Characterisation of proteolytic activity in thermally-treated milk for novel product development

A thesis submitted in fulfilment of the requirements for the degree of
Master of Applied Science

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## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xx</td>
</tr>
<tr>
<td>LIST OF SYMBOLS</td>
<td>xxiii</td>
</tr>
</tbody>
</table>
DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Baddegamage Anusha Lakmini Ranjith Buthgamuwa
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SUMMARY

Milk is one of the most nutritious and widely consumed foods, and a versatile raw material for the food industry. Industrial processing involves conversion of raw milk into a wide range of dairy-based food products such as beverages and desserts. Advanced processing technologies including utilizing ultra-high temperatures (UHT) that results in production and transportation efficiencies, extended shelf-life and product availability over a wide distribution area. There are various factors that relate to raw milk quality, processing and formulation principles controlling the quality and safety of produced milk or dairy-based products that use UHT and other thermal techniques. The objectives of this study were to (i) identify the raw material quality in UHT-treated milk through the monitoring of protease activity, (ii) develop shelf-stable formulations of dairy beverages enriched with protein and dietary fibre, and iii) utilise the protein enrichment concept to provide highly nutritious dairy-based desserts thus meeting consumer nutritional expectations.

The first experimental chapter in this Thesis focused on the determination of protease activity in UHT-treated raw milk in relation to age-gelation during storage at room temperature. Heat-stable bacterial proteases from psychrotrophic Pseudomonas spp., which are strongly associated with age-gelation in UHT processing, were extracted and added to raw skim milk at a range of concentrations from 0 to 0.005% (v/v). Identification of protease activity before and after UHT treatment (135°C/2s) was carried out using the FITC-casein assay. During storage, the microbial count and pH of the samples remained the same as that recorded immediately after thermal exposure. However, milk with added crude enzyme exhibited gelation within a 10-week trial.

The association between peptide generation and added enzyme extract in UHT-treated milk can be related to the shelf-life of UHT-milk. This was identified via the quantification of short peptides or free amino groups using the fluorescamine assay, which was shown to form a linear relationship. At the time that gelation was identifiable visually, the free amino groups in samples with added crude protease were found to have similar viscosities, irrespective of the added crude enzyme concentration. The work identified the minimal concentration (activity) of proteolytic enzymes sufficient to cause premature spoilage in milk and dairy systems. The relationship between threshold enzymatic activity and storage of the UHT-treated milk was estimated from an empirically derived equation (with an R² of 0.9943), in
which parameters are: \( y \) (shelf-life in weeks) = 9.6301\(x^{-2.12}\) and \( x \) was the enzyme activity in terms of the concentration of FITC isomer 1 (\( \times 10^4 \) nM).

Increasing consumer interest in a healthy diet in terms of protein, dietary fibre and energy intake provides opportunities for the development of novel dairy-based products. Thus, the second objective of this Thesis was to develop shelf-stable dairy beverages enriched with protein and dietary fibre. Reverse formulation engineering of selected commercial products was performed by investigating their nutritional composition and steady-shear flow behaviour leading to the development of formulations enriched with protein and dietary fibre. Only formulations that showed similar rheological properties to that of the commercial products were further chosen for production on a large scale using pilot plant UHT and pasteurisation techniques. Work revealed that UHT processing is advantageous, as compared to high temperature pasteurisation due to the high microbiological quality of the product produced. Characterisation of physicochemical properties (including colour evaluation) of novel formulations containing 6.1\% (w/w) protein and 2\% (w/w) dietary fibre and the commercial product indicated that both systems are similar; an outcome which was further confirmed from the results of sensory evaluation in which consumers found the novel dairy beverage to be acceptable.

The last experimental chapter targeted the development of protein-enriched dairy desserts and focused on benefits to senior nutrition by incorporating in products high levels of leucine. This amino acid has been found to be a potent agent for efficient muscle-protein synthesis. Similar to our earlier work, reverse formulation engineering on commercial dairy desserts was carried out by establishing a database of steady-shear flow and textural profile that had to be imitated in novel formulations. The novel formulations incorporated whey protein and/or casein to achieve high levels of protein (11.5\% w/w) and leucine (1.2\% w/w). Protein-enriched dairy desserts prepared in the laboratory were examined in terms of physicochemical characterisitics using steady shear viscosity, small deformation dynamic oscillatory in shear and texture profile analysis. Enriched products that had a similar textural behaviour to that of the commercial dairy dessert, even though the latter had less than 50\% of protein in the novel formulations, were developed on a large scale to imitate processing in the factory of the sponsoring company. Rheology results and microscopy images of both the commercial product and the protein-enriched dairy desserts exhibit similar structural features for all materials. Moreover, results from sensory evaluation revealed that the nutritious dairy desserts were liked by the consumer.
TABLE OF CONTENTS

CHAPTER 1 INTRODUCTION

1.1 Dairy industry in Australia 1

1.2 Composition and nutritional quality of milk 1
  1.2.1 Overall introduction 1
  1.2.2 Milk proteins 2
    1.2.2.1 Casein 2
    1.2.2.2 Whey protein 3
  1.2.3 Native proteolytic enzymes 5

1.3 Heat treatment techniques of milk and dairy products 5
  1.3.1 Overview of heat treatment techniques applied in dairy industry 5
  1.3.2 Application of different UHT methods and processing conditions 6
    1.3.2.1 Direct UHT treatment 7
    1.3.2.2 Indirect UHT treatment 8
    1.3.2.3 Combined direct-indirect heating systems 9
  1.3.3 Advantages & disadvantages of UHT-treatment 10

1.4 Age-gelation and factors affecting age-gelation 11
  1.4.1 Proposed mechanism of age-gelation 11
  1.4.2 Overall effect of ‘age-gelation’ on the commercial aspects of the dairy industry 14
  1.4.3 Involvement of psychrotrophic microorganisms in ‘age-gelation’ 16
  1.4.4 Commercial practices that can be used to moderate the problem of ‘age-gelation’ 19

1.5 Fortification and production of dairy-based products 23
  1.5.1 What is fortification? 23
  1.5.2 Commercially available fortified dairy-based food products 23
  1.5.3 Ingredients for fortification of dairy-based food products with protein and dietary fibre 25
  1.5.4 Quality characteristics of dairy beverages and desserts 26
  1.5.5 Methods for the production of fortified dairy-based food products 32
### CHAPTER 2 MATERIALS & METHODS

