, 1999) are unsuitable in the context of the applications discussed here, because with these assays, it is not possible to determine proteolytic activity, only the presence/absence of the specific enzyme that the product is being tested for.

2.7.2.1 Protein substrates

The proteins that have been used as protease substrates are diverse, and include bovine serum albumin (Garesse *et al.*, 1979), equine myoglobin (Bohlen *et al.*, 1973) and haemoglobin (Nakai *et al.*, 1964). However, not all of these are suitable for the assay of protease found in milk, whether this be native milk protease (plasmin) or that produced by contaminating bacteria. Casein has a major advantage as a substrate for proteolytic enzymes, in that it is

susceptible, due to its random and complex structure, to proteolysis by many proteases without denaturation (Reimerdes & Klostermayer, 1976). However, some authors have even reported denaturation of casein prior to use as a protease substrate (Wretlind & Wadstrom, 1977). While the use of whole casein is widespread (Kunitz, 1947; Chism *et al.*, 1979; McKellar, 1982), research has been carried out into the preference of particular proteases for specific caseins. Mitchell and Marshall (1989) have shown that while there is preferential hydrolysis of certain caseins, protease from four strains of *P. fluorescens* demonstrated most proteolytic activity against whole casein compared to individual caseins. This is in contrast to native milk protease, which preferentially hydrolyses β-CN over whole casein (Pande & Mathur, 1994). The results of Mitchell and Marshall (1989) also provided evidence that *P. fluorescens* proteases show limited activity towards the whey proteins tested, α-LA and β-LG. However, β-LG can be a suitable substrate for some proteases which can completely hydrolyse it, such as protease from *Bacillus licheniformis* (Madsen & Qvist, 1997). As expected, protease from *P. fluorescens* is inactive against proteins not encountered in milk, such as haemoglobin and ovalbumin (Patel *et al.*, 1983). Therefore, selection of such proteins as substrates for proteases native to milk or produced by milk-borne bacteria would be unjustifiable.

2.7.2.2 Protein derived substrates

2.7.2.2.1 Chromogenic labels

One of the most commonly used and widely researched chromogenic substrates is hide powder azure (HPA). It was developed by Cliffe and Law (1982). The technique involves addition of the HPA reagent directly to milk, with absorbance measurement at 595 nm. An early paper in this area covers work involving a labelled substrate of HPA and remazol brilliant blue (RBB), resulting in a highly sensitive procedure for detection of the activity of a range of proteolytic enzymes (Rinderknecht *et al.*, 1968). In a comparison study with the TNBS method, use of HPA as substrate was found to be similar in detectability (McKellar, 1984). However, in a later study, the HPA assay was found to

possess “poor sensitivity” as linear increases in protease concentration were not reflected by linear increases in detectable proteolysis (Rollema *et al.*, 1989). Suitability of azo-dye as a chromogenic label for protease substrates was reported in the mid 1940s, with the advent of azocoll (collagen with an azo-dye label) (Oakley *et al.*, 1946), which has been used as a protease substrate (Chavira Jr. e*t al.*, 1984). Another azo-labelled protein, azocasein, has also been in widespread research use. It has been used often as a substrate for detection of proteases from dairy isolates of *Pseudomonas* spp. (Ewings *et al.*, 1984; Mitchell & Ewings, 1985; Mitchell *et al.*, 1986; Mitchell & Marshall, 1989; Kohlmann *et al.*, 1991; Makhzoum *et al.*, 1996; Schokker & van Boekel, 1997; Bendicho *et al.*, 2002). Sensitivity of this assay was also poor, and similar to that of the HPA assay (Kroll & Klostemeyer, 1984). A further azo-based method, used less often compared to azocoll and azocasein, has azoalbumin as substrate (Tomarelli *et al.*, 1949. A commercially available protease test kit consisting of a chromogenic substrate has been produced (Royer *et al.*, 1996). The substrate is a mixture of albumin, gelatin and azoalbumin with spectrophotometric detection at 450 nm. Detection is of the azo dye-free amino acid complex. It has been described as being suitable for a wide range of proteases. This substrate has been used, in a study of the characteristics of proteases of *Bacillus* spp. (Bjurlin *et al.*, 2002). A major advance in development of chromogenic substrates was made by Wolf and Wirth (1996). Nearly three decades after the work of Rinderknecht *et al.* (1968) and first use of RBB, Wolf and Wirth (1996) used RBB to label casein, gelatin and collagen resulting in a soluble substrate. The versatility of this assay is such that it can be used as an agar plate assay for the detection of proteolytic microorganisms. This is in contrast to substrates such as HPA and azocoll, which are insoluble. Another little used chromogenic label for casein is 3,5-dinitrosalicylaldehyde (DNSA) (Gallegos *et al.*, 1996). DNSA was used to determine activity of subtilisin from *Bacillus subtilis*, by measurement at 366 nm following precipitation in 10% trichloroacetic acid (TCA) and adjustment to pH 13 with sodium hydroxide. An additional method which has not been adopted is that described by Nelson *et al.* (1961). Their chromogenic substrates were indigo

carmine labelled to fibrin and congo red-labelled hide powder. Also, use of various amino acid combinations with *p*-nitroanilide as label was carried out. This involves detection of the product of proteolysis, 4-nitroalinine, at 405 nm. One method, in which this substrate has been utilised, is for investigation of specificity of unknown proteases, by screening (Whitmore *et al.*, 1995). The substrate, such as casein, is cleaved by the protease.

In the peptides produced, the N-terminal end is blocked to prevent attachment of the added chromophore at that end. Upon addition of the chromophore to the solution, it attaches to the C-terminal end of the peptide. This peptide, with both ends blocked, could be used to screen proteases which possess identical specificity to the original protease as release of the chromophore indicates a matching specificity. The characterisation study of an *Aspergillus terreus* protease by Chakrabarti *et al.* (2000) included use of various *p*-nitroanilide-labelled substrates. Some chromogenic substrates have been developed for assaying activity of plasmin. Metwalli *et al.* (1998) details the use of Val-Leu-Lys-*p*-nitroanilide, for assessment of plasmin activity with spectrophotometric detection at 405 nm.

2.7.2.2.2 Fluorogenic labels

The first report of the demonstrated use of fluorescent-labelled proteins (immunoglobulins) was by Coons *et al.* (1942). Twenty years later, a paper was published reporting the use of fluorescein-labelled fibrin, in an assay for detection of streptokinase activity (Pappenhagen *et al.*, 1962).

Fluorescamine is the reagent commonly used to determine fluorogenically the amount of FAG present following proteolysis of a protein substrate. Another use for this reagent, as a fluorescent label, was reported by Sogawa and Takahashi (1978) who labelled casein with fluorescamine and used this substrate to assay for activity of trypsin, chymotrypsin and papain. A subsequent paper also details use of fluorescamine-labelled casein (Ichihara *et al.*, 1982). It was found that this assay had improved sensitivity for trypsin detection over radioactive methods.

As discussed earlier, esters of 4-methylumbelliferone, fluorogenic substrates, have found wide suitability in assays for lipase activity. Not until the early 1980s was the use of this substrate reported for detection of protease activity. Khalfan *et al.* (1983) used casein, labelled with 4-methylumbelliferone, as a substrate for trypsin, chymotrypsin, elastase and cathepsin D.

The first report detailing the use of fluorescein isothiocyanate (FITC) appears to be by de Lumen and Tappel (1970). These authors describe an assay for activity of trypsin and cathepsin D, using FITC-labelled haemoglobin as substrate. Twining (1984) introduced FITC-labelled casein for assay of trypsin and chymotrypsin activity. In subsequent investigations with FITC as substrate, Christen and Senica (1987) found it to be a good choice for detection of activity of protease produced by psychrotrophic bacteria. Of the three concentrations of FITC these workers investigated, the highest (58.4 μg/g solid) was found to be most sensitive.

BODIPY dyes (substituted 4-bora-3a,4a-diaza-*s*-indacene derivatives), a patented product, have been shown to have versatile applications in fluorogenic detection of proteases. In work by Jones *et al.* (1997), the dye and quencher are coupled to the substrate, which was virtually non-fluorescent. These workers detected a range of proteases with high sensitivity, using BODIPY dyes with different excitation and emission wavelengths and detection with different fluorometric instrumentation.

Additional fluorogenic substrates, which have not gained acceptance for routine research or industrial applications include: *N*-carbobenzyloxy-arginyl-arginyl-7-amido4-methylcoumarin, used to assay for activity of a protease from *Entamoeba histolytica* (Spinella *et al.*, 1999); 2-methoxy-2,4-diphenyl-3(2*H*)furanone-labelled casein and fibrin for detection of trypsin, chymotrypsin and elastase (Wiesner & Troll, 1982); casein labelled with l-anilino-8-naphthalensulfonate for determination of trypsin and pepsin activity (Spencer *et al.*, 1975); 7-glutaryl-phenylalaninamido-4-methylcourmarin as a peptide label which was used as a substrate for trypsin, chymotrypsin and elastase (Zimmerman *et al.*, 1977); NorFES labelled with tetramethylrhodamine as a substrate for serine protease elastase (Packard *et al.*, 1997); short peptides of

approximately ten amino acids labelled with iodoacetamidotetramethylrhodamine for the detection of protease from *Plasmodium falciparum* (Blackman *et al.*, 2002) and 3,5-diphenyl-5-hydroxy-2-pyrrolin-4-one, originally reported by Weigele *et al.* (1973).

Fluorescence polarisation was introduced by Maeda (1979), who reported the labelling of diverse substrates with FITC, with detection using a fluorescence spectropolarimeter. Shortly thereafter, the use of this technique in the assay for bovine plasmin and plasminogen was reported (Kinoshita *et al.*, 1980). Changes in molecular volume of a fluorescent labelled molecule are measured (Bolger & Checovich, 1994); the decrease in overall molecular weight indicates proteolysis (Maeda, 1979). An example of such a substrate is bovine serum albumin (BSA) labelled with 25 FITC residues per BSA molecule (Voss Jr. *et al.*, 1996) which are thus cleaved into FITC-labelled peptides. This technique appears to have been developed as a replacement for the use of radioactive substrates (Voss Jr. *et al.*, 1996).

2.7.2.3 Additional assays and techniques

2.7.2.3.1 Bioluminescence

Bioluminescence has potential for application in many areas of biology, both in industry and for research. The biochemistry of bioluminescence was elucidated between the 1950s and 1970s. The bioluminescence reaction can be summarised as follows, where $FMNH_2$ is an NADH-reduced dihydroflavin mononucleotide and RCHO is a long chain fatty aldehyde.

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + light$$

McCapra (1982) has detailed the various stages in somewhat more detail, indicating the various steps in the reaction, with luciferase catalysing the reaction.

$$NADH \rightarrow FMNH_2 + luciferase(E) \rightarrow FMNH_2.E$$

$$FMNH_2.E + O_2 \rightarrow FMNH(OOH).E$$

$$FMNH(OOH).E + RCHO \rightarrow FMN + RCO_2H + E + light$$

In the latter stages of the pathway, reduced flavin mononucleotide is oxidised by molecular oxygen (Cline & Hastings, 1972) with three intermediates being produced: i) the reduced enzyme, ii) reduced enzyme-oxygen adduct, iii) reduced enzyme-oxygen/aldehyde complex (Hastings & Gibson, 1963). Initial study into the potential of a luciferase-based protease activity assay was undertaken by Njus *et al.* (1974). In this investigation, bacterial luciferase was hydrolysed 40-fold faster than firefly luciferase. This first generation luciferase-based assay was reported by these same workers to possess comparable sensitivity to colourimetric assays. In further research into the application of luciferase as a protease substrate by Norton *et al.* (1981), the suitability of firefly luciferase was confirmed. These workers used a commercial source of luciferase, unlike the crude preparation used by Njus *et al.* (1974). As a result, high sensitivity could be achieved by Norton *et al.* (1981), detecting as little as 200 pg of trypsin. One of these applications could be detection of protease from psychrotrophic bacteria. Rowe *et al.* (1990) have described such an application. The principle of their assay is a reduction in bioluminescence proportional to the amount of protease present in a particular sample. The protease will hydrolyse the luciferase, thereby decreasing the amount of that enzyme present to catalyse the reaction that gives illumination of luciferin.

2.7.2.3.2. Radioactive labels

Protease activity assays incorporating radiolabelled substrates are very sensitive (Fairbairn, 1989) and some have been developed over the years, including ^{131}I-labelled casein (Katchman *et al.*, 1960), ^{14}C-labelled casein (Drucker, 1972), ^{125}I-labelled β-CN (Hofman *et al.*, 1979) and benzoyl-DL-arginine [^{3}H]anilide hydrochloride (Roffman & Troll, 1974). However, due to the hazards of radioactivity, these methods are not desirable. Some assays with radiolabelled casein appear to have been adopted as a substrate for routine research purposes (Exterkate, 1975).

2.7.2.3.3. HPLC

The determination of proteolysis using an HPLC technique based on a synthetic substrate is a relatively novel approach. Such work was carried out by O'Driscoll *et al.* (1999) giving a highly specific method. Cathepsin D was first found to clearly produce a specific product, identified by HPLC and MS, which possessed a molecular mass of around 481 Da. When an acid whey preparation from raw milk was used as the source of enzyme, one of the proteolytic products of the same substrate eluted at the same time and was of the same molecular mass as that produced by cathepsin D.

2.7.2.3.4 Reporter enzymes

Research on the use of reporter enzymes appears to have been conducted for a brief period during the early 1980s. The principle of the method was described by Andrews (1982a, 1982b). Casein or denatured haemoglobin is immobilised with sepharose and linked to a reporter enzyme. The reporter enzyme is ideally a globular protein, which is resistant to proteolytic activity. Incubation with the protease is performed next, for one to three hours. During this time, proteolysis results in release of the reporter enzyme. The released reporter enzyme then acts on its own substrate during a subsequent incubation step, for a specified time. A high sensitivity is achieved with this technique due to the "very substantial amplification factor" of the protease, which may be present at a low concentration.

2.7.2.4 Applications in dairy research

Use of protease activity assays (in dairy research) comprising chromogenic or fluorogenic protein-derived or protein substrates have usually been in studies that involve: i) characterisation of proteases or ii) assessment of spoilage. Jensen *et al.* (1980) used an azocasein substrate to characterise a protease from *Pseudomonas aeruginosa*. Characterisation of properties of a *P. fluorescens* protease has been carried out by several investigators: McKellar (1982) who used casein as substrate, Kohlmann *et al.* (1991) who used azocasein as substrate, and Koka and Weimer (2000b) who used BODIPY a dye-labelled substrate. In another similar characterisation study, a casein-

based protease assay was employed to investigate the influence of iron on protease production (Bjorn *et al.* 1979). A heat inactivation study was conducted by Kumura *et al.* (1999) by comparing differences in the proteolytic activity of the enzyme, with casein used as substrate. In an attempt to alter the functional properties of milk through the use of a protease, Barros *et al.* (2001). used azocasein as substrate to determine properties of the protease from *Cynara cardunculus*, a plant.

2.7.2.5 Assessment of spoilage with protease assays

While a specific activity of protease doesn't appear to be necessary for various spoilage outcomes to become obvious, a high level of protease in milk will accelerate time to onset of sensory defects. A direct relationship was found by Mitchell and Ewings (1985) between the amount of protease added to UHT milk and the time to onset of gelation. The work of Pande and Mathur (1994) indicated the importance of indigenous milk protease isolated from whey as it was very active against casein, in particular β-CN.

2.8 Prediction of shelf life of UHT milk

The approach adopted in the present study was to correlate hydrolysis of substrates in assays with hydrolysis of milk fat or protein in the stored UHT milk. For prediction of spoilage of pasteurised milk, good correlations have been observed using bacterial numbers (Griffiths and Phillips, 1988), an endotoxin (Bishop & White, 1985) and impedance (Bishop *et al.*, 1984; Kahn & Firstenberg-Eden, 1987) as indicators. Interestingly, Bishop and White (1985) reported very poor correlation when proteolysis was used as an indicator of spoilage in pasteurised milk and their conclusion was that proteolysis "is of little predictive value". Conversely, a good correlation was obtained between the progress of proteolysis by enzymes extracted from bacterial cells, or from leucocytes, with shelf life of UHT milk, denoted by the onset of age gelation (Keogh & Pettingill, 1984). Being a commercially sterile product, UHT-processed milk requires a completely different approach to that often adopted when attempting to predict the shelf life of pasteurised milk. Spoilage of UHT

milk is almost exclusively caused by the action of enzyme(s) remaining after processing (Stepaniak, 1991). Therefore, shelf life prediction based on progress of lipolysis and proteolysis is likely to be more reliable than other approaches. McKellar *et al.* (1984) found a possible way forward by drawing conclusions on likely development of bitterness by monitoring initial rates of proteolysis in stored UHT milk. They observed relatively strong correlations and based their conclusions on these. Work on modelling (in UHT milk) of action of lipases and proteases produced by spoilage bacteria (Braun & Fehlhaber, 2002) has given additional information on the action of these enzymes under various conditions. In addition, in that study, the enzymes were added to UHT milk and spoilage defects followed over time to establish an approximate time for onset of spoilage for a given enzyme under specific conditions. While the work of Braun and Fehlhaber (2002) makes major advances towards reliable and accurate spoilage prediction of UHT milk, it was not based on testing actual UHT milk samples, therefore could not be used to predict the spoilage potential of UHT milk.

2.9 Summary

The dairy industry is a vital rural industry, which includes production of commodities such as UHT milk and other long-life products. In commercially sterile UHT milk, hydrolytic enzymes are the main causes of spoilage. These enzymes degrade fat and protein, which comprise a substantial portion of the solids in milk. The most detrimental enzymes are heat-resistant lipases and proteases from psychrotrophic *Pseudomonas* spp. which are not completely inactivated by UHT processing. In the UHT-treated product, these enzymes are still active, which over the long storage times typical of these products, can manifest as various spoilage defects. The work described in this thesis is intended to add to the body of knowledge on detection of lipases and proteases of psychrotrophic *Pseudomonas* spp., biochemical characterisation of spoilage and identification of sources of these bacteria.

CHAPTER 3

DEVELOPMENT OF LIPASE AND PROTEASE ASSAYS UTILISING MILK-LIKE SUBSTRATES

3.1 Introduction

In the 1980s, shelf life prediction of UHT milk was recognised as a key research area (O'Donnell, 1987), and today this topic is still of much importance because reliable and rapid predictive assays are not yet available. There are many substrates available for the detection of the *Pseudomonas* lipases and proteases that may contribute to spoilage of long-life dairy products (Fairbairn, 1989; Stead, 1989). These may be categorised as natural or synthetic substrates where the natural substrates are lipids or proteins naturally occurring in milk or other natural sources and the synthetic substrates which are compounds with a chromogenic or fluorogenic label attached by an ester or a peptide bond which is the target of lipase and protease, respectively. The action of lipase and protease on natural substrates is indicated by release of free fatty acids (FFA) and free amino groups (FAG) while on synthetic substrates it is indicated by cleavage of the label to produce colour or fluorescence. Synthetic substrates are often preferred in practice as the end products are relatively easy to measure compared with those of the natural substrates; however, the sensitivity, stability and good correlation of synthetic substrate assays with dairy product spoilage has not yet been demonstrated. Deeth and Touch (2000) state that milk-based substrates would be preferred when testing long-life dairy products as they may correlate better with spoilage than assays that utilise synthetic substrates. During the course of the research described in this thesis, some milk-derived substrates have been investigated, such as trivalerin (Svensson *et al.*, 2006) and triheptanoin (Andrewes *et al.*, 2007), which definitely show promise for the use of non-labelled substrates for detection of lipase in long-life dairy products. However, the natural substrate assays remain largely undeveloped for long life dairy products, therefore their usefulness for this purpose is unclear.

The work presented in this chapter describes the development of natural substrate-based assays using crude milk fat (cream powder) or triolein (a C18:1 triacylglycerol) for lipase detection and crude milk protein (skim milk powder) or casein for protease detection. The approach was to prepare sterile UHT whole milk with added crude pseudomonad lipase and protease and to test this for

lipase and protease activity using the natural substrate assays. These were conducted at the temperature (37 ºC) and pH (7.0-8.0) which are known to be optimal for many of these enzymes (Cousin, 1982; Fox *et al*., 1989). Many factors may inhibit lipase and protease activity (Stepaniak & Sorhaug, 1989) including the buffer concentration required to achieve pH 8.0 in the protease assay. Therefore, a low and a high buffer concentration were included in the investigation. The milk with added lipase and protease was also incubated for a short period at three storage temperatures (7, 25 and 37 ºC) and the extent of lipolysis and proteolysis during storage was determined using tests for FFA and FAG. Linear regression analyses were performed to determine the correlation between the enzyme assays and milk storage results. The assays which were most sensitive and correlated well with milk fat and protein decomposition in the stored milk were identified.

3.2 Materials and Methods

3.2.1 Source of chemicals and microbiological media

Unless otherwise stated, all chemicals were of the highest grade available and purchased from Sigma-Aldrich Co. (Sydney, NSW). All microbiological media were purchased from Oxoid Australia Pty. Ltd. (Adelaide, SA).

3.2.2 Source of bacteria

Isolates of bacteria were obtained from the dairy culture collection at Food Science Australia in Melbourne. These were *Pseudomonas fluorescens* isolates 65, 117 and 328 and *P. fragi* isolate 102.

3.2.3 Preparation of crude *Pseudomonas* spp. lipase and/or protease

The cultures were stored at -80 °C in Nutrient Broth containing 20% glycerol. A small portion of the frozen culture was inoculated from a cryotube into 10 mL of Nutrient Broth and incubated at 25 °C for 24 h. The incubated culture was centrifuged at 3020 *g* for 10 min in a sterile centrifuge tube, before

being washed in sterile 145 mM NaCl (Chem-Supply; Adelaide, SA) and centrifuged again at 3020 *g* for 10 min. This wash and centrifugation step was repeated, before the supernatant was decanted and the pellet resuspended in 145 mM NaCl. The turbidity of the suspension was measured at 590 nm and the washed culture was added to UHT skim milk to achieve an inoculum of about 10^5-10^6 cfu/mL. The skim milk culture was incubated with circular agitation (150 rpm) at 4 °C for 8 d, before being centrifuged at 28 000 *g* for 40 min at 4 °C. The supernatant (crude enzyme) was then filtered through a 200 nm filter within a filter tower, and frozen at -72 °C or stored at -72 °C with cells present, and filter sterilised through a 200 nm filter (Millipore Corporation; Sydney, NSW) shortly prior to use. Each crude enzyme preparation was used at two concentrations. For lipase assays, the crude enzyme was added to the milk sample at 0.05% (for isolate 328), 0.10% (for isolate 328), 1% (for isolate 65), 2% (for isolates 65 and 102) and 3% (for isolate 102). Crude protease was used at 0.05% (for isolate 65), 0.10% (for isolate 65), 0.15% (for isolates 102 and 117) and 0.25% (for isolates 102 and 117).

3.2.4 Lipase assays

The lipase assays were based on the procedure described by Bucky *et al.* (1986) using either triolein (10.0%) or cream powder (16.5%) (supplied by a Victorian dairy factory) as substrate (final fat concentration of 4.4% in both assays). Fat concentration in the cream powder was 60.6%. The substrate was homogenised for 5 min at maximum speed in 10% gum arabic using a model T45/6 Ultra-Turrax homogeniser (IKA Werke GmbH & Co. KG; Staufen, Germany). The assay mixture was buffered to either pH 7.0, 7.5 or 8.0 with 300 mM Tris-HCl and contained thimerosal as a preservative at a final concentration of 1.2 mM. With some assay mixtures, homogeneity was not maintained throughout the incubation period. Separation of some assay mixture components was observed, particularly the fat fraction. Where such substrate instability was found, the gum arabic was replaced with xanthan gum or carboxymethyl cellulose and homogenised as described previously. In some experiments, the cream powder and gum arabic were sonicated using a model

400S laboratory sonicator (Hielscher Ultrasonics GmbH; Teltow, Germany) with a 22 mm focused sonotrode. The sonication conditions were 24 kHz/100 micron amplitude for approximately 3 min. The final assay mixture contained 2 mL of homogenised or sonicated substrate, 2 mL of buffer and 500 µL of sample.

3.2.5 Protease assays

The substrates used in the protease assays were bovine casein and skim milk powder (SMP) (supplied by a Victorian dairy factory). Protein concentration (casein 4.5%, whey protein 0.7%) was 5.2%. The casein was dissolved by stirring in buffer at a concentration of 1 %. The buffer used was Tris-HCl at a concentration of 100 mM (to achieve a final pH in the assay of 7.0) or 3 M (to achieve final pHs in the assay of either 7.0 or 8.0). Thimerosal was added as preservative to a final concentration in the assay mixture of 1.2 mM. SMP was prepared in a similar manner, at a concentration of 36 mg/mL. The concentration of protein in the casein and SMP assay mixtures was the same - 1%. The protease assay mixture comprised 900 µL of substrate and 300 µL of sample.

3.2.6 Assay conditions

The lipase and protease assays were performed with incubation at 37 ºC for 24 h. FFA or FAG concentrations were measured before and during incubation. An increase in FFA and FAG content during incubation of the assay, was reported for lipase and protease, respectively. All tests were performed in triplicate.

3.2.7 Determination of free fatty acids

FFA concentration was determined using the extraction/titration method of Deeth *et al.* (1975). Extraction mixture containing propan-2-ol (BDH; Melbourne, VIC), petroleum spirit (BDH; Melbourne, VIC) and 2 M sulphuric acid (BDH; Melbourne, VIC) in the ratio 40:10:1 was added to a stoppered test

tube with petroleum spirit, distilled water and sample in the ratio 10:6:4:3. The content of the tube was mixed thoroughly by shaking for 1 min. The layers were then allowed to settle and separate over at least 30 min, before the height of the top layer was measured. A volume of 5 mL from the top layer was transferred to a 50 mL flask and 500 μL of 400 μM bromothymol blue in propan-2-ol added as indicator. The sample was titrated with standardised 20 mM potassium hydroxide prepared in methanol, until the sample had just changed colour to green. To obtain an accurate concentration of potassium hydroxide in methanol, it was standardised against 20 mM potassium hydrogen phthalate. Using 5 mL of 20 mM potassium hydrogen phthalate, potassium hydroxide was titrated with 80 μL of phenolphthalein added as an indicator. The volume of potassium hydroxide to reach the end point was noted and the titration repeated twice. Using the formula below, the precise concentration of methanolic potassium hydroxide was calculated.

$$KHPthth = KOH$$

$$V_1N_1 = V_2N_2$$

$$N_2 = V_1N_1/V_2$$

Where: V_1 = volume of KHPthth

V_2 = KOH titre – blank

N_1 = molarity of KOH

To calculate the FFA in the sample, the volume of potassium hydroxide titrated was noted and the height of the remaining upper layer measured again. The proportion of the upper layer titrated was calculated by subtracting the upper layer remaining from the initial height of the upper layer and then dividing this by the total height of the upper layer to obtain the ratio of the height difference, which was multiplied by three. The blank titre was subtracted from the sample titre, and this value multiplied by 1000 and then multiplied again, this time by the calculated molarity of the potassium hydroxide titrant. Lastly, this was divided

by the ratio (which was previously multiplied by three). The formula used is shown below:

$$\frac{\text{(Sample titre – blank titre) x 1000 x calculated KOH molarity}}{\text{height difference ratio x 3}}$$

3.2.8 Determination of free amino groups

FAG content was determined using the 2,4,6-trinitrobenzenesulphonic acid (TNBS) and fluorescamine assays. The standard curves prepared for these assays are shown in Appendix 2.

3.2.8.1 2,4,6-trinitrobenzenesulphonic acid assay

The 2,4,6-trinitrobenzenesulphonic acid assay (TNBS) assay was performed using a modification of an assay described by Spadaro *et al.* (1979). A 2 mL volume of sample was added to 4 mL of 835 mM trichloroacetic acid (TCA) and incubated at 25 °C for 20 min. The acid treated sample was then filtered through No.1 filter paper (Whatman PLC; Brentford, England). A 10 µL aliquot of the filtrate was transferred to a well in a 96-well microtitre plate followed by 40 µL of 1 M potassium borate buffer at pH 9.2 and 40 µL of 5 mM TNBS. The plate was covered and incubated in the dark at 25 °C for 30 min. After incubation, 20 µL of 18 mM sodium sulphite and 2 M sodium phosphate was added to the well. The absorbance was then read at 405 nm with a microplate reader (Molecular Devices; Melbourne, VIC). Each time the assay was performed, a standard curve consisting of five glycine standards, at different concentrations, was constructed. The glycine standards were treated with 835 mM TCA, but were not filtered. The concentrations of these standards were 2.7, 5.3, 8.0, 11.7 and 13.3 mM.

3.2.8.2 Fluorescamine assay

The fluorescamine assay was performed as described by Kocak and Zadow (1985a), with minor modifications. A 500 µL volume of sample was mixed with 500 µL of 734 mM trichloroacetic acid (TCA) (BDH; Melbourne, VIC).

This was incubated for 20 min at room temperature and filtered through No. 41 filter paper (Whatman PLC; Brentford, England). A 12.5 µL aliquot of the filtrate was mixed with 1 mL 100 mM sodium phosphate at pH 8.0 and 500 µL of 719 µM fluorescamine in acetone and measured for intensity of fluorescence at an excitation wavelength of 390 nm and an emission wavelength of 475 nm using a model F-2000 fluorescence spectrophotometer (Hitachi High-Technologies Corporation; Tokyo, Japan).

3.2.9 Milk storage conditions and sterility tests

The UHT whole milk with added crude lipase and protease was stored in 10mL volumes in McCartney bottles at 7, 25 and 37 ºC for 168, 72 and 24 h, respectively. Sterility tests were performed after incubation using the spread plate method as described in AS 1766.1.4 (Standards Australia, 1991). The medium used was Plate Count Agar with incubation at 25 ºC for 72 h. Only results for sterile milk are reported.

3.2.10 Determination of lipolysis and proteolysis in stored milk.

The extent of lipolysis and proteolysis in the stored milk was determined by measuring the increase in FFA and FAG content before and after the storage period. Results are reported as an increase in FFA (measured in meq/L) or FAG (measured in Leu-Gly equivalents) content for lipolysis and proteolysis, respectively. All tests were performed in triplicate.

3.2.11 Statistical analysis

Differences between treatments were determined using analysis of variance (ANOVA). Correlation coefficients and ANOVAs were calculated using GenStat for Windows, Version 7.2.0.208 (VSN International; Hemel Hempstead, England). Bars above and below each data point on each graph represent the least significant difference (LSD) at $p<0.05$.

3.3 Results

3.3.1 Stability of lipase assay mixtures

Assay mixtures for the lipase tests comprised the prepared substrate, hydrocolloid, buffer and milk sample. Under all conditions tested, the triolein substrate remained stable in the assay mixture for the duration of the incubation period, regardless of the hydrocolloid used to disperse the substrate. However, assay mixtures containing cream powder did not remain stable and homogenous when gum arabic was used in the assay. This was seen in a clear cream layer developing over the 24 h incubation period when 100 mM Tris-HCl at pH 7.0 was used as the buffer. The stability was not improved by sonicating the hydrocolloid and substrate rather than using homogenisation. At this point, additional hydrocolloids were investigated for their ability to maintain homogeneity of the cream powder. These were carboxymethyl cellulose and xanthan gum. Both were used initially at 10%, which is the same concentration of gum arabic in the stabilisation of triolein-based lipase assays. At this concentration in the cream powder assay, the carboxymethyl cellulose separated more distinctly than the gum arabic colloidal dispersion and the xanthan gum was too viscous. Reduction of the concentration of the xanthan gum to 1% improved the liquidity of the assay and maintained homogeneity during incubation at 37 ºC for 24 h. Therefore, xanthan gum at 1% was selected for use in subsequent lipase assays.

3.3.2 Relationship between lipolysis in UHT whole milk and lipolysis in the lipase assay

3.3.2.1 Correlation coefficients

The lipase assays were initially performed at pH 7.0 and 8.0 using triolein and cream powder substrates. Linear regression analyses demonstrated that there was a good correlation between the progress of lipolysis in the assays and hydrolysis of the fat in the UHT whole milk samples when stored at 37 ºC for 24 h (Table 3.1). Under these conditions, the correlation coefficients ranged between r=0.86 to 0.88. The correlation was lower with decreasing milk storage temperatures (r=0.65 to 0.78 and 0.34 to 0.51 for milk stored at 25 ºC for 72 h and 7 ºC for 168 h respectively). The highest assay correlation coefficients were obtained with triolein at pH 7.0 and cream powder at pH 8.0 irrespective of

the milk storage temperatures. While these assays had the same correlation coefficient with the milk stored at 37 ºC (r=0.88), the cream powder assay at pH 8.0 and triolein assay at pH 7.0 had higher correlation coefficients for the milk stored at 25 and 7 ºC (r=0.78 and r=0.51 respectively).

In an attempt to further improve the correlation between the assays and milk storage results an additional assay pH was examined (pH 7.5). This improved the correlation when the milk was stored at 37 ºC (r=0.94 and 0.92 for triolein and cream powder, respectively) but did not substantially improve the correlation for the milk stored at 25 (r=0.76 and 0.73) and 7 ºC (r=0.50 and 0.54).

Table 3.1: Correlation between the results of the triolein and cream powder lipase assays and lipolysis in stored UHT milk.

Lipase assay		**Correlation co-efficients for milk stored at**		
Substrate	**pH**	**37 ºC for 24 h**	**25 ºC for 72 h**	**7 ºC for 168 h**
Triolein	7.0	0.88	0.73	0.51
	8.0	0.86	0.73	0.46
Cream powder	7.0	0.86	0.65	0.34
	8.0	0.88	0.78	0.46

3.3.2.2 Assay sensitivity

There was significant progress of lipolysis in all assays ($p<0.05$). When triolein was used as the substrate in the assays, isolates 102 and 328 produced the greatest response at pH 7.5 ($p<0.05$) while isolate 65 had a similar response at pH 7.0, 7.5 and 8.0 ($p>0.05$) (Table 3.2). In contrast, there was more variation in lipolysis when cream powder was used as the substrate. Although not always statistically significant at both concentrations, isolate 65 showed a higher response at pH 8.0 and isolates 102 and 328 at pH 7.0. At pH 7.5 there was no instance where cream powder would give higher results than triolein ($p>0.05$). The assay with triolein gave higher results ($p<0.05$) than cream powder for four out of the six treatment conditions studied.

3.3.3 Relationship between proteolysis in UHT whole milk and proteolysis in the protease assay

Initially, proteolysis was followed using the TNBS assay. However, owing to high background readings in assays buffered with 3 M Tris-HCl, this approach was abandoned. In contrast, the fluorescamine assay was not affected by buffer concentration, therefore this test was used to determine proteolysis.

Table 3.2: Increase in free fatty acids in triolein and cream powder lipase assays with crude lipase from different isolates of *Pseudomonas* spp.

Isolate	Concentration (%)	Triolein			Cream powder		
		pH 7.0	pH 7.5	pH 8.0	pH 7.0	pH 7.5	pH 8.0
65	1.0	3.8^{abc}	4.2^{abc}	5.0^{ac}	3.2^{b}	3.8^{abc}	5.2^{ac}
	2.0	7.9^{a}	8.3^{a}	8.5^{a}	6.4^{b}	5.9^{b}	8.2^{a}
102	2.0	0.5^{ac}	1.4^{bd}	0.6^{ac}	1.3^{bd}	1.0^{ef}	0.9^{cef}
	3.0	1.0^{a}	2.1^{b}	0.5^{c}	2.0^{b}	1.6^{d}	1.5^{d}
328	0.05	0.8^{a}	1.9^{b}	0.3^{c}	1.3^{d}	0.9^{a}	0.8^{a}
	0.10	1.4^{a}	2.2^{b}	0.6^{c}	2.4^{b}	2.1^{b}	1.6^{a}

Units for free fatty acids are meq/L
Different superscripts within each row indicate significant differences ($p \leq 0.05$)

3.3.3.1 Correlation coefficients

There was a very good relationship between proteolysis in UHT milk and in the casein and SMP assays (Table 3.3). In general, casein gave higher correlation coefficients than SMP, although there was a difference with regard to storage conditions of the milk. While all correlation coefficients were high, near perfect ($r=0.99$ to 1.00) correlation coefficients were observed with assays carried out at 37 ºC for 24 h when casein was utilised as substrate. For casein, the correlation coefficients decreased to between 0.96 to 0.98 and 0.84 to 0.89 for milk stored at 25 ºC for 72 h and 7 ºC for 168 h respectively. Overall, pH and buffer concentration did not have a marked effect on correlation coefficients, for either substrate.

3.3.3.2 Assay sensitivity

The proteases from isolates 65 and 117 were active in the assays; however, isolate 102 did not produce a significant response ($p>0.05$), except against SMP when the 3 M buffer at pH 7.0 was used. For the other two isolates, the highest results were observed with the 100 mM pH 7.0 buffer for

both casein and SMP substrates, with casein being preferred over SMP ($p<0.05$) (Table 3.4).

Table 3.3: Relationship between the casein and skim milk powder protease assays and proteolysis in stored UHT milk

Protease assay			Correlation coefficients for milk stored at		
Substrate	**Buffer concentration (M)**	**pH**	**37 ºC for 24 h**	**25 ºC for 72 h**	**7 ºC for 168 h**
Casein	0.1	7.0	1.00	0.98	0.89
	3.0	7.0	0.99	0.97	0.84
	3.0	8.0	0.99	0.96	0.88
SMP	0.1	7.0	0.98	0.95	0.92
	3.0	7.0	0.94	0.83	0.81
	3.0	8.0	0.84	0.90	0.73

SMP=skim milk powder

Table 3.4: Increase in free amino groups in casein and skim milk powder assays with crude protease from different isolates of *Pseudomonas* spp.

Isolate	**Concentration (%)**	**Casein**			**Skim milk powder**		
		pH 7.0 0.1 M	**pH 7.0 3.0 M**	**pH 8.0 3.0 M**	**pH 7.0 0.1 M**	**pH 7.0 3.0 M**	**pH 8.0 3.0 M**
65	0.05	6.2^{a}	0.8^{b}	1.0^{b}	4.1^{c}	3.5^{d}	1.5^{e}
	0.10	16.7^{a}	1.8^{b}	1.5^{b}	11.9^{c}	7.3^{d}	1.4^{b}
102	0.15	-0.2^{a}	0.2^{b}	0.2^{b}	-1.6^{c}	2.4^{d}	0.6^{e}
	0.25	0.8^{a}	0.1^{a}	0.1^{a}	-2.3^{b}	2.8^{c}	0.7^{a}
117	0.15	14.1^{a}	3.0^{b}	3.0^{c}	6.8^{d}	5.1^{e}	3.4^{c}
	0.25	19.5^{a}	4.1^{b}	4.1^{c}	14.5^{d}	7.3^{e}	4.0^{c}

Assays were buffered at 0.1 (pH 7.0) or 3.0 M (pH 7.0 and 8.0)
Units are µM Leu-Gly equivalents
Different superscripts within each row indicate significant differences ($p \leq 0.05$)

3.3.4 Use of the 2,4,6-trinitrobenzenesulphonic acid assay

The TNBS assay was unsuitable for use with the 3.0 M buffer employed in this investigation due to the presence of TNBS-reactive products in the assay, which are a normal component of the buffer. The level of FAG was typically between 9-10 mM glycine equivalents, in both the control and treatment samples. The photograph of the reacted standards in Figure 3.1 shows that the FAG content present was towards the upper range of the standard curve, which resulted in inability to clearly discern protease action. Furthermore, standard deviations between replicate values were higher,

typically at 7%, compared to the fluorescamine assay, where the standard deviations were approximately 5%.

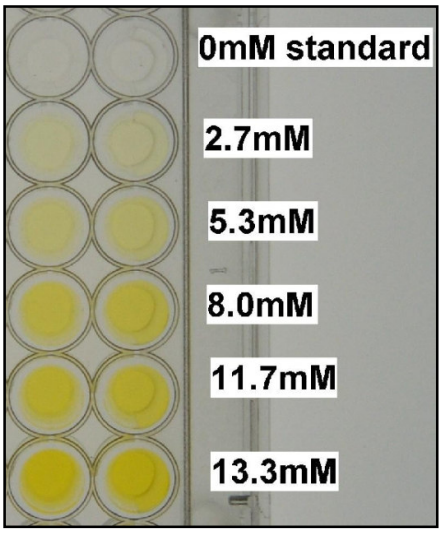


Figure 3.1: TNBS reacted glycine standards, with concentration within the standard curve.

3.4 Discussion

3.4.1 Correlation of lipolysis or proteolysis in stored milk with results of lipase and protease assays

The lipase and protease assays studied in this investigation all demonstrated good potential for estimating the extent of lipolysis and proteolysis in stored milk products. As the correlations between the assay and milk storage results diminished with storage temperature, it appears that the assays would be best suited to UHT milk which is stored at ambient temperature, as opposed to extended shelf life (ESL) or pasteurised milk which is refrigerated. Most lipases and proteases *Pseudomonas* spp. isolated from milk have optimum activity at 37 ºC (Cousin, 1982; Fox *et al.* 1989). At this temperature the rate of lipolysis and proteolysis should be highest. Although the correlation decreases at storage temperatures below 37 ºC, this may not be a great disadvantage as it relates to storage conditions which are most likely to be encountered. The various isolates tested using the protease assays showed a clear preference for substrate (casein) and pH (7.0), which is expected. This

is because these proteases show a distinct preference for casein as substrate in comparison to whey proteins (Mitchell & Ewings, 1985; Mitchell & Marshall, 1989). This result is consistent with the fact that although the protein concentration was the same in both casein and skim milk assay mixtures, the casein assay mixture contained a higher concentration of preferred protein. Demonstrating of preference for substrate of particular conditions was not as clear for lipase, as it was for protease. For the lipase result, this suggests that the crude preparations may contain multiple lipases and proteases, with variations in pH optima. In addition, incubation temperature has a more marked effect on variation in results of lipase assays than on the protease assays. The reasons for this were not investigated; however, it is known that milk fat melts over a wide temperature range (Mulder, 1953). As temperature decreases the fluidity of the fat decreases, with triacylglycerols of lower molecular weight having lower melting points (Taylor & MacGibbon, 2003) and therefore being available for the action of lipases over a wider temperature range.

In the present investigation, the applicability of the assays for future use in the prediction of UHT milk shelf life was determined, using, as a model, milk stored for a short period. The approach was used to establish correlation between hydrolysis of the substrate in the assays and hydrolysis of milk fat or protein in stored milk. It will be necessary to conduct further investigations to determine if the assays are sufficiently sensitive to indicate spoilage during the standard shelf life of UHT milk.

3.4.2 Buffering of lipase and protease assays

The pH values selected for all lipase and protease substrate mixtures were 7.0 and 8.0, as optimum pH for the lipases and proteases under investigation is slightly alkaline (Cousin, 1982; Fox *et al*, 1989). While the pH adjustment could be readily achieved for the lipase assay using 300 mM Tris-HCl buffer, this was not the case with the protease assays where a 3.0 M buffer was needed to obtain a pH of 8.0. This concentration was unsuitable as it clearly interfered with protease action. Furthermore, the 3.0 M buffer was incompatible with the TNBS assay for measuring protease activity in the assay. The fluorescamine

assay did not have this disadvantage. While the TNBS assay is convenient to perform, the fluorescamine assay is more rapid as the reaction between FAG and fluorescamine is completed within seconds, at room temperature (Bantan-Polak *et al*., 2001) and therefore has a clear time advantage. Furthermore, it is reported to be more reliable than the TNBS assay due to smaller standard deviations between replicate results (Kwan *et al*., 1983). This was also observed in the current study.

3.5 Conclusions

- Lipase (with triolein as substrate) and protease (with casein as substrate) assays are promising candidate tests to be used for the reliable prediction of the onset of enzymatic spoilage in UHT milk.
- The assays are less suited to the determination of hydrolysis in milk stored at refrigeration temperature (pasteurised and ESL milk) than to milk stored at ambient temperature (UHT milk).

CHAPTER 4

EVALUATION OF LIPASE AND PROTEASE ASSAYS AS TOOLS TO PREDICT SPOILAGE OF UHT MILK

4.1 Introduction

This chapter describes work to develop and evaluate assays utilising synthetic chromogenic or fluorogenic substrates, which release coloured or fluorescent labels when acted on by the lipases or proteases of psychrotrophic *Pseudomonas* spp. The rate of release of the labels can be related to lipase or protease activity. Sevier (1976) stated the criteria for an ideal protease assay, which can be applied to lipase assays as well. These are; I) high sensitivity, II) reproducibility, III) short incubation time and IV) low blank readings. Furthermore, Sevier (1976) pointed out that the assay substrate should resemble the enzyme's usual substrate.

With reference to the criteria of Sevier (1976), detailed above, assays which utilise synthetic substrates have advantages over those that utilise natural milk-based substrates, as well as limitations. For example, detection is often less tedious and rapid with synthetic substrates (using spectrophotometry, for example) and sometimes, a qualitative indication of the extent of lipase or protease present can be obtained visually, without the need for any equipment. Many chromogenic and fluorogenic assays have been proposed for lipase and protease detection, including β-naphthyl caprylate (β-NC) and 4-methylumbelliferyl oleate/caprylate for lipase and azocasein and FITC-casein for protease detection.

One of the first groups of synthetic substrates to be used in assays for detection of lipase or protease from *Pseudomonas* spp. were esters of 4-methylumbelliferone. Roy (1980) tested purified and concentrated *P. fluorescens* lipase (produced in synthetic media) with five esters of 4-methylumbelliferone (4-MU), viz.: butyrate, nonanoate, palmitate, oleate and elaidate, (the incubation time was not specified). In the same year, Fitz-Gerald & Deeth (1983) and Stead (1983) reported the use of the 4-methylumbelliferone ester-based assay (heptanoate and oleate esters) using crude lipase, while shortly thereafter Stead (1984a), published similar work, but involving 4-MU nonanoate as substrate. Stead (1983, 1984a) investigated the 4-MU oleate/nonanoate for determination of lipase activity in buffer and pasteurised

milk with added crude lipase while Fitz-Gerald and Deeth (1983) used the heptanoate and oleate esters of 4-MU to assess activity of a crude lipase preparation directly. Incubation times for all assays were short (less than two hours) and the expected lipase activity in the samples used was high. Nevertheless, Fitz-Gerald and Deeth (1983) commented on the high sensitivity of the method, especially with increased incubation time. However, various disadvantages exist, as stated by Stead (1984b): reduction of the measurable lipolysis by cream, inhibition of lipase by its association with casein and turbidity of milk. These problems can generally be overcome without too much effort.

Shortly after esters of 4-MU were characterised as substrates in assays for detection of pseudomonad lipases, McKellar (1986) published another synthetic-substrate assay method for detection of pseudomonad lipases - the colourimetric assay based on utilising esters of β-naphthol. Initially, three β-naphthol esters were investigated; butyrate, caprylate and myristate, with a crude lipase from *P. fluorescens* B52. As substantially higher activity was obtained with β-NC, McKellar and Cholette (1986) extended this work, with β-NC only, to detection of crude *P. fluorescens* B52 lipase in pasteurised skim milk. While sensitivity was not assessed in either work, due to the short (30 min) incubation times, the assay was simple to carry out and interpret, even though fat was a hindrance.

Azo dye-labelled protease substrates appear to have been used for a long time, including for milk and dairy products, without many changes to the method or reference to an original method. From previous applications, its suitability to detection of low protease activity in UHT milk was not demonstrated. For example, Mitchell and Ewings (1985) tested activity of a purified protease, while Ewings *et al.* (1984) and Kohlmann *et al.* (1991) tested activity of a crude preparation from synthetic media. Both of these would have been expected to have shown a high degree of proteolysis because they were not diluted. Bendicho *et al.* (2002), working on optimisation of the azocasein assay, tested skim and whole milk samples with a range of concentrations of added *Bacillus subtilis* protease. The work described in this paper is closest to the applications intended in the present investigation.