<table>
<thead>
<tr>
<th>Section</th>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Materials</td>
<td>44</td>
</tr>
<tr>
<td>2.2</td>
<td>Microbiological methodology used in the extraction of enzyme from <em>Pseudomonas fluorescens</em></td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>2.2.1 Culture Storage</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>2.2.2 Cell Growth</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>2.2.3 Cell enumeration</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>2.2.4 Agar Diffusion Method (ADM)</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>2.2.5 Extraction of protease from <em>Pseudomonas fluorescens</em></td>
<td>50</td>
</tr>
<tr>
<td>2.3</td>
<td>Ultra high temperature (UHT) treatment.</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2.3.1 Preparation of milk samples with added crude protease</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2.3.2 UHT treatment of raw milk with/without added crude protease</td>
<td>50</td>
</tr>
<tr>
<td>2.4</td>
<td>Analytical methods used to monitor protease level and activity during the shelf-life study of UHT milk samples</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>2.4.1 Fluorescein isothiocyanate (FITC)-Casein assay</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>2.4.2 Fluorescamine method</td>
<td>55</td>
</tr>
<tr>
<td>2.5</td>
<td>Formulation principles and preparation of dairy-based food products</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>2.5.1 Formulation principles</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>2.5.2 Samples preparation of dairy beverages and desserts</td>
<td>58</td>
</tr>
<tr>
<td>2.6</td>
<td>Rheology</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>2.6.1 Principles of rheology</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>2.6.2 Small deformation dynamic oscillation</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>2.6.3 Texture profile analysis (TPA)</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>2.6.4 Viscosity determination using Brookfield viscometer</td>
<td>66</td>
</tr>
<tr>
<td>2.7</td>
<td>Colour evaluation in food</td>
<td>67</td>
</tr>
<tr>
<td>2.8</td>
<td>Optical microscopy</td>
<td>71</td>
</tr>
<tr>
<td>2.9</td>
<td>Sensory evaluation of food</td>
<td>72</td>
</tr>
<tr>
<td>2.10</td>
<td>References</td>
<td>75</td>
</tr>
</tbody>
</table>
CHAPTER 3 CHARACTERISATION OF RAW MATERIAL QUALITY THROUGH DETERMINATION OF PROTEOLYTIC ACTIVITY IN UHT- TREATED MILK

3.1 Abstract
3.2 Introduction
3.3 Experimental protocol
  3.3.1 Materials
  3.3.2 Enzyme & sample preparation
  3.3.3 Assessment methods
3.4 Results and discussion
  3.4.1 The growth of Pseudomonas fluorescens and production of extracellular proteases.
  3.4.2 Quality of skim milk before UHT treatment.
  3.4.3 The initial effect of UHT-treatment on both milk quality and the activity of added crude enzyme extract
  3.4.4 Effect of storage time and enzyme (protease) concentration on the viscosity and free amino group concentration of UHT-treated skim milk
3.5 Conclusion
3.6 References

CHAPTER 4 DEVELOPMENT OF SHELF-STABLE DAIRY BEVERAGES ENRICHED WITH PROTEIN AND DIETARY FIBRE

4.1 Abstract
4.2 Introduction
4.3 Experimental protocol
  4.3.1 Materials
  4.3.2 Sample preparation
  4.3.3 Methods
4.4 Results and discussion
  4.4.1 Characterisation of the flow behaviour in the commercial beverage and the prototype beverage made at the research laboratory
  4.4.2 Characterisation of flow behaviour in dairy beverages prepared at the
CHAPTER 6 CONCLUSIONS AND FUTURE WORK

6.1 References
LIST OF FIGURES

Figure 1.1  Tertiary structure of β-lactoglobulin
Figure 1.2  Time temperature profile for direct UHT plant
Figure 1.3  Time temperature profile for indirect UHT plant
Figure 1.4  Model of age gelation of UHT milk showing (1) formation of the βκ-complex, (2) its dissociation from micelles during storage, and (3) subsequent gelation of the milk through crosslinking of the βκ-complex
Figure 1.5  Structure of starch molecule
Figure 1.6  Schematic representations of different ideal structures of carrageenan
Figure 1.7  General flow diagrams for the production of dairy-based food products
Figure 2.1  Typical growth curve for Bacteria
Figure 2.2  Micro centrifuge (Sigma 1-14K)
Figure 2.3  Agar diffusion plate; clear area around the well
Figure 2.4  Homogeniser FT9 (Armfield, UK)
Figure 2.5  UHT/HTST processing unit (FT74X, Armfield)
Figure 2.6  Bacteriological killing effects in heat-treated milk
Figure 2.7  Schematic diagram of cleavage of peptide fragments in FITC-casein assay
Figure 2.8  Luminescence Spectrometer (LS50B, Perkin Elmer)
Figure 2.9  Reaction of fluorescamine and amino group
Figure 2.10  Stephan Kettle
Figure 2.11  Advanced Rheometer Generation 2 (ARG-2)
Figure 2.12  Texture profile analyser (TA.XTplus)

Figure 2.13  A generalized TPA curve

Figure 2.14  Brookfield DV-I PRIME viscometer

Figure 2.15  CIE Chromaticity diagram

Figure 2.16  CIELAB uniform colour diagram representing relationship of red/green ($a^*+/-$) and yellow/blue ($b^*+/-$) opponent co-ordinates to lightness $L^*$, chroma $C^*$ and hue angle $h^*$

Figure 2.17  Minolta Chroma meter (CR-400)

Figure 2.18  Köhler illuminations

Figure 2.19  Optical Microscope (Leica DM 2500 M)

Figure 3.1  Standard curves of (a) FITC isomer 1 for the fluorescein isothiocyanate-casein (FITC-casein) assay and (b) Leu - Gly dipeptide for the fluorescamine assay

Figure 3.2  Growth of *Pseudomonas fluorescens* in NB at 4 ± 1°C. Each point is an average of two samples counted on NA plates incubated at 30 ± 1°C

Figure 3.3  Effect of UHT treatment on the activity of proteolytic enzyme in skim milk samples with added crude protease. Enzyme concentration ranged from 0%, (for the control sample) to 0.005% (% v/v). Where (◯) is before UHT treatment and (□) is after UHT treatment. n=2 and the vertical bars on the histograms represent the range in the data obtained

Figure 3.4  Relationship between the enzyme concentration (% v/v in raw skim milk) and the proteolytic activity (equivalent to FITC isomer 1 concentration in the FITC-casein assay) in UHT-treated milk. The FITC-casein assay involved an incubation period of 2h. The solid line represents the line of best fit.
Figure 3.5  Relationship between the enzyme concentration (%, v/v in raw skim milk) and the proteolytic activity (equivalent to fluorescein concentration in the FITC-casein assay) in UHT-treated milk. The FITC-casein assay involved an incubation period of 1 day (a) and 14 days (b) (Button et al., 2011).

Figure 3.6  Effect of storage time (at 20°C) on viscosity (a) and proteolysis of UHT–treated skim milk (b) with added crude protease enzyme (%, v/v) from *Pseudomonas fluorescens*: 0%, (control sample (♦)), 0.0001% (■), 0.0005% (▲), 0.001% (●), 0.005% (+)

Figure 3.7  Relationship between the concentration of free amino groups and the viscosity (at 20°C) in UHT-treated skim milk samples with added crude protease enzyme (%, v/v) of *Pseudomonas fluorescens*, where (a) was the lowest added enzyme concentration (0.0001%), and (b) was the highest added enzyme concentration (0.005%). The solid line represents the line of best fit.