Initial work on the use of fluorescein isothiocyanate (FITC)-casein was published by Christen and Senica (1987). Using an assay incubation period of 30 min, protease was detected in pasteurised milk inoculated with one isolate of a psychrotrophic bacterium, which included a *P. fluorescens* isolate. Unlike previous work with assays using synthetic substrates, Christen and Senica (1987) did not determine the activity of purified or crude enzyme. Instead, they adopted a more practical approach by directly determining the extent of proteolysis in pasteurised milk to which were added protease-producing psychrotrophic bacteria, and reported a high degree of sensitivity. Similar to previous investigations in this area, a short incubation time was used. However, a major step in improvement of this assay for detection of protease in UHT milk was the direct detection of the low level of protease that was present in the pasteurised milk used. Thus, high sensitivity and the potential for direct detection of protease produced in milk were demonstrated.

These assays can give rapid results (<1 h), but the work conducted to date has not established their sensitivity, stability and usefulness in predicting spoilage of long life dairy products. A useful assay would need to have minimal spontaneous substrate decomposition and be able to detect low lipase and protease activity that may cause spoilage during prolonged storage periods.

In addition to labelled substrates, other substrates have also been widely used for detection of lipase and protease. Triolein (systematic name 1,2,3-propanetriyl-tri-(Z)-9-octadecanoate, but referred to as triolein throughout this thesis) is used often in lipase detection assays, although olive oil (a mixture of various triacylglycerols) is commonly used as well. Following the incubation period, a titrimetric determination of the free fatty acids (FFA) is made.

Casein is one of the most common substrates for all types of proteases; in addition, it is the most abundant milk protein. Cream powder and casein are natural milk-based substrates, and their degradation as a result of proteolytic action exposes the free amino groups (FAG) of the resulting free amino acids or peptides, which are easily quantifiable. Due to their natural occurrence as components of milk or close resemblance to the natural substrates for these

enzymes, it is possible that the use of these substrates may lead to improved sensitivity of the assays that utilise them.

In the previous chapter, the use of various non-labelled substrates was examined and triolein and casein assays recommended for subsequent use. In the work conducted in this section, these non-labelled substrates will be evaluated and compared with chromogenic and fluorogenic substrates. Following a literature review of available chromogenic and fluorogenic substrates that have been used in assessment of lipolysis and proteolysis in milk, one chromogenic and one fluorogenic substrate were selected for each of lipase and protease detection. These were then compared with the assays utilising non-labelled substrates recommended in the previous chapter. Initial comparisons involved measurement of the extent of hydrolysis and correlations with lipolysis or proteolysis in milk (stored for up to one week), and then the most promising synthetic labelled substrate was selected and incubated in an assay for up to two weeks to assess stability of the substrate, sensitivity of the assay and its relationship to lipolysis and proteolysis in UHT milk stored for an extended period.

4.2 Materials and Methods

4.2.1 Source of chemicals

Unless otherwise stated, all chemicals were of the highest grade available and purchased from Sigma-Aldrich Co. (Sydney, NSW).

4.2.2 Lipase assays

4.2.2.1 β-naphthyl caprylate assay

The β-NC assay was based on the procedure of Versaw *et al.* (1989), with some modifications. Composition of the assay mixture was: 35 µL of sample, 50 µL of substrate (100 mM β-NC prepared in dimethylsulphoxide (DMSO) (Calbiochem; Melbourne, VIC) and 615 µL of buffer (50 mM Tris-HCl buffer at pH 7.5). After the incubation, 7 µL of 100 mM Fast Blue BB dissolved in DMSO was added and the incubation continued for a further 5 min. Proteins and large peptides were precipitated with 70 µL of 720 mM trichloroacetic acid

(TCA) (BDH; Melbourne, VIC) and then the assay mixture was clarified with 1.75 mL of a 1:1 (v/v) mixture of 95% (v/v) ethanol (CSR Distilleries; Melbourne, VIC) and ethyl acetate (Labscan Asia; Bangkok, Thailand). Suspended material visible after chemical clarification was removed with centrifugation at 18 000 *g* for 10 min. The absorbance of the supernatant was measured at 540 nm. Concentration of products of lipolysis was determined by comparing the results with absorbance readings of the chromogen, β-naphthol (prepared in DMSO), at concentrations of 0 (distilled water only), 2, 4, 6, 8 and 10 mM. The standard curve is included in Appendix 2.3.

4.2.2.2 4-methylumbelliferyl oleate and caprylate

The method for assays utilising 4-methylumbelliferone (4-MU) esters as substrate was based on the work of Fitz-Gerald and Deeth (1983). The substrate, either 4-methylumbelliferyl caprylate (4-MUC) (Research Organics Inc.; Cleveland, OH, United States) or 4-methylumbelliferyl oleate (4-MUO), was dissolved in DMSO to a final concentration of 150 nM and then diluted 1:10 in 100 mM phosphate buffer at pH 7.5. The assays contained 2.2 mL of 100 mM phosphate buffer at pH 7.5, 500 µL of substrate and 100 µL sample. After incubation, the fluorescence of the assay was measured using wavelengths of 325 nm (excitation) and 450 nm (emission). Extent of lipolysis was determined by comparing the results with predetermined fluorescence readings of 4-MU, prepared in 50 mM citrate (BDH; Melbourne, VIC) phosphate (Ajax Chemicals; Sydney, NSW) buffer at pH 6.5, at concentrations of 0 (distilled water only), 1, 2, 5, 7, 10 and 12 µM. The standard curve is shown in Appendix 2.4.

4.2.2.3 *p*-nitrophenyl caprylate and stearate

Assays utilising *p*-nitrophenyl esters were based on the procedure of Blake *et al.* (1996). Total volume of assay mixture was 900 µL and comprised 450 µL of sample, 360 µL of 50 mM sodium phosphate (Ajax Chemicals; Sydney, NSW) buffer at pH 7.2 and 90 µL of substrate (5 mM *p*-nitrophenyl caprylate or stearate dissolved in DMSO). Following incubation, assay mixtures were clarified by adding 173 mM SDS (900 µL). Absorbance was measured at

410 nm. Results were calculated by comparison with absorbance readings at 410 nm of *p*-nitrophenol, prepared in 50 mM sodium phosphate buffer (pH 7.2) at concentrations of 0 (distilled water only), 0.5, 1.0, 2.5 and 5.0 µM. The standard curve is shown in Appendix 2.5

4.2.2.4 Triolein assay

The triolein assay, buffered at pH 7.5 with 300 mM Tris-HCl, was performed as described in section 3.2.4, except that gum arabic was used to prepare the colloidal dispersion of triolein in the assay in some experiments. This change was made from previous work as better repeatability and higher sensitivity could be achieved using gum arabic instead of xanthan gum.

4.2.3 Protease assays

4.2.3.1 Azocasein assay

For colourimetric determination of proteolytic activity, the azocasein assay method of Christen and Marshall (1984) was used. Composition of assay mixture was 2 mL of substrate (1% azocasein in 100 mM Tris-HCl buffer at pH 8.0) and 1 mL of sample. To determine the amount of sulphanilamide released from azocasein, an equal volume of 306 mM TCA was added. Incubation at room temperature for 30 min followed, prior to filtration through No. 41 filter paper (Whatman PLC; Brentford, England). Centrifugation of the filtrate was carried out for 10 min at 18 000 *g* and then the absorbance measured at 440 nm. A standard curve of sulphanilamide was used to relate absorbance to extent of proteolysis. To construct the standard curve, sulphanilamide was prepared according to the method of Charney and Tomarelli (1947). Firstly, 2.5 g sulphanilamide was dissolved in 100 mL of 150 mM sodium hydroxide (BDH; Melbourne, VIC), followed by 1.1 g of sodium nitrate. A 9 mL volume of 5 M hydrochloric acid (BDH; Melbourne, VIC) was then added and the mixture stirred for 2 min before the addition of 9 mL of 5 M sodium hydroxide. The standard curve consisted of sulphanilamide at concentrations of 0 (distilled water only), 10, 20, 30, 40, 50, 60, 70 and 80 mM. The standard curve is shown in Appendix 2.6.

4.2.3.2 FITC-casein assay

The FITC-casein assay was first described by Twining (1984). That method, and a modification to make the assay applicable to milk by Christen and Senica (1987), formed the basis of the procedure described here. The composition of the FITC-casein assay mixture was 69 µL of 0.25% type III FITC-casein in 10 mM sodium phosphate buffer at pH 8.0 and 17 µL of sample. To enable measurement of the fluorescence of the liquid portion of the assay after incubation, casein and unreacted FITC-casein were precipitated by the addition of 14 µL of 306 mM TCA, followed by centrifugation at 13 000 *g* for 5min. Fifty microlitres of supernatant was then removed and mixed with 1.2 mL of 300 mM of phosphate buffer at pH 8.5, and fluorescence measured at wavelengths of 490 nm (excitation) and 515 nm (emission). Degree of proteolysis was calculated by relating intensity of fluorescence of the assay samples to the fluorescence of the free fluorogen, fluorescein, at different concentrations. The concentrations of fluorescein used in the standard curve were 0 (distilled water only) 0.11, 0.23, 0.38, 0.76, 1.14, 2.28 and 3.80 µM. The standard curve is shown in Appendix 2.7

4.2.3.3 Casein assay

The casein assay was carried out as described in section 3.2.5 and was buffered to pH 7.0 with 100 mM Tris-HCl.

4.2.4 Assay preparation and incubation

Crude preparations of *Pseudomonas* spp. lipase and protease were used as described in Section 3.2.3. These were from three isolates of *Pseudomonas* spp., at two concentrations, to initially ascertain substrate stability, correlation with fat and protein hydrolysis and to ensure the enzymes were being used within the range that could be detected in the assay. Once the preliminary work was completed, further investigations were carried out with *Pseudomonas* spp. lipase and protease from one isolate to determine assay sensitivity.

For the initial lipase work, with β-NC and 4-MUO as substrates, crude lipase from isolates 65 (1.0 and 2.0%), 102 (2.0 and 3.0%) and 328 (0.05 and 0.10%) were used, at 37 ºC for 24 h. Following the preliminary investigations, crude enzyme from isolate 328, the isolate with the highest lipolytic potential, was used at concentrations of 0.00025, 0.0005, 0.001 and 0.002% in the β-NC and 4-MUO/C assays. The assay mixtures were incubated at 37 ºC for up to 336 h (14 d).

For the initial protease work, with the azocasein and FITC-casein assays, crude protease preparations from isolates 65 (0.05 and 0.10%), 102 and 117 (0.05 and 0.10%) were used in assay mixtures incubated at 37 ºC for 24 h. Crude enzyme from isolate 117, the most proteolytic, was used with the azocasein and FITC-casein assays at concentrations of 0.0003, 0.002, 0.003 and 0.006%. They were all incubated at 37 ºC for up to 336 h (14 d).

In all assays, 1.2 µM thimerosal (final concentration) was added to the buffer to prevent bacterial growth during incubation. All tests were carried out in triplicate.

4.2.5 Storage of UHT milk

Crude preparations of lipase (from *P. fluorescens* 328) or protease (from *P. fluorescens* 117) were added to UHT whole milk (3.3% fat content) at the following concentrations: 0.00025, 0.0005, 0.001 and 0.002% for crude lipase, and 0.0003, 0.002, 0.003 and 0.006% for crude protease. These concentrations were selected after the preliminary work to determine the detection limit. The samples were then incubated at 25 ºC for either 180 d (to observe effects of lipolytic spoilage) or 366 d (to observe effects of proteolytic spoilage). In addition, hydrolysis of milk fat or protein in milk, stored for short periods as described in section 3.2.9, was correlated with hydrolysis of the substrate in the various assays investigated in this Chapter. Sterility tests were performed on incubated UHT milk on each test day as described in section 3.2.9. Only results for sterile milk are reported.

4.2.6 Determination of free fatty acids in UHT milk

FFA were determined in triplicate by the extraction/titration technique, as described in section 3.2.7.

4.2.7 Determination of free amino groups in UHT milk

FAG were determined in triplicate with the fluorescamine assay, as described in section 3.2.8.

4.2.8 Statistical analysis

Statistical analysis was performed as described in section 3.2.11.

4.3 Results

4.3.1 Evaluation of chromogenic and fluorogenic lipase assays

4.3.1.1 β-naphthyl caprylate

In the β-NC assay, the colour of the reaction mixture prior to incubation is yellow. With incubation, the intensity of the colour increases and changes to pink, red and finally to brown with increasing lipase activity due to the release of β-naphthol. When the β-NC assay was performed with buffer (buffer control) and UHT whole milk (milk control) containing no added lipase there was minimal or insignificant increase ($p<0.001$) in β-naphthol during incubation at 37 ºC for 24 h for the respective samples, demonstrating substrate stability in the assay under these incubation conditions (Figure 4.1). Increases in intensity of colour with increasing concentration of lipase were evident when the assay was tested with milk samples containing each of the three *Pseudomonas* spp. lipases. The distinction between the results was clear enough to be observed visually.

When the results from a short (24 h) incubation of the assay mixture at 37 ºC, were correlated with the results of short term storage of UHT milk at 37, 25 and 7 ºC for 24, 72 and 168 h respectively, the correlation coefficients were 0.82, 0.56 and 0.32 respectively.

4.3.1.2 4-methylumbelliferyl oleate and *p*-nitrophenyl caprylate/stearate

Assays utilising 4-MU esters yield blue fluorescence when the substrates are cleaved by lipase. Work was initially performed using the 4-MUO substrate (C18) with buffer and UHT whole milk samples containing no added lipase (buffer and milk controls), and UHT whole milk containing lipase at two

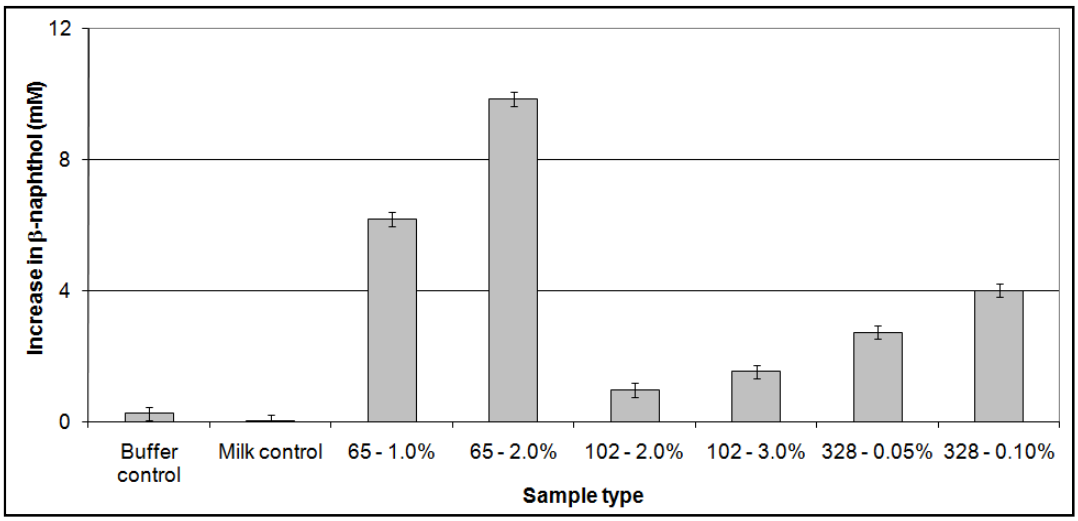


Figure 4.1: Extent of lipolysis in the β-naphthyl caprylate assay with UHT whole milk and added crude lipase from *Pseudomonas* spp. isolates 65, 102 and 328 at two concentrations at 37 ºC for 24 h. Controls contain no added lipase.

concentrations from three *Pseudomonas* spp. isolates. After incubation for 24 h at 37 ºC, the increase in fluorescence observed for the buffer control was significantly higher ($p<0.001$) than that of the milk control and the samples (Figure 4.2). There was no significant difference ($p>0.05$) in fluorescence increase between the milk control and any of the samples with added *Pseudomonas* spp. lipase. This higher background fluorescence in the assays for the buffer than for the milk control indicates that this assay is not suitable for detecting activity at the lipase concentrations tested.

The crude lipase prepared from isolate 65 (2%) was used in an assay under the same incubation conditions with another class of synthetic lipase substrate, esters of *p*-nitrophenol with both a short (C8) and a long chain (C18) fatty acid. The *p*-nitrophenol assay is a chromogenic method where an increase in the yellow chromogen, *p*-nitrophenol, reflects lipase activity. There was a significant increase ($p<0.001$) in *p*-nitrophenol in the *p*-nitrophenyl

stearate (C18) assay compared with the milk (no added *Pseudomonas* spp. lipase) control (Figure 4.3). However, there was a much larger increase in *p*-nitrophenol in the *p*-nitrophenyl caprylate (C8) assay, clearly indicating a preference for a shorter chain fatty acid. Therefore, a shorter carbon chain length 4-MU ester, 4-MUC, was used in further fluorogenic assay studies rather than the 4-MUO.

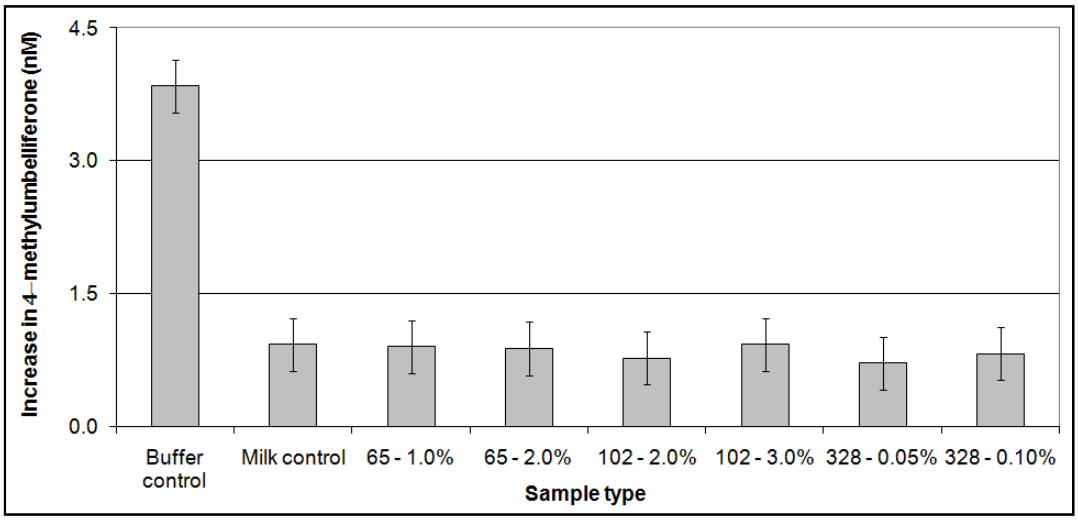


Figure 4.2: Extent of lipolysis in the 4-methylumbelliferyl oleate assay with UHT whole milk and added crude lipase from *Pseudomonas* spp. isolates 65, 102 and 328 at two concentrations, at 37 ºC for 24 h. Controls contain no added lipase.

4.3.2 Comparison of lipase assays

The sensitivities of assays utilising the β-NC, 4-MUC and triolein were compared by testing UHT whole milk with added *Pseudomonas fluorescens* lipase (isolate 328) at five concentrations. The assay mixtures were incubated at 37 ºC over 14 d. Initially, xanthan gum was used to disperse the substrate in the triolein assay mixture. However, results of this assay were inconsistent over the 14 d incubation period (Figure 4.4). This was seen in results not consistently increasing with lipase concentration and incubation time. The xanthan gum was then replaced with gum arabic, after which more consistent results and higher sensitivity was observed (Figure 4.5). Increasing concentration of added lipase resulted in an increase in FFA concentration. There were significant increases ($p<0.001$) in FFA concentration over the milk

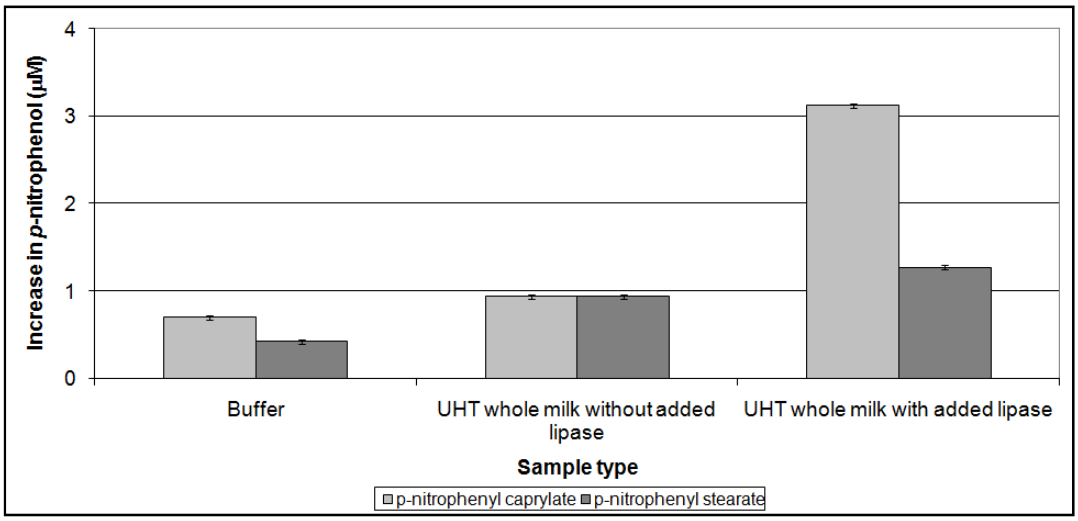


Figure 4.3: Extent of lipolysis caused by activity of crude lipase from *Pseudomonas* spp. isolate 65 in UHT whole milk tested in the *p*-nitrophenyl caprylate and stearate assays at 37 ºC for 24 h. Controls contain no added lipase.

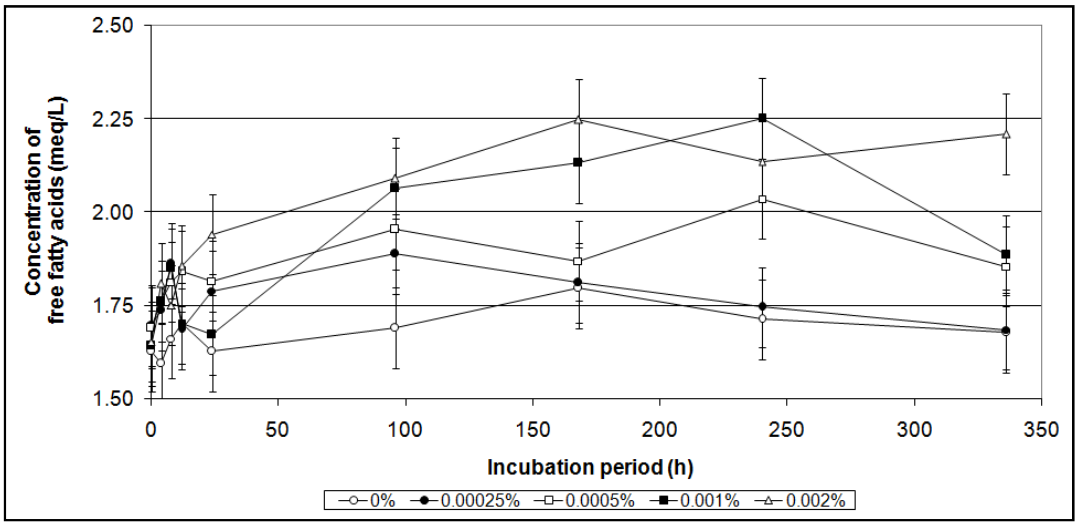


Figure 4.4: Progress of lipolysis in the triolein assay (substrate homogenised with xanthan gum) with UHT whole milk and lipase from *Pseudomonas fluorescens* isolate 328 at five concentrations, at 37 ºC for 336 h (14 d).

control, from 4 h onwards in the samples with 0.002% added lipase, from 24 h onwards for the samples with 0.001% added lipase and from 168 h onwards for the samples with 0.0005% added lipase. Even after the entire 14 d incubation, there was no significant ($p>0.05$) increase in the FFA concentration of the

sample containing 0.00025% when compared to the milk control. The FFA concentration in the milk control remained constant during this incubation period.

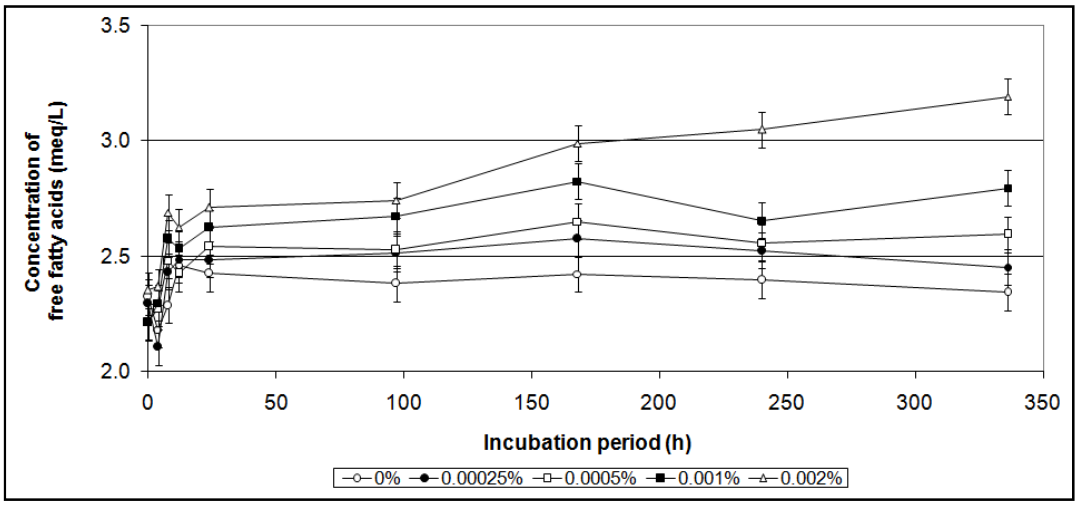


Figure 4.5: Progress of lipolysis in the triolein assay (substrate homogenised with gum arabic) with UHT whole milk and lipase from *Pseudomonas fluorescens* isolate 328 at five concentrations, at 37 ºC for 336 h (14 d).

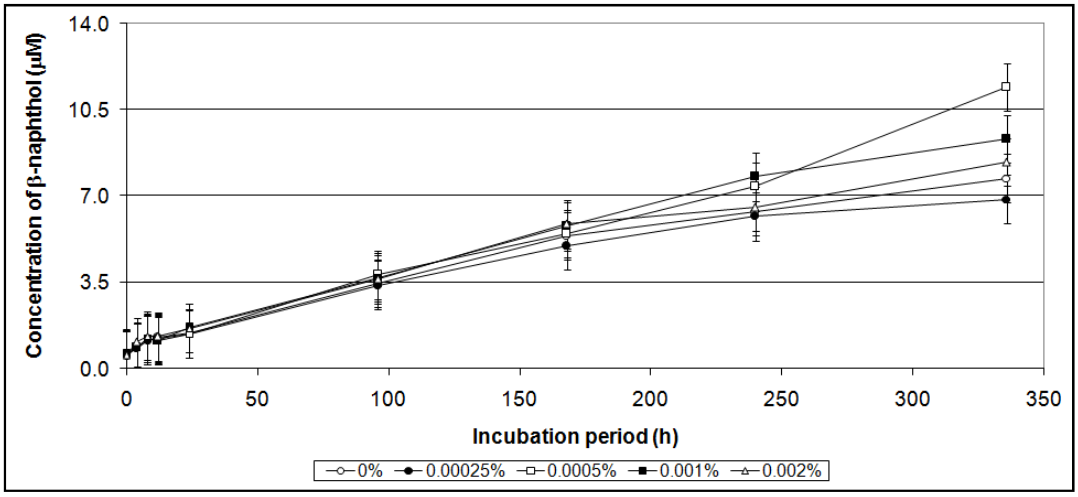


Figure 4.6: Progress of lipolysis in β-naphthyl caprylate assay with UHT whole milk and lipase from *Pseudomonas fluorescens* isolate 328 at five concentrations, at 37 ºC for 336 h (14 d).

In contrast, the β-NC assay demonstrated limited usefulness. *P. fluorescens* lipase could not be detected in any samples reliably, even with a 14 d incubation period. This is because no significant differences ($p>0.05$) were observed until day 14. However, even though there were significant differences

at day 14 ($p<0.001$), the most advanced lipolysis was not observed with the samples with the highest concentration of added lipase (Figure 4.6). The level of β-naphthol in the milk without added lipase increased significantly ($p<0.001$) over the incubation period at a steady rate and a clear relationship between concentration of added crude lipase and lipolysis was not observed.

The 4-MUC assay was also evaluated. Similar to the free chromogen in the β-NC assay, the fluorogen liberated in this assay increased significantly with incubation time in both the presence and the absence of added lipase (Figure 4.7). This corresponded to 95-100% of the substrate being decomposed with all samples and the milk control, over the incubation period. After 14 d incubation, the sample with the highest concentration of lipase had the highest concentration of 4-MU but the milk control did not have the lowest concentration of 4-MU.

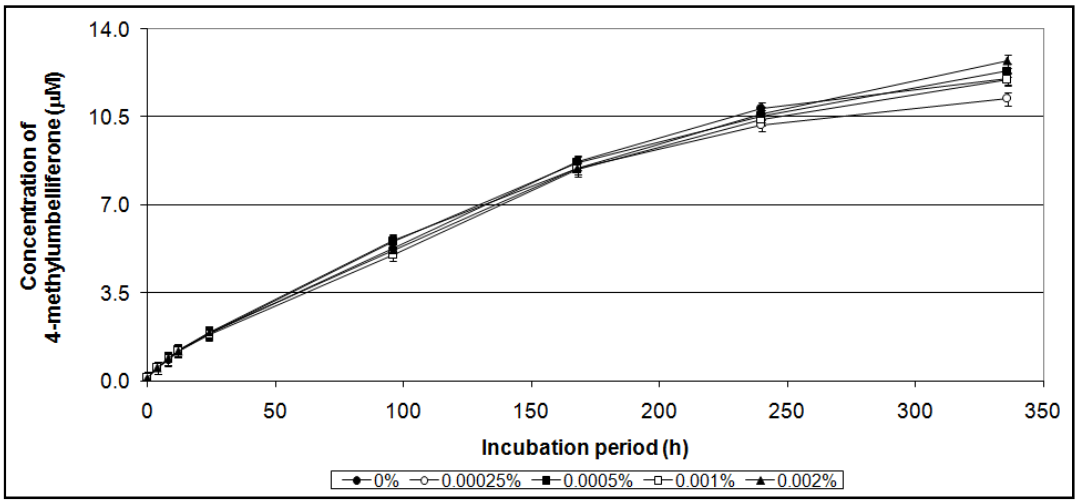


Figure 4.7: Progress of lipolysis in 4-methylumbelliferyl caprylate assay with UHT whole milk and lipase from *Pseudomonas fluorescens* isolate 328 at five concentrations, at 37 ºC for 336 h (14 d).

4.3.3 Increase in free fatty acids in UHT milk during storage

P. fluorescens lipase used in the assays incubated over 14 d was added at the same concentration to UHT whole milk and stored for 168 d at 25 ºC. The largest rate of increase in FFA occurred in the first month of incubation but

shortly thereafter the rate of increase of FFA diminished quite noticeably (Figure 4.8). Higher concentrations of added lipase resulted in larger monthly increases in FFA. At the lower concentrations of added lipase, increases from month to month were often insignificant ($p>0.05$). Concentrations of lipase at 0.0005% and above resulted in a significant increase in FFA from day 18 onwards, while 0.00025% did not increase significantly until 168 days storage. The FFA content remained consistent in the milk control (0%) during this time.

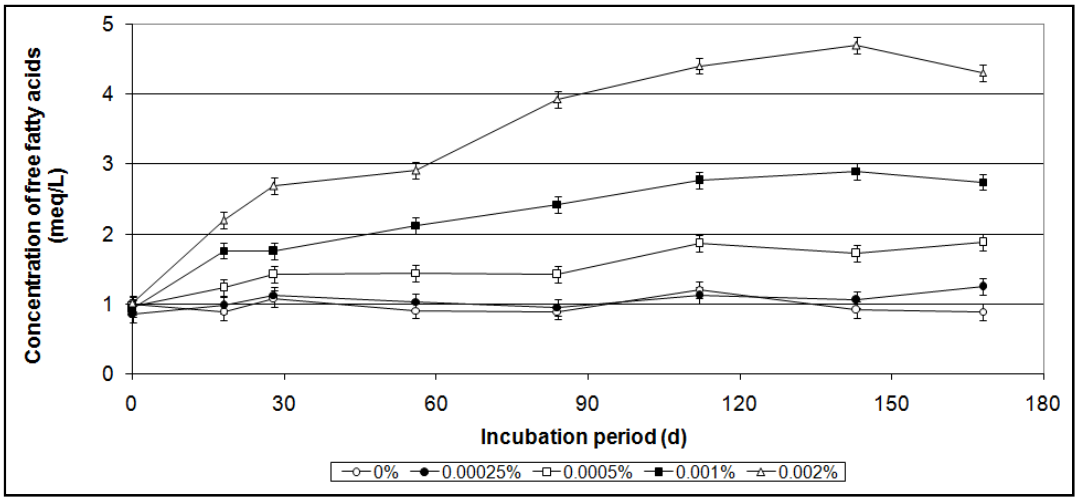


Figure 4.8: Lipolysis during storage of UHT whole milk with added lipase from *Pseudomonas fluorescens* isolate 328 at five concentrations and stored at 25 ºC.

4.3.4 Evaluation of chromogenic and fluorogenic protease assays

4.3.4.1 Azocasein

Proteolytic decomposition of the chromogenic substrate azocasein is characterised by release of sulphanilamide, an orange-coloured azo dye. When buffer and UHT whole milk without added protease was examined, there was a slight increase in sulphanilamide during incubation at 37 ºC over 24 h (Figure 4.9). Addition of *Pseudomonas* spp. protease to the milk resulted in a much larger release of the dye from the substrate ($p<0.001$) with protease from isolates 65 and 117, but not from isolate 102. The dye concentration rose with increased concentration of protease.

When the assay results, from 24 h incubation at 37 ºC, were correlated with the results of short term storage of UHT milk at 37, 25 and 7 ºC for 24, 72 and 168 h respectively, the correlation coefficients were 0.94, 0.92 and 0.80 respectively.

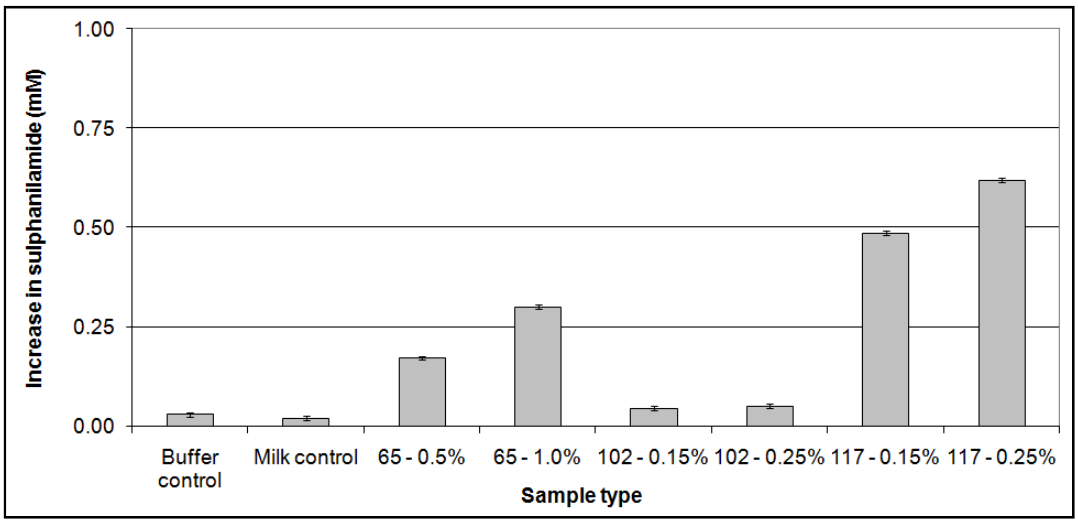


Figure 4.9: Extent of proteolysis in the azocasein assay with UHT whole milk and added crude protease from *Pseudomonas* spp. isolates 65, 102 and 117 at two concentrations, at 37 ºC for 24 h. Controls contain no added protease.

4.3.4.2 FITC-casein

Proteolysis releases fluorescein, which results in the characteristic fluorescent green colour. The concentration of fluorescein increased slightly ($p<0.001$) in the milk control without added protease over the incubation period, whereas no increase was seen in the buffer (Figure 4.10). Of the milk samples tested with added *Pseudomonas* spp. protease, no significant increase ($p>0.05$) was observed in the assays for samples with added protease from isolate 102. In contrast, there was an increase ($p<0.001$) in fluorescein between all other samples and both the buffer and milk controls. These were consistent with the increasing concentrations of protease tested.

When the assay results from 24 h incubation at 37 ºC were correlated with the results of short term storage of UHT milk at 37, 25 and 7 ºC for 24, 72 and 168 h, respectively, the correlation coefficients were 0.99, 0.99 and 0.91, respectively.

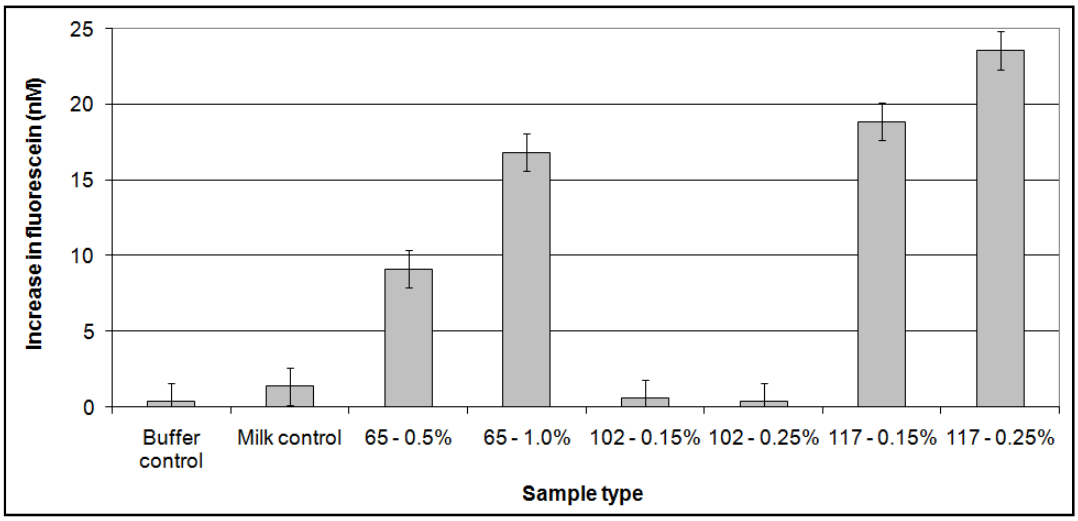


Figure 4.10: Extent of proteolysis in the FITC-casein assay with UHT whole milk and added crude protease from *Pseudomonas* spp. isolates 65, 102 and 117 at two concentrations, at 37 ºC for 24 h. Controls contain no added protease.

4.3.5 Comparison of protease assays

The progress of proteolysis in the casein and FITC-casein assays was compared at 37 ºC over an incubation period of 336 h (14 d). Both assays were most satisfactory, with regard to the distinction between results for samples with different concentrations of added crude protease. The lowest concentration of added protease (0.0003%) was detected (significantly different from the milk without added protease, $p<0.001$) at either 240 h (10 d) in the FITC-casein assay (Figure 4.11) or 336 h (14 d) in the casein assay (Figure 4.12). With the FITC-casein assay, it took 8 h until 0.003% and 0.006% added crude protease was detected (with casein, 0.003 and 0.006% were detected in 96 h - 4 d) while 12 h was necessary to detect 0.002% (compared with 96 h for casein). Clearly, FITC-casein is a more rapid assay than the casein assay. Both assays had similar sensitivity under the conditions studied (incubation up to two weeks).

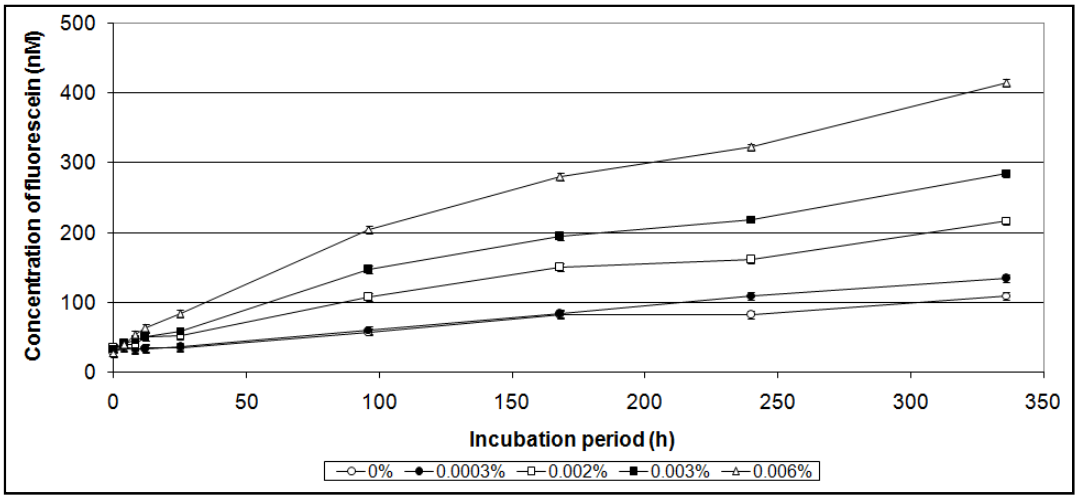


Figure 4.11: Progress of proteolysis in FITC-casein assay with UHT whole milk and protease from *Pseudomonas fluorescens* isolate 117 at five concentrations, at 37 ºC for 336 h (14 d).

4.3.6 Increase in free amino groups in UHT milk during storage

UHT whole milk, containing the same concentrations of crude protease that were tested in the various protease assays, was stored for 12 months. During the initial period of storage, there was a rapid increase in FAG in the UHT milk samples containing the three highest concentrations of added crude protease (Figure 4.13). For the two highest concentrations of added crude protease, the FAG content increased ($p<0.001$) at each time point until 140 d. Generally, after this time, the rate of increase slowed, although this was more pronounced in samples with higher concentrations of added protease. It was in these samples that decreases ($p<0.001$) in FAG were observed after 282 d. Milk with the lowest concentration of added crude protease continued to show modest, but steady increases in FAG over the entire incubation period. These monthly increases in FAG were not always significant ($p>0.05$), and it was in these samples that no rapid increase in FAG was observed over the storage period. After six and 12 months, the FAG increased from two to 13 and 21 µM Leu-Gly equivalents, respectively.

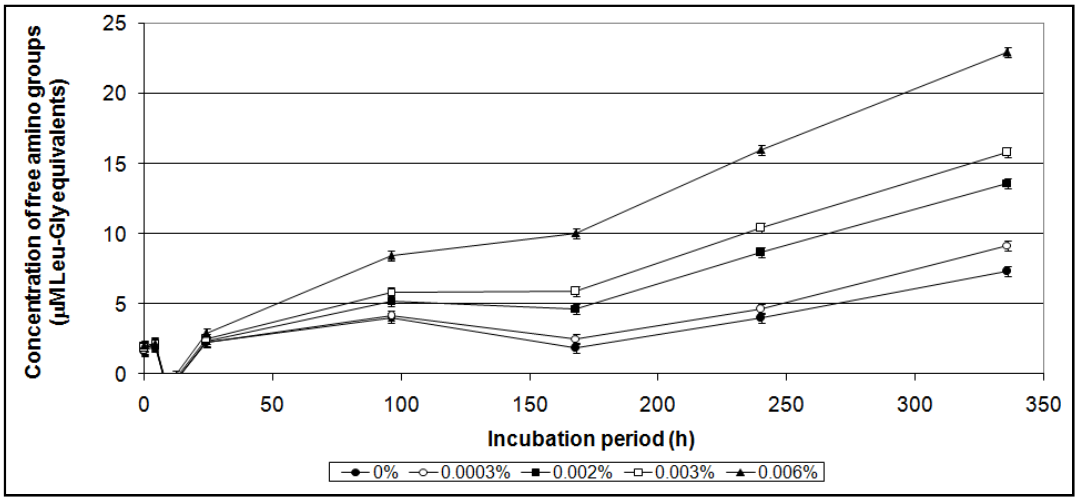


Figure 4.12: Progress of proteolysis in casein assay with UHT whole milk and protease from *Pseudomonas fluorescens* isolate 117 at five concentrations at 37 ºC for 336 h (14 d).

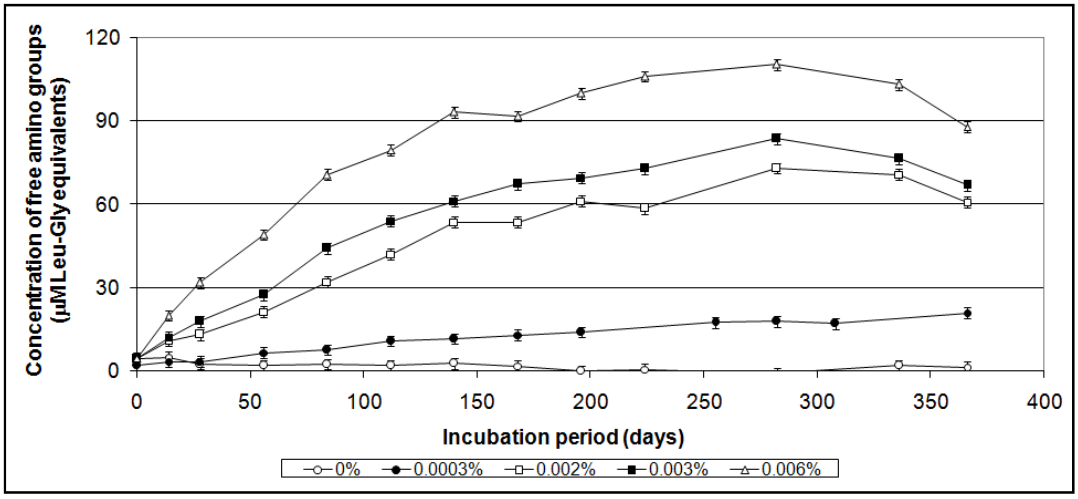


Figure 4.13: Proteolysis during storage of UHT whole milk with added protease from *Pseudomonas fluorescens* isolate 117 at five concentrations and stored at 25 ºC.

4.4 Discussion

4.4.1 Previous applications of the assays investigated in this study

Labelled substrates have been used extensively to study the lipases and proteases of *Pseudomonas* spp. isolated from dairy sources. However, in most investigations, the emphasis was on speed rather than sensitivity. For example, the 4-MUO (Stead, 1984b) and β-NC (McKellar, 1986) assays were designed to be used with a 30 min incubation period. The FITC-casein assay was also

originally designed, by Christen and Senica (1987), for a 30 min incubation period. In contrast, the azocasein assay has been used with an incubation period between 15 min (Bendicho *et al.*, 2002) and 2 h (Kohlmann *et al.*, 1991). These assays have been used previously to characterise pure lipase or protease and to detect relatively advanced hydrolytic breakdown (Mitchell *et al.*, 1986; Mitchell & Marshall, 1989; Makhzoum *et al.*, 1996; Schokker & van Boekel, 1998). Therefore, while these assays have been used previously, their purpose was quite different to the aim of this study, where detection of only limited lipolysis and proteolysis in milk stored over a period of months was required, and where the number of lipases and proteases present was not known (which is always the case in industrial practice). Consequently, no direct comparisons on performance of these assays can be made.

4.4.2 Substrate stability

Rising background levels of the chromogenic or fluorogenic label were a feature of the lipase substrates investigated. With the β-NC assay, these increases occurred both in milk samples with added lipase and in the milk control, which suggested that the substrate was decomposing, resulting in release of the chromogenic label. In the 4-MUO assay, more substrate decomposition appeared to occur in buffer than in milk with the 4-MUO assay which suggests either that, more decomposition was taking place in buffer than milk or that milk was quenching fluorescence. Alternatively, a combination of the two is also possible.

Unlike the lipase substrates investigated, high background levels were not observed in the FITC-casein assay.

4.4.3 Correlation of the assays with lipolysis and proteolysis in milk

In Chapter 3, the relationships between the results of triolein and skim milk powder assays with milk lipolysis and proteolysis were characterised. The correlation coefficients obtained for these assays using incubation conditions of 37 ºC for 24 h were 0.94 and 0.98, respectively. When the β-NC lipase and azocasein protease assays were examined under the same conditions the

correlations were lower, but still acceptable (r=0.82 and 0.94 respectively) because the r^2 values associated with these r values are well above 0.50 or 50%. This indicates that considerably more than half (67% for the β-NC assay and 88% for the azocasein assay) the variation in the milk storage results can be explained by the relationship between the milk storage results and the assay results. In contrast, both the FITC-casein and casein assays gave an excellent correlation coefficient (r=0.99).

4.4.4 Assessment of assay sensitivity

Sensitivity of the assays was determined by comparing the lowest amount of hydrolytic breakdown products that they could detect. The lowest amount of hydrolytic breakdown products that could be detected was identified as the UHT milk sample with the lowest concentration of added *Pseudomonas* spp. lipase or protease that yielded detectable hydrolytic products, which were significantly different from the milk controls without added lipase or protease. Incubation at 37 ºC for up to 14 d was employed in all assays.

4.4.4.1 Lipase assays

With the triolein assay (substrate homogenised in gum arabic), the crude lipase added at the lowest concentration did not produce a sufficient amount of FFA to be detected throughout the incubation period. Furthermore, there was no significant increase in FFA in the milk control with no added lipase. Crude lipase at all other concentrations was detected: 0.002% after 4 h, 0.001% after 24 h and 0.0005% after 168 h. In contrast, the β-NC assay did not reliably detect the *P. fluorescens* lipase in any of the samples. Furthermore, in this assay, there was a high degree of substrate decomposition, reflected in the high background level of the chromogen, β-naphthol. For the first 10 days of the 14 day incubation period, the concentration of β-naphthol released in the control was not significantly different from any of the samples with added lipase. The 4-MUO assay results were very similar to those of the β-NC assay, with very high background readings, suggesting substrate decomposition. It was not possible to detect the added lipase because of this decomposition. Reports in

the literature indicate that incubation periods in these assays were very short. As an example, McKellar (1986) incubated the β-NC assay mixture for 30 min and directly used a crude lipase preparation as sample. Only a short incubation period was required due to the very high lipase concentration; with such a short incubation period, no substrate decomposition was evident. Most, if not all original investigations of chromogenic and fluorogenic substrates employed this approach - a very high concentration of enzyme and a very short incubation period. This would easily mask any problems regarding stability of the substrate. Not only because of the short incubation time, but because the very high concentration of hydrolytic breakdown products could mean that the high levels of hydrolytic breakdown products appearing as a result of substrate decomposition would look low, in comparison.

4.4.4.2 Protease assays

The casein and FITC-casein assays were compared for sensitivity. Assays with both substrates could detect the lowest concentration of protease added (0.0003%); casein in 336 h (14 d) and FITC-casein in 240 h (10 d). This indicates that the FITC-casein assay is more rapid but both assays can be used to detect a similarly low level of proteolytic end products. With a shorter incubation time, the FITC-casein assay is more sensitive than the casein assay. The FITC-casein assay has been described previously as being the most sensitive protease assay available (Chen *et al.*, 2003).

4.4.5 Lipolysis and proteolysis during storage of UHT milk

Crude lipases and proteases from *P. fluorescens* were added to UHT whole milk at various concentrations and stored at 25 ºC to simulate conditions typical of long term shelf life product. To confirm the sensitivity of the assays for assessing spoilage in long life milk during its entire shelf life, the concentrations used included ones which resulted in minimal hydrolytic breakdown during the storage period. As expected, the higher added concentrations of lipase or protease resulted in more advanced hydrolysis of the fat and protein. Furthermore, there was a matching relationship between progress of hydrolysis

and concentration of added crude lipase/protease. Total FFA content is often measured with a solvent extraction followed by a titration. This is one of the common ways to estimate the total concentration of FFA present, and to generally determine if a milk product is undergoing lipolysis. FFA levels within a specified range are regarded as “generally acceptable” or “generally not acceptable”. Deeth and Fitz-Gerald (1976) provided a guide for interpreting results: milk with an FFA concentration above 1.3 meq/L is regarded as “generally unacceptable” to most people. The FFA concentration in the samples with the lowest (0.00025%) concentration of added lipase remained below this threshold throughout the storage period. The triolein assay did not detect lipase activity in this sample. Using the criterion of Deeth and Fitz-Gerald (1976), the samples with the second lowest concentration (0.0005%) of added crude lipase could be regarded as possessing lipolysed flavour and “generally” unacceptable” to most people after 28 d of storage. This FFA content could be detected in seven days, using the lipase assay with triolein as substrate. The FFA concentration in the milk remained stable between 28 and 112 days storage, but continued to increase after this time to reach 1.9 meq/L at the end of the experiment (168 d). Based on this observation, the triolein assay is a promising technique for assessing significant levels of lipase in UHT whole milk.

Elevated concentrations of FAG in milk samples are usually taken as an indication of proteolysis. However, no reports in the literature suggest a FAG content associated with milk spoilage due to proteolysis, whether bitterness or age gelation, which are the usual organoleptic defects associated with proteolytic spoilage. The FAG content may not be directly related to either bitterness or age gelation. Rather, accumulation of hydrophobic peptides results in bitterness (Ishibashi *et al.*, 1987a, 1987b) while protein and/or peptide interactions are likely to play a role in phenomena leading to age gelation (Datta & Deeth, 2001). However, it is reasonable to assume that increases in FAG, concurrent with the onset of age gelation and bitterness, can be indicators of proteolysis. In the next chapters, these areas will be explored in more detail when the protein fragments possibly responsible for gelation, due to proteolysis

caused by bacterial proteases, and the change in their concentration during shelf life are investigated. Furthermore, some inferences will be made on the involvement of particular peptides in the development of bitterness.

In the absence of a universally accepted test for proteolytic spoilage, increases in FAG concentration were used to assess the applicability of the casein and FITC-casein assays. The use of both tests allowed quantification of small increases in the FAG content over 168 days of storage, even at the lowest concentration of added crude protease. The FITC-casein assay gave more rapid results than the casein assay. Based on these results, both protease assays show promise for an application to assessment of significant levels of proteolysis in UHT milk.

4.4.6 Advantages and limitations of synthetic labelled lipase and protease substrates

Sensitivity is a key criterion that needs to be satisfied for an assay aimed at assessing the shelf life of UHT milk to be successful. From the industry perspective, cost is also an issue. Other considerations are the ease of carrying out the assay, whether special equipment is required, and stability of the substrate during incubation. These aspects will be discussed below.

Triacylglycerols are widely used as lipase substrates, with triolein among the most common. Since triolein comprises long-chain fatty acids, it is an ideal substrate for “true” lipases (E.C. 3.1.1.3). Tributyrin has also been used for the assay of lipases, but was not considered in the present investigation because it is not specific for lipases. Being a short-chain fatty acid, it is also an esterase substrate (Fojan *et al.*, 2000). The β-NC (C8) assay has a number of advantages as reported by McKellar (1986). These include simplicity, rapidity and the low cost of the test. These advantages would be similar to those offered by other well-described chromogenic lipase substrates, such as esters of *p*-nitrophenol. Drawbacks of these assays include a reduction in sensitivity due to FFA and milk fat that can interfere with the enzyme-substrate interaction (McKellar & Cholette, 1986). Up to 97% loss of activity was observed when whole milk (with a fat concentration of 3.5%) was included in the assay. No

solution has been developed to remedy this problem. Use of the C8 ester of β-naphthyl is based on the work by McKellar (1986) on a comparison of the preference for esters of different chain length. The C8 was found to be preferable when compared to esters of butanoic (C4) and tetradecanoic (C14) acids. β-NC is regarded as both a lipase and esterase substrate (Fojan *et al*., 2000); however, it is not known if microbial esterases would interfere with results in UHT milk. The triolein assay is based on the detection of FFA. The assay should not be subject to interference from fat as is the case in the labelled substrate assays. It would also not be subject to esterase hydrolysis.

The 4-MUO assay was developed by Roy (1980) who found that 4-MUO gave the highest sensitivity followed by 4-methylumbelliferyl nonanoate (4-MUN). These were preferred over other 4-MU esters, when using lipases of *P. fluorescens* and *Serratia marcescens* purified from minimal media supplemented with tryptone. Some of the other benefits of the use of 4-MUO are: low non-enzymatic hydrolysis, at least with short incubations (Stead, 1984a), and a higher correlation with hydrolysis of triacylglycerol substrates (Fitz-Gerald & Deeth, 1983). A method applicable to the assay of lipase present in skim milk was devised (Fitz-Gerald & Deeth, 1983; Stead, 1983) and extended to include skim milk powder, whey powder and whey protein concentrate (Stead, 1984c). An evaluation of the activity of lipases from eight *P. fluorescens* isolates and two *P. fragi* isolates towards either 4-MUO or 4-MUN was undertaken by Stead (1984b) in both a buffer-based system and a milk-based system. The results of that study indicated the applicability of 4-MUO as a substrate in a milk-based environment as activity of most lipases was substantially higher against 4-MUO than 4-MUN. A limitation to this method, as for β-NC is that fat (such as the presence of cream) reduces the measurable activity of the lipases being assayed (Stead, 1983). Interpretation (and relation to hydrolysis of a triacylglycerol substrate) of lipase activity results needs to be carried out with care, with reference to the particular 4-MU ester being used (Deeth & Touch, 2000). For example, 4-MUO correlated well (r=0.84) with hydrolysis of milk fat compared to 4-methylumbelliferyl heptanoate, which was inferior, with a correlation coefficient of r=0.69 (Fitz-Gerald & Deeth, 1983).

Sensitivity is a major criterion for selection of a substrate for the work undertaken in the present project, and the sensitivity of assays employing FITC-casein as substrate was excellent. Christen (1987) has observed that the sensitivity of this assay is similar to casein labelled with ^{14}C; assays based on radioactive substrates are particularly sensitive (Weihrauch, 1999). Further application of the Twining (1984) method, the original description of FITC-casein assay, to detect psychrotrophic bacterial proteases in milk was undertaken by Christen and Senica (1987) who evaluated the three commercially available concentrations of FITC attached to casein. The casein labelled FITC at the highest concentration of FITC was found to be most suitable as the greatest fluorescence intensity was seen. In addition, increasing concentrations of FITC attached to casein resulted in increased sensitivity (Lonergan *et al*., 1995). A comparison of the FITC-casein method with the then recently released protease assay based on bioluminescence (Rowe *et al*., 1990) was carried out by Sutherland (1993). It was found that the FITC-casein assay had a much higher sensitivity, by a factor of 100. As is the case with chromogenic protease substrates, many fluorogenic substrates for proteases have been developed, but very few are in use. Overall, the assay based on FITC-casein was found to be suitable for assessment of proteolysis in milk owing to its high sensitivity. In addition, it correlates well with hydrolysis of milk protein by proteases of psychrotrophic bacteria. Both the casein and FITC-casein assays are inexpensive, costing less than one cent per test to perform, but may require a capital outlay for a fluorescent spectrophotometer. Overall, the FITC-casein assay is less labour intensive than the casein assay.

Substrates utilising azo dyes as chromogens are widespread in their use and application. Azocasein has been described as useful in detection of general proteolytic action as it is not a specific substrate (Kohlmann *et al*., 1991). Therefore, azocasein has wide applicability to many proteases, which should include those under investigation in the present study. The proteases act primarily on casein with only limited hydrolysis of whey proteins (Nielsen, 2002). Practically, casein is almost the exclusive substrate. Therefore, its use as a substrate in bacterial protease assays is well justified. A major contributing

factor is that globular proteins, such as albumin, are more difficult for proteases to hydrolyse. Proteins with little or no tertiary structure, such as the caseins, are much more susceptible to proteolysis. Bendicho *et al.* (2002) reported that azocasein assays can be used with near-identical sensitivity with skim or whole milk, with a good precision. One possible disadvantage of azo dyes is that high background readings may result; these can be overcome with a particular procedure (Sigma-Aldrich, 2002), based on a series of steps involving dialysis, lyophilisation and centrifugation. High background readings were not observed in the present study, therefore further supporting the use of an azo dye-labelled substrate. However, the azocasein assay was inferior to the FITC-casein assay in that its results did not relate as well to proteolysis in milk.

4.5 Conclusions

- Assays based on triolein, homogenised in gum arabic, and casein or FITC-casein, are sensitive and reliable tools for the early detection of lipolysis or proteolysis, respectively, in UHT whole milk due to the action of lipases and proteases from *Pseudomonas* spp.
- At least ten days incubation is required (which is compatible with current microbiological testing in industry) to detect the lipolysis or proteolysis that will result in spoilage of UHT milk after three to six months of storage.
- Assays utilising the labelled substrates, β-NC and 4-methylumbelliferyl caprylate, are not sufficiently sensitive or stable for early detection of lipolysis.

CHAPTER 5

BIOCHEMICAL CHARACTERISTICS OF UHT MILK SPOILAGE

5.1 Introduction

Major biochemical changes take place in UHT milk upon spoilage, without accompanying microbial growth. The presence of specific hydrolytic products, resulting from lipase and protease action, can impart off-flavours or off-aromas to the product (Bassette *et al.*, 1986). In addition, structural defects such as age gelation may be seen, which is often attributed to proteolytic action (McMahon, 1996).

Action of lipase leads to various off-flavours in UHT milk, widely known and described in general as rancidity. Such terminology is ambiguous as rancidity can also refer to the spoilage defect resulting from lipid oxidation and therefore “lipolysed flavour” or “lipolytic rancidity” are the descriptive terms often given to the off-flavours resulting from enzymatic lipolysis (Shipe *et al.*, 1978). Lipolysis can occur in raw milk, and this type is termed spontaneous lipolysis, or, alternatively, heat-stable lipases can cause lipolysis in UHT milk (Deeth, 1993). The link between off-flavours and lipase action in UHT milk was demonstrated by the work of Adhikari and Singhal (1992) who found a positive relationship between FFA concentration and development of lipolysed flavour. Although capable of hydrolysing triacylglycerols containing short-chain fatty acids, esterases appear to contribute little to lipolytic spoilage. This has been shown by the work of McKay *et al.* (1995) who found the patterns of FFA released were the same in an esterase over-producer and an esterase deficient mutant when compared to the wild type. Phospholipase may also influence lipolytic spoilage. Chrisope and Marshall (1976) found that lipase action in milk can be elevated quite substantially through the presence of *Pseudomonas* spp. phospholipase C, due to the modifications to the MFGM that phospholipase brings about, which result in easier substrate access by the lipase. Griffiths

(1983) found, however, that phospholipase C from *Bacillus cereus* led to no increase in lipolysis caused by *P. fluorescens* lipase.

In UHT milk, age gelation is a serious problem (Fox, 1981) and has been known since the 1940s following the observations of Curran *et al.* (1944) and Deysher *et al.* (1944). This phenomenon, also known as "age thickening" and "coagulation" (Hong, 1982) among various other terms, is a quality defect characterised by the product increasing in viscosity and subsequently losing fluidity. It is a major quality defect, in which proteolysis is almost certainly a contributor. While the available evidence suggests that proteolytic activity plays a role in gelation, there are many other factors which determine time to onset and if gelation will actually take place. These factors are: UHT processing conditions (time and temperature) (Zadow & Chituta, 1975), the use of either direct or indirect UHT treatment (Datta *et al.*, 2002), stage of lactation (Zadow & Chituta, 1975; Auldist *et al.*, 1996) (early lactation milk is more likely to gel), proteolytic activity (Law *et al.*, 1977; Richardson & Newstead, 1979), temperature of milk storage (Samel *et al.*, 1971; Kocak & Zadow, 1985a; Manji & Kakuda, 1988) (proteolysis increases at higher temperatures but age gelation doesn't always take place at those temperatures), use of added chemicals such as sodium citrate, sodium hexametaphosphate and sodium polyphosphate (Kocak & Zadow, 1985b; 1985c), whey protein concentration (Hardham, 1999) and fat content (Garcia-Risco *et al.*, 1999) (time to onset of age gelation is longer in higher fat milks).

Bitterness in milk is a sensory perception attributed to hydrophobic peptides liberated by proteases (Datta *et al.*, 2002). A good correlation has been found between proteolysis and bitterness in UHT milk although this correlation was weaker in UHT milk samples processed using the indirect method of heating (McKellar *et al.*, 1984). There is little doubt that the action of *P. fluorescens* protease can lead to bitterness (Richardson & Newstead, 1979) as an increase in protease concentration results in more rapid development of bitterness (Adams *et al.*, 1975), with only a low level of protease required for bitter flavours to develop (Mitchell *et al.*, 1986).

Another spoilage phenomenon attributed to proteolytic action is sedimentation. It is often observed in UHT milk but is not a major spoilage problem (Burton, 1988). UHT milk sediment has been described as an aggregation of denatured protein and other components of milk that forms during heat processing (Datta *et al.*, 2002). Raw milk that has been UHT processed by the indirect method results in less sediment (Ramsey & Swartzel, 1984) and the onset of this defect can be delayed with various altered heat processing regimes (Newstead *et al.*, 2006).

The present Chapter reports the results of studies to characterise the changing nature of the hydrolytic products of *Pseudomonas* spp. lipase and protease activity in UHT milk in relation to spoilage. The approach taken was to incubate raw milk with pure cultures of two *P. fluorescens* isolates, one which was predominately lipolytic and the other predominately proteolytic, until the microflora produced lipases and/or proteases. Two incubation temperatures were used, one allowing higher lipase and protease production (raw milk sample incubated at 4 ºC – treatment sample group) while the other permitted lower production of lipase and protease (raw milk sample incubated at 1 ºC – control sample group). The raw milk was then UHT processed, and the UHT milk stored at ambient temperature. Studies were performed to determine how FFA, peptides and free amino groups (FAG) increase over time during storage. Furthermore, the applicability of the previously developed lipase and protease assays was assessed. Three separate trials were conducted with different concentrations of added *P. fluorescens* isolates.

5.2 Materials and Methods

5.2.1 Source of chemicals and microbiological media

Unless otherwise stated, all chemicals were of the highest grade available and purchased from Sigma-Aldrich Co. (Sydney, NSW). All microbiological media were purchased from Oxoid Australia Pty. Ltd. (Adelaide, SA).

5.2.2 Lipase and protease assays

The triolein (4.2.2) and FITC-casein (4.2.3) assays were used to assess lipolysis and proteolysis, respectively. The incubation time was 14 days.

5.2.3 Shelf life experiment trials

Three trials were undertaken, with slightly different methodologies. The first and second were carried out in 2005 with the third in 2006. During the first, raw milk was collected on May 6, incubated from May 6 to 10 and processed into UHT milk on May 10. The UHT milk was then stored for 182 d (26 weeks) until November 24. Raw milk for the second trial was collected on September 28, incubated from September 28 to October 5, and UHT processed on this date. Storage of the UHT milk was for a period of 14 d (2 weeks), concluding on October 20. The third trial, in 2006, commenced on February 28 with collection of the raw milk. Incubation of the raw milk was from February 28 to March 7, upon which time the milk was UHT processed. The storage time concluded on day 66 (9 weeks, 3 days), which was on May 12.

5.2.4 Raw milk collection, incubation and analysis

Approximately 70 L (Trial 1) or 80 L (Trials 2, 3) of raw milk was collected in seven (Trial 1) or eight (Trials 2, 3) containers of 10 L each, from a farm near Colac in the western region of Victoria. The milk was obtained immediately after milking from the refrigerated farm bulk tank and transported back to the laboratory at Food Science Australia in Melbourne at 0 ºC.

For trial 1, the milk was halved (35 L each), with one half incubated at 1±1 ºC (control group) and the other half at 4±1 ºC (treatment group) for five days. With trials 2 and 3, two separate cultures of *P. fluorescens* were added, isolates 328 (a known strong lipase producer) and 117 (a known strong protease producer). These isolates were inoculated separately into eight 20 mL volumes of Nutrient Broth and incubated at 25 ºC for 24 h, when the cell count reached approximately 10^7 cfu/mL. A 10 mL volume of each culture was then centrifuged for 10 min at 3000 *g*. The supernatant was discarded and the pellet resuspended in 10 mL of 145 mM NaCl (Chem-Supply; Adelaide, SA). This

washing procedure with 145 mM NaCl using centrifugation was repeated twice. The entire volume of each saline-washed culture was diluted 1:1000 by adding to each 10 L volume of raw milk, resulting in a final cell count of 10^4 cfu/mL each. The raw milk and added culture were mixed by inverting five times. The 80 L of raw milk plus added saline washed cultures were then incubated, 40 L at 1±1 ºC (control) and 40 L at 4±1 ºC (treatment) until the total count of the 4 ºC incubated culture had exceeded 10^7 cfu/mL for three days.

Various analyses of milk were carried out daily. A total plate count was conducted after mixing the milk and culture, as was measurement of pH (using a model PHM210 pH meter; Radiometer Analytical; Melbourne, VIC) and determination of FFA (see 3.2.7), FAG (see 3.2.8, using fluorescamine) and of ethanol stability.

5.2.4.1 Total plate count

Two dilution series' were prepared for each raw milk sample in 0.1% Bacteriological Peptone. The dilutions were enumerated using the spread plate technique with Plate Count Agar and incubated at 30 ºC for 72 h, based on AS 1766.1.4 (Standards Australia, 1991). Plates with between 20-200 colonies were counted.

5.2.4.2 Ethanol stability

Ethanol stability was assessed by mixing equal parts of milk and 70% ethanol (CSR Distilleries; Melbourne, VIC). The milk was considered to be heat-stable and suitable for UHT processing if a precipitate did not form (Horne & Muir, 1990).

5.2.4.3 Lipase and protease

Milk samples were collected daily and placed in an ultracold freezer (-80 ºC) for storage, so that lipase and protease (section 4.2.2) testing could be subsequently undertaken.

5.2.5 UHT treatment and milk storage

The raw milk was UHT processed using an indirect tubular pilot-scale UHT plant at the Food Processing Centre at Food Science Australia in Melbourne. The product was preheated to 75 ºC over 12 sec, followed by regenerative heating to 122 ºC over 10 sec. The next stage of heating was passage through the UHT heater for 12 sec, which heated the milk to 140 ºC, before holding at that temperature for 4 sec. Regenerative cooling took place next, which lowered the temperature of the UHT milk to 84-88 ºC over 30 sec. Further cooling took place to 50 ºC (in a water cooler) and then the milk was homogenised using a Schroder Lab 100 two-stage homogeniser (APV Australia Pty. Ltd.; Melbourne, VIC) with the first stage pressure being 17.24 mPa and the second stage being 3.45 mPa. Passage through a second water cooler lowered the temperature to 31-36 ºC, and then the homogenised UHT milk was aseptically filled into sterile 1 L airtight foil bags using an Intasept aseptic liquid packaging system (Rapak Asia Pacific; Melbourne, VIC). Plate heat exchangers were used in the preheating and UHT temperature stages while a tube heat exchanger was used for regenerative heating and cooling. All heat exchangers were obtained from Alfa-Laval Australia Pty. Ltd. (Melbourne, VIC). The flowrate through the UHT system was 1.5 L/min.

5.2.6 Assessment of UHT milk quality

5.2.6.1 Sterility and pH

Sterility of the milk samples was assessed by the spread plate method described in section 3.2.9. The pH of each UHT milk sample was tested in duplicate, at room temperature (18-21 ºC**)**, using a model PHM210 pH meter (Radiometer Analytical; Melbourne, VIC). The precision of the calibrated pH meter was ±0.02 of a pH unit.

5.2.6.2 Visual observation for age gelation

The milk samples were inspected for loss of fluidity while it was poured from the airtight foil bags. Any loss of consistent fluidity due to the presence of aggregations in the milk, but not at the base of the bag, was regarded as a gel.

5.2.6.3 Viscosity

Viscosity was measured using an LVTDV-II viscometer (Brookfield Engineering Laboratories; Middleboro, MA, United States). For most measurements, a sample volume of 30 mL was used, with the manufacturer's Ultra Low Adapter. This was because most samples measured were good quality milk, therefore a low viscosity was expected. The spindle speed was 30 rpm. When measuring high viscosity samples (gelled milk), spindle LV1, from the standard spindle set was selected. This spindle was also used at a speed of 30 rpm. Measurement of gelled milk was in a 300 mL tall beaker, using 250 mL of sample. All measurements, performed in duplicate, were made within a temperature range of 18-21 °C.

5.2.6.4 Determination of the end products of lipolysis

For an indication of FFA content, the extraction titration method was used (see 3.2.7). Determination of specific FFA was by gas chromatography/mass spectrometry (GC/MS), using a combined method of de Jong and Badings (1990) and Wijesundera and Drury (1999). Ten mL of 96.5% (v/v) ethanol, 1 mL of 2.5 M sulphuric acid (BDH; Melbourne, VIC) and 1 mL of internal standard were added to 10 mL of sample. The internal standard comprised 4.9 mM pentanoic acid (C5:0), 3.8 mM heptanoic acid (C7:0), 2.3 mM tridecanoic acid (C13:0) and 1.8 mM heptadecanoic acid (C17:0). A 15 mL volume of a mixture of equal volumes of diethyl ether (Merck; Melbourne, VIC) and heptane (BDH; Melbourne, VIC) was added next, for extraction. The sample was then centrifuged at 3000 *g* for 2 min before the upper layer was transferred to a 50 mL flask containing 1 g of anhydrous Na_2SO_4. This extraction step was repeated twice. The extract was passed through a 500 mg aminopropyl solid-phase extraction (SPE) Discovery tube (Supelco; Bellefonte, PA, United States) that had been preconditioned with 10 mL of heptane. Neutral lipids were eluted first, with dichloromethane/propan-2-ol (Merck; Melbourne, VIC) (2:1, v/v) and then FFA were eluted with 5 mL of diethyl ether/methanoic acid (97:3, v/v). For some samples, there were limited volumes available. On these occasions, all volumes above were halved.

For GC/MS analysis, a model 6850 Series II gas chromatograph (Agilent Technologies; Melbourne, VIC) was used with a model 5973 Mass Selective Detector (Agilent Technologies; Melbourne, VIC). The column was a HP-FFAP (Agilent Technologies; Melbourne, VIC) nitroterephthalic acid modified polyethylene glycol capillary column. Carrier gas was helium, used at a pressure of 57.6 kPa, a flow rate of 1 mL/min and at an average velocity of 36 cm/sec. The volume of sample injected was 1 µL at a split ratio of 20.0:1, with the split flow at 19.7 mL/min, total flow at 24.0 mL/min, pressure of 57.6 kPa and at a temperature of 250 °C. From an initial oven temperature 65 °C, the temperature was raised 15 °C per minute to a final oven temperature of 240 °C and held at the final temperature for 13 min. Tests were carried out in duplicate.

5.2.6.5 Determination of the end products of proteolysis

Four methods were used, the fluorescamine assay (section 3.2.8), high performance liquid chromatography (HPLC), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS).

HPLC analysis of peptides was based on the method of Le *et al.* (2006), with some modifications. An equal volume of trichloroacetic acid (TCA) (BDH; Melbourne, VIC) was added to UHT milk to give a final TCA concentration of 4%. The milk and TCA was mixed in a vortex mixer and incubated at room temperature for at least 60 min. Filtration was then carried out through No. 1 filter paper (Whatman PLC; Brentford, England) and the filtrate passed through an Acrodisc 220 nm syringe filter (Pall Corporation; Cornwall, England) into vials which were placed in the autosampler of the VP series HPLC, which incorporates an SIL-10AD autoinjector, DGU-14A degasser, FCV -10AL solvent delivery system, LC-10AT pump, CTO-10AS column oven and SPD-10AV UV-VIS detector (Shimadzu Scientific Instruments; Melbourne, VIC). The injection volume was 50 µL with separation in a Luna 5 µ-C18 reversed-phase column with dimensions of 150 x 4.6 mm (Phenomenex Inc.; Sydney, NSW) with a flow rate of 1mL/min. Two solvents were used for the gradient separation: solvent A

was 0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water (Millipore Australia Pty. Ltd.; Sydney, NSW) and solvent B was 0.1% (v/v) TFA in acetonitrile. At the commencement of the 55.06 min run, solvent B proportion was 20%, which was increased to 35% over the first 20 min. There was a further increase over the following five minutes with solvent B comprising 100% of the solvent at this point until the end of the separation, after a further 3 min. Commencement of column washing was carried out by decreasing the solvent B proportion to 0% in 0.6 sec and washing for 20 min in 100% solvent A. Solvent B was then increased to 20% for two minutes prior to equilibration in the same proportion of solvents for 5 min.

To study the extent of proteolysis, SDS-PAGE was also used. This was carried out with the Mini-PROTEAN 3 Electrophoresis System (Bio-Rad Laboratories Pty. Ltd.; Sydney, NSW). The manufacturer's instructions were used as a guide, with a discontinuous buffer system. Firstly, the 15% separating gel (5.0 mL 30% 2-acrylamide/bis solution - 37.5:1 mixture, 2.5 mL 1.5 M Tris-HCl at pH 8.8, 2.4 mL Milli-Q water and 100 μL 10% SDS) and the 5% stacking gel (2.85 mL Milli-Q water, 1.25 mL 500 mM Tris-HCl at pH 6.8, 850 μL 30% acrylamide/bis solution (37.5:1 mixture) - Bio-Rad Laboratories Pty. Ltd.; Sydney, NSW and 50 mL 10% SDS) were cast. Once the components of the gels were mixed together, each solution was filtered through No. 114 filter paper (Whatman PLC; Brentford, England) and then degassed for 10 min. Immediately prior to pouring each gel, 50 μL of 10% ammonium persulphate (Bio-Rad Laboratories Pty. Ltd.; Sydney, NSW) and 5 μL of N,N,N',N'-tetramethylethylenediamine (TEMED) (Bio-Rad Laboratories Pty. Ltd.; Sydney, NSW) were added. For sample preparation, reducing conditions were used. A 100 μL volume of sample was added to 200 μL of sample buffer. Composition of sample buffer, per 9.5 mL was as follows: 3.55 mL Milli-Q water, 2.5 mL glycerol, 2 mL 10% SDS, 1.25 mL 500 mM Tris-HCl buffer at pH 6.8, 200 μL 0.5% bromophenol blue. Immediately prior to use, β-mercaptoethanol was diluted 1:20 in the sample buffer. The sample and sample buffer were mixed with a vortex mixer and then heated at 95 ºC for 5 min. After this, the mixture of sample and sample buffer was mixed again with a vortex mixer and then 0.7 μL

was loaded into each well. A molecular weight standard (5 μL of a 14-116 kDa protein molecular weight standard from Fermentas International Inc.; Burlington, ON, Canada) was loaded into one well. The run was commenced with a constant application of 200 V from a PowerPac 3000 (Bio-Rad Laboratories Pty. Ltd.; Sydney, NSW) and continued until the stain was close to the bottom of the gel. At this stage, the protein bands were fixed for approximately 1 h with sideways agitation at 160 rpm in fixative solution (40% methanol, 10% acetic acid) before overnight staining in colloidal Coomassie solution (34% methanol, 17% ammonium sulphate, 3% phosphoric acid, 0.1% Coomassie G-250). Destaining was in 169 mM acetic acid for 4 h, with hourly changes of the destaining solution. The gels were then stored in 17 mM acetic acid and scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories Pty. Ltd.; Sydney, NSW). The bands were analysed using Quantity One software (Bio-Rad Laboratories Pty. Ltd., Sydney, NSW).

Peptides were discriminated by MALDI-TOF MS. The sample preparation procedure commenced with saturation of the resin bed of a C18 Zip Tip (catalogue number ZTC1 8M) (Millipore Corporation; Bedford, MA, United States) with 10 μL of 0.1% (v/v) TFA prepared in 50% (v/v) acetonitrile in double distilled water, which was then expelled and the action repeated three times. The tip was then equilibrated with four successive 10 μL volumes of 0.1% (v/v) TFA in double distilled water. Next, a series of washings were performed, with five 10 μL volumes of 0.1% TFA in double distilled water. Finally, elution was carried out by taking in and then expelling, 10 μL of 0.1% (v/v) TFA prepared in 50% (v/v) acetonitrile in double distilled water. The MALDI-TOF MS instrument used was a Voyager DE STR biospectrometry workstation (Applied Biosystems; Framingham, MA, United States) operated in linear mode for low-resolution protein analysis. One microlitre of α-cyano-4-hydroxycinnamic acid (CHCA), used as matrix, was spotted onto the sample and allowed to air dry. The sample (1 μL) was mixed with an equal volume of 0.1% (v/v) TFA prepared in 50% (v/v) acetonitrile in distilled water and spotted onto the dried matrix, and allowed to air dry. The data obtained from 500 laser shots (337 nm, nitrogen laser) were collected, the signals of the mass spectra averaged and then

processed with the Data Explorer software program (Applied Biosystems; Framingham, MA, United States).

Following MALDI-TOF MS analysis, the peptide mass to charge ratio (m/z) was used to obtain the likely primary sequence. This was carried out by entering the m/z of the peptide and the primary sequences of the various milk proteins into the FindPept tool (au.expasy.org/tools/findpept.html) on the ExPASy Proteomics Server. A mass tolerance of ±0.1 Da was used. The peptide with the smallest difference in m/z was taken as the most likely peptide, and its primary structure used for assessment of the hydrophobicity. The CC hydrophobicity scale of Tossi *et al.* (2002) was employed for quantification of hydrophobicity.

5.2.6.6 Sensory analysis

5.2.6.6.1 Electronic nose

The electronic nose was a FOX-4000 (Alpha M.O.S.; Toulouse, France), with a manual injection method permitting analysis of a large sample volume with a large headspace. Each 1 L volume of UHT milk removed from the 25 ºC storage room was refrigerated overnight at 4 ºC and then poured into a 2 L Schott bottle and covered with cling wrap. The samples were then warmed to room temperature over a period of at least 30 min. Headspace sampling was through electrical tape affixed to the cling wrap, to which domestic silicone sealant had been added at least the day before (Figure 5.1). Once the silicone had set, it provided an airtight seal through which manual sampling could be carried out. A manual sample of 10 mL was taken by inserting a 32 mm long 23G needle with an outside diameter of 650 μm (Terumo Corporation; Sydney, NSW) attached to a 10 mL syringe with an eccentric tip (Terumo Corporation; Sydney, NSW), through the silicone, electrical tape and cling wrap into the headspace. This headspace sample was then injected manually, directly into the gas supply line of the instrument. The pressure used for the instrument air gas supply was 40 kPa. Following the five minute analysis period, the sensors were cleaned with instrument air for 15 min. The results were statistically analysed, following sensory optimisation, with principal components analysis to

obtain the level of discrimination between the samples. Information from the instrument's manufacturer stated that the minimum Discrimination Index for a statistically significant difference ($p<0.05$) to be present, was 70.

Figure 5.1: Cling wrap, electrical tape and kitchen/bathroom silicone covering the top of a 2 L bottle, through which manual sampling is undertaken with a needle for electronic nose analysis.

5.2.6.6.2 Sensory panel

Sensory analysis was performed with a triangle test, based on that described in AS 2542.2.2 (Standards Association of Australia, 1983; Standards Australia, 2005). Approximately 30 mL of each sample of UHT milk was poured into 100 mL plastic cups. A lid was then placed on each cup to restrict release of volatile compounds, responsible for aroma of the sample, from the headspace. The samples were the UHT milk stored at 1 ºC prior to UHT processing (control sample) and the UHT milk stored at 4 ºC prior to UHT processing (treatment sample), with two of one of these samples and one of the other, in each of the three cups presented for sensory analysis. The standard response sheet is provided in Appendix 1. A second response sheet was developed for one-off analysis of gelled milk, also included in Appendix 1. The sensory panellists were instructed to rinse their mouth with the water provided between each tasting. A minimum of 24 panelists were used. Results were interpreted according to Roessler *et al.* (1978), who stated that a minimum of 13

panelists (out of a total of 24 or 25) must detect a difference in order for that difference to be statistically significant at $p<0.05$.

5.2.7 Statistical analysis

Differences between treatments were determined using analysis of variance with GenStat for Windows, Version 7.2.0.208 (VSN International; Hemel Hempstead, England). Bars above and below each data point on each graph represent the least significant difference (LSD) at $p<0.05$.

5.3 Results

5.3.1 Monitoring of raw milk quality prior to UHT treatment - Trial 1

5.3.1.1 Total plate count

The difference in total count between the two samples at the commencement of the incubation period was not significant ($p>0.05$) (Figure 5.2). Throughout the incubation, the total count of the control (incubated at 1 ºC) remained below 10^5 cfu/mL whereas the total count of the treatment sample (incubated at 4 ºC) increased above 10^5 cfu/mL after two days of incubation and was significantly ($p<0.05$) higher than the total count of the control sample. At the conclusion of the incubation period, the total count of the treatment sample (6.7×10^5 cfu/mL) was significantly higher ($p<0.05$) than the total count of the control sample (7.2×10^4 cfu/mL).

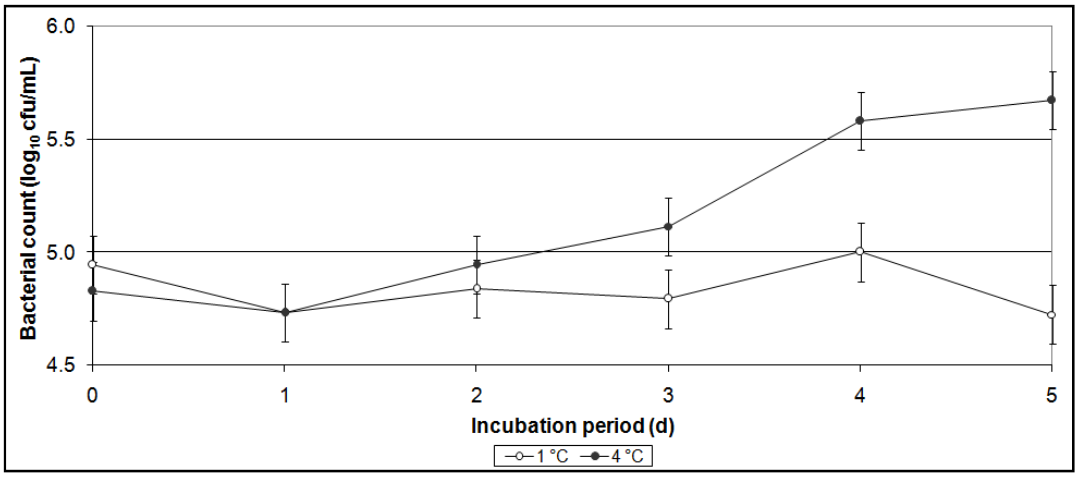


Figure 5.2: Total plate count of raw milk - Trial 1.

5.3.1.2 pH

The pH changed only marginally during incubation from days zero to five. Some fluctuations were seen, but the decrease in pH from day 0 (control sample, 6.74 and treatment sample 6.75) to day 5 (both control and treatment samples, 6.72) was not significant (Figure 5.3).

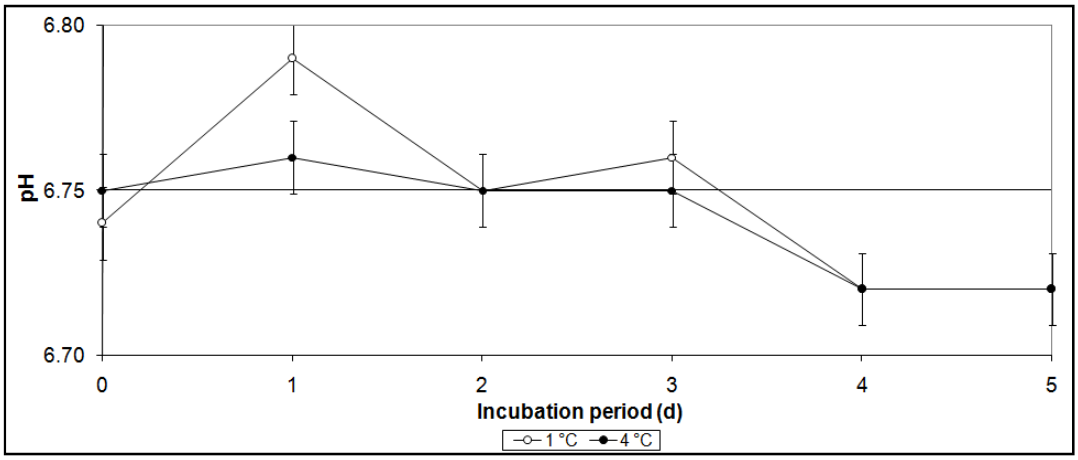


Figure 5.3: pH of raw milk during incubation - Trial 1.

5.3.1.3 Free fatty acids concentration

In the control sample, the FFA concentration did not increase over the course of the incubation while the treatment sample increased slightly (Figure 5.4). Only on day four was there a significant ($p<0.001$) difference between the control and treatment samples. As the concentration of FFA was used to indicate the presence of active lipase, it could be inferred that there was no lipase(s) in the control sample and limited lipolysis in the treatment sample.

5.3.1.4 Free amino groups concentration

While there were significant daily fluctuations ($p<0.001$) at times over the course of the incubation, at no time was there a significant difference ($p>0.05$) between the control and treatment samples. Furthermore, there was no significant change ($p>0.05$) in either sample between day 0 and day 5. The FAG concentration was used as an indication of proteolysis in the samples. No

increase in FAG concentration over time indicates the absence of detectable proteolysis.

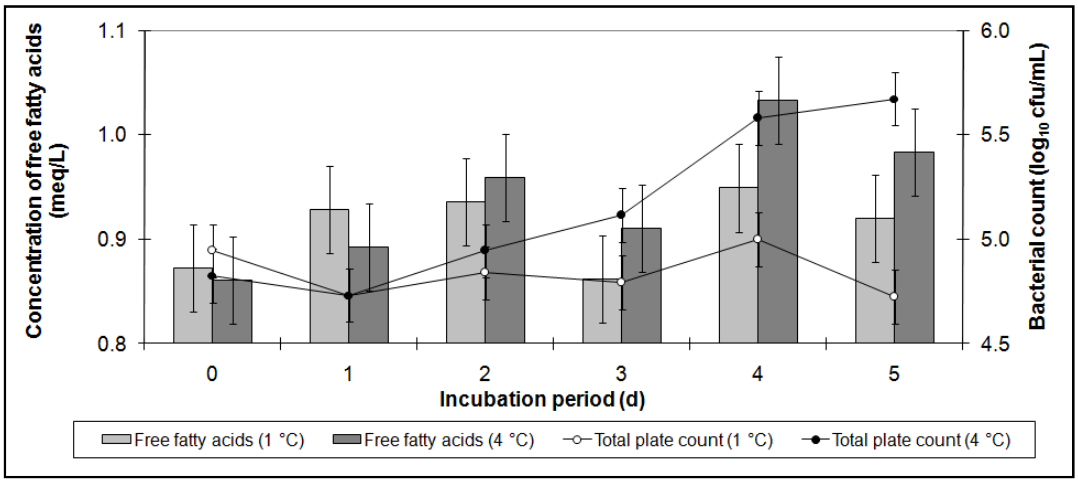


Figure 5.4: Total plate count and increase in free fatty acids during incubation of raw milk - Trial 1.

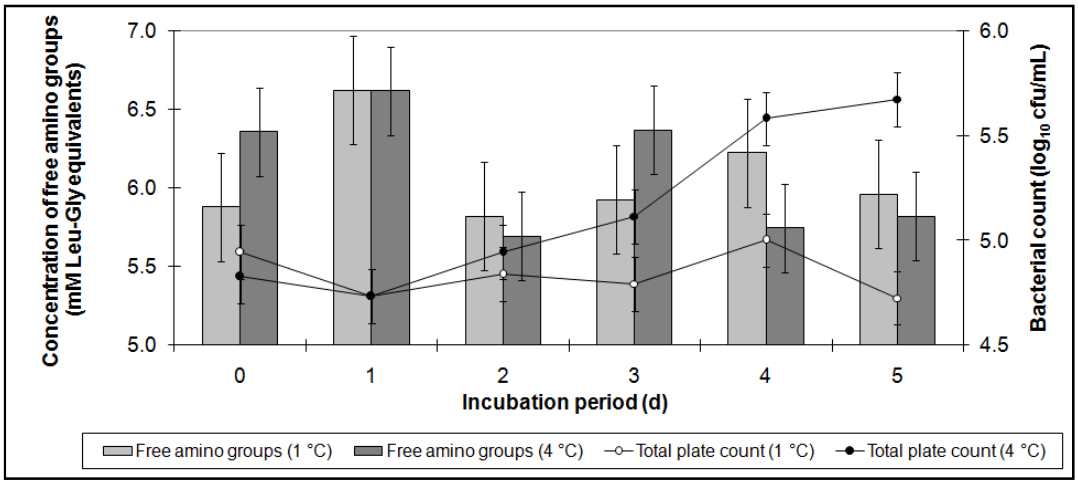


Figure 5.5: Total plate count and increase in free amino groups during incubation of raw milk - Trial 1.

5.3.2 Microbiological, chemical and physical analysis of UHT milk during storage - Trial 1

5.3.2.1 Sterility and ethanol stability

No viable bacteria were detected in the UHT milk during the shelf life trial and the milk was stable in ethanol.

5.3.2.2 pH

During the first two weeks, the pH of both samples decreased significantly ($p<0.001$), the decrease being approximately 0.2 (Figure 5.6). Following this decline, there was a period of approximately 100 d where the pH changed very little (mostly between 6.56 and 6.60). Then, over the course of the next month, a gradual decrease in pH was observed, to below 6.5, before an increase to between 6.5 and 6.6 until the end of the storage period. Although there were often significant differences ($p<0.001$) between the samples, and significant differences ($p<0.001$) between the time points, the differences were very small, typically 0.02 or less.

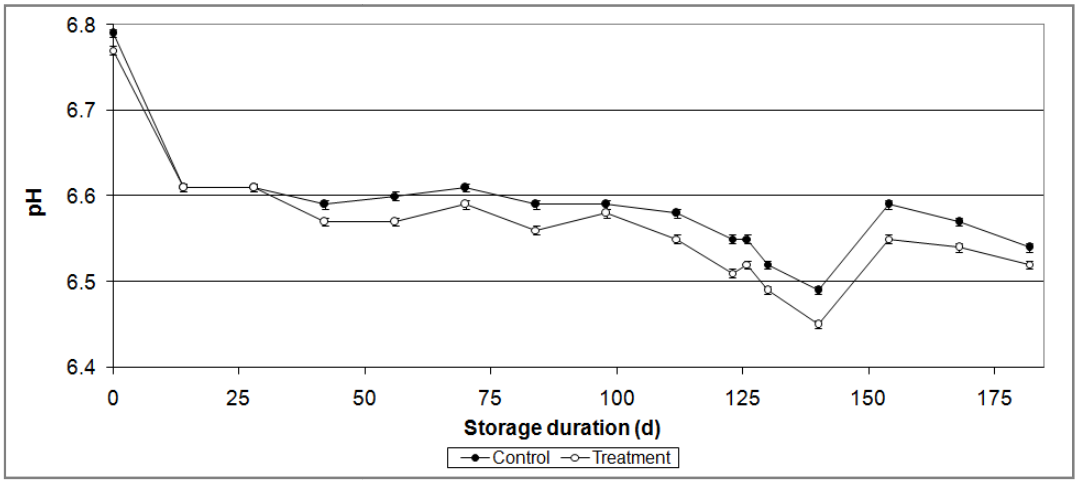


Figure 5.6: pH of UHT milk during storage - Trial 1.