Figure 3.8  The viscosity at the onset of gelation (a) and the corresponding free amino group concentration (b) of the UHT treated skim milk stored at 20°C, irrespective of the added crude enzyme concentration.

Figure 3.9  Relationship between the proteolytic activity and the onset of gelation (weeks at 20°C) of UHT–treated milk samples with added crude enzyme concentrations (%, v/v), namely: for 0.0001, 0.0005, 0.001, 0.005 (a), and for 0.0001, 0.001, 0.005 (b). The solid line represents the line of best fit.

Figure 4.1  Steady shear viscosity for the commercial dairy beverage (▲), and dairy beverage made at RMIT research laboratory according to the commercial formulation (■) at 4°C.
Figure 4.2  Steady shear viscosity for the commercial dairy beverage (▲), dairy beverage made at RMIT research laboratory according to the commercial formulation (■), and dairy beverages enriched with protein and dietary fibre produced at RMIT research laboratory with 3.5% Alacen 392 (whey protein concentrate), 2% inulin, 0.1% lecithin and 0.01% carrageenan (●), and 3.5% Alanate 180 (sodium caseinate), 0.1% lecithin, 0.01% carageenan, 2% inulin (♦) at 4°C

Figure 4.3  Steady shear viscosity for the commercial dairy beverage (▲), and dairy beverages enriched with protein and dietary fibre containing 3.5% Alanate 180 (sodium caseinate), 0.1% Lecithin, 0.01% carageenan, 2% inulin manufactured at RMIT pilot plant with UHT treatment (■), and high temperature pasteurisation using Stephan kettle (●) at 4°C

Figure 4.4  Sensory evaluation at hedonic scale (solid line) and assessment of intensity for the parameters of the dairy beverages (dash line): for the commercial dairy beverage (a); for the dairy beverage enriched with protein and dietary fibre (b); for the two beverages at preference scale (c); and for the two beverages assessing the intensity of the parameters (d). The commercial product is indicated as a red circles, whereas the novel formulation is presented as green diamonds

Figure 5.1  Steady shear viscosity (a) and elastic (G’, closed symbols) and viscous (G”, open symbols) moduli for the applied frequency range from 0.1 to 100 rad/s with 0.1% strain (b) for the commercial dairy dessert (♦), and dairy dessert made at RMIT research laboratory (■) and pilot plant (●) according to the commercial formulation at 22°C

Figure 5.2  Hardness (a) and adhesiveness (b) for the commercial dairy dessert, and dairy dessert made at RMIT research laboratory and pilot plant according to the commercial formulation at 22°C. n=2 and the vertical bars on the histograms represent the range in the data obtained
Figure 5.3  Steady shear viscosity for the dairy dessert made at RMIT research laboratory according to the commercial formulation (●), and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (♦), with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carrageenan, 0.6% HPDP (○), with 3% WPI, 7.1% Alacen 392, 0.37% gelatin, 0.6% HPDP (■), and 3% WPI, 7% Alacen 392, 0.37% gelatin, 0.04% carrageenan, 0.6% HPDP (▲) at 22°C.

Figure 5.4  Elastic ($G'$, closed symbols) and viscous ($G''$, open symbols) moduli for the dairy desserts made at RMIT research laboratory according to the commercial formulation (■,□), and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (♦,◊), with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carrageenan, 0.6% HPDP (▲,∆), with 3% WPI, 7.1% Alacen 392, 0.37% gelatin, 0.6% HPDP (●,○), and 3% WPI, 7% Alacen 392, 0.37% gelatin, 0.04% carrageenan, 0.6% HPDP (-,=) for the applied frequency range from 0.1 to 100 rad/s with 0.1% strain at 22°C with 0.1% strain at 22°C.

Figure 5.5  Hardness (a) and adhesiveness (b) for the dairy desserts made at RMIT research laboratory according to the commercial formulation, and protein-enriched formulations with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carrageenan, 0.6% HPDP, with 3% WPI, 7.1% Alacen 392, 0.37% gelatin, 0.6% HPDP, with 3% WPI, 7% Alacen 392, 0.37% gelatin, 0.04% carrageenan, 0.6% HPDP, and 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP at 22°C. n=2 and the vertical bars on the histograms represent the range in the data obtained.

Figure 5.6  Steady shear viscosity for the dairy dessert made at RMIT pilot plant according to the commercial formulation (●), and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (♦), with 6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatine, 2% HPDP (■), with 11% Lacprodan, 0.3% gelatin, 0.5% HPDP (-), with 2.2% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP (▲), and 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.6% HPDP and 0.04% carrageenan (+) at 22°C.
Figure 5.7 Elastic ($G'$, closed symbols) and viscous ($G''$, open symbols) moduli for the dairy dessert made at RMIT pilot plant according to the commercial formulation (●,○), and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (♦, ◊), with 6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatin, 2% HPDP(■,□), with 11% Lacprodan, 0.3% gelatin, 0.5% HPDP(▲,∆), with 2.5% sodium caseinate, 5.5 % calcium caseinate, 0.3% gelatin, 2.5% HPDP(▲,∆), and 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.6% HPDP and 0.04% carrageenan (▲,∆) for the applied frequency range from 0.1 to 100 rad/s with 0.1% strain at 22 °C.

Figure 5.8 Hardness (a) and adhesiveness (b) for the dairy dessert made at RMIT pilot plant according to the commercial formulation, and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatine, 0.5% HPDP, with 6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatin, 2% HPDP, with 2.2% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP, with 11% Lacprodan, 0.3% gelatin, 0.5% HPDP, and 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.6% HPDP and 0.04% carrageenan at 22 °C. n=2 and the vertical bars on the histograms represent the range in the data obtained.

Figure 5.9 Micrographs for the commercial dairy dessert (a), and protein-enriched formulations with 2.2% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP (b), with 6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatin, 2% HPDP (c), with 11% Lacprodan, 0.3% gelatin, 0.5% HPDP (d), with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (e), and with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.6% HPDP and 0.04% carrageenan (f) at 22°C (magnification is ×100).
**LIST OF TABLES**

**Table 1.1** Proximate composition of liquid milk (g/100g)

**Table 1.2** The heat stabilities of proteolytic enzymes extracted from *Pseudomonas fluorescens* isolated from raw milk

**Table 1.3** Effect of the growth of psychrotrophs in raw milk before heat treatment on the quality of dairy products

**Table 1.4** Effect of UHT processing type (Direct or Indirect) on age-gelation

**Table 1.5** Properties of the main hydrocolloids used in the dairy industry

**Table 2.1** Materials used for the shelf-life study of UHT-treated milk

**Table 2.2** Materials used for the development of protein-enriched dairy beverages and dairy desserts

**Table 2.3** Standard rheological parameters

**Table 2.4** Parameters measured by Texture Profile Analysis

**Table 3.1** Concentration of crude protease* added to raw skim milk samples for UHT treatment.

**Table 3.2** Skim milk quality

**Table 3.3** Estimated relationship between time for UHT-treated milk to gel and the activity of proteolytic enzymes present in the raw milk before UHT treatment.