5.3.2.3 Viscosity

Throughout the storage period, the viscosity of both the control and treatment samples was typically between 3.0-3.5 mPas (Figure 5.7). There were, however, some substantial variations towards the end of the storage, in which the viscosity increased above 3.5 mPas and decreased below 3.0 mPas. Similar to pH, variations between samples and between time points were often significant ($p<0.001$), but the actual values were small.

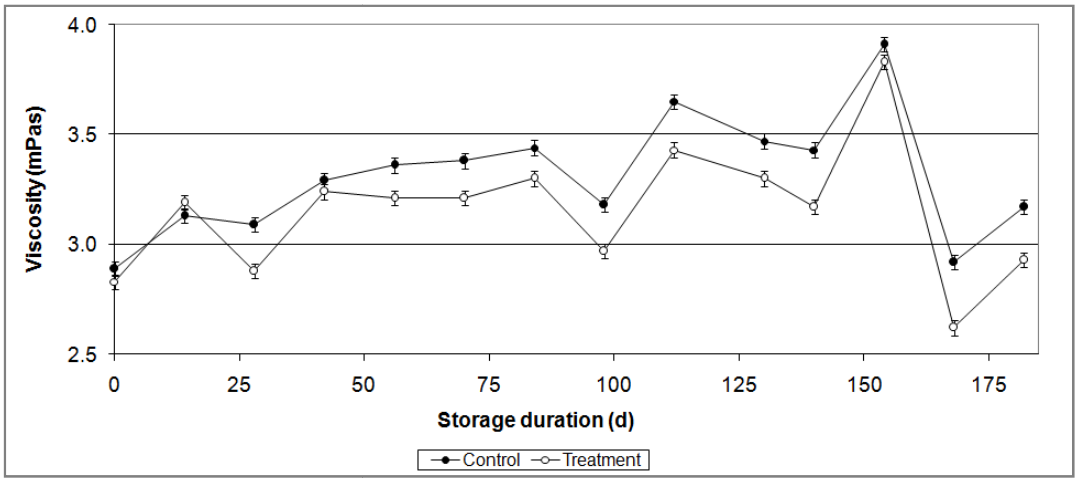


Figure 5.7: Viscosity of UHT milk during storage - Trial 1.

5.3.2.4 Free fatty acids concentration

Over the first 42 d, there was an upward trend in the concentration of FFA. The increase was from 0.86 meq/L (control sample) and 0.89 meq/L (treatment sample) (difference not significant, $p>0.05$) up to 0.99 meq/L (control sample) and 1.26 meq/L (treatment sample) (Figure 5.8). However, after 42 d, the FFA remained close to 1.00 meq/L (control sample) and close to 1.10 meq/L (treatment sample). Once the storage commenced, the concentration of FFA in the treatment sample was significantly higher than in the control sample ($p<0.001$) for the first 84 d, thereafter, the differences were mostly not significant ($p>0.05$).

5.3.2.5 Free amino groups concentration

Over the entire storage period, there was an evident upward trend, albeit with modest fortnightly increases (Figure 5.9). From a concentration of 3.3 µM Leu-Gly equivalents (both samples), the FAG in the control sample increased to 4.1 µM Leu-Gly equivalents, while the increase in the treatment sample was greater, with the final concentration of FAG being 6.2 µM Leu-Gly equivalents. There was a significant difference ($p<0.001$) in the FAG concentration between the samples throughout storage.

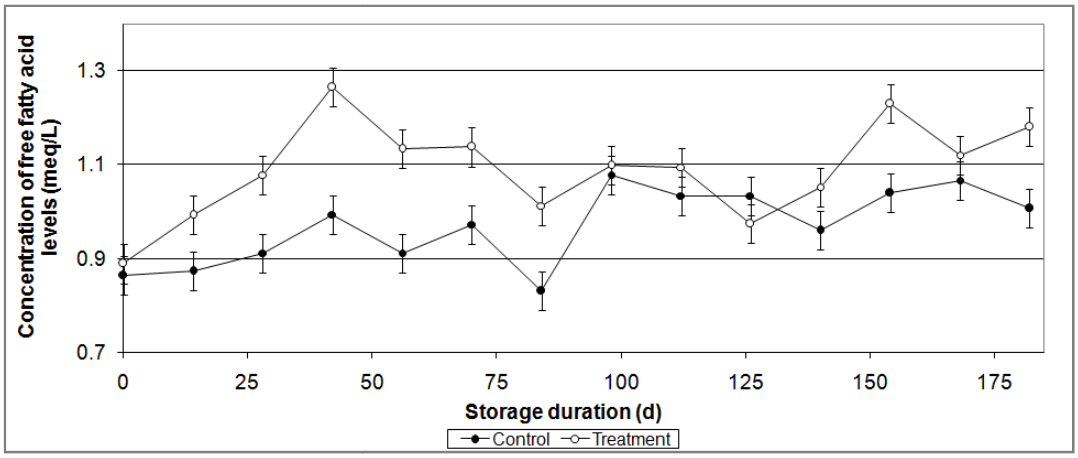


Figure 5.8: Free fatty acid content in UHT milk during storage - Trial 1.

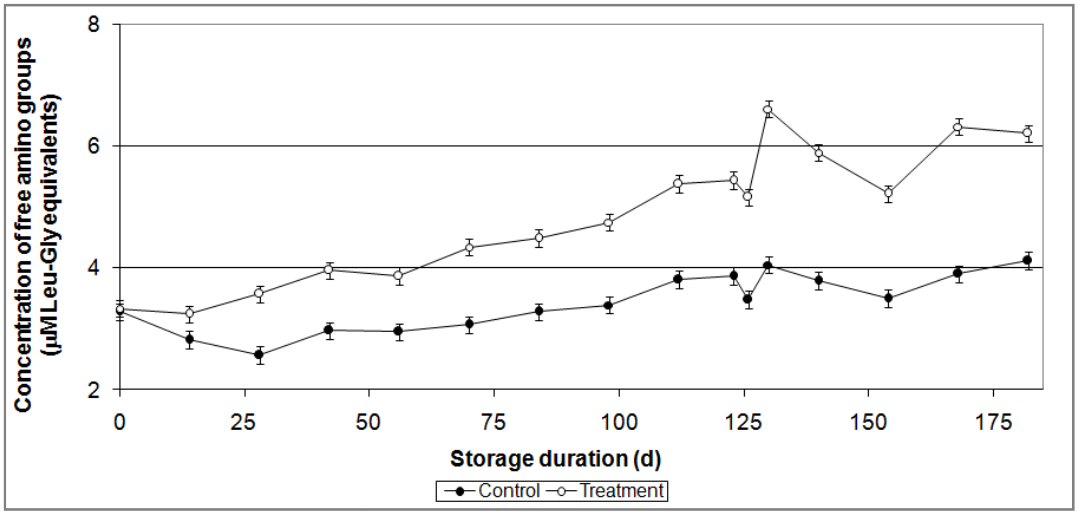


Figure 5.9: Free amino groups in UHT milk during storage - Trial 1.

5.3.2.6 Sensory analysis

The control and treatment samples could not be distinguished at any time throughout the storage period, whether the sensory analysis was by the electronic nose (tested fortnightly for 18 weeks) or sensory panel (tested fortnightly for 16 weeks) (Table 5.1). There was also much fortnightly variation, with both the electronic nose and sensory panel results.

Table 5.1: Sensory analysis of UHT milk during storage - Trial 1.

Length of storage (weeks)	Sensory panel analysis			Electronic nose (Discrimination Index)
	Number of panellists	Number of panelists who detected a difference*		
		Aroma	Flavour	
0	24	8	6	64
2	25	8	7	59
4	24	10	9	47
6	24	5	3	72
8	24	8	10	59
10	24	9	10	27
12	24	9	8	63
14	24	8	7	50
16	24	6	9	42
18	24	Not determined	Not determined	55

* Detection of a difference by a minimum of 13 panellists was required for a significant difference at $p<0.05$

5.3.2.7 Potential for lipolytic and proteolytic spoilage in UHT milk after six months of storage

Tests for lipase and protease in both the control and treatment UHT milk samples were undertaken after six months (26 weeks) of storage. This was because there had been no chemical or physical changes in either sample during the 26-week period to suggest that spoilage was imminent. Assays with 14 d incubation were used, triolein as substrate for lipase and FITC-casein as substrate for protease. The results suggested that very little lipolysis was evident as the increase in FFA concentration was 0.22 meq/L for the control sample and 0.32 meq/L for the treatment sample. This difference in lipolysis between the samples was not significant ($p=0.381$). The situation was similar with proteolysis. An increase in fluorescein in the FITC-casein assay of 38.5 µM for the treatment sample and 37.4 µM for the control sample (no significant difference, $p=0.902$) indicated very little additional proteolysis in the treatment sample. At the point of analysis (26 weeks), the FFA concentration of 1.2 meq/L and the FAG concentration of 6 µM Leu-Gly equivalents, was not sufficient to result in sensory defects. Furthermore, based on the lipase and protease assay results, there was only a very small potential that the treatment sample would spoil, and consequently, the experiment was terminated. A second trial was then set up, with a slightly different approach, as described in section 5.2.4.

5.3.3 Monitoring of raw milk quality prior to UHT treatment - Trial 2

5.3.3.1 Total plate count

For both samples, an increase in total count was observed, from a starting point of 1.1 x 10^4 cfu/mL in both samples to 1.0 x 10^7 cfu/mL for the control sample and to 8.0 x 10^7 cfu/mL for the treatment sample. (Figure 5.10). This difference, and the difference in the total count between the samples from days one to seven of the incubation, were significantly different ($p<0.001$). The increase in total count in the control sample was consistent over the seven day period, while in the treatment sample, a steeper increase was seen in the first three days, before the increases diminished with continued incubation.

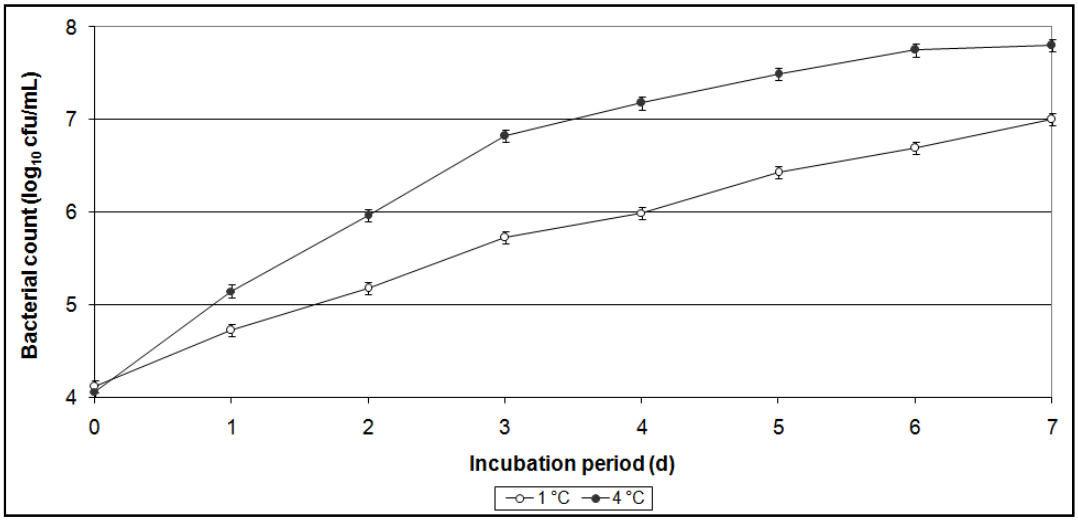


Figure 5.10: Total plate count of raw milk - Trial 2.

5.3.3.2 pH

Prior to incubation, the pH of the milk was 6.59 (control sample) or 6.58 (treatment sample) (Figure 5.11). By day one, the pH had increased substantially, by approximately 0.1 to 6.69. This was a significant increase ($p<0.001$). For the remainder of the incubation, the pH was consistently observed in the vicinity of 6.7, for both samples. While variations between days and between samples were typically very small, in the range of 0.01-0.02 of a pH unit, the differences were significant ($p<0.001$).

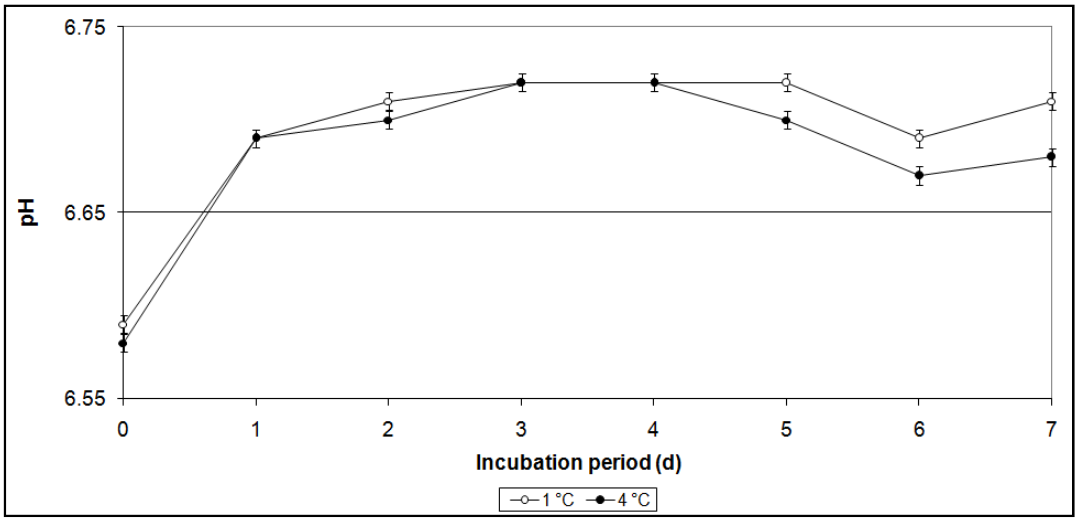


Figure 5.11: pH of raw milk during incubation - Trial 2.

5.3.3.3 Free fatty acids concentration

There was a modest, although significant ($p<0.001$) increase in FFA from days zero to seven (Figure 5.12). The actual increases in concentration of FFA were small, however, with 0.06 meq/L increase observed in the control sample and 0.13 meq/L increase observed in the treatment sample. During the incubation, there were significant daily fluctuations ($p<0.001$), but at no time did the FFA concentration in either sample increase over 1.0 meq/L.

5.3.3.4 Free amino groups concentration

The concentration of FAG changed only slightly over the first two days, with a larger increase seen on the third day of incubation (Figure 5.13). From this point until day seven, a modest increase was noted until the FAG concentration stabilised at approximately 6 µM Leu-Gly equivalents, in both samples, from a starting point of 4.5 (control sample) and 4.3 (treatment sample) µM Leu-Gly equivalents (this difference was not significant, $p>0.05$). For the most part (day one being the exception), differences between the samples were not significant.

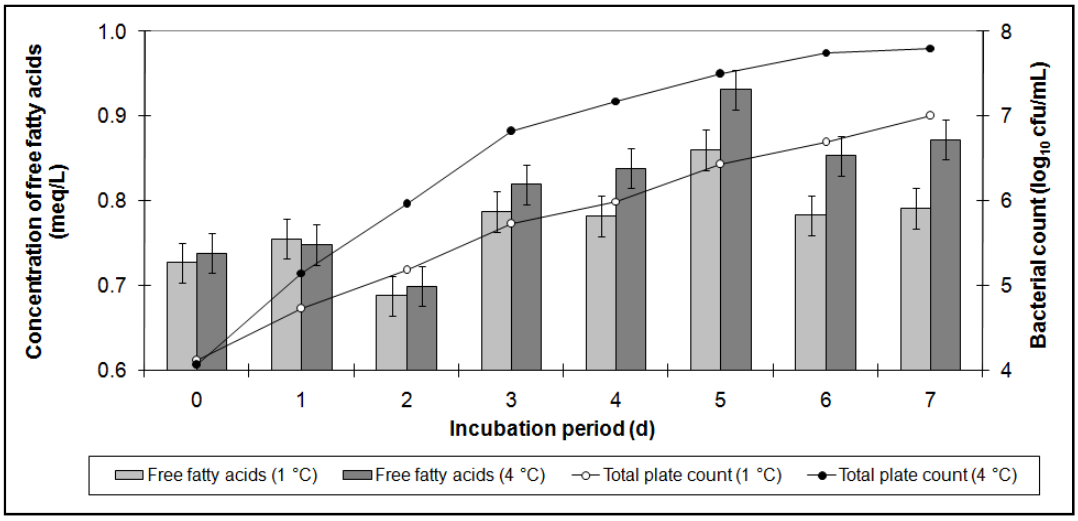


Figure 5.12: Total plate count and increase in free fatty acids during incubation of raw milk - Trial 2.

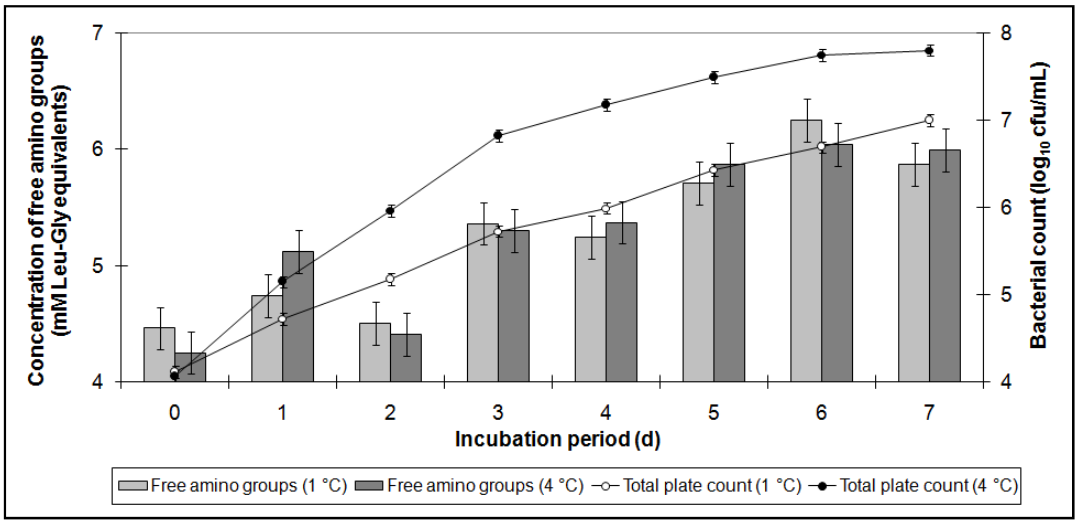


Figure 5.13: Total plate count and increase in free amino groups during incubation of raw milk - Trial 2.

5.3.3.5 Lipolysis and proteolysis in spoiled milk at the end of raw milk incubation

A 14 d incubation period was used for detection of lipase (triolein used as substrate) and protease (FITC-casein used as substrate). An increase in both lipolysis and proteolysis was observed from the control (incubated at 1 ºC) and treatment (incubated at 4 ºC) samples. FFA concentration in the control sample

was 6.44 meq/L, and in the treatment sample, 7.81 meq/L (difference significant, $p<0.001$) while the FAG concentration was 283 (control) and 300 (treatment) μM fluorescein (difference significant, $p<0.001$).

5.3.4 Lipase and protease inactivation during UHT processing - Trial 2

There was a considerable difference in heat stability of the lipases and proteases. The concentration of hydrolytic end products following action of the heated lipase was low in both samples and not significantly different ($p>0.1$) from one another. This level of hydrolytic end products in the control was 4.5% of that released by the unheated lipase, with the mean FFA increase in the triolein assay of 0.29 meq/L. However, in the treatment group, the FFA concentration was 6.7% of that produced by the unheated lipase, where the mean FFA increase in triolein assay was 0.52 meq/L. In contrast, the protease(s) were much more heat-stable, with increase in fluorescein in the FITC-casein assay being 67 μM (control sample) and 129 μM (treatment sample), a significant ($p<0.001$) difference. The level of proteolysis was therefore 23.7% (control) and 43.0% (treatment) of the level of proteolysis of the unheated protease.

5.3.5 Microbiological, chemical, physical and sensory analysis of UHT milk during storage - Trial 2

The treatment samples of UHT milk in the second trial had reached the end of shelf life sometime between days zero and 14, when the first analysis of the milk was undertaken. On day 14, extensive age gelation was observed (Figure 5.14); the gelled milk was sterile. Therefore, trends and observations on changes in milk quality indicators leading up to the end of shelf life are not available. In Table 5.2, information is presented to show the changes that took place in the first 14 days of storage. The most noticeable effects with the treatment sample were seen with viscosity and FAG. The viscosity increased from 2 mPas to 183 mPas while the FAG concentration increased from 4.4 μM Leu-Gly equivalents to 16.0 μM Leu-Gly equivalents. These increases were

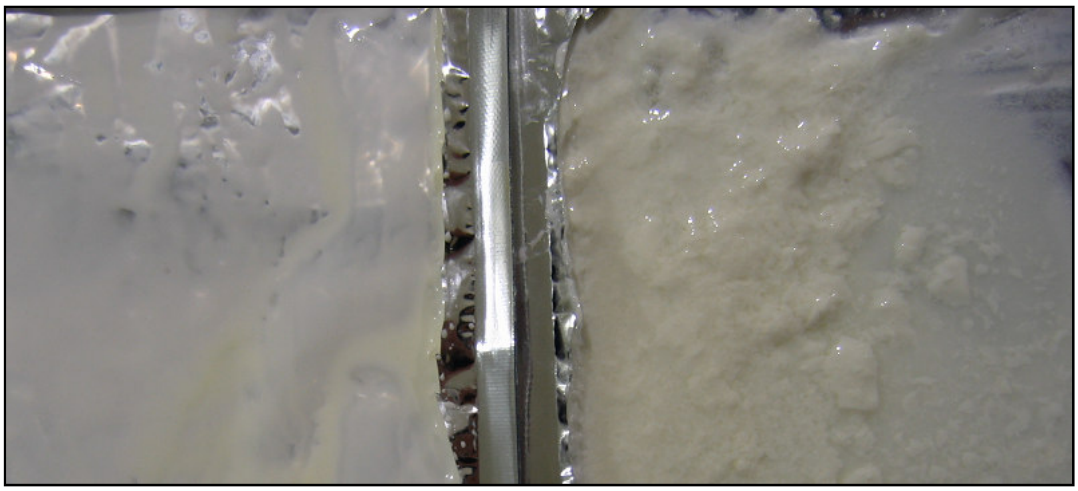


Figure 5.14: Non-gelled (left) and gelled (right) milk on day 14 - Trial 2.

significant ($p<0.001$) as was the increase in FFA and decrease in pH (for both, $p<0.001$). While a significant change from day zero to day 14 was seen with viscosity ($p<0.001$) of the control sample, along with pH ($p<0.001$), concentration of FFA ($p < 0.001$) and concentration of FAG ($p<0.01$), the actual changes were small.

Table 5.2: Milk quality indicators on days 0 and 14 - Trial 2.

Milk quality indicator	Day 0		Day 14	
	Control	Treatment	Control	Treatment
pH	6.67[a]	6.65[a]	6.65[b]	6.58[b]
Viscosity (mPas)	2.29[a]	2.08[a]	1.85[b]	145.00[b]
Free fatty acids (meq/L)	0.74[a]	0.88[a]	0.86[b]	1.47[b]
Free amino groups (μM Leu-Gly equivalents)	2.29[a]	4.29[a]	2.58[b]	16.01[b]

Identical superscripts for either the control or treatment samples indicate no significant difference ($p<0.01$) from day 0 to day 14 for each milk quality indicator.

In addition to a substantial increase in viscosity and FAG, the observation of age gelation on day 14 of storage was accompanied by extensive protein degradation and consequently, a considerable increase in the number and type of peptides produced (Figures 5.15, 5.16 and 5.17). A range of peptides of varying sizes appeared in the HPLC chromatograms (Figure 5.15). From the SDS-PAGE gel in Figure 5.16a, it can be seen that almost no κ-CN remained in the treatment samples after 14 d, with a decrease from 7.6% to zero, while there was a sizeable increase in para-κ-CN, from approximately

3.5% to 9.6% (Figure 5.16b). Furthermore, from Figure 5.16b, proteolysis appears evident in the raw milk, because the proportion of κ-CN was 10.9% in the control milk and 7.6% in the treatment milk. A large increase in peptides was also evident following MALDI-TOF MS analysis (Figure 5.17).

Sensory analysis on the milk at the commencement of the storage period revealed no difference between the samples. Eight (aroma analysis) or six (flavour analysis) panellists out of a total of 24 detected a difference between the samples, while the e-nose result was similar, with a discrimination index of 63. This discrimination index is above 70, the level stipulated by the manufacturer above which the samples can be regarded as different. Analysis by sensory panel was not performed on the gelled milk, after 14 days of storage, but the e-nose returned a discrimination index of 50.

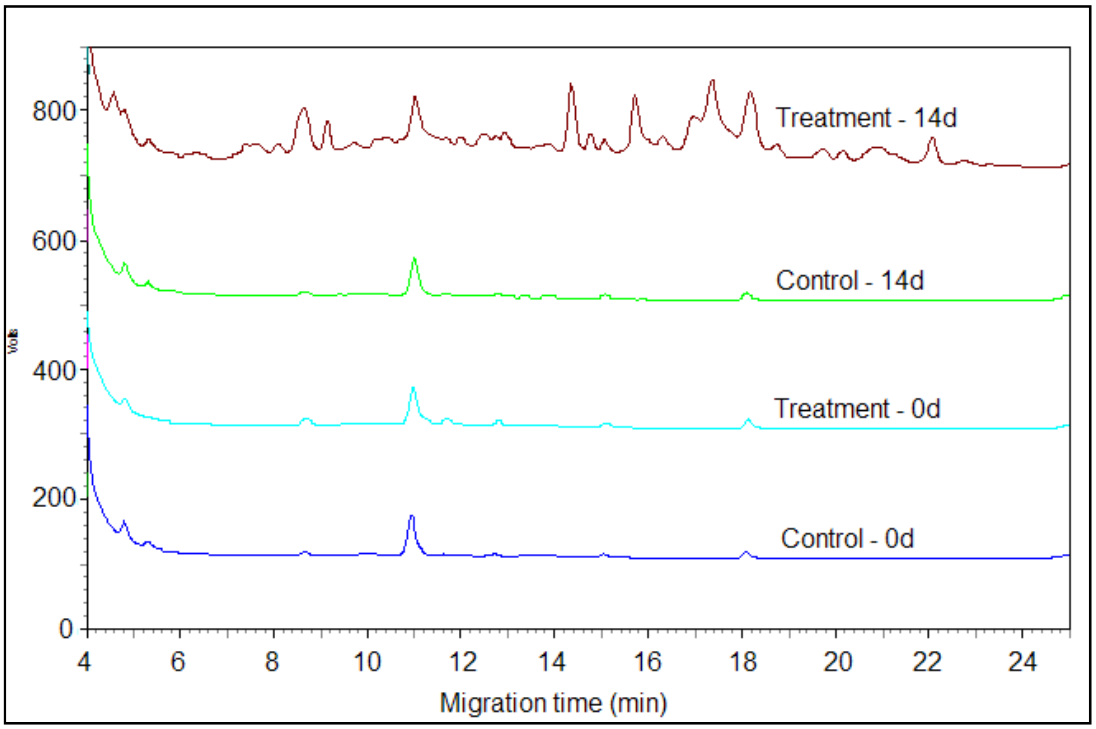


Figure 5.15: Peptide profile by RP-HPLC of control and treatment UHT milk - Trial 2. Note: the peak at 11 min originated from the mobile phase.

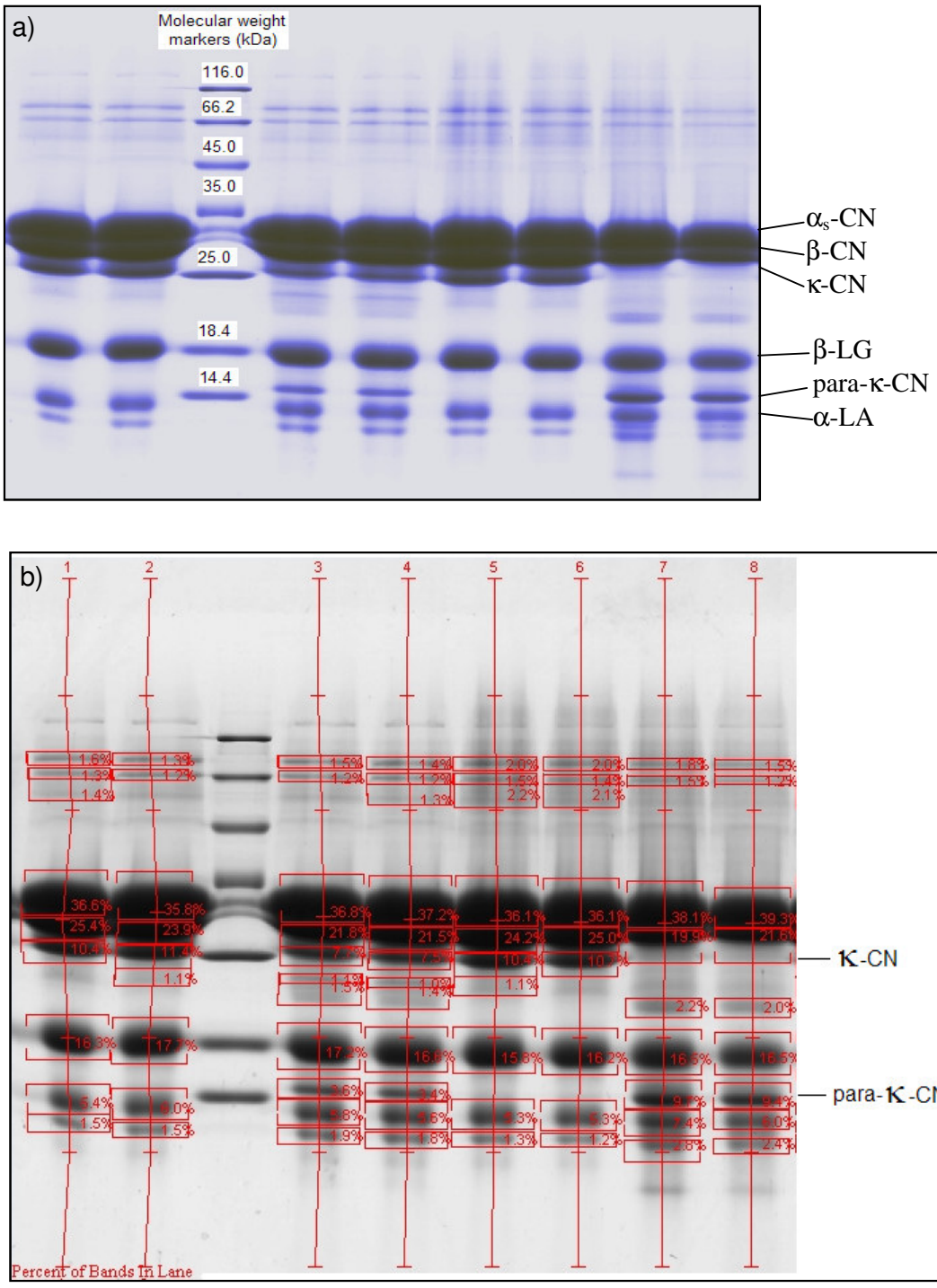
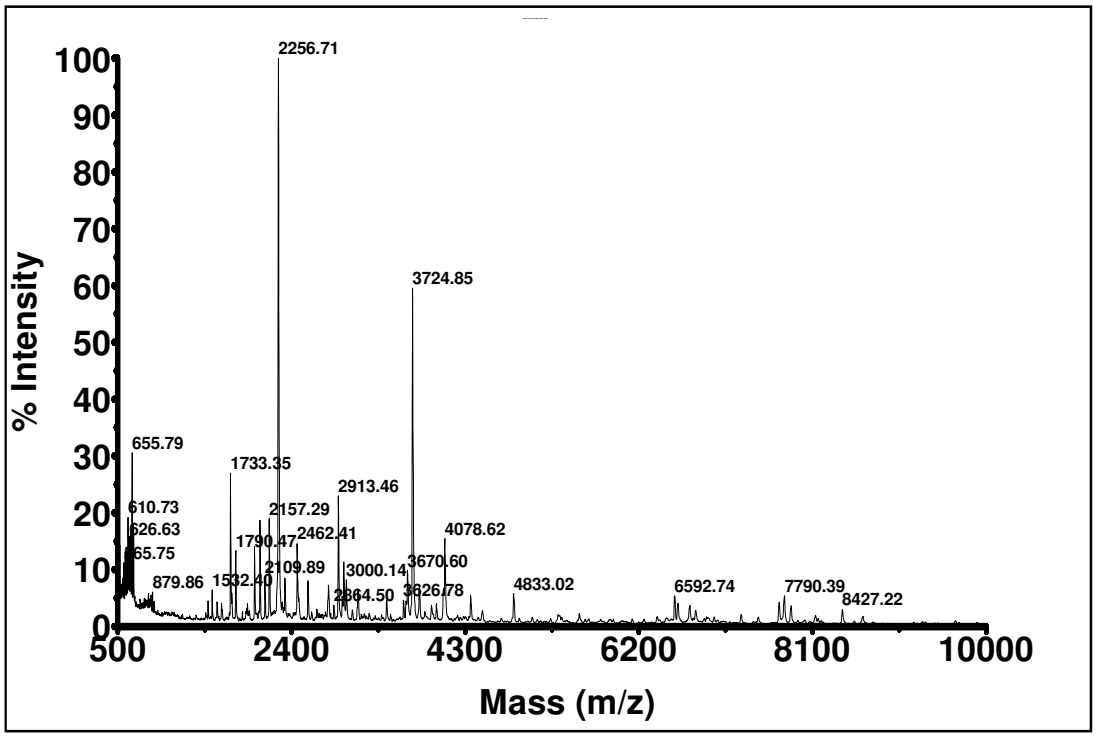


Figure 5.16: Protein degradation of UHT milk samples analysed with SDS-PAGE - Trial 2. a) Original unlabelled picture. b) Percent of bands labels added. Lane 1: Day 0 – control (replicate 1), Lane 2: Day 0 – control (replicate 2), Lane 3: Molecular weight markers, Lane 4: Day 0 – treatment (replicate 1), Lane 5: Day 0 – treatment (replicate 2), Lane 6: Day 14 – control (replicate 1), Lane 7: Day 14 – control (replicate 2), Lane 8: Day 14 – treatment (replicate 1), Lane 9: Day 14 – treatment (replicate 2).



b)

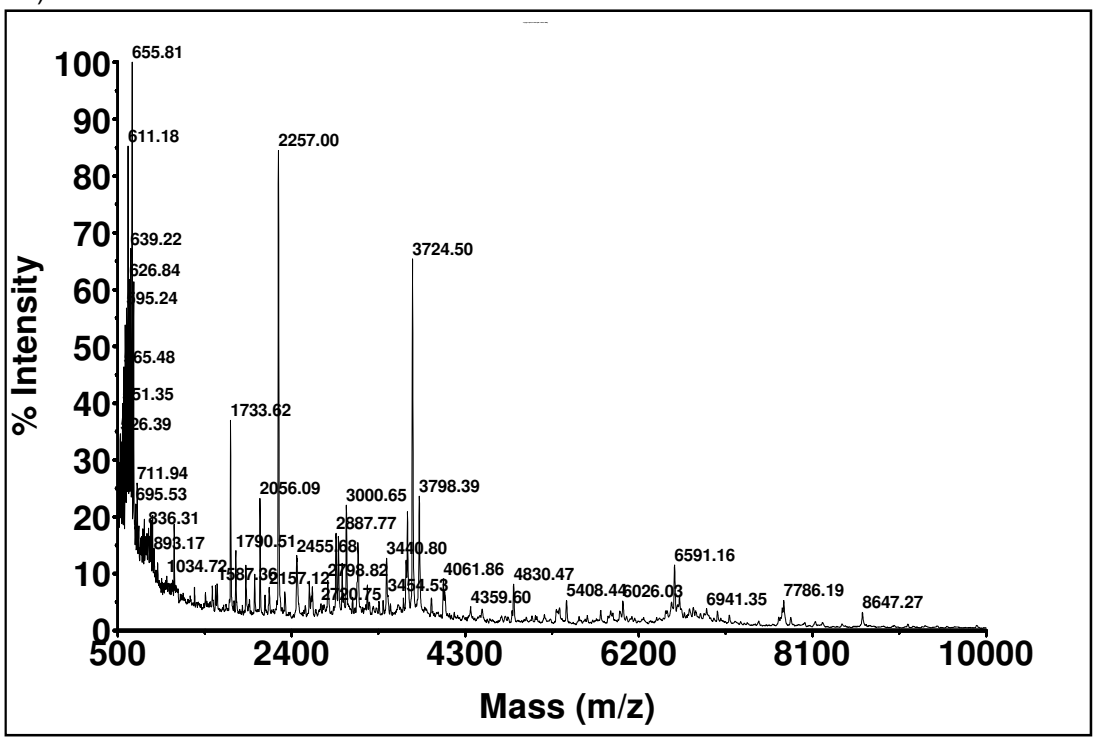


Figure 5.17: Mass spectra of peptides from MALDI-TOF MS analysis of UHT milk after 14 d of storage - Trial 2. a) Control. b) Treatment.

5.3.6 Monitoring of raw milk quality prior to UHT treatment - Trial 3

5.3.6.1 Total plate count

At the commencement of incubation, the number of bacteria in the raw milk was 4 x 10^4 cfu/mL (Figure 5.18). During the 7 d incubation period the numbers of bacteria in the milk increased steadily. This occurred more quickly with the treatment milk samples compared with the control milk samples. There was no lag phase observed for either storage temperature, but rather a gradual log phase until growth slowed slightly after 3 and 5 d storage at the respective storage temperatures. At both temperatures, the total count was significantly different ($p<0.001$) from day one. On day two the difference was one log. This difference between the samples remained approximately the same until the time of UHT processing on day seven.

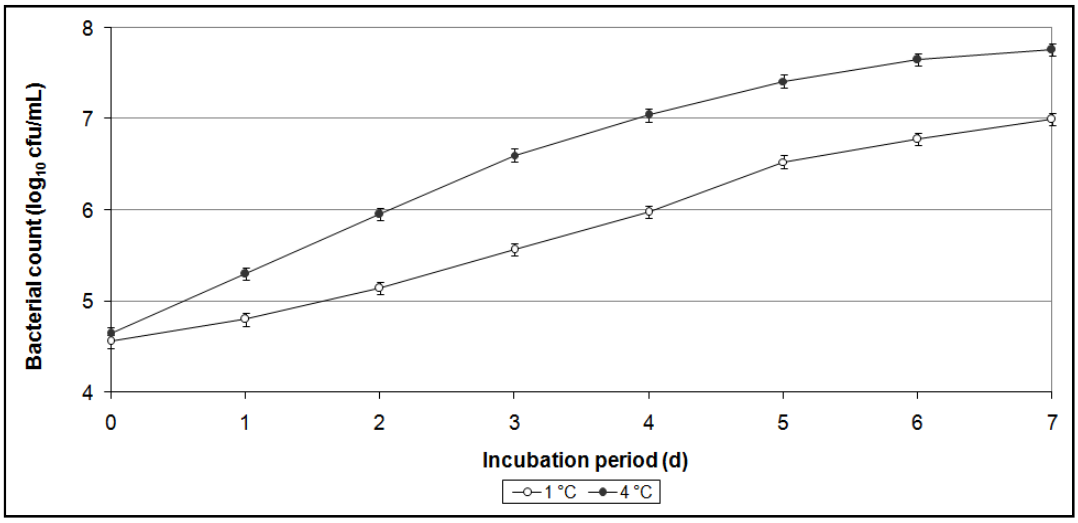


Figure 5.18: Total plate count of raw milk during incubation - Trial 3.

5.3.6.2 pH

The pH over the week-long incubation period remained relatively constant, varying between 6.63 and 6.69 (Figure 5.19). There was a large increase in pH ($p<0.001$) after the second day, but thereafter, did not vary greatly. On some days, there were significant differences ($p<0.001$) between the control and treatment samples, although the actual differences in pH were quite small. Overall, the temperature of storage did not affect the pH as there

was no significant difference ($p>0.1$) in pH between the milk stored at the two temperatures for seven days.

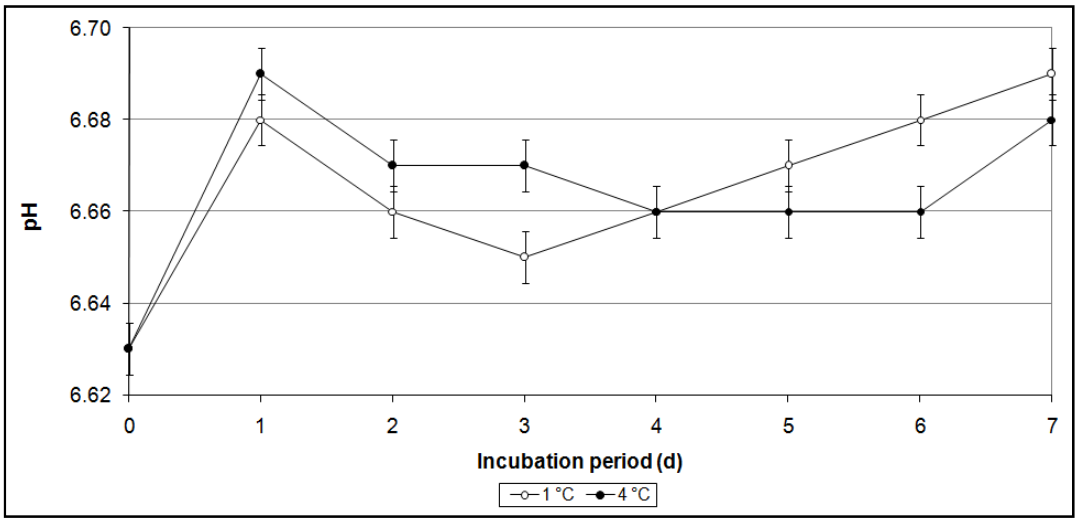


Figure 5.19: pH of raw milk during incubation - Trial 3.

5.3.6.3 Free fatty acids concentration

The FFA content of the raw milk was very low. For both the control and treatment samples, it was under 0.7 meq/L when storage commenced and even after seven days, it increased to only 0.9 meq/L (Figure 5.20). Throughout the incubation, there were no significant daily differences ($p>0.1$) in FFA content of the control and treatment raw milk samples. Although there was not a large increase over the course of the incubation period, there was a clear upward trend and a significant increase ($p<0.001$) from day zero to after six days incubation for both samples.

5.3.6.4 Free amino groups concentration

The FAG concentration was not significantly different ($p>0.1$) in the control and treatment samples of raw milk on any day through the 7 day incubation period. There were, however, significant fluctuations in FAG on various days for both storage temperatures ($p<0.001$) (decrease in FAG at day one and increase in FAG at day two of storage) before a significant upward trend which continued for both samples over the remaining days of incubation

(Figure 5.21). On day four, the FAG content was significantly different from day zero for both temperatures. Overall, the increase in FAG was small (from 4 to 6 µM Leu-Gly equivalents).

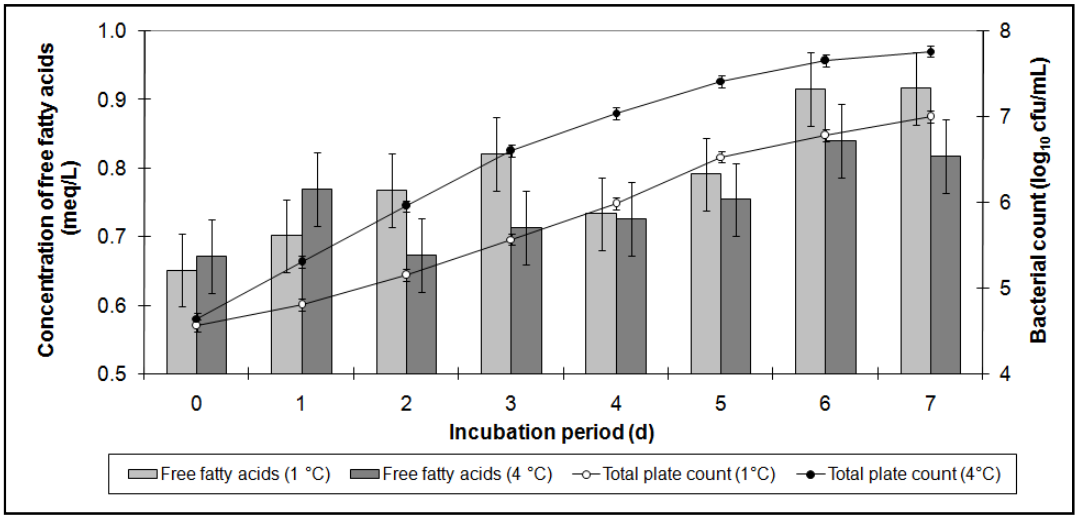


Figure 5.20: Total plate count and increase in free fatty acids in raw milk during incubation - Trial 3.

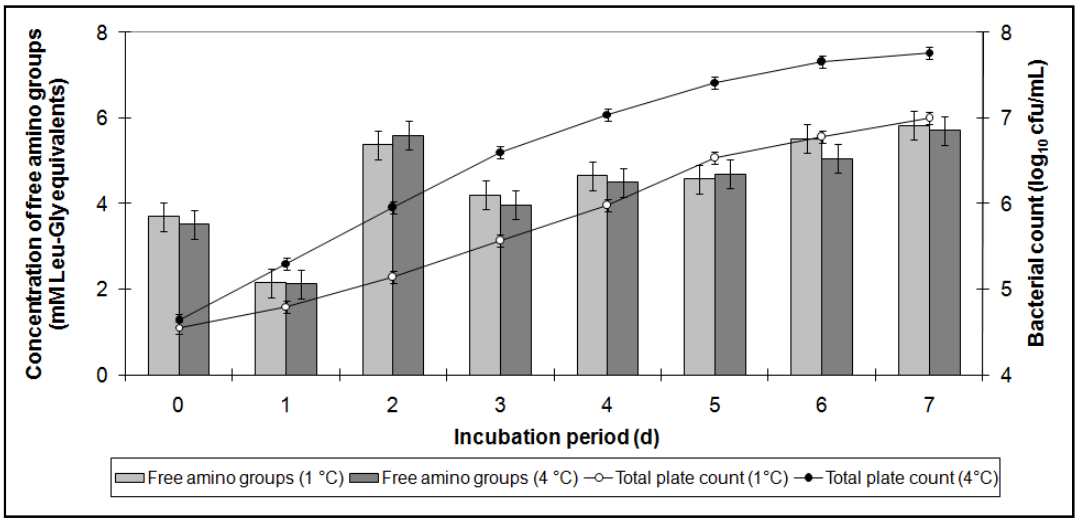


Figure 5.21: Total plate count and increase in free amino groups during incubation of raw milk - Trial 3.

5.3.6.5 Ethanol stability

Throughout the incubation period, both the control and treatment samples remained stable when analysed in the ethanol stability test.

5.3.7 Lipase and protease inactivation during UHT processing - Trial 3

Prior to processing, the FITC-casein assay detected a fluorescein increase of 268 and 261 μM in the control and treatment raw milks respectively. This difference was significant ($p<0.001$). Immediately following UHT processing, the FITC-casein assay results were lower ($p<0.001$). For the control sample, the fluorescein increase in the assay was 48 μM while it was higher ($p<0.001$) in the treatment sample, at 85 μM. This was a decrease in proteolysis by 82% for the control sample and 67% for the treatment sample.