**Table 3.4** Threshold proteolytic enzyme values for UHT-treated milk for age-mediated gelation from the literature.

**Table 4.1** Formulations of dairy beverages enriched with protein and dietary fibre made at the RMIT research laboratory and pilot plant
**Table 4.2** Effect of processing parameters on total bacteria count of the dairy beverage enriched with protein and dietary fibre made at the RMIT pilot plant

**Table 4.3** Colour parameters of the commercial dairy beverage and product enriched with protein and dietary fibre made at the RMIT pilot plant

**Table 4.4** Taste panel data (%) for (A) description of 4 sensory parameters on a 9 point scale for two products, namely: Commercial beverage (C), and protein & fibre-enriched formulation (EF)

**Table 4.5** Taste panel data (%) for (B) Product acceptability based on a hedonic scale (ranging from extremely like (9) to extremely dislike (1)) for two products, namely: Commercial beverage (C), and protein & fibre-enriched formulation (EF)

**Table 5.1** Formulations (percentage) of protein-enriched dairy desserts made at RMIT research laboratory

**Table 5.2** Formulations (percentage) of protein-enriched dairy desserts made at RMIT Pilot Plant

**Table 5.3** Percentage preference for taste panel one and taste panel two on hedonic scale score ranging from extremely like (9) to extremely dislike (1)
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ANZFA</td>
<td>Australia New Zealand Food Authority</td>
</tr>
<tr>
<td>ARG-2</td>
<td>Advanced Rheometer Generation-2</td>
</tr>
<tr>
<td>AUDS</td>
<td>Australian dollar</td>
</tr>
<tr>
<td>BCAAs</td>
<td>Branched chain amino acids</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony-forming units</td>
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<td>CIE</td>
<td>Commission Internationale de l'éclairage</td>
</tr>
<tr>
<td>CIP</td>
<td>Clean-in-place</td>
</tr>
<tr>
<td>cP</td>
<td>Centipoise</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DF</td>
<td>Dietary fibre</td>
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<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<tr>
<td>EU</td>
<td>European union</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture organisation</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FSANZ</td>
<td>Food Standards Australia New Zealand</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HPDP</td>
<td>Hydroxypropyl distarch phosphate</td>
</tr>
<tr>
<td>HTST</td>
<td>High temperature short time pasteurisation</td>
</tr>
<tr>
<td>IDF</td>
<td>International Dairy Federation</td>
</tr>
<tr>
<td>IUTM</td>
<td>Instron Universal Testing Machine</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilo gram</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilo Pascal</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Leu-Gly</td>
<td>Leucine-Glycine</td>
</tr>
<tr>
<td>LTLT</td>
<td>Low temperature long time pasteurisation</td>
</tr>
<tr>
<td>LVR</td>
<td>Liner visco-elastic region</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
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<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>Milli molar</td>
</tr>
<tr>
<td>mPa.s</td>
<td>Milli Pascal second</td>
</tr>
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<td>mths</td>
<td>Months</td>
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<td>Mw</td>
<td>Molecular weight</td>
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<td>N</td>
<td>Newton</td>
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<tr>
<td>n</td>
<td>Number</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient Agar</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ions</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NB</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td>NC</td>
<td>Not counted</td>
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<tr>
<td>ng</td>
<td>Nano gram</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National health and medical research council</td>
</tr>
<tr>
<td>nm</td>
<td>Nano meter</td>
</tr>
<tr>
<td>nM</td>
<td>Nano molar</td>
</tr>
<tr>
<td>NR</td>
<td>Not reported</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascal</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>Ps.</td>
<td><em>Pseudomonas</em></td>
</tr>
<tr>
<td>rad</td>
<td>Radian</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>RDI</td>
<td>Recommended daily intake</td>
</tr>
<tr>
<td>RMIT</td>
<td>Royal Melbourne Institute of Technology</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Spp.</td>
<td>Species</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>TCA</td>
<td>Tri chloro acetic acid</td>
</tr>
<tr>
<td>TPA</td>
<td>Texture profile analyser</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra-high temperature treatment</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight by volume</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight by weight</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WPC</td>
<td>Whey protein concentrate</td>
</tr>
<tr>
<td>WPI</td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
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<tr>
<td>κ</td>
<td>Kappa</td>
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## LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>$a^*$</td>
<td>Red-green axis, from –60 (green) to +60 (red)</td>
</tr>
<tr>
<td>$b^*$</td>
<td>Yellow-blue axis, from –60 (blue) to +60 (yellow)</td>
</tr>
<tr>
<td>$C^*$</td>
<td>Chroma</td>
</tr>
<tr>
<td>$G$</td>
<td>Modulus</td>
</tr>
<tr>
<td>$G'$</td>
<td>Shear storage modulus</td>
</tr>
<tr>
<td>$G^*$</td>
<td>Complex modulus</td>
</tr>
<tr>
<td>$G''$</td>
<td>Shear loss modulus</td>
</tr>
<tr>
<td>$h_{ab}$</td>
<td>Hue angle</td>
</tr>
<tr>
<td>$L^*$</td>
<td>Lightness axis, from 0 (black) to 100 (shiny)</td>
</tr>
<tr>
<td>$N$</td>
<td>RPM (In equation 2.4)</td>
</tr>
<tr>
<td>°C</td>
<td>Centigrade</td>
</tr>
<tr>
<td>$R^2$</td>
<td>Coefficient of determination for a regression curve or line</td>
</tr>
<tr>
<td>$R_b$</td>
<td>Radius of spindle</td>
</tr>
<tr>
<td>$R_c$</td>
<td>Radius of beaker</td>
</tr>
<tr>
<td>$\dot{\gamma}$</td>
<td>Shear rate</td>
</tr>
<tr>
<td>$\tan \delta$</td>
<td>Phase angle</td>
</tr>
<tr>
<td>$U$</td>
<td>Enzyme units</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Shear strain</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>Difference</td>
</tr>
<tr>
<td>$\Delta E$</td>
<td>Colour difference</td>
</tr>
<tr>
<td>$\Delta E^*_{\text{max}}$</td>
<td>Maximum possible colour difference</td>
</tr>
<tr>
<td>$\Delta H$</td>
<td>Hue difference</td>
</tr>
<tr>
<td>$\eta'$</td>
<td>Dynamic viscosity</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
</tr>
<tr>
<td>$\eta^*$</td>
<td>Complex viscosity</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Shear stress</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Frequency</td>
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</table>
CHAPTER 1
INTRODUCTION

1.1 Dairy industry in Australia

As the third largest agricultural industry, the revenue from the dairy industry contributes a gross farm gate value of over AUD$4 billion annually to the Australian economy. This industry produces in excess of 9.48 billion litres of milk annually with 38% of this being exported to predominantly Asian countries. These exports represent 7% of the world’s dairy trade. Of the total milk produced, 25% is consumed as drinking milk, whereas the rest is utilised for the manufacturing of other dairy-based food products (Dairy Australia, 2012).

1.2 Composition and nutritional quality of milk

1.2.1 Overall introduction

Bovine milk is a liquid product that contains a wide range of nutrients such as protein, fat, lactose, vitamins, minerals and water. It meets the nutritional requirements of neonates and thus provides an outstanding nutritional profile for the diet. It is often described as a well-balanced food (Jensen, Ferris & Lammi-keefe, 1991). The proximate composition of bovine milk is presented in Table 1.1. It should be noted that milk composition varies and is dependent on the breed, stage of lactation, season, hormone level, feed and intervals between milking (Auldist, Walsh & Thomson, 1998; Mackle, Bryant, Petch, Hill & Auldist, 1999).

Table 1.1 Proximate composition of liquid milk (g/100g) (Adapted from Deeth & Hartanto, 2009)

<table>
<thead>
<tr>
<th>Product</th>
<th>Water</th>
<th>Fat</th>
<th>Protein</th>
<th>Carbohydrate</th>
<th>Minerals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk</td>
<td>87</td>
<td>3.7</td>
<td>3.3</td>
<td>4.8</td>
<td>0.70</td>
</tr>
<tr>
<td>Skim milk</td>
<td>90</td>
<td>&lt;0.1</td>
<td>3.4</td>
<td>4.9</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Dairy products like milk are also a good source of calcium, phosphorus, magnesium and protein, all of which are essential for healthy bone growth and development. Adequate consumption of milk from early childhood and throughout life contributes to strengthening bones and as a result provides protection against ailments like osteoporosis (a debilitating, brittle bone disorder). The calcium and phosphorus in milk are also beneficial for the development and maintenance of healthy teeth. Studies suggested that milk also reduces the effect of cariogenic foods when consumed together in the diet. Moreover, several investigations also linked milk consumption with reduced risks of cardiovascular disease, type 2 diabetes, and colorectal and breast cancer (Fox, 2009).

1.2.2 Milk proteins

Proteins are naturally occurring compounds that are essential to all life processes as they perform a variety of functions in living organisms ranging from providing structure to reproduction. Milk proteins represent one of the significant contributions of milk to human nutrition. Furthermore, research and commercial activities have shown that many unique properties of milk proteins have been successfully utilised in the production of a range of dairy-based products, such as cheese, and fermented dairy products (Fox, 1989). This is despite the fact that bovine milk only contains approximately 3.5% protein which consists of two major fractions, namely: casein and whey protein in the proportions of 80:20, respectively. It is well understood that the nutritional and physicochemical characteristics of casein and whey protein varies significantly (Raikos, 2010). Key characteristics of the milk proteins are presented in the following sections.

1.2.1.1 Casein

Casein is a phosphoprotein that contains chemically bound phosphoric acid and is present in milk in the form of a calcium salt. This salt is a mixture of casein proteins that form a casein micelle. The diameter of casein micelles range from 50 to 500 nm (average of 120 nm) with molecular weights ranging from $10^6$ to $3\times10^9$ Da. The major components of casein proteins are $\alpha_s1$, $\alpha_s2$, $\beta$ and $\kappa$-caseins that represent about 38, 10, 35 and 12% of the phosphoprotein, respectively (Marchesseau et al., 2002). Some research (Dalgleish, 1998; Holt, 1992; Horne, 1998; Walstra, 1979) supports the idea that casein micelles consist of spherical sub micelles. However, Horne (2002) suggested a model that has been described as “dual-binding”. In that model, calcium phosphate nanoclusters and hydrophobic associations
play a main role in holding the micelle structure. What is particularly intriguing about the casein micelle is that some of its components are calcium sensitive i.e. they can be precipitated by calcium salts. Yet this does not occur in the calcium-rich milk. The reason is that the calcium sensitive components are ‘shielded’ from the calcium by the non-calcium sensitive components. This explains why $\kappa$-casein (a non-calcium sensitive component) is located on the outer surface of the micelles and the presence of spherical micelles.

However, the forms of casein proteins differ because of differences in genetic origin and variation in phosphorylation. Moreover, the major components of the casein proteins have different hydrophobic and hydrophilic regions along their protein chain. Unlike $\alpha$ and $\beta$ casein, $\kappa$-casein is less phosphorylated (one phosphate residue per molecule) which improves its stability and protects other caseins from precipitation (Phadungath, 2005). A study utilising immobilised enzymes also showed that the stability of casein micelles mainly depends on $\kappa$-casein which is located at the outer surface of the micelles (Fox, 1989), as stated above.

Studies show that the components of the casein form varies from one sub-micelle to another. Nevertheless, at the pH of raw milk (i.e. pH 6.6 ± 0.1) the casein micelle has a net negative charge providing electrostatic repulsion between the casein micelles (Helstad, Rayner, Vliet, Paulsson & Dejmek, 2007). Neutralisation of this charge occurs at the isoelectric point of milk, i.e. at pH 4.6, at which the casein micelles form a coagulum. This process is the basis of casein acid coagulation (Bringe & Kinsella, 1987; Tuinier & de Kruif, 2002). The casein micelles structures are very stable due to hydrophobic interactions, van der Waals interactions and salt bridges (Gaucher, Molle, Gagnaire & Gaucheron, 2008). Understanding the composition of casein and interaction among casein molecules is important as the characteristics of the casein micelle influences the organoleptic properties of milk (Fox, 1989).

1.2.2.2 Whey protein

As mentioned in the previous section, caseins account for 80% of the total protein whereas the remainder of the protein is whey protein (20%). There is growing interest in utilising whey protein as emulsifying and foaming agents in various food applications due to its versatile functional properties as well as its high nutritional values (de Wit, 1998).

Furthermore, Madureira, Pereira, Gomes, Pintado & Malcata (2007) have stated that whey protein might provide a wide range of biological functions such as antimicrobial activity, immune system modulation and transportation of vitamins. The major constituents of whey proteins are $\beta$-lactoglobulin, $\alpha$-lactalbumin, immunoglobulins and bovine serum
albumin. Among the constituents of whey proteins, $\beta$-lactoglobulin and $\alpha$-lactalbumin are highly sensitive to heat denaturation and responsible for the hydration, gelling and surface-active properties of whey protein ingredients (Damodaran, Parkin & Fennema, 2008).