Compared with protease inactivation, lipase inactivation was more pronounced ($p<0.001$) after UHT processing. Before processing, there was an increase in FFA concentration of 4.6 meq/L in the control sample with the corresponding value higher ($p<0.001$) in the treatment sample, at 5.6 meq/L. After UHT processing, the increase in FFA concentration in the lipase assay was 0.3 meq/L in the control sample and 0.6 meq/L in the treatment sample, which was a significant decrease ($p<0.001$). Therefore, in the control sample, a 93% decrease in lipolysis was observed, and a 89% decrease with the treatment sample.

5.3.8 Microbiological, chemical, physical and sensory analysis of UHT milk during storage - Trial 3

5.3.8.1 Sterility and ethanol stability

No viable bacteria were detected in the UHT milk during the shelf life trial and the milk was stable in ethanol.

5.3.8.2 pH

Throughout the storage period, there was only a slight variation in pH with both the control sample, which varied between 6.56 and 6.63, and the treatment sample, which varied between 6.52 and 6.60 (Figure 5.22). At the

commencement of storage, the pH of both samples was identical, but when the milk gelled, at 19 d (see section 5.3.6.3), the pH of both samples was lower ($p<0.001$) than when storage commenced and they were different ($p<0.001$) from one another. From 19 d to 66 d, the pH was less variable, for both samples, but significant ($p<0.001$) changes were still observed.

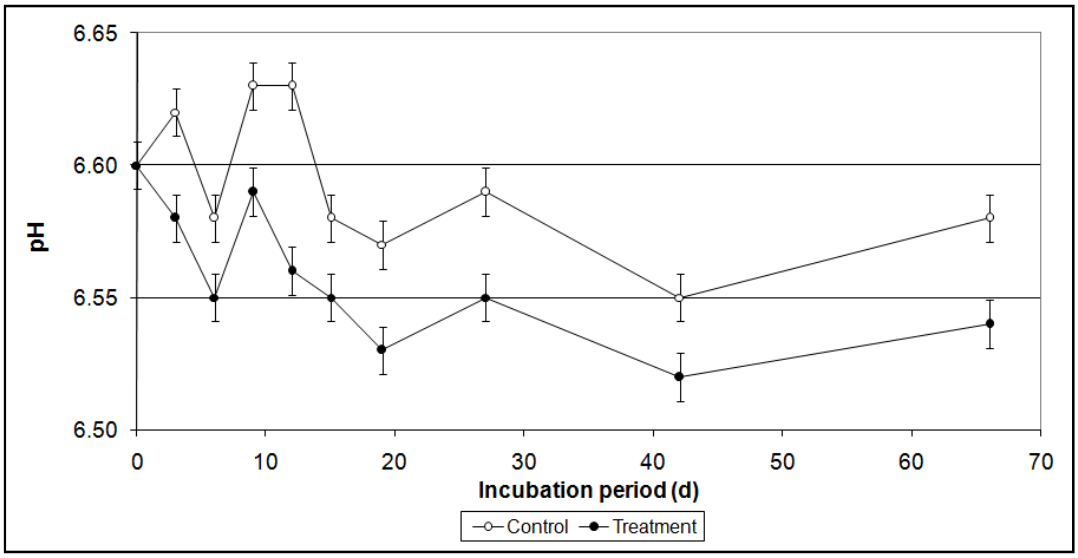


Figure 5.22: pH of UHT milk during storage - Trial 3.

5.3.8.3 Viscosity

The viscosity of the UHT milk, prepared from raw milk stored at 1 (control) and 4 ºC (treatment) prior to processing, was constant for the first 19 d of incubation after processing ($p>0.05$) (Figure 5.23). Age gelation occurred for the treatment milk between 15 d and 19 d. It was during this time period that there was a substantial increase in viscosity for this milk, which was different from that of the control milk ($p<0.001$). This substantial difference continued for at least a further 47 d when no further measurements were taken. Age gelation was not observed in the control samples. This milk was incubated for 66 d during the experimental period. After 10 months storage, the control milk had not gelled.

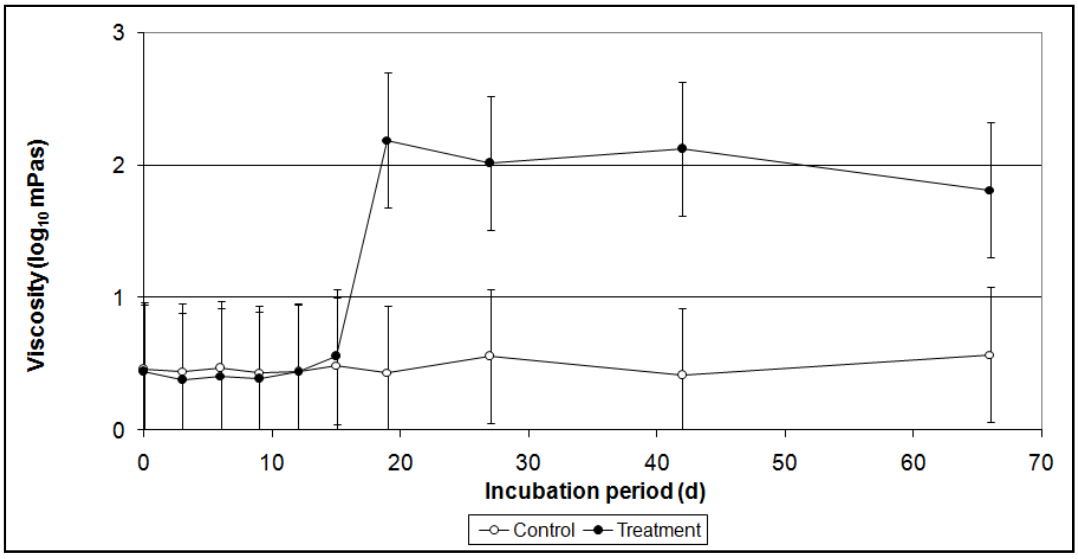


Figure 5.23: Viscosity of UHT milk during storage - Trial 3. The LSD bars are from a one-way ANOVA analysis and enable comparisons only between the control and treatment groups and not between timepoints.

5.3.8.4 Free fatty acids concentration

Over the course of the storage period, the FFA in the control milk remained low, between 0.7-0.9 meq/L, but did increase ($p<0.001$) (Figure 5.24). In contrast, there was a substantial increase in FFA in the treatment milk ($p<0.001$) during the storage period. At the point gelation was observed, day 19, the FFA content was 1.3 meq/L, a concentration just below where most people can perceive presence of lipolytic rancidity (Deeth & Fitz-Gerald, 1976). In contrast, the FFA content was 0.8 meq/L at the commencement of incubation. At the commencement of storage, there was a significant difference ($p<0.001$) between the concentration of some of the specific types of FFA in the control and treatment samples (Figure 5.25). Butanoic (C4:0) and tetradecanoic (C14:0) acids dominated both the control and the treatment samples, with the domination most pronounced in the UHT milk treatment samples. Octanoic (C8:0), decanoic (C10:0), dodecanoic (C12:0) and hexadecanoic (C16:0) acids comprised a small percentage of the FFA in either sample. Upon storage, butanoic (C4:0), tetradecanoic (C14:0) and *cis*-9-octadecenoic (C18:1) acids increased to the largest extent in the treatment milk samples while no FFA increased substantially in the control milk (Figure 5.26).

In the treatment sample, butanoic acid (C4:0), increased steadily from 48 µM to 88 µM in the first 27 d of storage and then increased by a further 50 µM to 138 µM between days 27 and 42. In a similar manner, *cis*-9-octadecenoic acid (C18:1) increased from 25 µM to 57 µM from days zero to 27 and then over the next 15 d, reached 87 µM, then further increasing to 109 µM by day 66 of storage. In contrast, tetradecanoic acid (C14:0) varied between 38-71 µM over

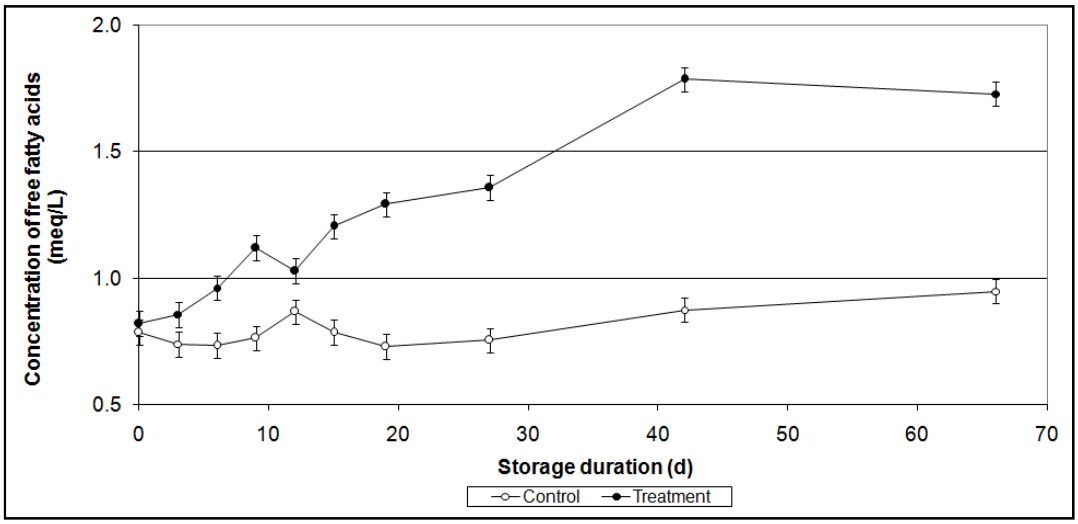


Figure 5.24: Free fatty acids concentration in UHT milk during storage - Trial 3.

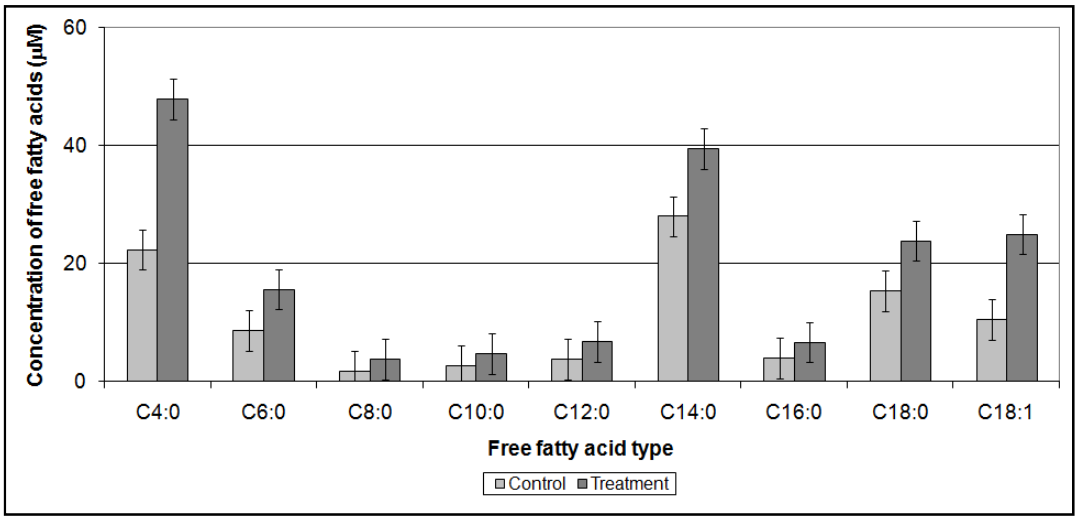
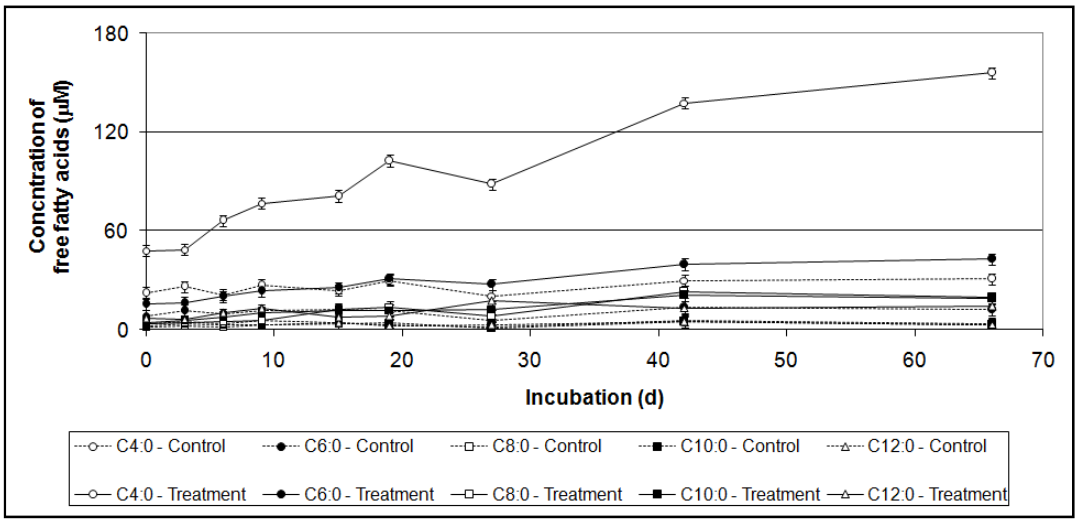


Figure 5.25: Free fatty acids present in UHT milk at the commencement of storage in the samples prepared from raw milk incubated at 1 (control) and 4 ºC (treatment) prior to UHT processing - Trial 3.

a)



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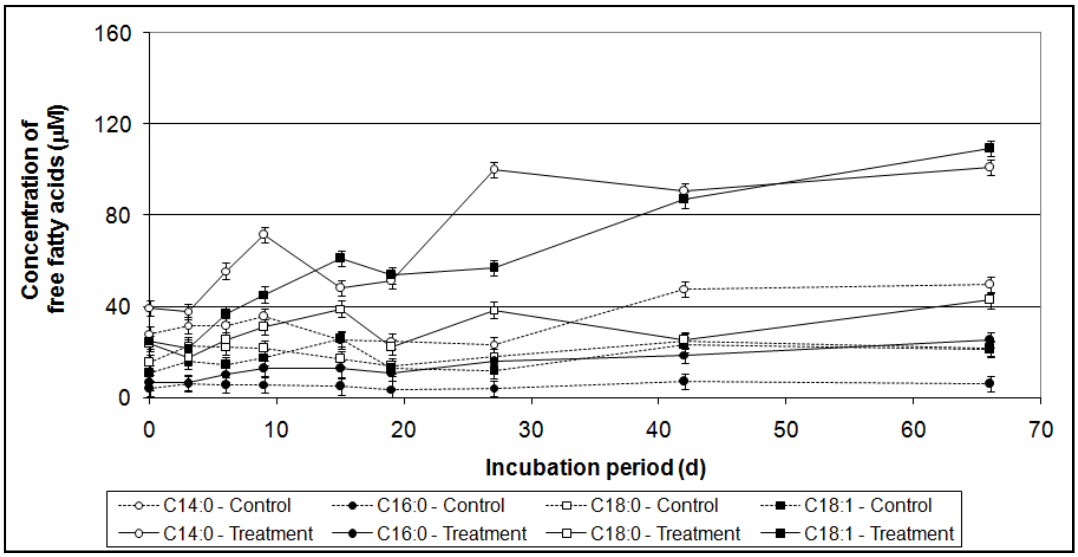


Figure 5.26: Concentration of C4 to C12 a) and C14 to C18 b) free fatty acids during storage of UHT milk - Trial 3.

the first 19 days of storage, before increasing to 100 µM by day 27. Then, between days 27 and 66, there was no significant increase ($p<0.001$) in the tetradecanoic acid (C14:0) concentration. The major increase in C4:0, C14:0 and C18:1 in the treatment sample was reflected in the increase of total FFA seen. While the FFA present in the three highest concentrations in the treatment sample all exceeded 100 µM, the FFA present in the three highest

concentrations in the control sample reached only 49 μM (tetradecanoic acid - C14:0), 31 μM (butanoic acid - C4:0) and 22 μM (octadecanoic acid - C18:0). Across the control and treatment samples, octanoic acid (C8:0) showed the smallest increase over the 66 d storage period, from 2 μM to 3 μM in the control samples and from 3 μM to 4 μM in the treatment samples. Chromatograms from the GC/MS analysis of FFA at day zero and day 66 are shown in Appendix 3.

5.3.8.5 Free amino groups concentration

At the commencement of storage, the FAG concentrations in the control and treatment milk samples were similar (Figure 5.27). The level in the control samples remained consistently low throughout the 66 d storage period, varying between 2.3-2.9 μM Leu-Gly equivalents. In contrast, the treatment samples showed a larger increase. Initially, this was from 3.5 μM at the commencement of storage to 6.1 μM after three days. Then there was a period of a relatively small increase from 6.1-8.2 μM from days three to 15. Following this, a steep increase in FAG content was seen between days 15 and 19, from 8.2 μM to 12.0 μM, upon which time age gelation was observed. At this point, the FAG concentration in the treatment sample (12.0 μM) was more than four-fold higher than the FAG concentration in the control sample (2.7 μM). After 10 months storage, the FAG content of the control sample reached 3.5 μM Leu-Gly equivalents.

5.3.8.6 Peptide profile and protein degradation

There was a substantial difference in the peptide profile and protein degradation of the control and treatment samples, as demonstrated by HPLC (peptide profile) and SDS-PAGE (protein profile). The control sample showed a limited number of peptides with little increase, even after 66 d of storage (Figure 5.28a). Although the increase was minimal, an increase in accumulation of peptides could nevertheless be seen, indicating that proteolysis was taking place in these control samples, albeit at a considerably lower rate. In contrast, there was a major increase in the number and concentration of peptides during

the storage period of the treatment samples (Figure 5.28b). This increase in peptides corresponded to substantial degradation in particular proteins, such as κ-CN and appearance of para-κ-casein (para-κ-CN) (Figure 5.29). At the commencement of storage, κ-CN comprised 9.9% of the total protein, while para-κ-CN totalled 1.0%. Up until age gelation was observed, a major shift in these proportions was seen. On the day that age gelation was observed (day 19), 2.7% of the protein present was κ-CN while the proportion of para-κ-CN

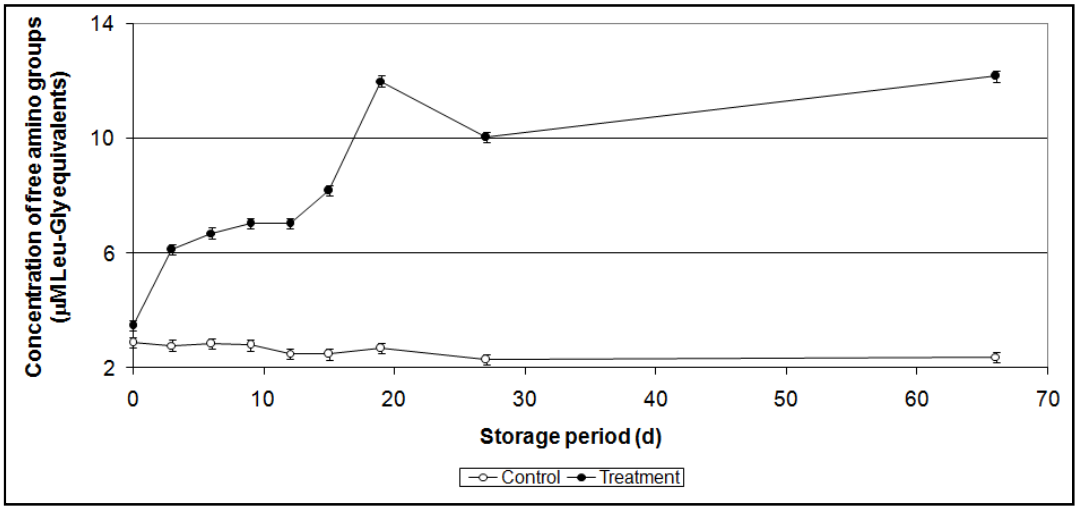
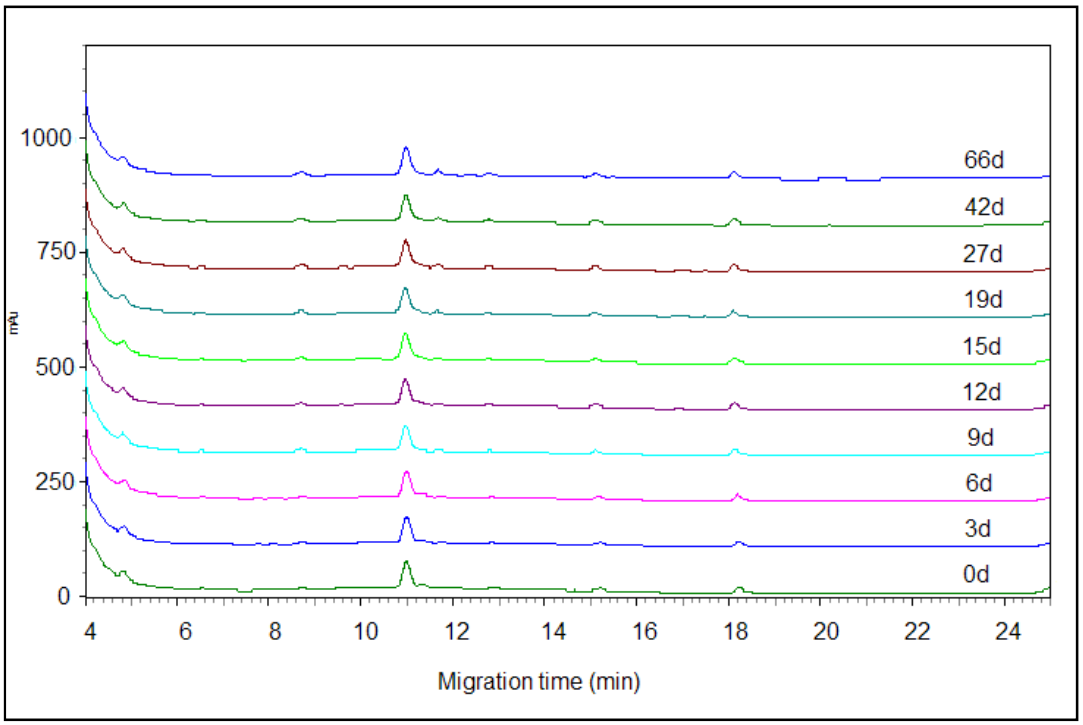


Figure 5.27: Free amino groups levels in UHT milk during storage - Trial 3.

had increased 10 fold, to 10%. There was much less change with the other proteins. Variations were also noted in the rate of proteolysis, as seen by the rate of appearance of different peptides. For example, in the HPLC analysis, there was a rapid appearance of the peaks between 17.5 and 18.5 min (Figure 5.28b), within the first three days of storage, and then minimal change to the accumulation of these peptides during the remaining 63 d. In contrast, there was a gradual accumulation of other peptides, such as represented in the peaks between 14 and 16 min. There was a modest increase for the first 15 d, and then the largest single increase was observed, between 15-19 d, which coincided with the onset of gelation. Different again was the peak at approximately 8.5 min, which showed gradual accumulation,

a)



b)

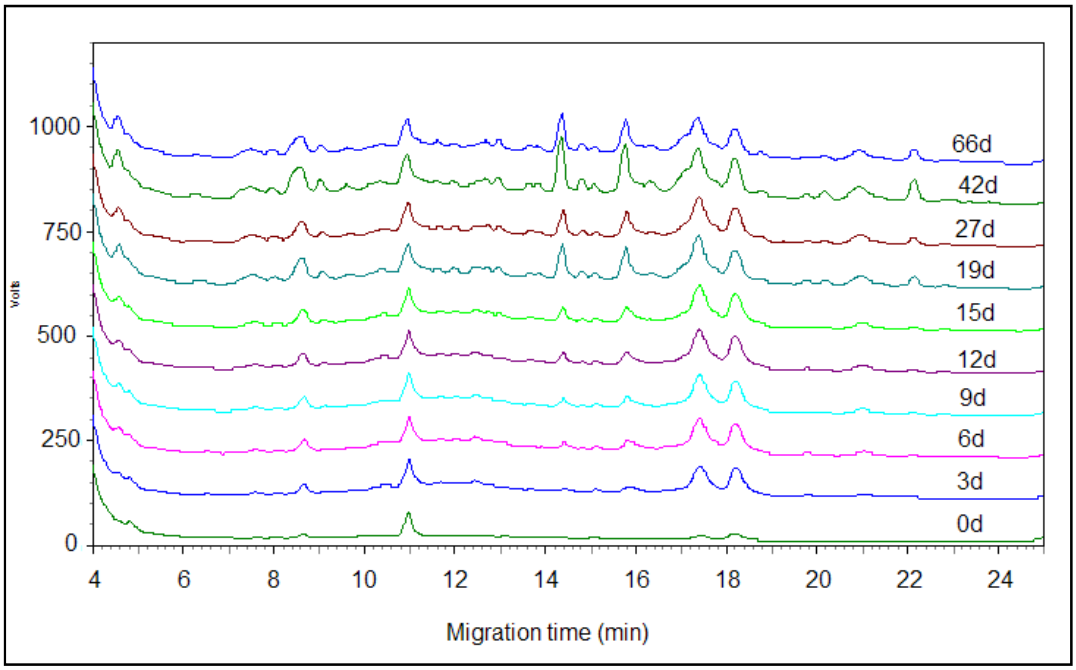
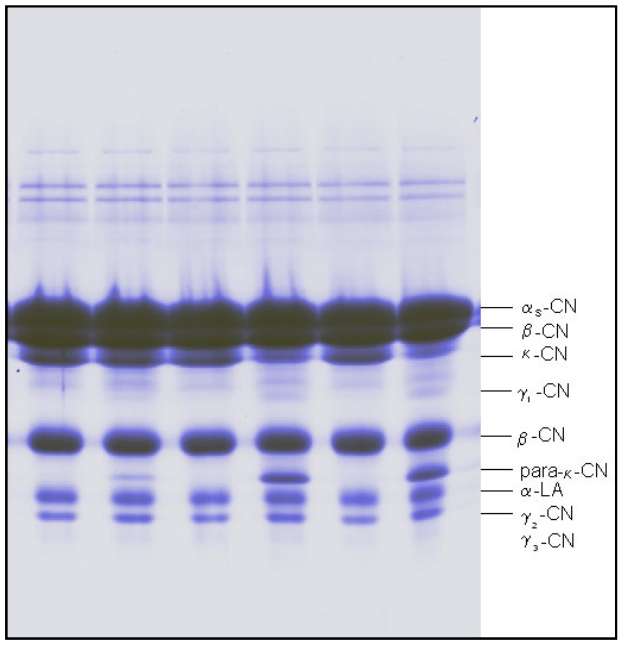


Figure 5.28: Peptide profile of UHT milk during storage at 25 ºC obtained by RP-HPLC - Trial 3. Samples are control a) and treatment b). From bottom to top, traces are 0 d, 3 d, 6 d, 9 d, 15 d, 19 d, 27 d, 42 d, 66 d. Note: the peak at 11 min originated from the mobile phase.

a)



b)

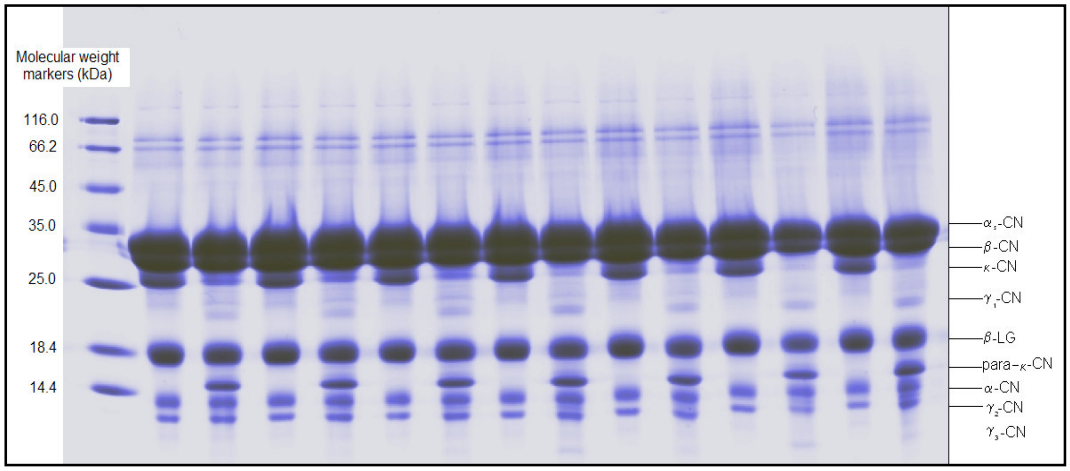
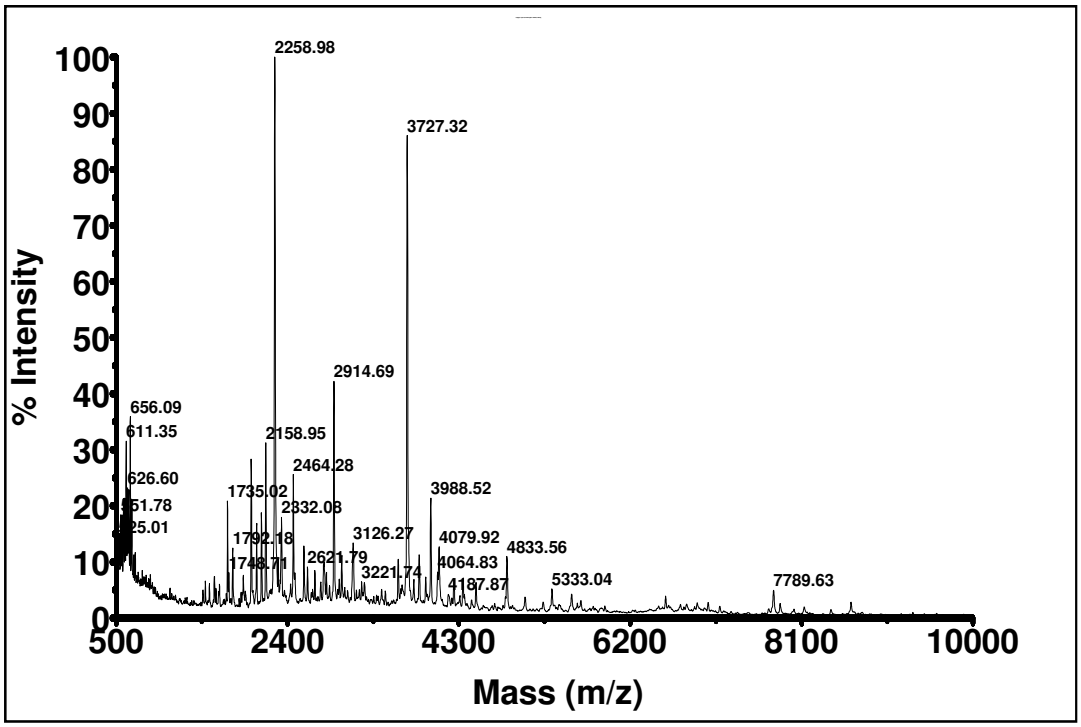


Figure 5.29: Protein degradation of UHT milk samples analysed with SDS-PAGE - Trial 3. a) Lane 1: Day 0 - control, Lane 2: Day 0 - treatment, Lane 3: Day 3 - control; Lane 4: Day 3 - treatment, Lane 5: Day 6 - control, Lane 6: Day 6 - treatment. b) Lane 1: Molecular weight markers, Lane 2: Day 9 - control, Lane 3: Day 9 - treatment, Lane 4: Day 12 - control, Lane 5: Day 12 - treatment, Lane 6: Day 15 - control, Lane 7: Day 15 - treatment, Lane 8: Day 19 - control, Lane 9: Day 19 - treatment, Lane 10: Day 27 - control, Lane 11: Day 27 - treatment, Lane 12: Day 42 - control, Lane 13: Day 42 - treatment, Lane 14: Day 66 - control, Lane 15: Day 66 - treatment.

a)



b)

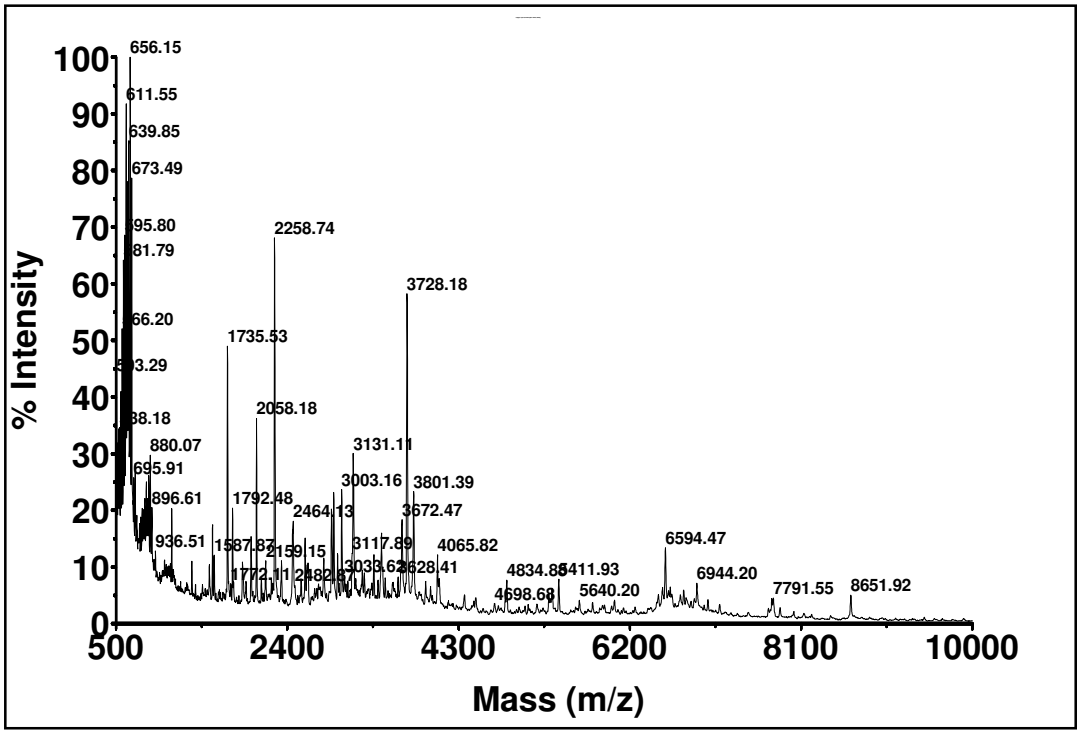


Figure 5.30: Mass spectra of peptides from MALDI-TOF MS analysis of UHT milk after 19 d of storage - Trial 3. a) Control. b) Treatment.

but not a large increase in peptides between days 15 (non-gelled) and 19 (gelled). Furthermore, there was a noticeable difference between the samples at the commencement of storage, with peptides obvious in the treatment sample (Figure 5.28b) compared with the control sample (Figure 5.28a) along with the κ-CN degradation product, para-κ-CN (Figure 5.29).

Overall, there were more peptides in the treatment sample (Figure 5.30b) compared to the control sample (Figure 5.30a). The most abundant peptide in the gelled milk (treatment sample) originated from κ-CN. From its mass to charge ratio (m/z), of 656.15, it was determined to be a short peptide, of five amino acids, corresponding to residues 147-151. The sequence of this peptide was EDSPE, and was hydrophillic with a mean hydrophobicity of -5.88 on the CCS.

5.3.8.7 Aroma and flavour analysis

Sensory analysis for aroma and flavour was undertaken with the panel from days zero to 12. There was a significant difference ($p<0.05$) between the flavour (and not the aroma) of the control and treatment, only at the commencement of the storage period (day zero). The number of panellists detecting a difference for aroma and flavour analysis was constant throughout the sensory analysis period, being just below the number required for statistical significance at $p<0.05$. During the initial stages of the storage period, when all other indicators suggested both samples of milk to be of good quality, there were isolated reports of various off-flavours, some which would normally be regarded as being associated with spoiled milk. A limited (with regard to number of participants) sensory session was held to ascertain the presence of off-aromas and/or off-flavours in the gelled milk by a panel previously trained for participation in other sensory panels and a panel of untrained participants. At that point, unpleasant aromas and flavours were still generally not present.

5.4 Discussion

5.4.1 Microbiological, chemical and biochemical changes in raw milk during refrigerated incubation and their effect on the quality of UHT milk during storage at 25 ºC

Even though, in all three trials, both control and treatment raw milk samples had viable counts of bacteria associated with spoilage (Garbutt, 1997), they were still of high enough quality to be heat processed, as suggested by their ethanol stability. It has been suggested by Zadow (1993b) that the ethanol stability test has limited value as a tool to assess the likelihood of a given raw milk sample to withstand heat processing; however, in the present study it proved to be sufficient as the raw milk was heat-stable. An alternative heat stability test, the phosphate test developed by Ramsdell *et al.* (1931), could have been used instead.

Psychrotrophic bacteria predominate in raw milk after low temperature storage (1-4 ºC) (Lafarge *et al*., 2004). This was observed in the present study, in each trial. As the predominant psychrotrophs in raw milk (*Pseudomonas* spp.) do not utilise lactose (Kives *et al*., 2005), there was little variation in pH in the milk samples during the seven day incubation period. Lactic acid bacteria, responsible for most acidification of raw milk due to the production of lactic acid following metabolism of lactose, are normally present in lower numbers in freshly drawn raw milk (Ternstrom et *al*., 1993; Desmasures *et al*., 1997a). As they are mesophilic, they would not be expected to grow at refrigeration temperatures. Therefore, little decrease in pH would occur. FFA can also contribute to low pH in raw milk. However, the FFA content in both samples was typical of good quality (unspoilt) milk (Deeth & Fitz-Gerald, 1976) and would contribute only slightly to the acidity of both raw milk samples. There were no apparent defects in either raw milk sample due to lipolysis or proteolysis and any other organoleptic defects due to the action of lipase or protease was unlikely as the concentration of FFA and FAG remained low and perceptible flavour defects were generally not observed by the sensory panel after processing.

The observation in each of the three trials that no significant difference existed between the FFA/FAG concentrations at either raw milk incubation temperature suggested that comparable concentrations of active lipase(s) and protease(s) were present and/or there was comparable total lipase and protease activity in the raw milk at the different storage temperatures. As there was no more than a one log difference between the total counts in the samples, such similarities in lipolysis and proteolysis don't seem unreasonable, especially considering that more lipase and protease may be produced per cell at lower temperatures (Sorhaug & Stepaniak, 1991). It was clear that lipases and proteases were present in the raw milk, as the FFA and FAG concentrations increased significantly during the incubation. Even so, the milk was still of an acceptable quality at the time of processing. It was organoleptically acceptable after processing.

The UHT milk in trial 1 did not gel, while the UHT milk in trials 2 and 3 did gel. Therefore, it is worthwhile to consider any differences that might be present in the raw milk which may have contributed to age gelation. However, the results indicate more similarities than differences. The FAG concentration throughout the incubation was very similar across all three trials and there wasn't a large difference in FFA concentration, with marginally more present in the first trial. Consequently, the major contributing factor is likely to be the more lipolytic and proteolytic isolates added into the raw milk in the second and third trials. While the similar concentration of enzymatic end products present in the raw milk might indicate a similar concentration or similar activity of enzyme, it would appear that during storage of UHT milk, at a high temperature, the enzyme's activity increased substantially. It would seem that these added highly lipolytic or proteolytic cultures, made the difference between non-spoilage and spoilage and supports the body of evidence for the involvement of proteolysis in age gelation.

5.4.2 Inactivation of lipase and protease after UHT processing

A substantial reduction in proteolysis following the indirect UHT processing used in this experiment, was expected. Mottar (1981) investigated

lipase and protease inactivation following both direct and indirect UHT treatment and found greater inactivation with milk UHT treated by the indirect method (87% for lipase, 85% for protease), compared to the indirect method (95% for lipase, 92% for protease). Substantial protease inactivation, after direct UHT processing at 149 ºC for 10 sec., has been reported by Adams *et al.* (1975) who observed a mean of 47% reduction (range 8 to 71%) with ten isolates of *P. fluorescens*. The significant difference ($p<0.001$) in the degree of inactivation between the control and treatment samples in both trials 2 and 3 (an enzyme inactivation study was not carried out for trial 1), was unexpected. If proteolytic action contributed to the age gelation observed in the current study, then it is clear that the most important factor in the gelation observed in the treatment sample and the lack of gelation in the control sample not gelling, was the inconsistency in protease inactivation reduction with UHT treatment. This is because the degree of protease action in both samples of raw milk was similar immediately prior to UHT processing.

From the work conducted in Chapter 4, it was observed that during the long term storage (Figure 4.13), 140 d elapsed before the milk samples with the lowest concentration of added protease (0.0003%) reached an FAG level of 12 µM Leu-Gly equivalents. It was possible to detect this FAG level in 14 days with the casein assay and 10 days with the FITC-casein assay. Two other preliminary UHT milk storage trials were carried out. In the second of these, age gelation was first observed at an FAG concentration of 16 µM Leu-Gly equivalents, in the treatment sample. In the first trial, the FAG concentration in the treatment sample did not rise above 7 µM Leu-Gly equivalents, even after eight months of storage. Gelation did not occur in that trial. Of importance is the observation that age gelation occurred between 12-16 µM Leu-Gly equivalents. In this experiment, 19 d of storage was required for that level to be attained. The time taken to reach this level of FAG is most likely dependent on the protease activity in that particular milk sample and/or the total contribution of proteases. Therefore, with a lower concentration of added protease, the time to reach that FAG content would be longer and consequently, the time taken for

age gelation to be observed, is likely to be longer. This is, of course, assuming a relationship always exists between proteolysis and age gelation.

5.4.3 Biochemical changes in UHT milk during storage

As commercial sterility was achieved during the UHT processing of this product, the changes observed were not caused by microbial growth.

5.4.3.1 Selection of storage temperature

UHT milk shelf life is usually determined by the onset of gelation (Garcia-Risco *et al*., 1999) and in this experiment, storage of UHT milk was at 25 ºC because gelation occurs most rapidly between 25 ºC and 30 ºC (Zadow & Chituta, 1975) and within this range, 25 ºC is closest to the typical storage temperature of UHT milk.

5.4.3.2 pH

The degree of variation in pH observed during the storage period in trials 1 and 3 was similar to that reported by Topcu *et al.* (2006) during storage of UHT milk, at the same temperature as used in the present project (25 ºC). When the UHT processing temperature was 150 ºC, the pH varied between 6.62 and 6.79, while a UHT processing temperature of 145 ºC resulted in a pH of between 6.59 and 6.72. Therefore, lowering the UHT processing temperature appears to lower the pH, and with the UHT processing temperature of 140 ºC used in this investigation, a variation of pH between 6.52 and 6.63 appears consistent with that reported by Topcu *et al.* (2006). Comparable minimum pH results were also reported by Adhikai and Singhal (1992) where pH 6.52 was the minimum pH observed during storage of UHT milk at 22 ºC.

5.4.3.3 Viscosity

Datta and Deeth (2003) measured viscosity in both high quality UHT milk samples (3 mPas), and those that had gelled due to the action of bacterial protease (100-110 mPas). These results were similar to those reported here, for both trials (second and third) in which age gelation was observed. McKellar

et al. (1984) also reported comparable results, with age gelation observed at a viscosity of 100 mPas. The typical prolonged period of little change in viscosity followed by a sudden and substantial increase, originally described by Harwalker (1982) and subsequently by Kocak and Zadow (1986) was seen.

5.4.3.4 Lipid degradation

The FFA profile observed in UHT milk immediately following processing, in the third trial, was somewhat different to that reported in various papers, albeit with different processing conditions. However, it should be remembered that the fatty acid content of raw milk can vary due to various factors, such as breed (Zegarska e*t al.*, 2001) or diet (Collomb *et al.*, 2002; Reklewska *et al.*, 2002). Hexadecanoic (C16:0) and *cis*-9-octadecenoic (C18:1) acids did not dominate, comprising 15% of the FFA in the control sample and 18% in the treatment sample. The reported combined percentages (in UHT milk immediately following processing) of these two FFA are 48.7% (Withycombe & Lindsay, 1969), 49.0% (Choi & Jeon, 1993) or 51.9% with limited seasonal variation (Lindmark-Mansson *et al.*, 2005). In the present study, tetradecanoic acid (C14:0) comprised a substantial proportion (22.7% in the control sample and 28.9% in the treatment sample) of the FFA, but has been reported to represent 7.7% (Withycombe & Lindsay, 1969) or 8.4% (Choi & Jeon, 1993) of the FFA UHT milk immediately following processing. There were also variations in concentrations between the same FFA immediately following UHT processing, depending on the temperature of incubation of the raw milk. These differences could have been due to either or both of two factors; i) lipolysis in the raw milk and ii) variation in FFA losses during UHT processing. The level of FFA increase in the control samples was minimal, increasing from 0.8 meq/L at day 0 to 1.0meq/L at day 66. A similar degree of FFA increase was observed by Choi and Jeon (1993), as measured by acid degree value (ADV). Over a 12-week storage period, the ADV increased from 1.4 (equivalent to 0.9meq/L) to 1.5 (equivalent to 1.0 meq/L) in commercial samples of UHT milk (UHT processed at 138 ºC for 10 sec), upon storage at 23 ºC. The UHT milk samples presumably were produced from good quality raw milk, and therefore, may be

compared to some extent with the control samples in the present study. In another paper (Adams & Brawley, 1981), results of increase in ADV are presented, in UHT whole milk samples stored at 25 ºC and 40 ºC, produced from raw milk of varying microbiological quality (7.4×10^{3} - 7.8×10^{5} cfu/mL) and with varying UHT processing conditions (138 ºC/21 sec, 143 ºC/7 sec, 149 ºC/3 sec). However, these results are difficult to compare due to the differences in total counts and FFA measurement methods.

Choi and Jeon (1993) reported that the largest increases in FFA were with hexadecanoic (C16:0) and octadecanoic (C18:0) acids while Ouattara *et al.* (2004) noted the largest increases with hexadecanoic (C16:0) and *cis*-9-octadecenoic (C18:1) acids, with lipase from *P. fluorescens*. This is partly in contrast to the treatment samples in the present study, where the largest increases observed were butanoic (C4:0), tetradecanoic (C14:0) and *cis*-9-octadecanoic (C18:1) acids.

5.4.3.5 Protein degradation

The lack of substantial increase in FAG in the control milk for all three trials, was expected and reflected in the lower bacterial counts of that milk at the time of UHT processing. In the treatment sample, increasing FAG concentration was observed after months of storage (Trial 1), at the end of shelf life (Trial 2), or as the end of shelf life approached. This suggests a link between proteolysis and age gelation, with a threshold range of values for its occurrence, evident. In fact, the most proteolysis, as indicated by the steepest increase in FAG (in Trial 3), was seen in the four days prior to age gelation. Unlike the step-like increase in FAG until the point of age gelation seen in the third trial, McKellar *et al.*, (1984) observed a linear increase in the concentration of FAG as the point of age gelation approached. In samples which did not gel, McKellar *et al.* (1984) still saw an increase in FAG, albeit limited. Such a result was seen in the first trial.