$\beta$-lactoglobulin

$\beta$-lactoglobulin represents 50% of whey proteins which is approximately 12% of the total milk protein. $\beta$-lactoglobulin is a globular protein whose native structure is a dimer with two disulphide bonds and a free thiol group in the pH range of 5.0-8.0 (Cayot & Lorient, 1997) (Figure 1.1). The secondary structure of this protein has approximately 6.8% $\alpha$-helix, 51.2% $\beta$-sheets, 10.5% $\beta$-turns and 31.5% random coils (Damodaran et al., 2008). Upon heating, $\beta$-lactoglobulin tends to denature by losing its secondary structure and as a result, it can undergo aggregation to form a polymeric network (Fox, 2009).

![Figure 1.1 Tertiary structure of $\beta$-lactoglobulin (Papiz et al., 1986)](image)

$\alpha$-lactalbumin

$\alpha$-lactalbumin is the second major fraction of whey protein. It is comprised of 123 amino acid residues (Fox, 2009). Globular structure of $\alpha$-lactalbumin is stabilised with four disulphide bonds at pH range of 5.4 to 9.0 (Madureira et al., 2007).

Bovine serum albumin and immunoglobulin

Bovine serum albumin is found in bovine milk as a result of passive leakage from the blood stream. This globular protein possesses an oblate shape containing three domains which are stabilised by seventeen disulphide bonds. It has a molecular weight of 66.5 kDa comprising of 583 amino acid residues (Madureira et al., 2007). Bovine immunoglobulin’s accounts for 1.9 – 3.3% of the total proteins in milk. The main classes of immunoglobulins
found in milk are IgM, IgA and IgG which are classified as glycoproteins. The primary function of immunoglobulins is to provide passive immunity for neonate (Fox, 2009).

1.2.3 Native proteolytic enzymes

Milk contains about 70 indigenous enzymes with plasmin, an alkaline serine proteinase, being the most important proteolytic enzyme. Plasmin consists of precursor plasminogen, plasminogen activators which convert plasminogen to plasmin, and inhibitors for plasmin and plasminogen activators (Fox, 2009). Raw milk contains approximately 0.3 mg/L of plasmin and 2.7 mg/L of plasminogen. Even though the amount of plasmin and plasminogen in milk is low, they still play an important role in milk products (Datta & Deeth, 2001).

This is because plasmin is capable of hydrolysing α and β-caseins to cause the coagulation of milk (Politis, Lachance, Block & Turner, 1989). Furthermore, research revealed that pasteurisation and sterilisation temperatures had a little effect on plasmin. Hence, plasmin could retain about 4% of its residual activity after indirect UHT treatment at 140°C for 5 seconds (Datta & Deeth, 2001).

Consequently, it is expected that the heat stability of the proteolytic enzymes will potentially create aggregates in milk and dairy-based products during extended storage. However, these effects are governed by the concentration of plasmin present which is highly variable in milk and is dependent on the mammary glands, somatic cell count and the stage of lactation (Politis et al., 1989).

1.3 Heat treatment techniques of milk and dairy products

1.3.1 Overview of heat treatment techniques applied in dairy industry

The main objective for the application of heat treatment in dairy industry is to inactivate pathogenic and spoilage microorganisms in raw milk to a level at which they do not constitute a health hazard or / and limit the shelf-life. Heat treatment techniques have been widely used in the food industry such as cheese and yoghurt manufacturing, and production of evaporated and dried milk (Lewis, 1986).

According to the International Dairy Federation (IDF), pasteurisation is defined as “a process applied to a product with the object of minimising possible health hazards arising from pathogenic microorganisms associated with milk by heat treatment, which is consistent
with minimal chemical, physical and organoleptic changes in the product”. There are two types of pasteurisation techniques utilising different time-temperature combinations, namely; low temperature long time pasteurisation (LTLT) and high temperature short time pasteurisation (HTST) with time-temperature combinations of 63°C/30 min and 72°C/15 sec, respectively (Ledenbach & Marshal, 2009).

Unlike pasteurisation, sterilisation is capable of more extensive killing of microorganisms, including bacterial spores, which can compromise the shelf-life of a product. To that end, 30 min at 110°C (in-container sterilization), 30 sec at 130°C, or 1 sec at 145°C usually will suffice (Lewis, 1986). The latter two are examples of the so-called UHT (ultra high temperature) treatment. Aseptic packaging following UHT treatment is required to produce a “commercially sterile” product which should have an extended shelf-life. UHT treatment involves heating the product in a continuous flow process at a very high temperature over a short time (140°C/2-5 sec) followed by rapid cooling (Andrews, 1984).

Prolonged heating at 110°C for 30 min (in-container sterilization) causes extensive Maillard reactions, enzyme inactivation, formation of off-flavour compounds, loss of some vitamins, changes in the size of the casein micelle, and reduction in pH of around 0.2. By contrast, UHT process with a shorter time and a higher temperature profile (e.g. 145°C/1 sec) does not cause severe chemical reactions that would result in undesirable flavour, colour, and nutritive losses. Furthermore, this time-temperature profile does not inactivate all enzymes, such as plasmin, bacterial lipases and proteinases. Therefore, UHT process provides a similar sterilisation effect to that of in-container sterilisation, but with less nutritional losses and chemical changes (Datta & Deeth, 2007).

1.3.2 Application of different UHT methods and processing conditions

The development of UHT process begun in 1893 and in 1912 the first direct heating method was patented. However, the commercialisation of UHT products was hindered for long period due to the limitations of aseptic packaging systems (Chavan, Chavan, Khedkar & Jana, 2011). There are two heating systems used in UHT, direct and indirect heating, with the latter being more popular in the Australian dairy industry. The typical steps involved in UHT processing are preheating, holding at the preheat temperature, reaching sterilisation temperature, holding at sterilisation temperature, cooling and aseptic packaging. In addition, the homogenisation process can be carried out before preheating or after holding at sterilisation temperature (Datta, Elliott, Perkins & Deeth, 2002).
1.3.2.1 Direct UHT treatment

Direct heating includes two main methods, steam infusion and steam injection. In the steam injection method, the superheated steam is injected into the product, whereas in the steam infusion the product is sprayed as a thin film or fine stream into superheated steam in an infusion chamber. In direct heating process, the product comes into a direct contact with the heating medium, and as a result the product is diluted. This extra water is vaporised and removed (generally after sterilisation) when the heated milk is cooled very rapidly in a vacuum chamber. In order to ensure that there is no dilution of the final product, its temperature on leaving the flash vessel should be approximately the same as the preheated milk temperature. In practice, the total solids of the incoming product and the processed product are monitored and the temperature in the vacuum chamber adjusted (during commissioning of the plant) so that these total solids levels are equal (Datta et al., 2002; Lewis, 1986). Figure 1.2 illustrates time-temperature profile of plant utilising direct UHT heating. It is shown in that figure that the temperature of the product is raised almost instantaneously by transferring the latent heat of vaporization of the steam to the product, and afterwards the product is rapidly cooled in the vacuum chamber (Rerkrai, Jeon & Bassette, 1987).

Figure 1.2 Time temperature profile for direct UHT plant (Datta & Deeth, 2007)
1.3.2.2 Indirect UHT treatment

Indirect heating system involves no direct contact between product and the heating medium. Instead, it utilises a heat exchanger to transfer the heat across a partition between the product and heating medium. Heating medium used in this method can be either steam or super-heated water. The latter is preferred since it reduces the amount of burn–on and flavour impairment due to the smaller temperature difference between the heating medium and the product (Dentener, 1984). The time temperature profile of indirect heating is presented in Figure 1.3. In contrast to direct heating, indirect heating (plate and tubular) requires a longer time to heat the product from around 70°C to 140°C and to subsequently cool it to the same temperature as shown in Figure 1.3.

![Time temperature profile for indirect UHT plant](image)

**Figure 1.3** Time temperature profile for indirect UHT plant (Datta & Deeth, 2007)

Tubular and plate are the two types of heat exchangers generally used in indirect heating. Both heat exchangers are susceptible to burn-on (product burnt onto the surface) and fouling (insoluble deposit of the product reducing space for product flow) because of the contact of the product with the heating surfaces. However, the fouling is more pronounced in plate systems than tubular systems because of the narrower spaces between the plates. The deposit build-up restricts the flow of product and increases the backpressure that may exceed the safety limit of the gaskets. This increase in backpressure is less prominent in tubular heat exchangers due to their ability to accommodate more viscous deposit before the product flow is disrupted. Burn-on occurs due to an excessive temperature difference between the heating
medium and the product. Thus, preheating of product is commonly practiced to reduce the burn-on especially in indirect heating (Datta & Deeth, 2007).

In commercial plants, the tubular systems are more efficient compared with the plate heat exchangers as there is less frequent cleaning required. As explained earlier, tubular heat exchangers are more suitable for medium and high-viscosity products than plate heat exchangers. The cooling section is particularly vulnerable to these problems because the product’s viscosity increases as it cools (Datta et al., 2002).

The instantaneous heating profile in direct heating causes less chemical changes to the product compared with indirect heating, especially in relation to flavour and water-soluble vitamins. Therefore, direct UHT-treated products may have better consumer acceptance in terms of sensory attributes and nutritional value. However, indirect heating offers greater stability in relation to age-gelation and sediment formation (Datta & Deeth, 2007). It should be noted that one of the main disadvantages of direct heating is the high-energy cost caused by energy losses which cannot be recovered meaningfully by regeneration (Scott, 2008).

1.3.2.3 Combined direct-indirect heating systems

The most recent application of UHT process in industry is the combined direct-indirect heating systems. Combination of the two conventional methods provides advantages in food applications such as higher rate of heat regeneration and reduced chemical damage, especially flavour change and burn-on. Examples of commercial combination systems are the High-Heat Infusion™ system of APV (Fredsted, 1996; Fredsted, 1997; Jensen, 1996; APV, 1999; Kjaerulff, 2000) and the Tetra Therm® Aseptic Plus Two of Tetra Pak (Bake & Theis, 1995; Bake, 1997; Tetra Pak, 1999). In both systems, indirect pre-heating is extended to about 120°C compared with 80-90°C in conventional systems. High Heat Infusion™ uses steam infusion for the sterilisation step, whereas Tetra Therm® Aseptic Plus Two uses steam injection (Datta et al., 2002). The key features of the two systems can be summarized as follows:

- The high-heat section (>120°C) utilising direct heating allows high temperatures to be reached, up to 150°C, without causing excessive chemical change in the product but still allowing for the inactivation of heat-resistant spores, such as *Bacillus sporothermodurans*. 
• Energy recovery of ~75%, intermediate between indirect (~90%) and direct systems (~50%), thus, combination systems are more cost-effective than direct systems but less economical than indirect systems.
• Less fouling than in the indirect systems, which enables extended run times. This is because there is little or no contact with hot surfaces during high-temperature heating.
• Upstream homogenisation in High Heat Infusion™ system eliminates the need for aseptic homogeniser, vacuum chamber and pumps.

1.3.3 Advantages & disadvantages of UHT-treatment

In comparison to conventional pasteurisation methods, the key advantage of UHT products is their extended shelf-life at room temperature due to the fact that UHT treated products are commercially sterile. Consequently, this advantage reduces energy requirements during storage and distribution and provides a wide range of product options for industry such as flavoured milk, dairy desserts and sports drinks without introducing any additional preservatives (Scott, 2008).

Another advantage of UHT technology is its uniform product quality and the diverse range of packaging opportunities that is not practical for the in-container sterilisation method. Maillard reactions, which occur during in-container sterilisation and pasteurisation processes, cause an alteration in the important quality attributes of colour and flavour of the finished product, whereas UHT process, by comparison, results in less changes in these quality parameters (Dunkley & Stevenson, 1987). However, the high temperatures of UHT treatment can cause some changes in the structure of proteins (denaturation of some whey proteins) that can further affect the product’s functionality (Foster & Jelen, 1981; Jelen, 1983). A study by Ranieri (1979) indicated that formulations containing high protein, starch and other food hydrocolloids could exhibit considerable thickening upon processing and cause severe burn-on problems in the indirect UHT system with plate heat exchangers. These problems could be overcome by amending the product formulations and changing the process design e.g. direct steam infusion and tubular heat exchangers (Jelen, 1983).

The main drawback of UHT technology is the incomplete inactivation of heat-resistant proteolytic enzymes present in the raw milk, and consequently this leads to textural changes during storage of UHT dairy-based products as discussed in the next section.
1.4 Age-gelation and factors affecting age-gelation

The first question to address is what is age-gelation? It is visually identified as a congealing of the milk sometimes accompanied with off-flavours or off-odours (Garcia-Risco, Ramos & Lopez-Fandino, 1999) and is associated with UHT treated products. This ‘sounds’ no different from the general spoilage of milk. It is correct to state that raw or pasteurized milk also can and does undergo similar changes. However, those changes in pasteurized and raw milk have been created by a decrease in pH which in turn results in the denaturing and coagulation of the casein micelles. Age-gelation, by contrast, is not pH ‘driven’, in fact monitoring the pH can help distinguish milk gelation due to ‘age’ from that associated with raw and pasteurized milk spoilage. In age-gelation, the increase in viscosity only occurs after an extended storage (>3 weeks) period and if the milk has undergone ultra-high temperature (UHT) treatment (Garcia-Risco et al., 1999). Furthermore, as will be explained in the section titled ‘age-gelation mechanism’, the mechanism of gelation as it occurs in UHT milk and as it occurs in pasteurized milk is very different.

The salient point here is that the advantages conferred by UHT treatment, long shelf-life and room temperature storage, are the exact parameters that allow age-gelation to manifest itself.