The peptide profiles, following RP-HPLC analysis of UHT milk from Trials 2 and 3, were as expected, and similar. This suggests a similar means of peptide formation in both trials. In the control samples, there were limited

peaks, representing comparatively few peptides, the result of a lower extent of proteolysis. The treatment samples were very different, with many peaks, indicating extensive proteolytic action during the storage period. Elution of the increased number of peptides in the latter part of the run of the treatment samples indicated that there were more hydrophobic peptides present (due to the decreasing polarity of the mobile phase). As most peptides were eluted within the first 20 min, this indicated that bacterial protease and not plasmin was responsible for the proteolysis (Lopez-Fandino *et al.*, 1993a; Datta & Deeth, 2003) because of the specificity of plasmin compared to bacterial protease and the peptides likely to result. The peptide profiles of proteolysed milk, particularly with reference to age gelation, have been studied in depth. Early work in the area by Stepaniak *et al.* (1989) was on the characterisation of peptides by action of proteases of *Pseudomonas* spp. and plasmin, on both whey proteins and caseins, using RP-HPLC. Lopez-Fandiño *et al.* (1993a, 1993b) further demonstrated the suitability of RP-HPLC to the study of casein proteolysis in UHT milk, in particular for differentiation of proteolysis due to the action of plasmin and bacterial protease and to follow proteolysis during storage. The application of RP-HPLC for investigation of UHT milk proteolysis was extended to whey proteins by Recio *et al.* (1996) who compared HPLC with capillary electrophoresis (CE) for analysis of peptides from UHT milk, following preliminary work with CE for this application by Kristiansen *et al.* (1994). Recio *et al.* (1997) then investigated the use of CE more comprehensively and demonstrated its suitability for quantification of proteolysis of both caseins and whey proteins. CE was then applied to the analysis of protein degradation in stored UHT milk by Garcia-Risco *et al.* (1999). Para-κ-CN results from the action of chymosin (Fox & McSweeney, 1998) or bacterial proteases on κ-CN (Lopez-Fandino *et al.*, 1993b; Miralles *et al.*, 2003). This specificity was exploited by Miralles *et al.* (2003) in the development of a technique combining RP-HPLC-ES-MS and CE to analyse bacterial proteolysis in milk. At the same time, Haryani *et al.* (2003) used HPLC to investigate proteolysis in refrigerated raw milk due to psychrotrophic bacterial proteases. That study focused on production of the protease(s), because HPLC analysis was carried out only after

incubation of the raw milk at 40 ºC for 24 h, following a set period of refrigerated storage. Many peaks were observed between 5-25 min elution time, as was seen in the current work. Sensitivity, however, may be questioned, because a large amount of protease could be present (following prolonged, 10 d, refrigerated storage of the raw milk) and a large amount of peptides could be present (following incubation close to the temperature optimum of the proteases under investigation). A subsequent study by the same research group (Le *et al.*, 2006) resulted in a paper describing proteolysis and appearance of peptides over time in UHT milk, supplemented with a substantial addition of sterile crude protease preparation from a psychrotrophic *Pseudomonas* spp. and a pure, commercial preparation of a serine endopeptidase. The proteolytic action was at a sufficiently high level to see a peptide profile similar to that observed in UHT milk in the present study over 66 d, in one to two hours. Again, the issue of sensitivity could be raised. However, that study did demonstrate again the potential for following proteolysis in milk by measuring appearance of peptides. While various aspects have been investigated, there have been no reports of the change in the peptide patterns during shelf life of UHT milk without added protease. Therefore, the present work demonstrated the suitability of RP-HPLC to detect relatively small changes in peptide concentrations that occur following limited proteolysis after the substantial inactivation of protease during UHT processing.

The results from the SDS-PAGE gel complemented the RP-HPLC results in the second and third trial. While the RP-HPLC results showed appearance of peptides, the SDS-PAGE results showed degradation of proteins. This degradation was most evident with κ-CN. In Trial 3, the κ-CN band diminishes visually in intensity from day three of storage, while at the same time, the para-κ-CN band increases in intensity. As the degradation of the other caseins was minimal, as assessed by band intensity, this demonstrates the preferential proteolysis of κ-CN compared to the other caseins. This is consistent with the study of casein degradation in gelled milk by Mitchell and Ewings (1985), who observed 80 to 100% degradation of κ-CN for almost all of the *P. fluorescens*

proteases under investigation, with limited little degradation of α_s-casein (α_s-CN).

Age gelation is the major factor limiting the shelf life of UHT milk (Datta & Deeth, 2001), which was the organoleptic defect that signalled the end of shelf life in the current study. This is because sensory analysis in the third trial by an untrained consumer panel did not detect any difference in aroma nor flavour up until the point the milk gelled. Even when the milk had gelled, no consistent off-aromas or off-flavours were detected by a trained sensory panel, which suggests that the chemical compounds responsible for such organoleptic defects were present at low concentrations.

5.4.4 Merits of techniques for free fatty acid determination in milk

FFA determination by solvent extraction followed by a titration, provides an estimate only, of the FFA concentration. These methods therefore, have a limitation in that the compounds measured in the titration may be acids other than fatty acids. The lack of clear discrimination between components of a mixture can be overcome by using GC/MS, and was used to quantify the specific FFA present in the UHT milk in Trial 3 only. Using a GC/MS method, the exact components of a mixture can be identified, and with a high degree of certainty.

It has been reported that the short and medium chain length FFA contribute most to lipolysed flavour in UHT milk (Cheeseman, 1975). Of these, only butanoic acid (C4:0), was present in a significantly higher concentration in the treatment samples compared to the control (Figure 5.27). The concentrations of the C6:0-C12:0 FFA were significantly higher ($p<0.0001$) in the treatment group compared to the control, and as they did not increase substantially (Figure 5.27), would not be expected to contribute to lipolysed flavour. The C14:0 to C18:1 FFA contribute little to lipolysed flavour (Wadhwa & Jain, 1989), but were present in higher concentrations than the shorter chain FFA. Some, tetradecanoic (C14:0) and *cis*-9-octadecanoic (C18:1) acids, were present in the treatment sample, at concentrations significantly higher ($p<0.0001$) than all the C6:0 to C12:0 FFA. This would suggest that the FFA

determination methods that cannot be used to quantify individual FFA present in the sample, are of limited use in ascertaining if a milk sample has lipolysed flavour. Deeth and Fitz-Gerald (1976) state that the FFA threshold at which most people will find milk unacceptable is 1.4 meq/L. Even though from day 27, the FFA concentration in the treatment sample was 1.4 meq/L or greater, perceivable lipolysed flavour in that sample would not be expected because the concentration of only butanoic acid was elevated. All other FFA known to contribute to this flavour defect, which have taste thresholds higher than butanoic acid (IDF, 1991), were present in relatively low concentrations. There was limited increase in FFA beyond 42 d, in comparison to the storage period up until that time. This was reflected in no significant increase ($p<0.001$) in the overall estimated concentration of FFA by the extraction/titration technique.

All FFA measurement procedures based on extraction of the FFA with organic solvents will be limited by the efficiency of the solvent extraction. A review of methods for FFA extraction (IDF, 1991), outlines the large difference in recovery of different FFA with a particular solvent extraction method and additionally, the large differences between methods, due to the variation in the extraction procedure. The method of Dole and Meinertz (1960) was one of the earliest in which FFA extraction is described. In this extraction technique, recovery of saturated FFA from C14:0-C20:0 was at or close to 100% while the recovery decreased substantially with decreasing chain length; 89% for C12:0, 77% for C10:0, 66% for C8:0, 45% for C6:0 and 24% for C4:0. In the 1970s, Deeth *et al.* (1975) modified the method of Dole and Meinertz (1960). There were similar recoveries of FFA from C12:0-C18:0, but improved recovery of C10:0 (85%), C8:0 (73%), C6:0 (57%) and C4:0 (34%). Deeth *et al.* (1975) compared their modified method with the widely used Bureau of Dairy Industries (BDI) technique developed by Thomas *et al.* (1955). The comparison demonstrated that the recovery of FFA by the BDI method was very poor. No more than 81% (C18:0) of any FFA was recovered, with the recovery of C6:0 (4%) and C4:0 (2%) being especially meagre. Regardless of the low recoveries, the BDI method has been suggested recently as the standard reference method by the International Dairy Federation for determination of FFA

in dairy products (Evers, 2003). Such a recommendation is surprising when Evers (2003), in a review of the theory and practice of the BDI method, reports the percentage recovery of saturated FFA from C4-C18 to range from zero to 69% (IDF, 1991), which is much lower than the comparable recovery by the Deeth *et al.* (1975) method (34 to 102%). Still higher recoveries for the same FFA (63 to 97%) have been have been reported from very early work by Perrin and Perrin (1958). Furthermore, the method used in this study (de Jong & Badings, 1990) to extract FFA for GC/MS analysis has a recovery of 79 to 104% for the C4-C18 saturated FFA.

5.4.5 Relationship of free fatty acid content to lipolysed flavour

For the approximate designation of lipolysed flavour, the criteria of Deeth and Fitz-Gerald (1976) are often applied. Using criteria based on the total concentration of FFA, the milk in Trial 1 would have been perceived as spoiled by some people on day 42 only, but very few detected a difference and identified it as related to spoilage. In Trial 3, the UHT milk would have been perceived as spoiled by some people after 6 d, and perceived by most people as spoiled after 27 d. Considering the total concentration of FFA found in the milk at 27 d and the perceived off-aromas and off-flavours, the solvent extraction and titration method along with the criteria for interpretation used in this investigation would appear a good choice for UHT milk. A comprehensive study of the sensory thresholds of lipolytic and proteolytic spoilage in pasteurised milk by Santos *et al*. (2003), indicated somewhat lower levels of perceivable spoilage. In that investigation, approximately 95% of sensory panellists perceived spoilage at 0.95 meq/kg while approximately 85% perceived spoilage at 0.55 meq/kg. Although, lower thresholds are reported, the quantification of the FFA is based on the copper soap method (Ma *et al*., 2003) which is insensitive (Deeth & Touch, 2000). A good relationship between FFA and lipolysed flavour in milk has been reported by Connolly *et al.* (1979), who used the method of Lindqvist *et al.* (1975), resulting in a much better recovery of all FFA. Lipolysed flavour was not observed in that study until the FFA concentration was at least 2 meq/L. Another related measurement of FFA

concentration which can be linked to sensory perception is acid degree value (ADV). It was reported as being the most widely used in the 1970s (Deeth & Fitz-Gerald, 1976) and is currently a standard method in the United States (Hooi *et al.*, 2004). Developed by Thomas *et al.* (1955), the ADV was shown by these authors and others including Janzen (1971) to relate well to sensory perception of lipolysed flavour in milk. However, it has also been demonstrated that ADV does not relate well to lipolysed flavour in milk (Kintner & Day, 1965; Rerkrai *et al.*, 1987; Duncan *et al.*, 1990; Duncan *et al.*, 1991) due to the lack of recovery and therefore measurement of all FFA, in particular, those responsible for sensory defects (Duncan & Christen, 1991). As a result of the questionable value of relating ADV to lipolysed flavour, Bandler (1982) states that milk with a high ADV must be confirmed as rancid by sensory analysis. In general, no firm conclusions can be drawn on the sensory importance from any chemical determination of FFA (Bills *et al.*, 1969), although this mainly relates to techniques which do not yield information allowing qualification and quantification of individual FFA.

5.4.6 Individual free fatty acids associated with perceived quality defects

Not all FFA contribute to lipolysed flavour, and those that do, do not contribute equally. This is because sensory thresholds vary between FFA. Patton (1964) describes a reduced threshold in milk of hexanoic (C6:0) in comparison to butanoic acid (C4:0) while a more comprehensive investigation by Scanlan *et al.* (1965) revealed that the lowest threshold for lipolysed flavour to occur in milk was with octanoic acid (C8:0) (22.5 mg/kg milk). The thresholds decreased from butanoic acid (C4:0) (46.1 mg/kg milk) to hexanoic acid (C6:0) (30.4 mg/kg milk), then increased after octanoic acid (C8:0). The threshold of decanoic acid (C10:0) was 28.1 mg/kg milk, dodecanoic acid (C12:0) was 29.7 mg/kg milk, tetradecanoic acid (C14:0) was 80.5 mg/kg milk and there was a large increase to hexadecanoic acid, with a threshold of 244.5 mg/kg milk. The 18-carbon chain-length fatty acids did not follow the trend of C4:0-C16:0 as the threshold of octadecenoic acid (C18:0) was 142.1 mg/kg milk and *cis*-9-octadecenoic acid (C18:1) was 222.1 mg/kg milk.

In the absence of sensory assessment, qualitative and quantitative results for individual FFA can aid greatly in determining the likely organoleptic properties of the product. The most important FFA that contribute to lipolysed flavour are the C4:0-C12:0 fatty acids (Cheeseman, 1975). However, in the milk samples from Trial 3 in the present study, it was the longer chain FFA that were present in the largest proportions as they comprised between 57.9% (control sample) and 63.9% (treatment sample) of the total FFA measured. As these longer chain FFA, C14:0 and longer, contribute little to aroma or flavour (Wadhwa & Jain, 1989), it would be expected that there would not be a substantial lipolysed flavour perceived. It is worth noting that, when decanoic (C10:0) and dodecanoic (C12:0) acids contribute most to lipolysed flavour (with flavours being rancid, unclean, bitter, soapy) (Al-Shabibi *et al.*, 1964; Woo & Lindsay, 1983), these were present at a low concentration and proportion in both control and treatment samples. Therefore, the lack of pronounced off-aromas or off-flavours reported in the third trial, was expected. Hexanoic (C6:0) and octanoic (C8:0) acids are perceived as cowy and/or goaty flavours (Al-Shabibi *et al.*, 1964; Woo & Lindsay, 1983), and as these FFA were present at low concentrations and proportions too, it was not unexpected that these flavours were not perceived at all. In some situations, only very small amounts of FFA can result in spoilage, for example, bitter flavours (Bassette *et al.*, 1986). This may have occurred in the present study because decanoic (C10:0) and dodecanoic (C12:0), which Al-Shabibi *et al.* (1964) report can result in a bitter flavour, when present at a very low concentration. The selective contribution to lipolysed flavour of specific FFA requires careful consideration in any qualitative and quantitative study and especially if relationships to organoleptic defects are sought. The use of chromatography (as in the present study) would be preferred in this regard, as such techniques allow both qualification and quantification of the individual FFA.

5.4.7 Relationship of free amino groups concentration to the onset of spoilage

An indication of the extent of proteolysis is often obtained by quantification of the FAG. However, by the nature of such a measurement,

establishing links to bitterness and age gelation is difficult, if not impossible, as specific peptides or degradation of specific proteins takes place in these quality defects. Furthermore, the lack of consistent units of measurement makes comparisons complicated. The present work (combined results of Trials 2 and 3) has shown that an FAG concentration between 8-12 μM Leu-Gly equivalents could be linked to age gelation. A quarter of a century ago, Harwalkar (1982) stated that the mechanism of age gelation had not been elucidated. This is still true. Even the contribution of proteolysis is not fully understood. Moreover, as bitterness results from the presence of peptides containing particular amino acids (Pedersen, 1994), rather than the total concentration of an easily quantifiable chemical compound, relationship to the FAG concentration may be tenuous. This is especially so, when peptides of varying length, comprising various combinations of amino acids can potentially result in the same degree of bitterness (Shinoda *et al.*, 1986). However, establishing a relationship to organoleptic defects was attempted by Rollema *et al.* (1989). That study concluded that an increase in FAG, in the order of 25% of the control, could be related to symptoms of spoilage. Based on such a criterion, the Trial 3 treatment milk samples in this work would have organoleptic defects consistent with proteolysis at around 4.4 μM Leu-Gly equivalents, whereas age gelation took place with at least 8.3 μM Leu-Gly equivalents observed. Throughout the shelf life, and even beyond the shelf life of these treatment samples, no consistent reports of bitter flavour were made during sensory analysis, and these samples would definitely not be rejected on the basis of bitter flavour, even at a FAG concentration in excess of 12 μM Leu-Gly equivalents. Therefore, the criterion of Rollema *et al.* (1989) did not apply in the samples investigated in this experiment. Generally, it could be said that while an increase in the FAG content in a milk sample will indicate proteolysis, little relationship can be drawn between the concentration of the FAG and the various organoleptic defects that arise as a result of proteolytic action. However, it is clear that protease action must be present for these to occur and that sensitive protease tests are of value in assessing the potential for spoilage of UHT milk.

5.4.8 Peptides: content and type associated with perceived quality defects

A major organoleptic defect resulting from proteolysis action is bitterness. A peptide's average hydrophobicity can be indicated by a Q value (Ney, 1979). With any given Q value, hydrophobicity was regarded as being solely responsible for bitterness when the Q value is over 1400 (Ney, 1979). However, from the work of Hill and Van Leeuwen (1974) the Q value above 1400 was not shown to be related to intensity of bitterness. Consequently, the Q value scale, although widely used, may not always reflect bitterness reliably. Overall, many scales have been developed to determine the hydrophobicity of a peptide. One such scale, possibly the most accurate because it incorporates most of the previously developed scales, is the Combined Consensus hydrophobicity Scale (CCS). This scale, developed by Tossi *et al*. (2002) utilises 193 hydrophobicity scales. While this might be an excellent means to ascertain hydrophobicity, the problem of direct relationship to bitterness still exists. This is because the presence of peptides containing strongly hydrophobic amino acids is only partly responsible for mean hydrophobicity (Ishibashi *et al*., 1987a; 1987b). The conformation of an amino acid can also give rise to bitter flavours (Ishibashi *et al*., 1987a; 1987b) while the intensity of the bitter flavour can be affected by position of the D and L isomers of the same amino acid at the C-terminal end (Shinoda *et al*., 1986) and the distance between the hydrophobic amino acid residues (Tamura *et al*., 1990). Total hydrophobicity of the peptide is also influenced by the amino acids present (isoleucine results in more intense bitterness than leucine for example), and the number of carbon atoms on the side chain of the amino acid, with at least three required (Ishibashi *et al*., 1988a). Examples of amino acids which are strongly hydrophobic are tryptophan, tyrosine and phenylalanine while valine and lysine are examples of amino acids with a lower hydrophobicity (Tanford, 1962). Peptides containing less hydrophobic residues, such as valine, can still impart bitterness (Ishibashi *et al*., 1988a). In milk, bitter peptides are more likely to originate from α_{s1}- and β-CN as these caseins contain more hydrophobic amino acids, with higher hydrophobicities (Koka & Weimer, 2000a).

Sensory thresholds are mostly available in the literature for short, synthetic peptides which have been produced to ascertain the contribution of particular amino acid residues and/or their position within the peptide to bitter flavour and its intensity (Matoba & Hata, 1972; Otagiri *et al.*, 1985; Shinoda *et al.*, 1986; Ishibashi *et al.*, 1987a,b; Shinoda *et al.*, 1987; Ishibashi *et al.*, 1988a,b). However, sensory assessment of peptides was obtained by Minamiura *et al.* (1972a,b) following digestion of whole casein, and was bitter at a concentration of 22 μM. In particular, the peptide described by Minamiura *et al.* (1972b) contained equal molar concentrations of leucine and tryptophan and was distinctly bitter at a concentration of 22 μM.

Age gelation is suggested to occur from the association of whey proteins (primarily β-LG) and casein (primarily κ-CN) which form complexes (βκ-complexes), and ultimately, a three-dimensional protein matrix (Datta & Deeth, 2001). While a link has been demonstrated between age gelation and proteolysis (Law *et al.*, 1977; Richardson & Newstead, 1979), appearance of peptides, as seen in a HPLC chromatogram, cannot be directly linked to the appearance of age gelation. Conversely, a technique which shows protein degradation, rather than appearance of peptides, would be more useful. This is where the benefits of SDS-PAGE are demonstrated, as degradation of proteins can be observed clearly. For example, the substantial degradation of κ-CN in the treatment samples in the second and third trials could not be followed with RP-HPLC, but was evident in the SDS-PAGE analysis. Furthermore, the degree of degradation could be quantified, and this can possibly be linked to a critical level, below which age gelation may take place.

Bitter peptides resulting from proteolytic activity are the usual cause of bitterness in milk (Deeth, 2006). Regardless of the presence of the increasing number and concentration of hydrophobic peptides in the treatment samples from Trials 2 and 3, there were no consistent reports of bitter flavours at the end of shelf life and there was no increase in bitter flavours reported leading up to the end of shelf life. This indicates that the peptides were present at a concentration below that required for a definite bitter taste to be imparted. The reasons for this could be that their hydrophobicity was not above the critical

value (Q value of 1400) for bitterness to result (Ney, 1979), or if it was, the position of particularly hydrophobic amino acids (Shinoda *et al.*, 1986) or the conformation of the molecule (Ishibashi *et al.*, 1988b) may have reduced the intensity of bitterness produced by those peptides. These results indicate that the hydrophobicity of the peptides observed in RP-HPLC analysis can only be used as a guide as to the sensory status of that particular milk sample. This is supported by the conclusions of various studies into the cause of bitter flavour, which indicate that no single factor is responsible, although hydrophobicity of peptides plays a key role.

5.4.9 Lipolysis and proteolysis in Trial 3 UHT milk versus predictive power of lipase and protease assays

In the shelf life experiment (Trial 3) described in this Chapter, the FFA concentration attained the threshold level of 1.4 meq/L on day 27. This degree of lipolysis, seen against the background of the experiment described in section 4.2.5 (in which the same lipase source was used), could be predicted with the triolein assay recommended in section 4.5, in 168 h. Age gelation was observed in Trial 3 on day 19 when the FAG concentration was 12 μM Leu-Gly equivalents. From the results presented in Figure 4.13, this corresponds to approximately 0.002% protease preparation. This degree of proteolysis could be predicted in 12 h using the FITC-casein assay.

5.4.10 Origin of protease present in UHT milk from Trial 3

One of the objectives of the experiment described in this Chapter was to observe changes in the protein fraction in UHT milk due to the action of heat-stable extracellular proteases produced by psychrotrophic *Pseudomonas* spp. The experiment was set up to give this scenario every chance to take place. While one of the expected outcomes was ultimately met, spoilage likely due to the action of protease, it is actually unclear where the protease originated. There are five possibilities - I) from the milk itself (plasmin), II) somatic cells, III) extracellular protease produced by bacteria growing in the raw milk, IV) intracellular protease produced by bacteria growing in the raw milk and V)

interactions between bacterial protease or somatic cell protease and the plasmin system in milk.

Plasmin, on its own, was the first possibility to be excluded. Datta and Deeth (2003) identified some clear differences in the peptide profile following proteolysis with plasmin and bacterial protease. The peptide profile (HPLC results) and the protein degradation pattern (SDS-PAGE results) in the current work resembled what Datta and Deeth (2003) have found to be characteristic of bacterial protease action, therefore, this was a strong indication that plasmin was not involved.

The second possible source is somatic cells. The somatic cell count, observed at 186 000/mL in the third trial, is considered to be at a medium level (Green *et al.*, 2006). A substantial increase in proteolysis has been observed in milk with elevated somatic cell counts due to mastitis (Verdi *et al.*, 1987; Saeman *et al.*, 1988). Some somatic cells, leucocytes, have been shown to lead to only slight proteolysis in comparison to bacterial protease and plasmin (Grieve & Kitchen, 1985), but overall, proteases originating from somatic cells can degrade casein (Haddadi *et al.*, 2006). In contrast, Senyk *et al.* 1985, reported a weak relationship between somatic cell count and proteolysis. In fact, it has been found that addition of leucocytes to UHT milk lengthened the time taken until the onset of age gelation (Keogh & Pettingill, 1984). Therefore, the contribution of protease originating from somatic cells would appear insignificant in the present study. This is because it is known that the milk was not obtained from mastitic cows, and it would appear unlikely that the somatic cells could contribute sufficient protease to influence age gelation.

The next two possibilities are bacterial proteases, either extracellular or intracellular. It seems probable that extracellular protease is not responsible, and it is possible that the cells produced only intracellular protease. This is due to the observation that proteolysis in the raw milk did not increase with increasing length of incubation. The intracellular protease theory fits with the observations. Firstly, there was no increase in proteolysis in the raw milk during incubation, as evidenced by increases in fluorescein concentration in the FITC-casein assay. Then cell lysis during heat processing of the milk could have

released the proteases, more of which would have been present in the treatment milk stored at 4 ºC prior to UHT processing, compared to the control (stored at 1 ºC prior to UHT processing). The difference in proteolysis seen immediately after UHT processing may simply reflect a high concentration of intracellular protease due to a higher cell count. This only partly matches the observations, however. During storage of UHT milk, there was a substantial increase seen in proteolysis in the seven days immediately prior to the onset of age gelation. Bacterial protease concentration can only increase in milk due to their production by the bacterial cells, and in a sterile UHT milk sample, such a possibility could not occur. Therefore, this leaves the last option as a possibility: interactions between bacterial protease or somatic cell protease and the plasmin system. During the first 12 days of storage, bacterial protease could have been the only active protease present, or there could have been a combination of bacterial protease and a protease from somatic cells. From that point for the next seven days, the bacterial (Farjardo-Lira and Nielsen, 1998) or somatic cell (Verdi & Barbano, 1991) protease may have stimulated the plasmin system, for example, by release of plasminogen and plasmin from the casein micelle, as suggested by Farjardo-Lira and Nielsen (1998). Then, at the point where sufficient proteolysis for age gelation to occur was attained, the activation ceased, age gelation took place, and the level of protease in the now gelled UHT milk, stabilised and remained constant for the rest of the storage period. The combined intracellular bacterial protease and activation of the plasmin system by bacterial protease theory would seem to be most in agreement with the observations made during the trial. However, although various lines of evidence may point towards a combined bacterial protease and plasmin cause, the peptide profile is the major result which indicates this is not true.

5.4.11 Assessment of sensory analysis methods for detection of off-flavours and off-aromas associated with lipolytic and/or proteolytic action

Sensory analysis of food has traditionally been performed with either “consumer” or “expert” panels. The choice of which panel is largely dependent on the intention of the sensory assessment. Sensory analysis for product

development purposes is best carried out with an expert panel. However, if the purpose of the analysis is assessment of sensory attributes of a more general nature, by a panel representative of the general population, then a consumer panel should be used. The relatively large number of sensory panellists required, is one disadvantage of a consumer panel. However, for the triangle test, replicate testing by panellists is statistically valid (Kunert & Meyners, 1999) and can in fact improve performance of non-experienced panellists (Dacremont & Sauvageot, 1997), such as those typically recruited for a consumer panel. In the present investigation, the goal was to ascertain the point of perceivable spoilage by the general population, hence a consumer panel was most applicable. At each point during shelf life, the only information desired was differentiation or not between the control and treatment samples. The discriminatory power of the duo-trio test is lower than the triangle test (Ennis, 1993), which in turn is lower than the 3-alternative forced choice (3-AFC) and 2-alternative forced choice (2-AFC) (O'Mahony & Rousseau, 2003). However, a drawback of the 2-AFC and 3-AFC tests is that a particular attribute must be specified in that test (Rousseau *et al*., 2002), and the specific difference was unknown in the current work. The triangle test is preferred when the experimenter does not know the nature of the difference between the samples (Francois & Sauvageot, 1988). Therefore, the triangle test, typically undertaken with a large number of untrained panellists (Smith, 1981), was selected for this simple discriminatory analysis. Triangle tests are satisfactory to demonstrate the existence of a difference, if the number of panellists that can detect a difference is more than the number of differences detected due to chance (MacRae, 1995). Furthermore, they are performed better than similar tests in which the difference is stated, when the differences are subtle and may overlap (O'Mahony, 1995). Electronic sensory analysis systems, such as the electronic nose used in this project, are an alternative to sensory panels. The electronic nose dates from the early 1980s when Persaud and Dodd (1982) developed an electronic olfactory system. Although sensory panels can be used for routine quality control in industry (Nakayama & Wessman, 1979), the e-nose has a particular advantage over panels in this area due to the shorter time and lower

cost at which analyses can be undertaken (Ampuero & Bosset, 2003). Further advantages are the avoidance of other problems such as individual variability, subjectivity and fatigue (Sarig, 2000), although conversely, the results from instrument analysis can be too objective (Piggott, 1995). A further consideration with sensory panel testing is how the panellists are to record their results. A study by Armstrong *et al.* (1997) found that for the triangle test, manual recording of results by the sensory panel was significantly better than computerised recording of results, as more panellists could detect a difference when a difference was present. Furthermore, sensitivity of the human nose can be improved on, in some instances, for example, for detection of aromas of various solvents (Doleman & Lewis, 2001). With regard to dairy products, differentiation of milk containing various concentrations of pure bacterial cultures has been achieved with an e-nose (Korel & Balaban, 2002). Following UHT processing, cooked aromas are present initially (Mehta & Bassette, 1978). The e-nose has been shown to identify UHT milk with cooked aromas (Di Natale *et al.*, 1998; Mulville, 2000) and to relate well to sensory panel results in the detection of those cooked aromas in UHT milk (Di Natale *et al.*, 1998). More recently, development of off-aromas in both pasteurised and UHT milk have been followed and clearly distinguished with an e-nose (Capone *et al.*, 2001). However, regardless of these promising results, and various improvements to the electronic olfactory systems (Visser & Taylor, 1998; Brudzewski *et al.*, 2004) and introduction of novel electronic nose systems, such as that based on solid phase microextraction and mass spectrometry (Marsili, 1999), the technology does not appear at a stage where it can be used reliably to distinguish UHT milk samples with off-aromas, in a manner superior to a sensory panel, as seen from results obtained in this project.

5.5 Conclusions

- *Pseudomonas fluorescens* produces more lipase and protease in milk stored at 1 ºC than at 4 ºC, and/or greater inactivation of these enzymes during UHT processing occurs if the raw milk was stored at 1 ºC compared to that stored at 4 ºC.

- Age gelation of UHT milk may not be accompanied by bitterness and other off-flavours or off-aromas which result from proteolysis.
- The total concentration of FFA does not relate well to lipolysed flavour in UHT milk; attention should be paid to those individual fatty acids which are most readily detected organoleptically.
- The FITC-casein assay can be used for detection of the degree of proteolysis that is associated with limiting the shelf life of UHT milk.

CHAPTER 6

SOURCES OF LIPASE AND PROTEASE CONTAMINATION

6.1 Introduction

It is interesting to speculate on and to investigate the phenotypes, genotypes and ultimately particular conditions or environments that contribute to the dominance of certain traits. This is related to evolutionary theory, the basis of which is the genetic alteration in a species. Mechanisms for such change are natural selection, random genetic drift, mutation and gene flow (Skipper Jr. & Millstein, 2005). With natural selection, a genotype (such as the presence of genes for lipase production and expression) that encodes a phenotype which improves an individual's survival (such as lipase and protease production), by ultimately resulting in a means to outdo other individuals (such as faster growth rate), will be selected for. These strains may also be expected to have improved utilisation of the products of lipolysis and proteolysis, therefore possibly gaining a further advantage over strains which do not possess such a genotype. Eventually, this may be expected to lead to a domination of lipase and/or protease producing isolates, if that population is present in an environment where such a genotype offers a selective advantage. This could be the dairy environment in general, or possibly even cells in a biofilm. These isolates would dominate, because in this example, lipase production confers a survival advantage over non-producers of lipase and ultimately the genotype of the population would shift and lipase production would become increasingly common. This scenario could take place on any farm, thereby gradually increasing the proportions of lipase-producing strains on a given farm, over time. The same scenario could see an increase in protease production in strains on a farm in due course.

Many types of microorganisms are present in the milk collection environment (Mahari & Gashe, 1990; Jayarao & Wang, 1999) and diversity in the raw milk microflora is typical, without dominance of a single species

(Hutchison *et al.*, 2005). The proportion of psychrotrophic bacteria in raw milk can vary widely and is associated with the level of farm hygiene (Thomas & Thomas, 1973). Studies in Europe have shown that typically, no more than 10% of the flora of good quality milk will be psychrotrophic (Suhren, 1989) with *Pseudomonas* spp. comprising a substantial proportion of these (Muir, 1996). *Pseudomonas fluorescens*, the most common species of the genus present in raw milk (Juffs, 1973), has been involved in bacterial spikes (sudden elevations in total bacterial count) in farm bulk tank milk (Hayes *et al.*, 2001). Psychrotrophic *Pseudomonas* spp. play an important role in spoilage of UHT milk through the production of heat-stable lipases and proteases in raw milk that retain activity following UHT processing (Sorhaug & Stepaniak, 1997). Lipase and protease, produced by psychrotrophic *Pseudomonas* spp. are detected when the cell count exceeds 10^6 cfu/mL (Sorhaug & Stepaniak, 1997). Prolonged refrigerated (4 ºC) storage of raw milk increases the proportion of *Pseudomonas* spp. as do slightly higher temperatures (for example 6 ºC) over a shorter period of time (Griffiths *et al.*, 1987). This in turn increases the likelihood that they will produce heat-stable lipases and proteases. Furthermore, temperature fluctuations have been shown to occur in farm bulk tank milk (Oz & Farnsworth, 1985), and a survey of the literature by Thomas *et al.* (1971) noted that the temperature of raw milk at the time of collection can vary widely, both further compounding the problem of *Pseudomonas* spp. proliferation in raw milk.

Various studies have been undertaken to investigate how widespread lipase and/or protease production is among *Pseudomonas* spp. isolated from raw milk. Dempster (1968) and Shelley *et al.* (1987) found that a large proportion of the lipolytic psychrotrophic flora of raw milk was *Pseudomonas* spp., of which *P. fluorescens* was the species most often identified. Although less commonly found in raw milk, *P. fragi* is possibly more important in lipolytic spoilage. This is because it is strongly lipolytic (Shelley *et al.*, 1987). *Pseudomonas* spp., particularly *P. fluorescens,* are the most often isolated proteolytic flora of raw milk (Ewings *et al.*, 1984; O'Connor *et al.*, 1986) and are more likely to be proteolytic than lipolytic (Wang & Jayarao, 2001). However, a

high proportion of *Pseudomonas* spp. isolated from raw milk have been found to produce both lipases and proteases (Muir *et al.*, 1979).

Spoilage can originate from a small group of farms, or even a single farm. Once this poorer quality milk has been mixed with milk collected from other farms, it is impossible to identify the farm(s) contributing to the problem, unless individual sampling has been conducted at each farm. The specific sources of contaminating organisms in milk can be diverse. The on-farm contamination sources include teats and udders of the cow, particularly for *Pseudomonas* spp. (Desmasures *et al.*, 1997b). Improperly cleaned milking equipment has also been shown to be a significant source of psychrotrophs in farm milk (Thomas *et al.*, 1971). Additional points along the pre-processing line may contribute too (Roberts, 1979), as a result of biofilm development. It can be useful to track spoilage organisms through the pre-processing chain to determine which locations need to be addressed with regard to hygiene, because this information can be used to reduce the risk of isolates with greater spoilage potential being present which leads to improved raw milk quality and consequently improved UHT milk quality.

Traditional identification methods are based on phenotype, such as biochemical and microbiological characteristics. However, identification methods based on phenotypic characteristics have limitations, including lack of reproducibility and discriminatory power as well as being ineffective at providing a link between results obtained from different samples (Dogan & Boor, 2003). Hunter and Gaston (1988) state that “discrimination, reproducibility and typability” (genetic relatedness) are the most important requirements to consider when assessing typing methods. While genotypic typing is not necessary to provide identification below subspecies level (Tenover *et al.*, 1995), only genotypic methods can best satisfy these three requirements. Molecular typing methods have emerged as important techniques in determining the genetic relatedness of bacteria, especially in epidemiology studies for tracking sources of pathogenic and spoilage bacteria (Wiedmann *et al.*, 2000). Pulsed field gel electrophoresis (PFGE), developed by Schwartz and Cantor (1984), was the only one of 13 typing methods described by Maslow and Mulligan (1996), which

was ranked in the top tier for all the three important criteria stated by Hunter and Gaston (1988). In addition, it is regarded as the "gold standard" for molecular typing of *Pseudomonas* spp. (Maslow & Mulligan, 1996), although such reports are usually based on epidemiological studies of pathogenic species, typically *P. aeruginosa*. Although PFGE is rather expensive compared with some other molecular typing methods, such as ribotyping (Wiedmann *et al*., 2000), RAPD and PCR, and it takes a lengthy period for the entire analysis to be completed, it is considered the best technique for bacterial genotypic characterisation (Olive & Bean, 1999).

Molecular identification methods have value in the typing of spoilage bacteria to identify sources of contamination of the product (van der Vossen & Hofstra, 1996). Such an approach has been used for typing of pseudomonads contaminating milk by Dogan and Boor (2003). They used a molecular typing technique (ribotyping), identified types of *Pseudomonas* spp. in various areas within the dairy environment (raw milk, factory environment and pasteurised milk) and assessed their genetic diversity and lipolytic and proteolytic potential. Jayarao & Wang (1999) investigated the diversity of *P. fluorescens* in farm bulk tank milk using phenotypic typing methods. Earlier, Ralyea *et al.* (1998) used ribotyping to track *P. fluorescens* in a dairy production system. These investigations demonstrated the suitability of molecular typing for *Pseudomonas* spp. within the dairy environment (bulk raw milk, pasteurised milk and various locations on the farm and in the factory) because the source of contamination was identified and the technique demonstrated a high discrimination index.

The aim of the present study was to identify sources of lipase and/or protease producing psychrotrophic *Pseudomonas* spp. at various pre-processing locations using pulsed field gel electrophoresis (PFGE), and to track the types identified through the pre-processing environment. Incubation of raw milk was also carried out to simulate possible scenarios where the raw milk may be stored on the farm and in the silo prior to UHT processing. This enabled enrichment for spoilage bacteria and studies to identify sources of microorganisms that may contribute to lipolysis and proteolysis in raw and subsequently UHT milk or other long life dairy products. The impact of various

storage conditions on the different Pulsed Field (PF) types of importance, with regard to lipase and protease production was also assessed.

6.2 Materials and Methods

6.2.1 Source of chemicals and microbiological media

Unless otherwise stated, all chemicals were of the highest grade available and were purchased from Sigma-Aldrich Co. (Sydney, NSW). All microbiological media were purchased from Oxoid Australia Pty. Ltd. (Adelaide, SA).

6.2.2 Source of raw milk

Five raw milk samples, each of approximately 500mL in total, were collected. Three were from farms in the area around Cardinia and Bayles, just beyond the south eastern suburbs of Melbourne. Milk was collected daily from two of these farms (Farms 2 and 3), while from the other (Farm 1), milk was collected every second day. Farm samples were obtained by sampling directly from the bulk tank, either through the roof access hatch or through the door near the bottom of the tank. Another sample was taken through the top access hatch in the milk tanker used to transport the raw milk from these farms to the milk processor. The tanker had been used previously to collect milk from other farms and had not been washed before collection of milk from Farms 1, 2 and 3. The final sample was from the silo at the milk processing site. This silo contained raw milk from this single delivery of milk only, and was cleaned prior to filling with this milk.

6.2.3 Incubation to achieve spoilage levels

A volume of 20mL of milk was incubated statically in a McCartney bottle to achieve spoilage levels under various conditions. All farm milk was incubated at 4 ºC for 7 d (daily enumeration), 10 ºC for 4 d (every second day enumeration) or at 4 ºC for 2 d followed by 10 ºC for 2 d (every second day enumeration). Silo milk was incubated at 4 ºC for 4 d (daily enumeration) or 4

ºC for 2 d and then 10 ºC for 2 d (every second day enumeration). Milk collected from the tanker was not incubated.

6.2.4 Enumeration of aerobic mesophiles and psychrotrophic *Pseudomonas* spp.

Two dilution series' were prepared for each raw milk sample in 0.1% Bacteriological Peptone. The dilutions were enumerated using the spread plate technique, based on AS 1766.1.4 (Standards Australia, 1991). Plate Count Agar, incubated at 30 °C for 72 h, was used to determine total plate count, while *Pseudomonas* Agar with C-F-C supplement was used for isolation of psychrotrophic *Pseudomonas* spp. Incubation for isolation of psychrotrophs was at 7 °C for 10 d (Juffs, 1972). Plates with between 20-200 colonies were counted, unless the range of dilutions spread did not yield a colony count within this range. Where this occurred the counts are reported as an approximation.

6.2.5 Isolation and presumptive identification of psychrotrophic *Pseudomonas* spp.

Pseudomonas spp. were isolated on *Pseudomonas* Agar Base plus C-F-C supplement. Isolates were taken from all farm, tanker and silo unincubated milk samples and from samples of incubated milk from each farm and the silo when the total plate count had reached 10^6 cfu/mL. The relative proportions of each morphologically distinct colony type on the counted plates were recorded and one of each type selected for further investigation. Initially, this involved purification of the culture on non-selective media by subculturing into 10 mL of Nutrient Broth, incubating at 30 ºC for 24 h before streaking for single colonies onto Nutrient Agar and incubating at 30 ºC for 24 h. After this, Gram staining was carried out as was a test for the presence of oxidase. Oxidase testing was performed by transferring two or three colonies with a toothpick onto No.1 filter paper (Whatman PLC; Brentford, England) impregnated with 1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride. Colonies were recorded as oxidase positive if they developed purple colour within 15 sec.. Pure isolates

which were oxidase positive Gram-negative rods were considered to be psychrotrophic *Pseudomonas* spp.

6.2.6 Culture maintenance

Each pure culture was subcultured bimonthly on Nutrient Agar slopes and stored at 4 °C. Beads (Microbank PL.160) (ProLab Diagnostics; Richmond Hill, ON, Canada) were used for long term storage in an ultracold freezer at approximately -80 °C. Cultures on beads were recovered through inoculation of Nutrient Broth and then incubation at 30 °C for 24 h. They were then transferred to Nutrient Agar slopes.

6.2.7 Screening of bacterial isolates for lipase and protease production

An agar diffusion method based on Christen and Marshall (1984) and Craven (1993) was used to screen the isolates for lipase and/or protease activity. Nutrient Agar plates containing either 0.1% triolein (for lipase) or 1% low-fat milk (for protease) were used. The agar was poured in equal layers of 10 mL each. Portions of the top layer of the agar were removed using a 6 mm sterile cork borer. A 10 µL volume of a Nutrient Broth culture incubated at 25 ºC for 24 h (containing approximately 10^8 cfu/mL) was added to each well. The plates were incubated for 168 h at 4 ºC with observations of zones of clearing around the wells recorded after 93 h, as well as at the end of the incubation period. Measurements were taken to the edge of the zone from the edge of the well. The largest zone size at 168 h for each test (17 mm for lipase production and 28 mm for protease production) was divided by three. This decided the designations for weak, moderate and strong producers. Isolates tested for lipase production were recorded as weak if the size of the zone, measured in the manner described above, was between one and five millimetres, moderate if the zone was between six and 11 millimetres and strong if the zone was between 12 and 17 millimetres. Isolates tested for protease production were recorded as weak if the zone was between one and nine millimetres, moderate if the zone was between 10 and 19 millimetres and strong if the zone was between 20 and 28 millimetres.

6.2.8 Extraction of genomic DNA and restriction endonuclease digestion

Cultures were inoculated into 10 mL of Tryptic Soy Broth and incubated at 25 ºC for 35 h. A 1.5 mL volume was centrifuged for 2 min at 10 000 *g.* The supernatant was discarded and the pellet resuspended in 1 mL of SE buffer (75 mM NaCl, 25 mM EDTA - pH 7.4) before identical centrifugation. Again, the supernatant was discarded and the pellet resuspended in 500 µL SE buffer. An equal volume of 2% (w/w) Sea Plaque agarose (BioWhittaker Molecular Applications; Rockland, ME, United States) was prepared in SE buffer and mixed with the cell suspension in SE buffer. Two plugs were immediately prepared in a gel mould, using 200 µL (100 µL each) of the agarose cell suspension. The plugs were allowed to set and up to 15 pieces between approximately 500-750 µM wide were sliced with a sterile size 22 razor blade (Keisei Medical Industrial Co. Ltd.; Tokyo, Japan). All slices were immersed in 1 mL of lysis solution (500 mM EDTA at pH 9.5, 500 µg/mL proteinase K and 34 mM N-lauroylsarcosine) and incubated at 55 ºC for 16 h. Following incubation, the slices were rinsed with 1 mL of SE buffer and then incubated for 15 min in SE buffer containing 1 mM phenylmethanesulphonyl fluoride (PMSF). Two further 15 min incubations were carried out with fresh 1 mM PMSF in SE buffer. After the last washing step, the 1 mM PMSF in SE buffer was discarded and replaced with 1 mL TE buffer (10 mM Tris base, 10 mM EDTA - pH 7.4). Slices were stored up to one week at 4 ºC in TE buffer, prior to use. Restriction endonuclease digestion, using one slice, was carried out with *Swa*I (New England Biolabs; Beverly, MA, United States) according to the manufacturer's instructions.

Two isolates were used as controls for reference purposes. These were *Pseudomonas fluorescens* ATCC948 and *Pseudomonas fluorescens* SBW25 (obtained from the Department of Plant Sciences, University of Oxford). The genome of one of these, isolate SBW25, has been sequenced recently, and therefore use of this isolate would potentially allow for more precise determination of the number of fragments, expected based on the recognition sequence of the restriction endonuclease used.

6.2.9 Pulsed field gel electrophoresis

One percent Pulsed Field Certified Agarose (Bio-Rad Laboratories; Sydney, NSW) was prepared in 0.5X TBE buffer (composition per litre - 5.4 g Tris base, 275 mg boric acid and 40 mL 25 mM EDTA) (Peacock & Dingman, 1967). The equipment used was the Bio-Rad CHEF-DR® II PFGE system (Bio-Rad Laboratories; Sydney, NSW). Temperature of the run buffer (0.5X TBE) was maintained at 14 ºC. The initial switch time was 1.79 sec and was ramped linearly to a final switch time of 1 min 33.69 sec. Gradient was at 6 V/cm and the inclined angle was 120 º. Total run time was 26 h 56 min.

6.2.10 Band visualisation and data analysis

The gel was stained in 1% ethidium bromide for 30 min followed by a brief rinse (30-60 sec) in distilled water. A model TM-36 Chromato-Vue UV transilluminator (Ultra-violet Products; San Gabriel, CA, United States) was used to visualise the ethidium bromide-strained bands. Photographs were then taken with a model DC290 camera (Kodak [Australasia] Pty. Ltd.; Melbourne, VIC) operated through Kodak 1D Image Analysis Software (Eastman Kodak Company; New Haven, CT, United States), and saved as TIF images. The "Ethidium Bromide" option was selected from the "Sample type" with exposure of 4.5 sec and bracket of 1.125 sec. Analysis of the gel image was with the GelCompar II (Applied Maths BVBA; Sint-Martens-Latem, Belgium) software program. Dendrograms were constructed using Jeffrey's X and unweighted pair-grouping. Band matching was carried out with 1.7% position tolerance and 0% optimisation. Isolates with a similarity of at least 80% were grouped into the same PF Type.

6.3 Results

6.3.1 Colony counts on fresh raw milk

The total counts across the five raw milk sampling sites (three farms, their milk collection tanker and the factory silo) ranged between 7.0×10^2 cfu/mL (Farm 2) and 9.0×10^3 cfu/mL (Farm 3) with a mean of 4.2×10^3 cfu/mL (Table 6.1). This mean value is close to counts obtained from the silo and the tanker.

The psychrotrophic *Pseudomonas* spp. counts were all lower than the total counts and were lowest in milk from Farms 1 (on every second day collection) and 2 (on daily collection), which had similar counts. The psychrotrophic *Pseudomonas* spp. count was similar in the samples from Farm 3 (on daily collection), the tanker and the silo, which were approximately one log higher than Farms 1 and 2. A large variation was seen in the proportion of psychrotrophic *Pseudomonas* spp. compared with the total plate count. On Farm 1, these organisms comprised approximately 4% of the flora while in the silo, approximately 50% of the microbes encountered were psychrotrophic *Pseudomonas* spp.

Table 6.1: Bacterial counts of raw milk on day of collection.

Source	**Number of bacteria (cfu/mL)**	
	Total count	**Psychrotrophic *Pseudomonas* count**
Farm 1	2.5×10^3	~ 1.0×10^2 (~ 4.0)
Farm 2	7.0×10^2	< 1.0×10^2 (~ 14.3)
Farm 3	9.0×10^3	~ 1.7×10^3 (~ 18.9)
Tanker	5.0×10^3	~ 2.0×10^3 (~ 40.0)
Silo	4.0×10^3	~ 2.0×10^3 (~ 50.0)

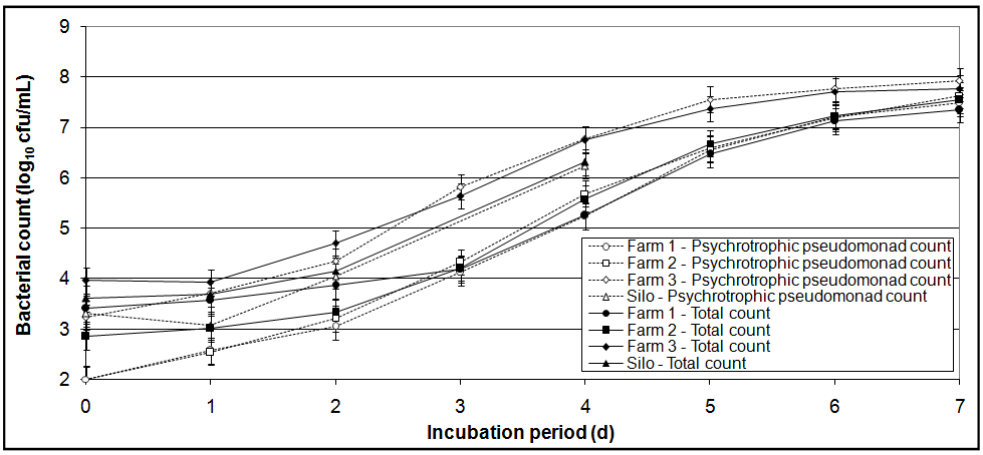
Numbers in brackets indicate proportion of the total count as a percentage.

6.3.2 Growth of raw milk microflora during storage

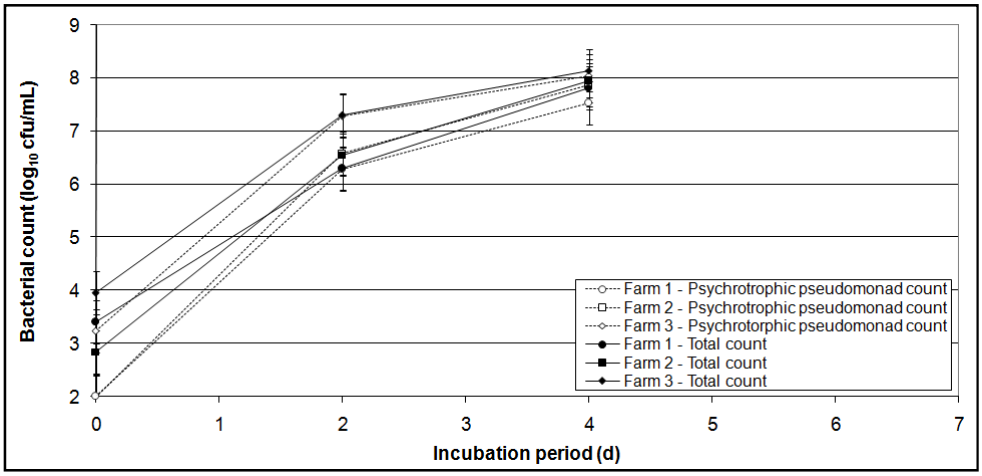
At the commencement of incubation, the counts of psychrotrophs in the raw milk were substantially lower than the total counts. However, after incubation for two to three days, the psychrotrophs were the predominant microflora present at all storage conditions.

When the milk was incubated at 4 ºC (Figure 6.1a), the bacterial count reached 10^6 cfu/mL (generally regarded as the level where production of lipases and proteases is detectable) in three days for milk from Farm 3, four days for the silo milk and between four and five days for milk from Farms 1 and 2. Following incubation at 10 ºC, all samples contained more than 10^6 cfu/mL after two days, with the milk from Farm 3 having the highest counts of bacteria at this time (Figure 6.1b). The other farm samples contained similar counts of bacteria. The fluctuating temperature of 4 ºC for two days

a)



b)



c)

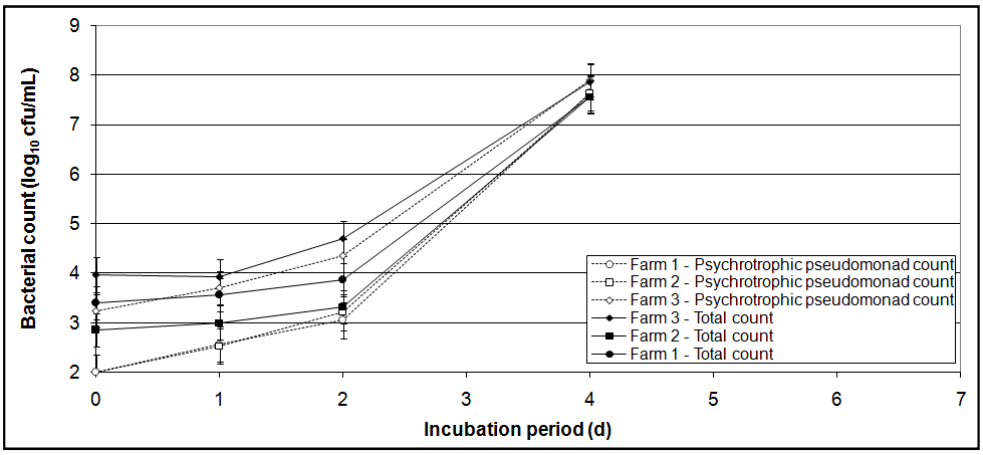


Figure 6.1: Total and psychrotrophic *Pseudomonas* spp. count of raw milk during incubation at 4 ºC a), 10 ºC b) and 4 ºC (0-2 d) followed by 10 ºC (2-4 d) c).

followed by 10 ºC for two days led to the highest counts of bacteria in milk from Farm 3, followed by Farms 1 and 2, after two days incubation (Figure 6.1c). The numbers of bacteria present exceeded 10^7 cfu/mL in all samples after 4 days incubation.

6.3.3 Identification of pulsed field Types and their sources

A total of 45 isolates were collected from milk from three farms, their farm milk collection tanker and the silo at the factory as described in section 4.2.5. There was much diversity in the psychrotrophic pseudomonad flora, with 39 pulsed field (PF) Types among the 45 isolates (Figure 6.2). Of the samples examined, there was most diversity in the Farm 3 milk, with 16 PF Types identified from 16 isolates across all incubation conditions. Similarly, all eleven Farm 2 PF Types were from eleven isolates and all eight silo PF Types were from eight isolates. There was slightly less diversity among the Farm 1 isolates, with eight PF Types from nine isolates. The two reference isolates, *P. fluorescens* ATCC948 and SBW25, were quite different from most of the isolates obtained from raw milk in this study.

6.3.3.1 Unincubated raw milk

There were eight isolates from fresh, unincubated raw milk, all of which were from different PF Types. There was one isolate from Farm 1 (PF Type 24), two isolates from Farm 2 (PF Types 8 and 31), two isolates from Farm 3 (PF Types 23 and 33), one isolate from the tanker (PF Type 36) and two isolates from the silo (PF Types 25 and 34).

6.3.3.2 Raw milk incubated at 4 ºC

Upon incubation at 4 ºC, there was little change in the number of PF Types with milk from Farms 1 and 2 as there were two PF Types (27, 28) identified from Farm 1 and three PF Types (7, 11, 16) identified from Farm 2. The situation was quite different for Farm 3 and the silo with six PF Types present in milk from each source (Farm 3-3, 5, 9, 31, 38, 39 and the silo-1, 3,

14, 31, 35, 37). PF Type 31 was the only PF Type present in unincubated milk, that was also present in the 4 ºC incubated milk. It was present prior to

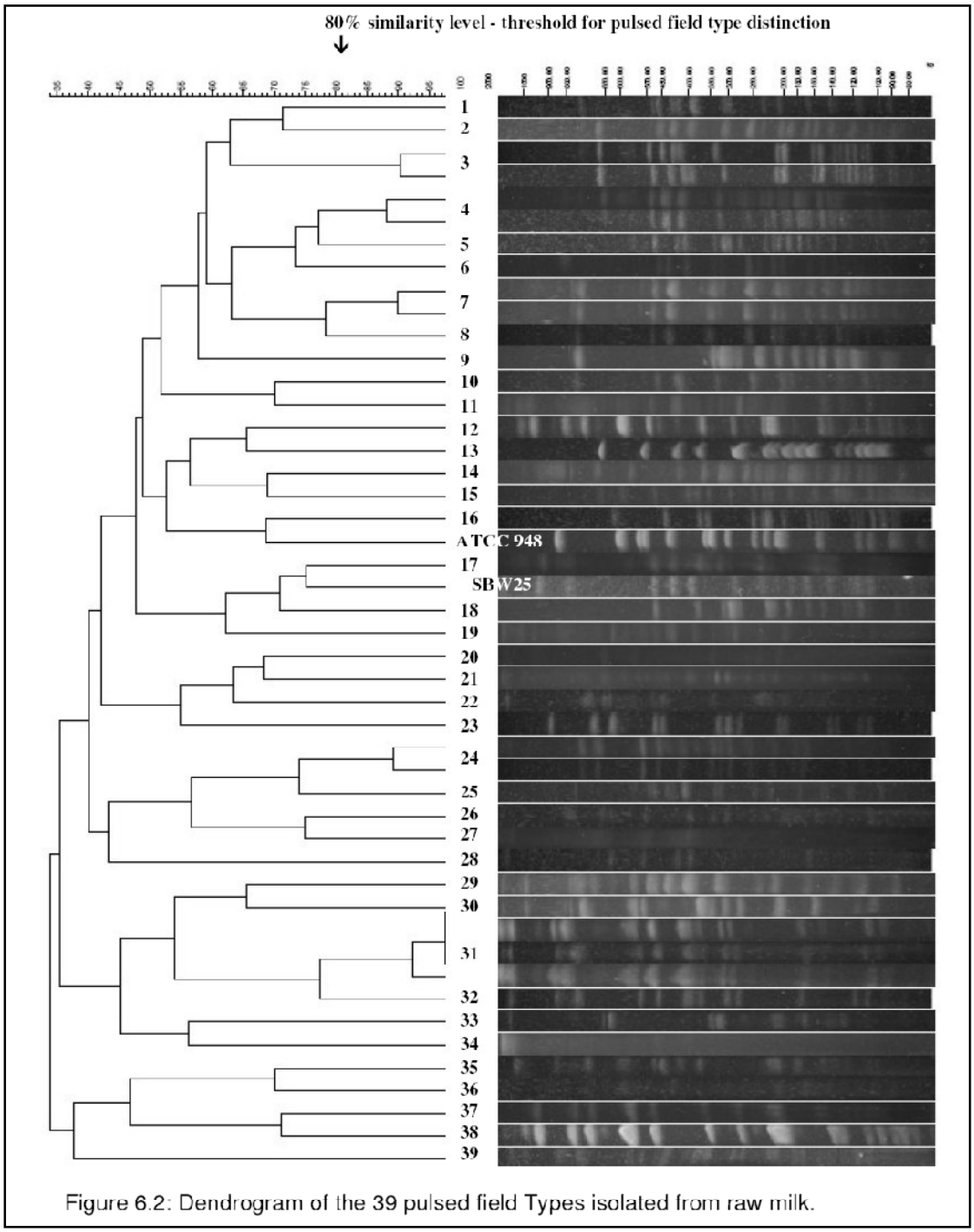
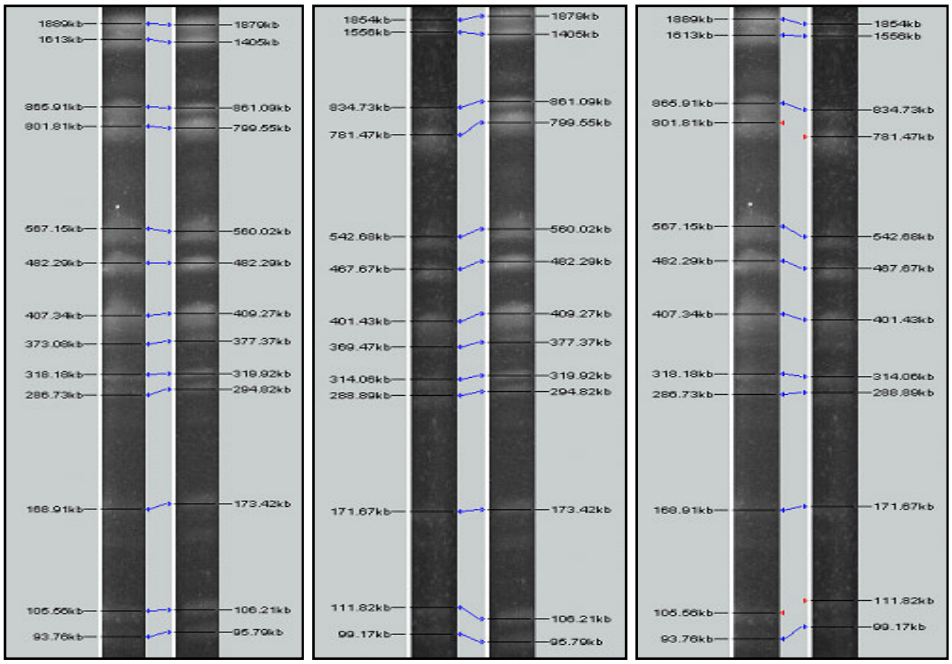


Figure 6.2: Dendrogram of the 39 pulsed field Types isolated from raw milk.

incubation in Farm 2 milk and then after 4 ºC incubation, it was found in Farm 3 and silo milk. Of these three isolates that comprised PF Type 31, there was a 100% similarity (based on the criteria used for band matching) between isolates 18311 (silo) and 74425 (Farm 3) and between isolates 251 (Farm 2) and 18311 (silo). The *Swa*I DNA restriction patterns for the three isolates within PF type 31 and displayed in Figure 6.3 a, b and c. There was 83% similarity between isolates 251 (Farm 2) and 74425 (Farm 3). PF Type 3 was not present in unincubated milk, but was observed in milk from Farm 3 and the silo after 4 ºC.



a) b) c)

Figure 6.3: *Swa*I DNA restriction patterns of isolates from PF type 31. a) Isolates 74425 (Farm 3) (L) and 18311 (Silo) (R). b) PF type 31 - Isolates 251 (Farm 2) (L) and 18311 (Silo) (R). c) PF type 31 - Isolates 74425 (Farm 3) (L) and 251 (Farm 2) (R).

6.3.3.3 Raw milk incubated at 10 ºC

There was little difference in diversity of PF Types between milk incubated at 10 ºC, compared to 4 ºC. Both Farm 1 (PF Types 24 and 26) and Farm 2 (PF Types 10 and 21) milk contained two PF Types each while four PF Types (12, 15, 20, 32) were found in Farm 3 milk incubated at this temperature. All PF Types were unique among all of the 10 ºC samples, that is, they were not

found in other samples. One PF Type, 24, isolated from unincubated Farm 1 milk, was also isolated from 10 ºC incubated milk from that same farm, with 89% similarity between the isolates.

6.3.3.4 Raw milk incubated at 4 ºC followed by 10 ºC

PF Types obtained from farm milk incubated at 4 ºC for 48 h followed by incubation at 10 ºC for 48 h, were very similar across all farms, with four PF Types from each farm milk. PF Types 4 (isolated twice), 13 and 19 were from Farm 1; PF Types 6, 7, 17 and 18 were from Farm 2 while PF Types 2, 22, 29 and 30 were from Farm 3. Most were unique, except that two isolates, with 88% similarity and belonging to the same PF Type (4) were found in Farm 1 milk. None of the PF Types present after the 4 ºC then 10 ºC incubation were present in the unincubated milk, but PF Type 7 was also isolated from milk from Farm 2 after incubation at 4 ºC with 92% similarity.

6.3.4 Sources of moderately and strongly lipolytic and proteolytic *Pseudomonas* Types

Table 6.2 presents a summary of the sources of *Pseudomonas* PF Types identified from isolates that were moderately and strongly lipolytic and proteolytic. The isolates that were not lipolytic/proteolytic or that demonstrated weak lipolysis/proteolysis were not considered in these results because these isolates are potentially of little practical significance in the spoilage of UHT milk.

6.3.4.1 Unincubated raw milk

Six of the eight PF Types from the unincubated raw milk were moderate or strong lipase and/or protease producers. Four were isolated from Farm 3 (2) and silo (2) milk and the other two were from Farm 2 (1) and the tanker (1). Four of the six PF Types in this group had both lipase and protease activity and two had protease activity only.

6.3.4.2 Raw milk incubated at 4 ºC

Of the 17 PF Types isolated from the milk after incubation at 4 ºC, 12 were moderate or strong lipase and/or protease producers. Again, these originated mostly from Farm 3 (6) and the silo (6). All PF Types from Farm 3 were proteolytic and three of these were also lipase producers. Five PF Types from the silo were proteolytic and four were lipolytic. Three were both lipolytic and proteolytic.

Table 6.2: Origin of *Pseudomonas* spp. that were moderately and strongly lipolytic and proteolytic.

Sample	Milk incubation temperature (ºC)	PF[1] Type designations		Total number of PF Types[2]
		Lipase	Protease	
Farm 1	Not incubated			1
	4	27	27	2
	10	26	26	2
	4/10		13	3
Farm 2	Not incubated		8	2
	4		7, 11	3
	10	21	10, 21	2
	4/10		6, 7, 17, 18	4
Farm 3	Not incubated	23, 33	23, 33	2
	4	9, 31, 39	3, 5, 9, 31, 38, 39	6
	10	12, 32	12, 15, 32	4
	4/10	30	2, 29, 30	4
Tanker	Not incubated	36	36	1
Silo	Not incubated	34	25, 34	6
	4	3, 31, 35, 37	1, 3, 14, 31, 35	1

[1]Pulsed field gel electrophoresis
[2] Total number of PF Types irrespective of whether they showed lipolytic and/or proteolytic activity.

6.3.4.3 Raw milk incubated at 10 ºC

PF Types with moderate or strong lipase and protease activity were isolated from milk incubated at 10 ºC from all three farms (one, two and three PF Types from Farms 1, 2 and 3 respectively). Four of the six PF Types were both lipolytic and proteolytic and two were proteolytic only. A total of eight PF Types were isolated from milk incubated at 10 ºC.

6.3.4.4 Raw milk incubated at 4 ºC followed by 10 ºC

Most of the PF Types isolated from milk incubated at 4 ºC followed by 10 ºC were not lipolytic (7 of 8 PF Types). The PF Types that were moderately and strongly proteolytic originated from Farm 1 (1), Farm 2 (4) and Farm 3 (3). Farm 3 had the only PF Type with both lipase and protease activity.

6.4 Discussion

6.4.1 Microbial composition of fresh raw milk

The raw milk obtained during this investigation was of high quality, with total counts ranging from 7.0×10^2 cfu/mL to 9.0×10^3 cfu/mL, depending on sampling location. From a survey of the literature by Thomas *et al.* (1971), most raw milk freshly drawn from healthy cows contains total microflora in the range from 5.0×10^2 to 5.0×10^3 cfu/mL. A later study by Senyk *et al.* (1982) reported that the total count of 86% of bulk tank milk samples was in the range 1.0×10^3 to 5.0×10^4 cfu/mL, while 92% of the psychrotrophic counts were less than 1.0×10^4 cfu/mL. At less than 2.0×10^3 cfu/mL, all milk samples in the current study were within this range. The tanker and silo total counts were also low. Some previous reports indicated that milk sampled from silos or from tankers contains higher total counts (Fryer & Halligan, 1974; Mahari & Gashe, 1990) due to contamination from the tanker or pumping and related equipment (Thomas, 1974). However, this was not observed in the present study.

In freshly drawn, good quality raw milk, psychrotrophic *Pseudomonas* spp. are generally present in low numbers, and are far from being the dominant microorganisms. With increasing refrigerated storage of raw milk, psychrotrophic organisms increase in proportion to dominate the flora (Cousins *et al.*, 1977). In the current investigation, between 4% and 19% of the total count were psychrotrophic *Pseudomonas* spp., in the farm samples. Similar results have mostly been reported in the literature. However, some uncharacteristic results, by Twomey and Crawley (1968) and Chye *et al.* (2004), have also been observed. In those studies, psychrotrophic bacteria comprised less than 0.1% of the total count. The result of Chye *et al.* (2004) may reflect higher ambient temperatures in the milk collection areas. More typical values

are quoted by Desmasures and Gueguen (1997), who sampled monthly from the bulk tank on four farms over two years. The mean observation was that *Pseudomonas* spp. accounted for between four and 23% of the total count, with two of the four farms averaging under 5% pseudomonads. Similar low values were observed by Jaspe *et al*. (1995), with pseudomonads comprising 5% of the total count, and psychrotrophs 6%. In a smaller study by Desmasures *et al.* (1997a), there was a much higher incidence of *Pseudomonas* spp., with these organisms comprising a higher proportion of the total count in winter (28%), compared to the warmer period of the year (21%).

In the present investigation, larger proportions of psychrotrophic pseudomonads were recovered from the tanker (40%) and silo (50%) samples than from the farm samples. Pseudomonads are among the organisms which commonly form biofilms on food contact surfaces (Salo *et al*., 2006) including stainless steel (Hood & Zottola, 1997). Therefore, milk contact surfaces on the farm, such as the bulk tank, may be expected to develop biofilms. In fact, on the farm, biofilms have been known to form on milking equipment (Teixeira *et al*., 2005). Furthermore, mixed cultures of species (as is present in raw milk) have been found to stimulate each other's capability to form biofilms (Kives *et al*., 2005) and to resist sanitisers (Lindsay *et al*., 2002). In the food processing environment, biofilms are of serious concern (Mosteller & Bishop, 1993) and with biofilms difficult to remove (Kumar & Anand, 1998), this level of concern may be justified throughout the pre-processing environment where suitable surfaces are available, including the tankers and silos, which are also made of stainless steel.

6.4.2 Change in cell count of raw milk after storage simulation and the possible effects on manufactured dairy products

During the storage simulation experiments, the proportion of psychrotrophs rose with increasing cold storage, as would be expected. This is because lower temperatures favour their growth. Similar results, albeit over a shorter simulated storage period, were reported by Fryer and Halligan (1974). Senyk *et al.* (1988), who investigated change in the microflora after storage at

temperatures between 1.7 and 10.0 ºC, found that psychrotrophs comprised a substantial portion (>70%) of the raw milk only when the incubation temperature was 7.2 or 10.0 ºC. After 48 h incubation at 4.4 ºC, the psychrotroph proportion was 22%, similar to the level (26%) after 24 h incubation. The lack of prominent lag phase, observed in the present study, has also been reported (Griffiths *et al*., 1987). In that study, psychrotrophs were observed to comprise 41% of the total microflora at the commencement of the incubation period; however, after 18 h at 5 ºC, they had increased their proportion to 54% while after storage at 10 ºC, they made up 84%.

Until the second day of storage at 4 ºC, the psychrotrophic *Pseudomonas* spp. count was considerably lower than the total count, suggesting that the total count was dominated by psychrotrophic *Pseudomonas* spp., from that point. This is not surprising because pseudomonads have been shown to outdo other psychrotrophic bacteria at refrigeration temperatures due to the shorter generation times (Jooste & Fischer, 1992). Within the first two days, the population of mesophilic aerobic bacteria would not grow, but may remain viable. This is reflected in there being no increase in the total count during this period. However, the psychrotrophic *Pseudomonas* spp. count increased, from the commencement of storage in most instances, and it took approximately two days until they outnumbered the other flora.

When incubated at 10 ºC, a substantial change in the time frame of the growth curve is immediately recognisable. Similar to 4 ºC, the psychrotrophic *Pseudomonas* spp. dominated the raw milk stored at this temperature, but reached levels of 10^6 cfu/mL sooner, in approximately half the time. This is consistent with the growth pattern of psychrotrophic bacteria, which have shorter generation times as temperature increases during refrigeration (Greene & Jezeski, 1954), with the generation times of mesophilic and psychrotrophic bacteria being approximately equal only above 15 ºC (Bester *et al*., 1986). Psychrotrophic and mesophilic bacteria have an optimum growth temperature within the ambient range but only psychrotrophs are capable of growth at normal refrigeration temperatures (Adams & Moss, 1995). Therefore, with an increase in temperature, both psychrotrophic and mesophilic bacteria will

increase in growth rate. The observation that both the total and psychrotrophic *Pseudomonas* spp. counts were nearly identical would suggest that psychrotrophic bacteria dominate the flora, particularly during the second half of the incubation.

The storage simulations in this study have demonstrated that 10^6cfu/mL spoilage threshold can be attained by psychrotrophic *Pseudomonas* spp. in three to five days at 4 ºC or in under two days at 10 ºC. Previous work has indicated that the initial cell count (Dommett & Baseby, 1986; Guinot-Thomas *et al.*, 1995b) and/or storage temperature (Griffiths *et al.*, 1987) are contributing factors to the time required to reach spoilage levels, and this was observed in the present investigation. The contribution of low quality (high microbial content) raw milk to the quality of the heat-processed product has been recognised (Griffiths *et al.*, 1988). As an example of the effect of high counts, the difference in psychrotrophic *Pseudomonas* spp. counts in fresh raw milk of about 1.5 log cfu/mL between Farm 1 and Farm 3 is sufficient for there to be a day difference in reaching 10^6 cfu/mL at 4 ºC. Higher temperatures (for example 10 ºC versus 4 ºC) had a similar effect in shortening the time to reach the reported 10^6 cfu/mL spoilage threshold. As every second day collection of milk from farms is not uncommon (Oz & Farnsworth, 1985) and with raw milk storage at the factory prior to processing generally 24 h or longer (Celestino *et al.*, 1996), storage of raw milk for three days prior to processing is common (Guinot-Thomas *et al.*, 1995b). As a result, the potential of the psychrotrophic *Pseudomonas* spp. count in raw milk to attain the 10^6 cfu/mL spoilage threshold is clearly evident.

A change in the composition of the raw milk microflora following growth has been widely reported. Due to competition and adaptation to the prevailing conditions, some populations do not persist at their original proportions and some may disappear altogether (Lafarge *et al.*, 2004).

6.4.3 Pulsed Field Types in raw milk: Variation and potential impact on manufactured dairy products

It was clear from the PFGE Typing results that there was much diversity among the psychrotrophic *Pseudomonas* spp. flora owing to both the sample location and the incubation conditions applied to the milk, as the 45 isolates from incubated milk could be assigned into 39 PF Types. A high degree of genetic diversity has been reported in pseudomonads, based on the results of two molecular typing methods. These were (I) ribotyping, used by Dogan and Boor (2003) in a study of isolates from milk (raw from farms and pasteurised) as well as from the farm and factory environment, and (II) the random amplified polymorphic DNA (RAPD) technique, used by Martins *et al.* (2006) to characterise isolates from raw milk, from unspecified location(s).

Overall, in the present study, a greater proportion of PF Types which were moderately or strongly lipolytic or proteolytic were obtained after incubation of the milk. This demonstrates the significance of cold storage in selecting for the development of spoilage bacteria. This was particularly evident for the milk from Farm 3 and the silo, where the initial level of psychrotrophs was relatively high. Storage at 4 ºC resulted in a greater proportion of bacteria with higher lipolytic and proteolytic potential than the higher incubation temperatures.

Overall, less PF Types demonstrated lipase production compared to protease production. This may suggest lipolytic spoilage was potentially of lower importance. An interesting observation is that strong lipase producers were also strong protease producers but strong protease producers were not always strong lipase producers. Also, without exception, PF Types devoid of proteolytic activity also lacked lipolytic activity. Therefore, there is a strong association between strong production of both lipase and protease or between absence of lipase and protease production. In general, the moderate and strong protease producing PF Types predominated, thereby increasing the likelihood of proteolytic spoilage in manufactured dairy products.

Percentages of *Pseudomonas* spp. isolates from raw milk reported to produce lipase and/or protease are variable, from the reports in the literature.

Wang and Jayaro (2001) also observed a higher proportion of protease producing isolates, compared to lipase producing isolates, in their samples of farm bulk tank milk in South Dakota and Minnesota, in the U.S. Conversely, in studies reported by Muir *et al.* (1979), Muir and Banks (2000) as well as Muir and Banks (2003), lipase production was much more common, particularly among non-fluorescent *Pseudomonas* spp. isolates. This is in contrast to the findings of Dogan and Boor (2003), who found lipase and protease production fairly equally distributed among raw milk *Pseudomonas* spp. isolates from dairy processing plants in New York state. Clearly, microflora can differ at various locations, which necessitates specific tracking studies to investigate and rectify quality problems such as lipase and protease contamination of milk.

6.4.4 Importance of raw milk microflora from farm 3 and the silo

Farm 3 appeared to be an important farm, with regard to contamination of raw milk with psychrotrophs. The highest psychrotrophic count was observed in samples from this farm compared with the others, despite the fact that milk was collected daily from this farm. Furthermore, some of the PF Types from this farm appeared to have been transferred to the silo. This is consistent with Farm 3 milk containing the highest psychrotrophic count, and therefore would contribute more psychrotrophs to the silo milk than the other two farms. However, most of the PF Types from the silo were unique to this source indicating this to be a significant source of contamination in addition to Farm 3. Although not proven in the present study, it could be expected that the tanker might also contribute new PF Types of psychrotrophs to the silo milk. This is because it is now accepted that biofilms formed by bacterial colonies do persist on surfaces of milk storage or processing equipment. These could have come from other farms as the tanker was not washed from a previous milk collection run. Only one isolate from the tanker milk was tested in the present study. Further samples or incubation of the milk may have provided more information on the significance of this source. However, storage of tanker milk is not an industry practice and was not included in the present investigation.

After the Farm 3 and silo milk was incubated at 4 ºC, a high proportion of strongly lipolytic and/or proteolytic PF Types were isolated. This demonstrates that if the Farm 3 or silo milk had been stored at this temperature in practice, the possibility of product contamination with heat-stable lipases and proteases from these sources would be high. It also reinforces the need to thoroughly clean refrigerated milk storage equipment to prevent the proliferation of these bacteria on surfaces which may contaminate subsequent batches of milk.

6.4.5 Selection of restriction endonuclease

Choice of restriction endonuclease is very important (McClelland *et al.*, 1987) because PFGE is a technique that requires a small number of DNA fragments to allow accurate interpretation. The widely adopted interpretation criteria of Tenover *et al*. (1995) (which are based on the number of band differences and how these band differences relate to similarity between isolates) cannot be applied easily if there are too many or too few fragments generated. It will be difficult to identify individual bands if the number of fragments are too numerous, thereby leading to a false number or position of bands. Consequently, selection of a rare-cutting restriction endonuclease can alleviate this problem (Allardet-Servent *et al.*, 1989). If there are too few bands, identifying individual bands will not be of concern, but the application of the Tenover *et al.* (1995) criteria could be equally difficult. This is because those criteria are based on the number of band differences, with a seven band difference sufficient to demonstrate unrelatedness. If a given restriction endonuclease results in fewer than ten fragments, these criteria cannot be applied reliably (Tenover *et al.*, 1995). The ideal maximum number of bands for accurate analysis of DNA restriction patterns following PFGE is between 25 (Goering, 2004) and 60 (Romling, 2004). There are, however, mathematical models available for the determination of the optimal number of bands (Mendez-Alvarez *et al.*, 1997), but these models are difficult to apply because they are too complex and cumbersome for routine use. The number of band differences need to be viewed in context of the genetic diversity of the organism (Barrett *et al.*, 2006). Indistinguishable band patterns do not mean a great deal

when an organism is genetically homogeneous, but are of much importance when an organism is genetically diverse (Barrett *et al.*, 2006). When interpreting band patterns, consideration needs to be given to factors which can influence the separation and appearance of bands. For example, Barrett *et al.* (2006) explains how the presence of a plasmid, or multiple plasmids, can alter a restriction pattern enough to distinguish otherwise indistinguishable isolates as can deletions or insertions into the DNA which would result in a restriction pattern containing multiple bands of a similar size that cannot be resolved. Therefore, the criteria of Tenover *et al.* (1995) cannot be universally applied, and the information gathered from PFGE typing needs to be considered with all phenotypic and other information. Selection is made considerably easier with the complete genome sequences of many bacteria, and other microorganisms, known. Furthermore, on-line restriction digest simulators with a wide array of restriction endonucleases (Vincze *et al.*, 2003; Bikandi *et al.*, 2004) make the task of selection relatively straight-forward. The starting point for enzyme selection is the G+C content of the genome. For example, a genome with a high G+C content will be digested best with an enzyme with an A+T recognition sequence, since such bases are rarer in the genome. Moreover, particular sequences are rare in some genomes (such as CTAG or CCG/CGG in genomes with over 45% G+C content) (McClelland *et al.*, 1987) along with length of the recognition sequence - the longer the recognition sequence, the rarer the frequency of cutting (Romling *et al.*, 2004). This means that enzymes that recognise an eight base-pair sequence are going to cleave the DNA less frequently than an enzyme that recognises a six base-pair sequence. The G+C content of *P. fluorescens* is 63.3% (Paulsen *et al.*, 2005), therefore this species is considered G+C rich. Selection of a restriction endonuclease with an 8-bp recognition sequence of only (*Pac*I, *Swa*I) or mostly (*Pme*I) A or T residues would ensure infrequent cutting. This was confirmed with the on-line restriction endonuclease digestion simulators. A further point to consider is that additional restriction endonucleases can often be useful, in order to confirm the results or to identify a difference between isolates based on increasing discrimination. Such an approach would have been useful in the present study where there

was one PF Type isolated from different farms (PF Type 31). This result, although possible, would be quite unexpected, unless transfer of isolates between farms was likely. PF Type 31 could be traced to one of the two farms (Farm 3) based on its lipase and protease production (Table 6.2).

6.4.6 Pulsed field gel electrophoresis for molecular typing of *Pseudomonas* spp.

Many methods are available for molecular typing, the choice of which depends on a variety of factors. While PFGE is currently the best available method for typing of bacteria, it would not be the method of choice under all circumstances. As stated earlier, the three key criteria for a reliable molecular typing method are the typability, reproducibility and discriminatory power (Hunter & Gaston, 1988). In comparison with other molecular typing techniques, PFGE is often unsurpassed. PFGE, along with PCR, was stated as the best molecular typing technique for *Pseudomonas* spp. by Maslow and Mulligan (1996) who rated PFGE "excellent" for the three criteria above. In comparison, they rate PCR "excellent" for typability and reproducibility with unknown discriminatory power. Ribotyping, which has been used for molecular typing of dairy isolates of *Pseudomonas* spp (Ralyea *et al*., 1998; Wiedmann *et al*., 2000; Dogan & Boor, 2003) has "excellent" typability and reproducibility but only "good" discriminatory power. However, there are limitations to the PFGE technique. For example, some isolates cannot be typed due to DNA degradation during the electrophoresis run (Lukinmaa *et al*., 2004) and comparisons between gels are difficult (Gurtler & Mayall, 2001). These difficulties together with the associated "technical demands" of the procedure and the high cost of the equipment are disadvantages in the application of PFGE (Tenover *et al*., 1997). Technically, the long procedure is laborious (Cox & Fleet, 2003) and one of its most important disadvantages is the time, typically five days (Goering, 2004). Although set-up costs can be slightly higher than those of other molecular typing methods (Olive & Bean, 1999; Wiedmann *et al*., 2000), the cost per isolate compares favourably with PCR and RFLP (Olive &

Bean, 1999), but is considerably more expensive than ribotyping (Wiedmann *et al*., 2000).

6.5 Conclusions

- Much genetic diversity is evident among the psychrotrophic *Pseudomonas* spp. isolated from farm bulk tank milk and other sources within the pre-processing environment.
- Low temperature (4 °C) storage of raw milk promotes the growth of lipolytic and proteolytic isolates of psychrotrophic *Pseudomonas* spp..
- Isolates of psychrotrophic *Pseudomonas* spp. are transferred between locations within the pre-processing environment.
- Proteolytic spoilage is potentially more likely to occur than lipolytic spoilage in long-life dairy products produced from the raw milk collected in this study.
- PFGE can be used successfully for tracking of psychrotrophic *Pseudomonas* spp. originating in the dairy environment.
- In future studies of the genetic diversity of *Pseudomonas* spp. in raw milk, collecting multiple samples would give higher numbers of isolates, allowing a deeper insight into their genetic diversity, revealing potentially higher genetic diversity in these populations.

CHAPTER 7

SPECIFICITY OF PROTEASE FROM *PSEUDOMONAS FLUORESCENS* B52 TOWARDS α_s- AND β-CASEIN

7.1 Introduction

Classification of proteases is based on specificity for the particular type of bonds that are hydrolysed (Webb, 1992). For example, chymosin (E.C. 3.4.23.4), a neonatal gastric protease, is known to attack the Phe105-Met106 bond of κ-CN (Delfour *et al.*, 1965) while particular peptides are recognised as major products of chymosin proteolysis of other caseins, such as the α_{s1}-CN fragment 1-23 and the β-CN fragment 193-209 (Minkiewicz *et al.*, 2000). Conversely, elastase from bovine milk is relatively non-specific towards the caseins, with the resulting peptides similar to proteolytic action of other indigenous milk proteases (Considine *et al.*, 1999).

Plasmin specificity towards α_{s1}-CN has been reported by Aimutis and Eigel (1982). The resulting fragments, first reported by Eigel (1977), were originally referred to collectively as λ-CN (Long *et al.*, 1958). Snoeren and van Riel (1979) identified fragments of α_{s2}-CN that resulted from plasmin action. Plasmin demonstrates a high preference for bonds on either side of lysine residues and to a lesser extent, arginine residues (Le Bars & Gripon, 1989). As lysine residues are more common in the fragment 110-207 of α_{s2}-CN (Brignon *et al.* 1977), more peptides result from that part of the sequence, due to plasmin action. Likewise, lysine residues tend to be between amino acids 1-50 and 80-135 in α_{s1}-CN (Mercier *et al.*, 1971), and consequently, this is where the most products of plasmin proteolytic action originate from (McSweeney *et al.*, 1993). Plasmin specificity has been reported for other caseins. For example, fragment 29-105 from β-CN is one of the major peptides resulting from plasmin action on this casein (Andrews & Alichanidis, 1983). In liberating this peptide, plasmin acts on the C-terminal side of lysine residues, at Lys29-Ile30 and Lys105-His106 bonds.

While extensive investigations have been carried out into the specificity of plasmin, there has been limited study regarding the specificity of bacterial proteases often present in raw milk. In the late 1980s, Stepaniak *et al.* (1989) characterised the peptide profile, by HPLC, after proteolysis of various caseins with *P. fluorescens* protease, while Mitchell and Marshall (1989) characterised action of partially purified *P. fluorescens* protease on ribonuclease A. Neither of these workers characterised the peptides. Likewise, Aroonkamonsri (1996) determined the peptide profile of α, β and κ-CN hydrolysed with protease from *P. fluorescens*, but did not identify the fragments. While the workers in all of the other studies mentioned here have investigated extracellular proteases, Gobbetti *et al.* (1998) looked at the intracellular protease of *P. fluorescens* ATCC948. They observed a low specificity when various peptides originating from α_{s1}-CN and β-CN were used as substrates. Subsequently, a comprehensive study by Recio *et al.* (2000) characterised the peptides present following the action of the well characterised protease from *P. fluorescens* B52 on β-CN. In that study, protease from *P. fluorescens* B52 was similar to chymosin in its action, but was not as specific. Since its isolation from raw milk in the late 1970s by Richardson and Te Whaiti (1978), *P. fluorescens* B52 and its hydrolytic enzymes (including a known single protease) have been the subject of extensive investigation. Consequently, it is one of the most widely studied and well characterised psychrotrophic *Pseudomonas* spp. raw milk isolates.

Mass spectrometry is an ideal tool for the study of peptides. In particular ESI-MS and MALDI-TOF MS are regarded as extremely valuable techniques for peptide analysis (Michalski & Shiell, 1999). Owing to various factors, MALDI-TOF MS was considered most suitable for the current investigation. One main reason is the fact that samples for analysis by MALDI-TOF MS can contain a mixture of compounds. This reason, along with its high sensitivity, were key factors for its selection in this work. The technique, which was first reported by Karas and Hillenkamp (1988) and Tanaka *et al.* (1988) revolutionised protein studies. It has subsequently found application in analysis of peptides from

bovine milk (Catinella *et al.*, 1996; Soeryapranata *et al.*, 2002; Schmelzer *et al.*, 2004).

The aims of the work reported in this chapter were to characterise the peptides produced by the action of *P. fluorescens* B52 protease on α_s-CN and β-CN and to evaluate the potential relationship of these peptides to bitter flavour in UHT milk.

7.2 Materials and Methods

7.2.1 Source of chemicals and microbiological media

Unless otherwise stated, all chemicals were of the highest grade available and were purchased from Sigma-Aldrich Co. (Sydney, NSW). The substrate used, α_s-CN, was stated by the manufacturer as being 60% α_s-CN with most of the remainder β-CN. All microbiological media were purchased from Oxoid Australia Pty. Ltd. (Adelaide, SA).

7.2.2 Isolate origin and protease characterisation

The isolate used, *P. fluorescens* B52, obtained from the culture collection at Food Science Australia in Melbourne was originally isolated from raw milk in New Zealand by Richardson and Te Whaiti (1978) where partial purification of its protease was reported. A complete purification and characterisation of the single protease from *P. fluorescens* B52 was reported by Richardson (1981) with specificity towards κ-CN undertaken by Recio *et al.* (2000).

7.2.3 Culture growth and preparation of crude protease

A 10mL volume of NB was inoculated with a bead from an ultracold stock of *P. fluorescens* B52, held in the culture collection at Food Science Australia in Melbourne. The inoculated NB was incubated at 25 ºC for 24 h. Following incubation, the culture was centrifuged at 3020 *g* for 10 min in a sterile 10 mL centrifuge tube, washed in sterile 145 mM NaCl and centrifuged again at 3020 *g* for 10 min. This washing and centrifugation step was repeated, before the supernatant was decanted and the pellet resuspended in 145 mM NaCl. The procedure of Richardson (1981) was used as a guide for the growth of the

culture for preparation of crude protease. One millilitre of the washed saline culture was added to 99 mL of UHT skim milk (fat content 0.1%) in a 250 mL flask and incubated at 22 ºC for 96 h with 150 rpm circular shaking. Following this incubation period, the UHT skim milk culture was centrifuged at 35000 *g* for 10 min at 4 ºC. The supernatant was filter sterilised through a 220 nm filter and the filtrate collected was the sterile crude protease used.

7.2.4 Digestion of casein

α_s-CN was dissolved in 100 mM potassium phosphate buffer at pH 6.8 to a final concentration of 0.3%, as used by Recio *et al.* (2000). Crude protease, prepared as described in 5.2.1, was added to a final concentration of either 0.0067% or 0.0800%. The digestion mixtures were then incubated at 45 ºC. This temperature lies within the optimal range for activity of this protease (Richardson, 1981). Samples were taken for later analysis at the commencement of incubation, after 2 h and at the end of the incubation period.

7.2.5 Analysis of peptides and protein degradation

Two techniques were used to analyse peptides resulting from the digestion, SDS-PAGE (performed as described in section 4.2.6.2) and HPLC (performed as described in section 4.2.6.3).

7.2.6 Discrimination and identification of peptides and amino acid sequence

The procedure described in 5.2.6.5, based on MALDI-TOF MS, was used for discrimination and identification of peptides and for determination of the amino acid sequence.

7.2.7 Confirmation of amino acid sequence

A HPLC technique followed by ESI-MS/MS was the method of choice for confirmation of the amino acid sequence of peptides following analysis by MALDI-TOF MS.

An Agilent 1100 capillary liquid chromatography system (Agilent Technologies; Melbourne, VIC) that consisted of a capillary pump with micro

vacuum degasser, thermostated micro well-plate sampler, a thermostated column compartment with a two-position/six-port microvalve, a diode-array detector and a thermostated fraction collector was used. Separation of peptides from the digestion mixture was carried out on a Zorbax 300SB C18 (150 × 0.3 mm internal diameter) capillary column (Agilent Technologies; Melbourne, VIC) packed with 3.5 μm particles. Both the eluent A (water) and eluent B (acetonitrile) contained 0.1% FA as ion pairing reagent. Samples injected were first loaded with eluent A onto an enrichment column (Zorbax 300SB C18, 5 × 0.3 mm internal diameter, 3.5 μm particles) by using an Agilent binary pump (Agilent Technologies; Melbourne, VIC) at a flow rate of 10 μL/min. The enrichment column and the analytical column were connected through the microvalve in the column compartment with the temperature controlled at 30 ºC. The microvalve was switched 15 min after sample injection to direct the flow from the capillary pump through the enrichment column and the analytical column. The concentrated and desalted peptides were then eluted from the enrichment column and sequentially separated by the analytical column with a linear gradient of five to 65% B over 60 min (1% B/minute) at a flow rate of 4 μL/min. UV detection was at 214 nm.

For mass spectrometry analysis, ESI-MS/MS analysis was employed, and carried out in the positive ion mode with an Agilent 1100 Series LC/MSD-SL ion trap mass spectrometer (Agilent Technologies; Melbourne, VIC) through an Agilent G1607A orthogonal electrospray interface (Agilent Technologies; Melbourne, VIC). The electrospray voltage was 3.5 kV and ion trap mass spectrometer was operated in full scan mode in the range of 100 to 1800 m/z. The nebulising gas (N_2) pressure, the drying gas (N_2) flow rate, and the drying gas temperature were set at 69 kPa, 5 L/min, and 300 ℃, respectively. The target mass was set at 800 m/z, the compound stability was set at 50% and the trap drive level was set at 100%. The ion accumulation time was automatically adjusted using the Ion Charge Control (ICC) feature of the instrument. The maximal accumulation time was set at 300 ms and the ICC target was set at 30000. For the tandem mass spectrometry experiments, the instrument was automatically switched from MS to MS/MS mode when the intensity of a

particular ion exceeded the preset threshold. All system control and data acquisition were conducted with Agilent ChemStation (Agilent Technologies; Melbourne, VIC) and MSD Trap Control software.

7.3 Results

7.3.1 Peptide pattern after proteolysis

Clear differences in the degree of protein degradation were observed when the crude protease was used at 0.0067% and at 0.08%, a 12-fold higher concentration. The SDS-PAGE gel (Figure 7.1) and the densitometry analysis of lanes 7 and 11 from that gel (Figure 7.2) demonstrate the depletion of caseins with the simultaneous appearance of many peptides resulting from casein hydrolysis. Peptides resulting from the proteolysis were not particularly hydrophobic as only a few peaks were seen towards the end of the HPLC run. The most abundant peptides were observed between 15 and 21 min (Figure 7.3). Four peptides in particular dominated the profile, at a relative front of approximately 0.48, 0.52, 0.69 and 0.75 (Figure 7.3).

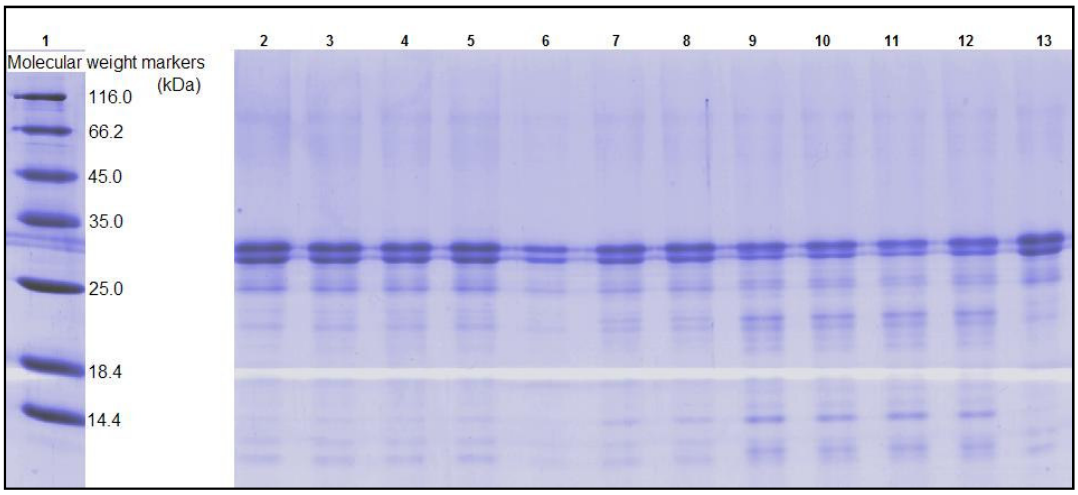


Figure 7.1: Protein degradation in a_s-casein/β-casein digests analysed with SDS-PAGE. Lane 1: Molecular weight markers, Lane 2: Replicate 1 of 0 h - 0.0067%, Lane 3: Replicate 1 of 2 h - 0.0067%, Lane 4: Replicate 2 of 2 h - 0.0067%, Lane 5: Replicate 1 of 4 h - 0.0067%, Lane 6: Replicate 2 of 4 h - 0.0067%, Lane 7: Replicate 1 of 0 h - 0.08%, Lane 8: Replicate 2 of 0 h - 0.08%, Lane 9: Replicate 1 of 2 h - 0.08%, Lane 10: Replicate 2 of 2 h - 0.08%, Lane 11: Replicate 1 of 4 h - 0.08%, Lane 12: Replicate 2 of 4 h - 0.08%, Lane 13: Replicate 1 of 0 h - 0.0067%

Only a few peptides, that is those with higher hydrophobicity, were observed towards the end of the RP-HPLC run. Most proteolysis appeared to have taken place in the first two hours, because, as seen in Figure 7.3, there was little visible change in the peptide profile between two and four hours.

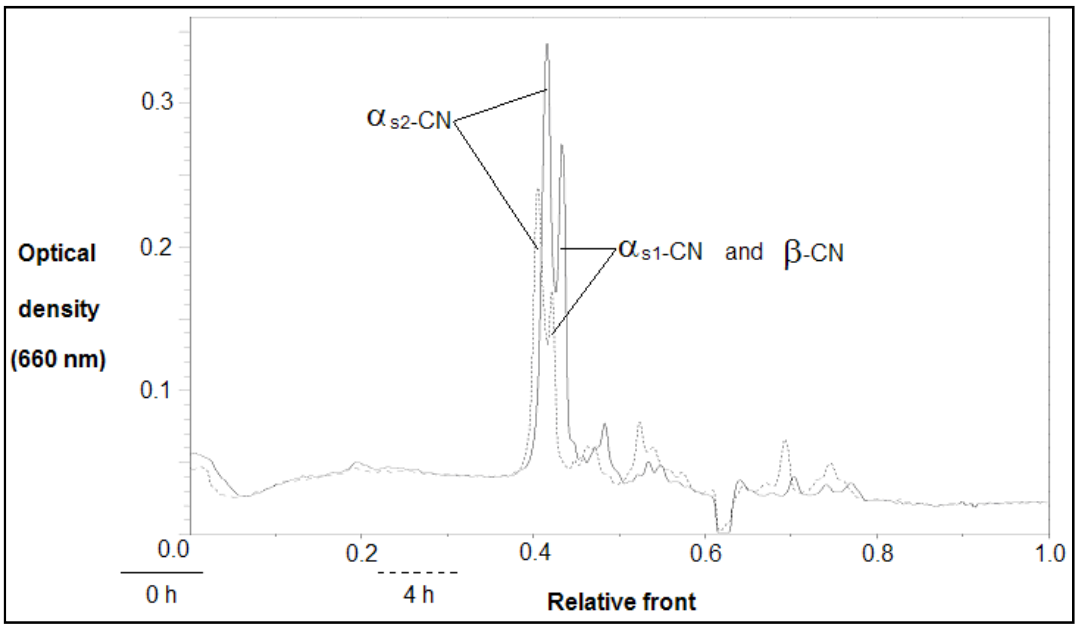


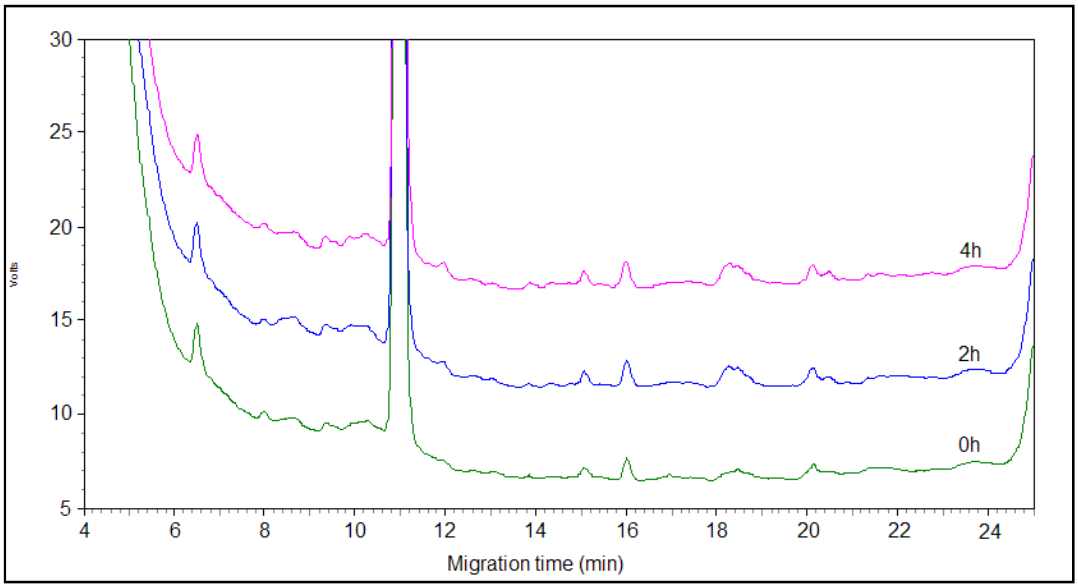
Figure 7.2: Quantification of protein bands from α_s-casein/β-casein digests by densitometry. The 0 h and the 4 h traces correspond to lanes 7 and 11 respectively from Figure 7.1.

7.3.2 Identification and characterisation of peptides arising from α_s-casein and β-casein following digestion with *Pseudomonas fluorescens* B52 protease.

At the commencement of the incubation period, few peptides were present. The only one present in a considerable quantity was f97-104 (m/z of 898.5064) from β-CN (Figure 7.4a). These results of the 2 and 4 h digestions are consistent with those presented in the previous section, where there was substantial proteolysis in the first 2 h and then negligible additional proteolysis between 2 and 4 h (Figure 7.4b,c). The most notable result was that in the 2 h digestion mixture, the peptide with an m/z of approximately 1485.726, which is α_{s1}-CN f138-149 (NQELAWFYPEL), was nearly as abundant as the peptide with an m/z of approximately 2054.101, which is β-CN f95-112

(VSKVKEAMAPKEMPFP). However, in the 4 h digestion mixture, the α-$_{s1}$-CN f138-149 peptide was present at less than half the intensity of the β-CN f95-112 peptide.

a)



b)

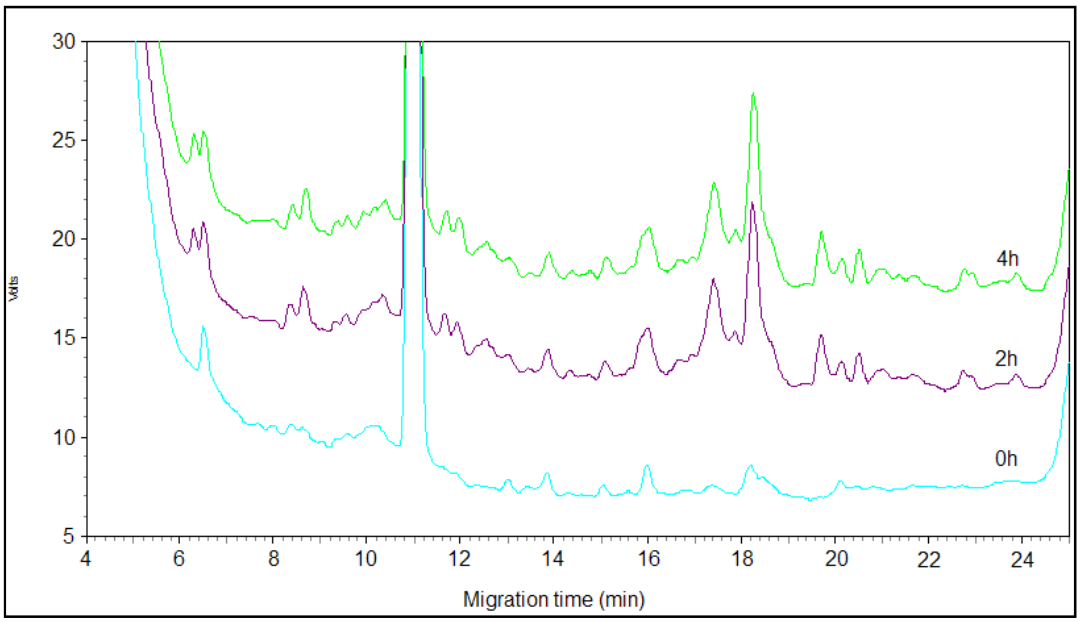
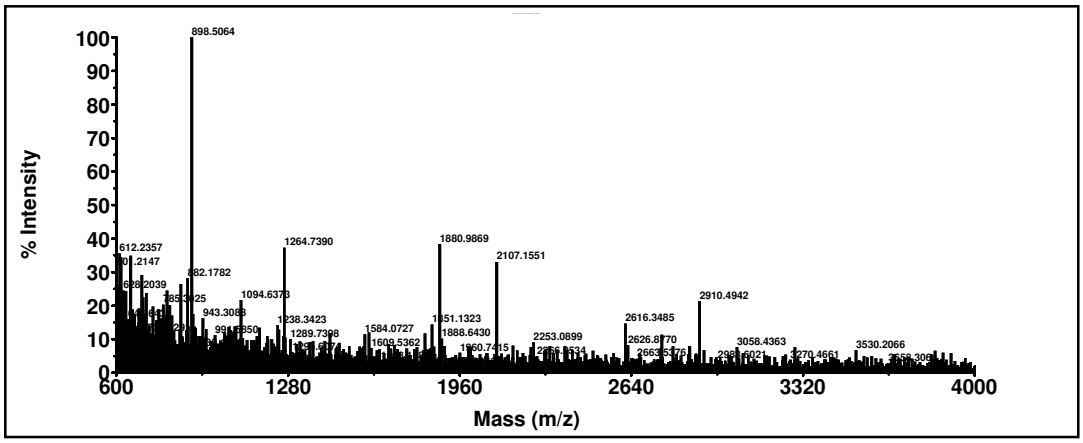
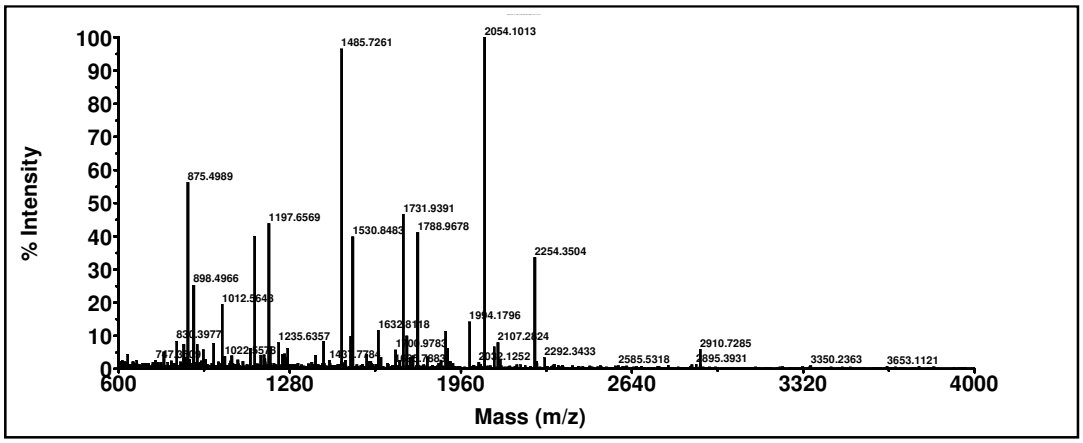


Figure 7.3: Peptide profile of α_s-casein digest by RP-HPLC, with 0.0067% a) and 0.08% b) added crude protease. Traces are of the different incubation periods. Bottom - 0 h, Middle - 2 h, Top - 4 h.

a)



b)



cC

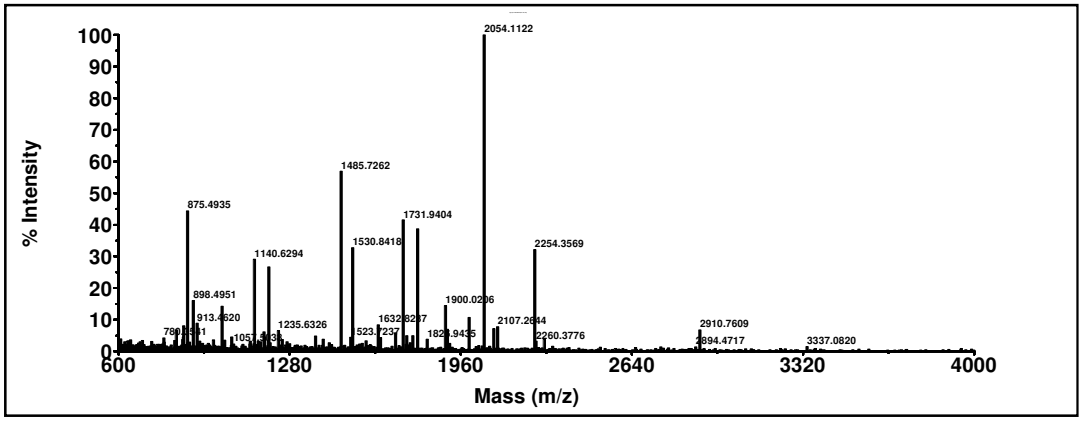


Figure 7.4: Mass spectra of peptides from MALDI-TOF MS analysis before a) and after a digestion of 2 h b) and 4 h c) with protease from *Pseudomonas fluorescens* B52.

7.3.3 Confirmation of sequence of α_{s1}-CN/β-CN peptides

By using ESI-MS/MS to confirm the presence or absence of particular peptides originating from α_{s1}-CN, doubts were raised over the identity of two peptides. These were the peptides with an m/z of 875.499 and 1731.939. From MALDI-TOF MS analysis, the closest match was to a peptide originating from α_{s1}-CN. However, ESI-MS/MS analysis of the sample, did not reveal any peptides of that sequence, even though that peptide was a perfect match, with regard to m/z. Therefore, the peptide with an m/z which was the next closest match that could be confirmed by ESI-MS/MS was α_{s1}-CN f11-24.

7.3.4 Specificity of *Pseudomonas fluorescens* B52 protease.

Visual analysis of the cleavage locations from the most abundant peptides after four hours of digestion did not yield any clear overall specificity, particularly at the N-terminal end of the peptide. By inspecting the amino acid residues at the N-terminal end of the peptides, and the one immediately upstream of the peptide, the cleavage appeared random with regard to the particular amino acid residues present (Table 7.1). However, the cleavage at the C-terminal end seemed to be specific for residues with non-polar side chains (especially leucine residues) immediately upstream of the cleavage site.

Table 7.1: Cleavage sites of the most abundant peptides after 4 h digestion.

Relative abundance	Protein of origin	Peptide sequence	Residues at cleavage site	
			N-terminal	C-terminal
1st	β	VSKVKEAMAPKHKEMPFP	G-V	P-K
2nd	α_{s1}	VNQELAYFYPEL	G-V	L-F
3rd	α_{s1}	KYKVPQL	K-K	L-E
4th	α_{s1}	IKHQGLPQEVLNENL	P-I	L-L
	α_{s1}	KHQGLPQEVLNENLL	I-K	L-R

7.4 Discussion

Under the conditions that the protein digestion was carried out, it was apparent that the proteolysis was almost completed within the first two hours. This could be because of a decrease in efficiency of proteolysis or that the specific bonds required for hydrolysis had all, or almost all, been hydrolysed, within the first two hours. The increase in peptides, resulting from proteolytic

action, was not noticeable between two and four hours, whereas it was substantial between zero and two hours. This was observed with both concentrations of added protease, indicating that the substrate concentration was not the limiting factor. Therefore, the enzyme may lose activity when kept at 45 ºC, the assay temperature, for more than 2 h. Alternatively, the action of pseudomonad protease on some caseins may be a factor in the slow progress of proteolysis beyond two hours.

It has been demonstrated that proteases from raw milk pseudomonad isolates exhibit a preference for both β-CN and κ-CN before α_{s1}-CN and α_{s2}-CN (Gebre-Egziabher *et al.*, 1980; Mitchell & Marshall, 1989), which suggests that they are less efficient at hydrolysing α_s-CN compared to the other two types of casein. As stated in section 7.3.2, the peptide VNQELAYFYPEL, cleaved from α_{s1}-CN, was almost as abundant as peptide the VSKVKEAMAPKHKEMPFP, liberated from β-CN. However, after four hours, peptide VNQELAYFYPEL was present at less than half the amount of peptide VSKVKEAMAPKHKEMPFP. This result confirms that β-CN rather than α_{s1}-CN is a preferred substrate for the protease from *P. fluorescens* B52.

Neither of these two most abundant peptides was hydrophobic and therefore would not be expected to be bitter, and consequently would not be expected to contribute to bitter flavour in UHT milk. In fact, the most abundant peptide, VSKVKEAMAPKHKEMPFP from β-CN, is moderately hydrophilic with its hydrophobicity being -2.22 on the CCS. The second most abundant peptide, VNQELAYFYPEL from α_{s1}-CN, had a hydrophobicity of 0.62 on the CCS, thereby not demonstrating any real hydrophilicity or hydrophobicity. The mean hydrophobicity of the ten other most abundant peptides was 1.09, which indicates only slight hydrophobicity and therefore they would not be expected to be bitter.

7.5 Conclusions

- The action of protease from *P. fluorescens* B52 does display some degree of specificity in its cleavage of peptide bonds in α_s-CN and β-CN.

- *P. fluorescens* B52 protease is unlikely to liberate hydrophobic sequences, therefore, it is unlikely to cause bitterness as a result of hydrolysis of α_s-CN and β-CN.

CHAPTER 8

GENERAL DISCUSSION

As presented earlier in this thesis, spoilage of UHT milk as a result of the action of heat-stable lipases and proteases produced by psychrotrophic *Pseudomonas* spp., is of concern to the dairy industry. The risk of UHT milk spoilage by the action of heat-stable lipases and proteases will continue for as long as psychrotrophic *Pseudomonas* spp. contaminate the raw milk supply. It is unlikely, at least in the foreseeable future, that lipolytic and proteolytic spoilage of UHT milk can be eliminated. Consequently, strategies to reduce contamination risk need to be considered. The present study addresses this problem, with various approaches suggested which could be used to potentially reduce or eliminate the occurrence of enzymatic spoilage of UHT milk.

It is critical to identify sources of the bacteria or industry practices likely to contribute the enzymes that can lead to spoilage. Results obtained from this, and related studies reported in the literature (Dogan & Boor, 2003), have identified the sources and/or industry practices along with providing information on the most suitable methods for this purpose. The current work suggests that particular environmental conditions may favour the development and persistence of more lipolytic and proteolytic populations of psychrotrophic pseudomonads. This is because, at least at certain points in the raw milk collection chain, there are high numbers of psychrotrophic *Pseudomonas* spp. likely to contribute to lipase and protease contamination in UHT milk. Some points in the milk collection chain (for example, farm 3 in the survey undertaken in the present work) may contribute more psychrotrophic *Pseudomonas* spp to the milk supply than others. If these bacteria are strongly lipolytic and/or proteolytic, then the problem is further compounded. Consequently, if high levels of spoilage bacteria with clear lipolytic and/or proteolytic spoilage potential are not addressed, then use of milk from those farms would need to be restricted, and not be processed into UHT milk. While it is apparent in the present limited investigation where the more important sources of contamination

arose, a widespread study, over many regions and with more samples, could be useful so that trends and likely sources of contamination could be identified. In addition, industry practices may influence the likelihood of bacterial lipase and protease contamination of UHT milk, as found in the present study, where 4 ℃ incubation appeared to "select" for isolates with more potential to contribute bacterial lipases and/or proteases to UHT milk. If other environmental conditions or specific sources of contamination which cause similar effects to those found for 4 ℃ in this work, are identified, then this could lead to better control of bacterial lipase and protease contamination of UHT milk, and hence, lipolytic and/or proteolytic spoilage of UHT milk. Furthermore, the most suitable typing technique(s) need to be identified. At present, both ribotyping, as evident from work by Wiedmann *et. al.* (2000) and Dogan and Boor (2003) and pulsed field gel electrophoresis (investigated in the present project) may be equally well suited for this application. Ribotyping technique may be more cost effective than PFGE.

Even if a reduction in the degree of lipolysis and proteolysis in UHT milk is brought about by improved general hygiene, it would still be worthwhile to determine the extent of lipolysis or proteolysis present in any given batch of UHT milk as unexpected breakdowns in hygiene and temperature control may occur. Moreover, lipolysis and proteolysis could be followed in raw milk to monitor the success of measures implemented to prevent lipase and protease contamination. The triolein (lipase) and casein or FITC-casein (protease) assays, presented in Chapters 3 and 4, offer a reliable means of identifying UHT milk with protease and lipase contamination and therefore identifying batches at risk of premature spoilage. Furthermore, the assessment of and adjustments to lipase and protease assays, along with the UHT milk storage results presented in Chapter 5, demonstrate the promise of that approach for the purposes of shelf life assessment of UHT milk. This is related to the observation that the lipolysis and proteolysis associated with perceivable spoilage over three to five months storage, can be detected in 10 to 14 days. While this seems a lengthy time for an enzyme assay, it falls within the current approximate timeframe for industry quality control testing of UHT milk. At

present, industry quality control programs incorporate microbiological testing of UHT milk. This testing takes approximately ten days. Therefore, no further time is committed to clearing a batch of UHT milk, as the suggested FITC-casein test requires incubation for ten days. For lipase detection, additional four days are required for the triolein assay.

The lipase and protease detection methods developed in the present project can be used as a basis for further work on shelf life assessment of UHT milk, for example by providing a comparison point for the possible development of more rapid assays such as those based on antigen-antibody recognition. If models could be developed to accurately determine the shelf life of UHT milk, then the anticipated lipolysis and proteolysis in the UHT milk during storage could be quantified prior to its release into the marketplace. Thus, more accurate “best before” dates may be determined. This would be of particular benefit for export markets, as batches of UHT milk could be exported to specific markets, with the location dependent on the expected shelf life of the product. A strategy like this could ensure that UHT milk with a high level of lipase and/or protease, and therefore with more potential for spoilage, would be exported to nearby or cooler locations or, if the spoilage risk is too high and therefore unacceptable, the product would not be released to the market. If milk with a high potential for lipolytic and/or proteolytic spoilage was exported to distant locations or to locations with a hot climate, the shelf life may be further reduced because higher temperature accelerates enzyme action. In addition, longer transit time would mean a shorter perceived shelf life by the consumer. This may harm the reputation of UHT milk as a long-life product, as potentially more batches will spoil or they will be marked with a “best before” date that is shorter than that generally regarded as standard for UHT milk. While not eliminating the bacterial lipases and/or proteases from UHT milk, improved detection methods could prevent release into the marketplace of milk with a high spoilage potential and therefore reduce the occurrence of lipolytic and proteolytic spoilage of UHT milk.

Age gelation is a major spoilage defect in UHT milk. As outlined earlier in this thesis, the mechanisms culminating in this phenomenon remain to be

elucidated. Age gelation was observed in two of the shelf life trials undertaken in this project, with protein and peptide studies providing information necessary for an improved understanding of the sequence of events leading to this important spoilage defect. The results presented in Chapter 5 are in agreement with the hypothesis of McMahon (1996), in which three major steps have been postulated, starting with the casein micelle with κ-CN on its surface, viz.: I) covalent bonding of β-LG to κ-CN on the surface of the micelle to form beta-kappa (βκ) complexes, II) dissociation of the βκ complex from the micelle, by proteolysis, III) cross-linking of the βκ complex to form aggregations and finally a gel. The particular result obtained in the present study, consistent with this hypothesis, is the virtual complete degradation of κ-CN; subsequently, the most abundant peptide found in milk originated from κ-CN. It is consistent with this hypothesis because κ-CN: I) plays a major role in steric stability of the micelle and II) κ-CN is part of the βκ-complex, postulated to form the major part of the protein network in gelled milk. Furthermore, while there is widespread acknowledgement of the possible role of proteases from *Pseudomonas* spp. in age gelation, the contribution from proteases of other bacteria present in raw milk should not be discounted (Keogh & Pettingill, 1982).

While age gelation is an important defect in UHT milk, other defects, including undesirable flavours, are also important. To complement the wider spoilage investigation work, supplementary experiments were undertaken in order to explore some of the possible mechanisms of the development of bitter flavour in UHT milk. In this work, a well characterised raw milk isolate of *P. fluorescens*, known to produce only one protease, was used. The results obtained with the model system suggest that it is unlikely that hydrolysis of α_s- and β-CN by this particular protease would contribute to bitterness in UHT milk because the majority of peptides liberated were marginally hydrophobic. It is possible, though, that bitter flavour in UHT milk may originate from hydrolysis of other caseins and/or from the action of other proteases.

The practical usefulness of the work carried out during this study is highlighted by the real potential it offers industry for improved shelf life estimation, compliance testing and validation of quality control procedures.

This, along with improved knowledge of sources of strongly lipolytic and proteolytic milk spoilage bacteria and industry practices that can contribute lipases and proteases to UHT milk, resulting in perceived sensory defects, are the strengths of this work.

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

In the present study, assays were identified that could be used to detect low levels of lipase and protease, and confirmed that results obtained in these assays can be used to indicate premature spoilage of UHT milk caused by the continuing lipolysis and/or proteolysis in this long shelf life product. A general perception that labelled substrates offer improved assay sensitivity over milk-like substrates was not confirmed in the present study. The minimal concentrations of hydrolytic breakdown products detected by the selected assays were sufficient to cause premature spoilage of UHT milk, after three to five months storage. Peptides and FFA accumulate in UHT milk at different rates during storage. Determination of the concentration of the individual FFA in UHT milk provides a more reliable indication of the likelihood of development of lipolysed flavour than does the total FFA content.

An association was identified between the pattern of κ-CN degradation and the development of age gelation. It is possible that there is a certain threshold of κ-CN degradation, above which age gelation occurs. This is supported by the observation that the largest extent of κ-CN degradation was evident in the few days prior to age gelation.

There was considerable genetic diversity among the isolates from the various sources where raw milk was collected. The prevalence of lipolytic and proteolytic psychrotrophic *Pseudomonas* spp. in raw milk from one of the farms included in the study, and in the silo, was observed. These sources had more potential to contribute lipases and proteases to UHT milk. There was no relationship between the frequency of raw milk collection by the tanker and the total bacterial count nor with the psychrotrophic *Pseudomonas* spp. count of the raw milk. Prolonged storage of raw milk at 4 °C, rather than at 10 °C, or at combined 4 °C then 10 °C, promoted the emergence of moderate to strong lipase and protease producing isolates of psychrotrophic *Pseudomonas* spp.

Protease from *P. fluorescens* B52 shows limited specificity towards α_s and β-CN, liberating hydrophilic peptides from these caseins. These peptides are unlikely to cause bitterness in UHT milk.

9.2 Recommendations

While the assays selected in the present study have sufficient sensitivity to detect the lipolytic and proteolytic activity that could result in spoilage of UHT milk after three to five months of storage, they are not sensitive enough to detect the activity that would result in spoilage of UHT milk during the standard shelf life period of at least six months. Therefore, further investigations to optimise the assays, or even to identify other assays, that could be used to detect even less advanced lipolysis and proteolysis, should be carried out. Once such assays have been identified, they should be tested with crude enzyme preparations from a diverse pool of isolates of lipolytic and proteolytic bacteria. This would provide a robust measure of the suitability of these assays, with a wide variety of enzymes being tested. Once such reliable assays are available, they could be used to detect the level of lipolysis and proteolysis in UHT milk that would result in spoilage after a minimum of six months, allowing models to be developed and used in shelf life prediction studies.

The work conducted in the present study has contributed to the elucidation of the mechanisms of age gelation. However, further studies should be carried out on the molecular mechanisms of the phenomena leading to formation of protein gel networks in UHT milk. To this end, detailed proteomic analyses of the intact proteins, free peptides and protein aggregates need to be carried out as the gel network forms. An investigation of this type could provide useful information on the fate of the various proteins and peptides at each stage of gel formation, and assessing the contribution of different proteins, peptides and also of different proteases. The use of diverse proteases in these studies, with different properties and specificity, may also be valuable in identifying the conditions and/or type of protein breakdown products which play a significant role in the events leading to age gelation.

It is well known that storage of raw milk below 5 ℃ does improve its microbiological quality, and that this leads to improved quality of the heat-processed product. A wider sampling schedule of milk, from different locations, would be of benefit in future work to ascertain how important low storage temperature is in selecting for moderate to strong lipase or protease producers in various geographic locations. While industry regulations are in place to reduce prolonged raw milk storage, these regulations should be strictly enforced for the raw milk destined for processing into UHT milk.

Further studies should be carried out to identify the most suitable molecular typing method for psychrotrophic *Pseudomonas* spp. Various genotypic typing methods, including ribotyping and pulsed field gel electrophoresis, have been used for molecular typing of psychrotrophic *Pseudomonas* spp. While PFGE is generally regarded as the “gold standard” for molecular typing of *Pseudomonas* spp., its applicability to psychrotrophic *Pseudomonas* spp. typically found in the dairy environment needs to be assessed further. Both ribotyping and PFGE should be compared under identical conditions, with identical samples, and in the same study, in order for accurate conclusions to be drawn about the merits of each technique.

Overall, there is clearly a need to continue the directions of research initiated in the present study.

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APPENDICES

Appendix 1: Sensory response sheets

A1.1: Regular sheet

UHT milk shelf-life experiment

Researcher: Philip Button from The University of Melbourne/Food Science Australia
E-mail: philip.button@csiro.au, Telephone: 9731 3420

Date: 06.10.05 - 0 weeks Name.....

Test type: Aroma

One of the three samples presented is different from the other two.

Please examine in the order provided and place a circle around the code number of the sample which you consider different. If you consider the different sample to be of unacceptable quality, please indicate this by writing "U" beside the code number.

_____ _____ _____

You must make a choice

Comments:

Test type: Flavour

One of the three samples presented is different from the other two.

Please examine in the order provided and place a circle around the code number of the sample which you consider different. If you consider the different sample to be of unacceptable quality, please indicate this by writing "U" beside the code number.

_____ _____ _____

You must make a choice

Comments:

Thank you for your participation

A1.2 : Special sheet for gelled milk

UHT milk shelf-life experiment

Researcher: Philip Button; The University of Melbourne/Food Science Australia
E-mail: pbutton@unimelb.edu.au, Telephone: 9731 3420

Date: 04.04.06 Name.....

Please test the aroma and flavour of the gelled UHT whole milk sample. In the blank spaces provided, describe all aromas/flavours that you detect and how pronounced they are. A reference/control sample of UHT whole milk is provided for comparison purposes.

Test type: Aromas

	Very slight	Slight	Noticeable	Pronounced	Very pronounced
_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____

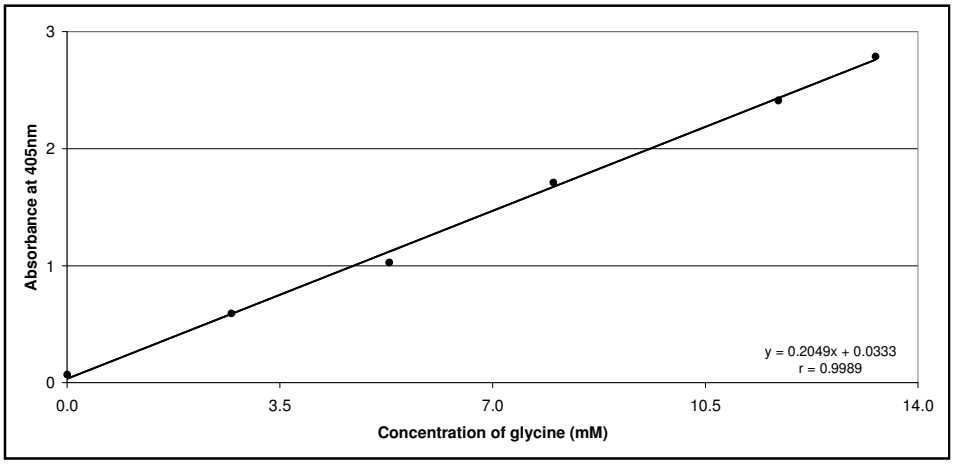
Test type: Flavours

	Very slight	Slight	Noticeable	Pronounced	Very pronounced
_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____

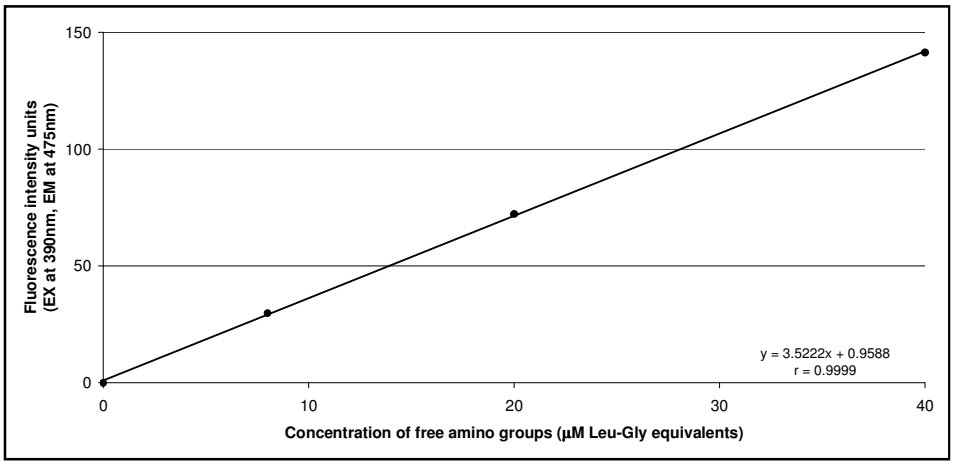
Thank you for your participation

Appendix 2: Standard curves

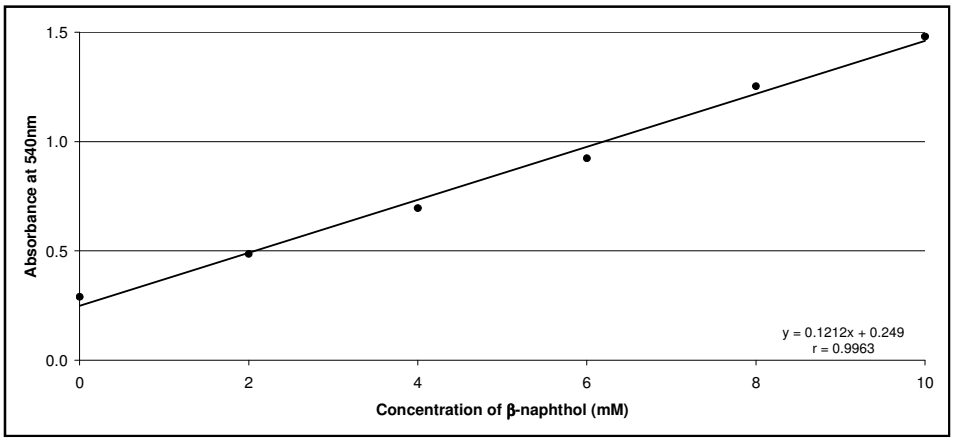
A2.1: Glycine (TNBS assay)



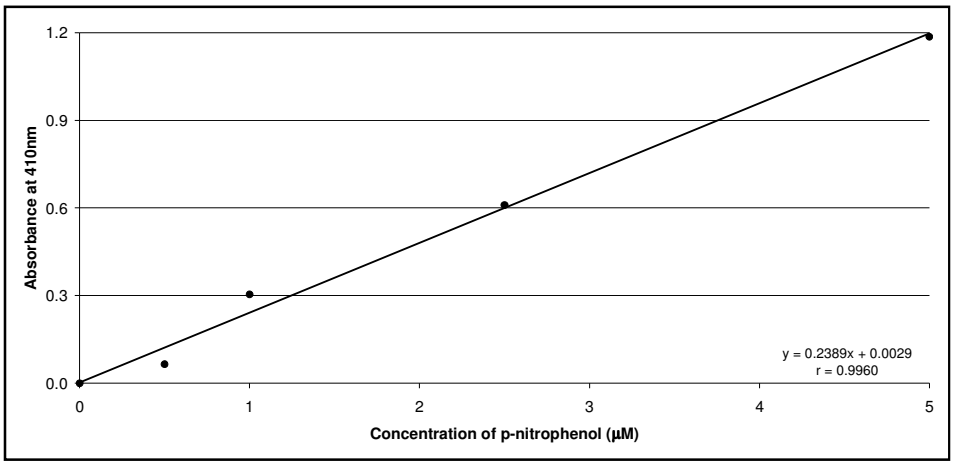
A2.2: Leucine-Glycine dipeptide (fluorescamine assay)



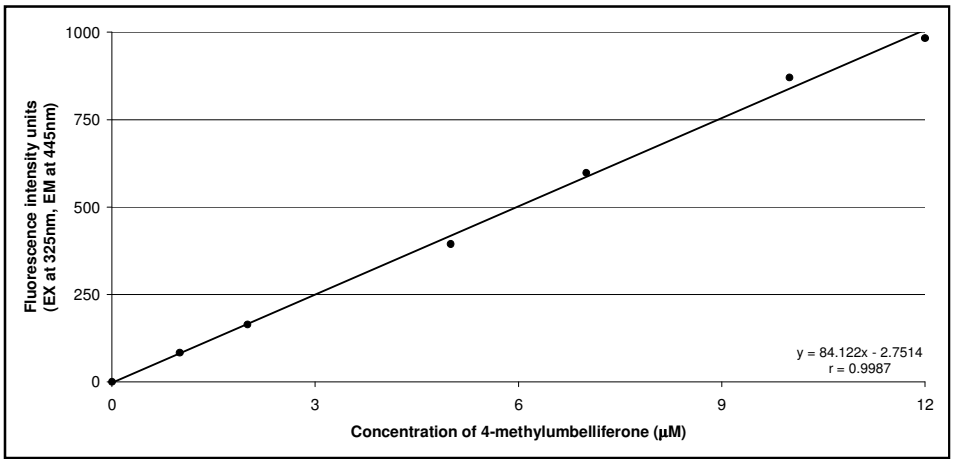
A2.3: β-naphthol (β-napthtyl caprylate assay)



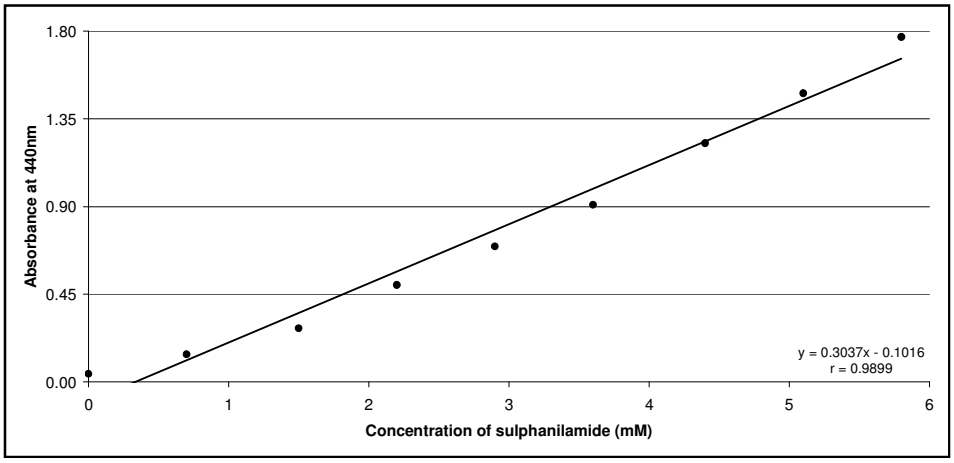
A2.4: *p*-nitrophenol (*p*-nitrophenyl caprylate and stearate assays)



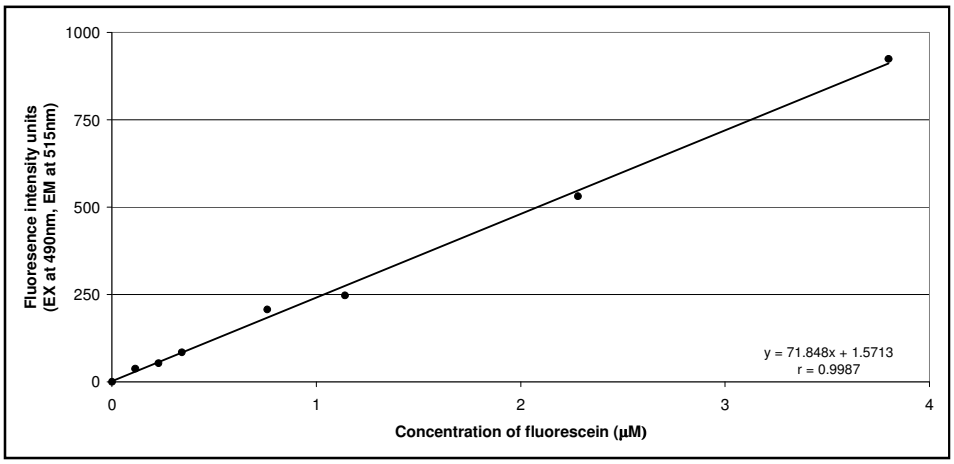
A2.5: 4-methylumbelliferone (4-methylumbelliferyl caprylate and oleate assays)



A2.6: Sulphanilamide (azocasein assay)

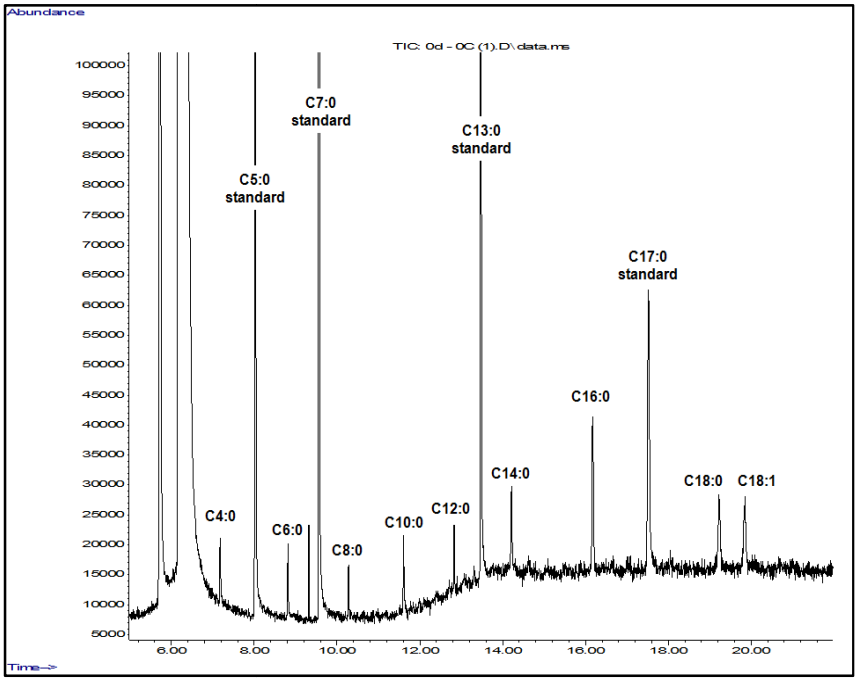


A2.7: Fluorescein (fluorescein isothiocyanate-casein assay)

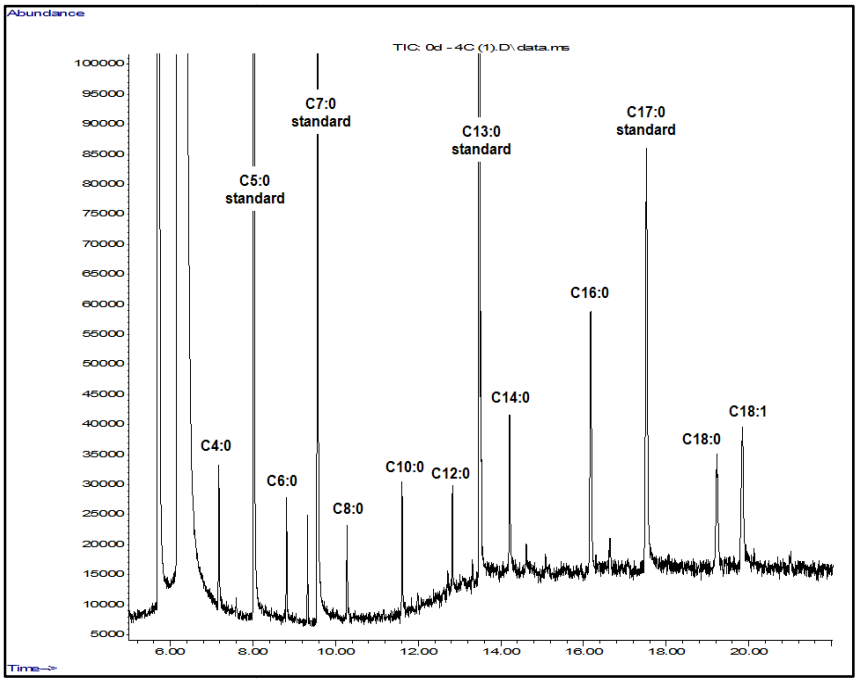


Appendix 3: Chromatograms of free fatty acid content of UHT milk samples at the commencement and conclusion of the storage period (Trial 3)

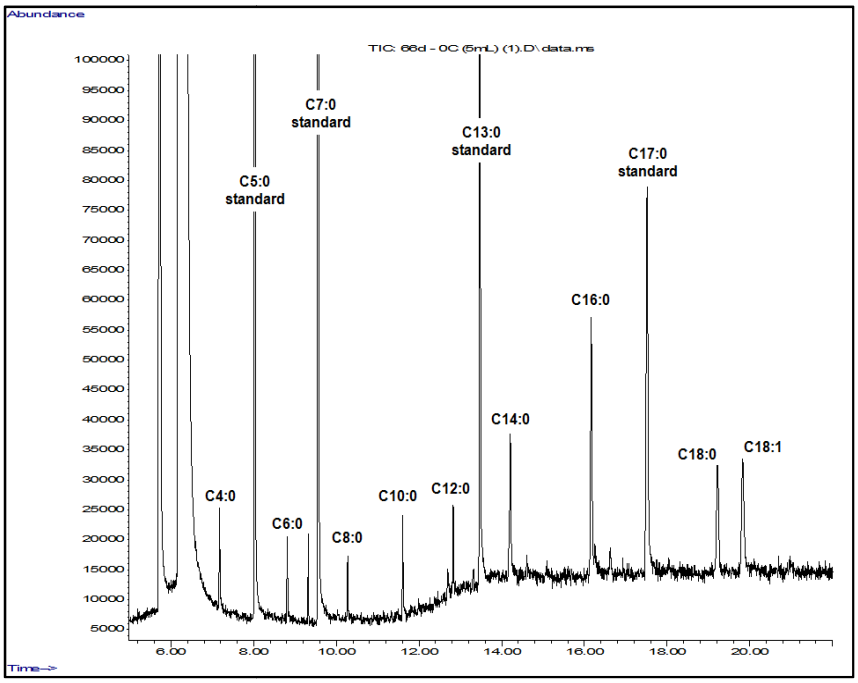
A3.1: Control UHT milk, 0 d



A3.2: Treatment UHT milk, 0 d



A3.3: Control UHT milk, 66 d



A3.4: Treatment UHT milk, 66 d