1.4.1 Proposed mechanisms of age-gelation

It is accepted that when milk is heated to temperatures >55°C, globular β-lactoglobulin will unfold and in doing so the cysteine disulphide bonds are ruptured creating reactive thiol groups in their place (Edwards, Creamer & Jameson, 2009). These thiol groups are now capable of a number of activities such as, forming β-lactoglobulin aggregates or inter-molecular reactions including disulphide bonds between β-lactoglobulin and κ-casein (Chavan et al., 2011). It has been postulated that the consequence of the disulphide bond between β-lactoglobulin and κ-casein is a ‘flow on’ weakening of the interactions between κ-casein and the other caseins (β etc.) in the micelle resulting in a change in the conformation of the casein micelle (Anema, 2009).

The above reactions to heat as depicted in Figure 1.4 (1) are associated with all heated (>55°C) milks. It is used to advantage when manufacturing products like yoghurt which need to ‘trap’ a considerable amount of water relative to the amount of protein present. In that case, the bound β-lactoglobulin κ-casein as a unit is more effective at binding water than when the two components are separate (Lee & Lucey, 2004). However, the improved capacity for
binding water is not required of UHT treated milks. It is worth noting, that if the binding of \(\beta\)-lactoglobulin to \(\kappa\)-casein was the only effect to occur, there would not be a problem called ‘age-gelation’.

![Diagram of age gelation of UHT milk](image)

**Figure 1.4** Model of age gelation of UHT milk showing (1) formation of the \(\beta\kappa\)-complex, (2) its dissociation from micelles during storage, and (3) subsequent gelation of the milk through crosslinking of the \(\beta\kappa\)-complex (Adapted from Datta & Deeth, 2001)

Overall, any proposed mechanism of age-gelation needs to be able to explain (a) the source and (b) the cross-linking of free peptide groups to create the increase in viscosity associated with age-gelation in UHT milks. The source of free peptide groups, step (a), is two-fold, namely: naturally occurring enzymes called plasmins and their precursors, plasminogens (introduced in section 1.2.3) and/or proteolytic enzymes produced by bacterial activity (McMahon, 1996; Topcu, Numanoglu & Saldamlı, 2006).

The mechanism of action of these two sets of enzymes is similar but only in general terms. The general terms are that they both hydrolyse a component of or an attachment to the casein micelle, to produce a free peptide group which eventually cross-links to form a gel. But the point of hydrolysis in the casein micelle is not the same, the nature of the free peptide groups released is consequentially not the same nor are the characteristics of the gel formed. Which of the two enzyme systems (plasmin versus bacterial) play the more important role in causing age-gelation is not clear. This is because the bacterial enzymes’ proteolytic activity
extends to activating plasminogen to produce the active plasmin (Kohlmann, Nielsen, Steenson & Ladisch, 1991). For this reason as well as the fact that the bacterial enzymes are more heat resistant than the native enzymes (Driessen, 1983) and have their own direct proteolytic activity, that the bacterial proteases can be considered as the more important (of the two) causes of the ‘age-gelation’ problem. So, based on these points, the remaining sections will deal predominately with age-gelation as mediated by bacterial proteases.

The free peptides are referred to as the $\beta\kappa$-complexes (step 2 in Figure 1.4). The nature of the free peptides released by bacterial proteolytic activity and the remaining ‘casein micelle’ is subject to discussion. The lack of evidence of any major disruption to the casein micelle suggests that there is only a minor, if any, change to the casein micelle when the $\beta\kappa$-complex is released. So, it could be that the attachment of $\beta$-lactoglobulin to $\kappa$-casein due to heat is somewhat reversed by the action of the bacterial proteolytic enzyme with possibly a minor effect on the casein micelle. Yet again, if the release of $\beta\kappa$-complexes was the last step, the problem of ‘age-gelation’ would not occur.

As an aside, the activity of plasmin is somewhat different to that of the bacterial proteases in that there are considerable changes to the casein micelle on the release of the peptides and the peptides released are larger (than those released by bacterial proteases). The differences in the nature of the peptides released by the proteolytic activity and the subsequent changes to the casein micelle can help explain the differences in the appearance of the gels produced by these two enzyme systems. The gel descriptors are ‘custard like’ throughout the sample for the bacterial enzyme-mediated gel and curd-like at the surface for the plasmin-mediated gel (Harwalkar, 1982; Hardham, 1988).

The actual cause of age-gelation is the cross-linking of these $\beta\kappa$-complexes, the free peptides produced (step 3 in Figure 1.4). It is the cross-linking that creates the 3D network that is the gel. The mechanism or ‘drive’ to cross-link is not covered in any detail in the literature. The simplistic explanation would be that the ‘concentration’ effect could be at play here, i.e. more $\beta\kappa$-complexes leads to an increased chance of contact and therefore bonding. However, the link between proteolysis and gel formation is doubted by Datta & Deeth, (2001). They base their statement on the fact that at 40ºC the rate of proteolysis is the fastest (compared with 4 to 40ºC) whereas the gel formation rate is less than that at 30ºC (Manji, Kakuda & Arnott, 1986). There is the argument that at 40ºC, proteolysis maybe the fastest but the nature of that proteolysis is such that the free peptides produced are ‘impaired’. Datta & Deeth, (2001) explained that “lack of gelation at the higher temperatures may be due to the high degree of protein decomposition producing extensively degraded proteins which are unable to form a stable gel matrix”.

13
There are a number of proponents supporting an explanation of the mechanism for age gelation that is non-enzymatic mediated. The reason for this approach is the doubt surrounding the connection between proteolysis and gelation. The proposed mechanisms range from the Maillard reactions leading to the polymerization of casein with whey proteins (Andrews & Cheeseman, 1971), to changes in free energy of the casein micelles (Harwalkar, 1992). However, the mechanism that has been discussed and examined in the literature more extensively is the proteolytic enzyme-mediated age-gelation explanation.

Whether enzyme-mediated or not, the mechanism for the ‘age-gelation’ problem is vastly different from that associated with pH-mediated gels. Examples of pH-mediated gels includes the gelling associated with pasteurized milk as in the case of milk spoilage and the production of yoghurts or cheeses. The acidification of the milk results in the neutralization of the surface charge on the casein micelles (specifically κ-casein) reducing the repulsion that keeps the casein micelles apart (Bringe & Kinsella, 1987; Tuinier & Kruif, 2002). The source of the pH decrease can either be direct acidification or the bacterial activity of converting lactose (or added sugar) to lactic acid. The latter occurs in yoghurt manufacturing and milk spoilage. In hard cheese manufacturing, the acidification assists the activity of an added enzyme, rennet. The rennet in acid conditions is able to hydrolyse κ-casein from the casein micelle to produce para-κ casein. The casein micelle structure and the nature of the consequential gels formed in these pH-mediated (± rennet) gels are very different to that produced by the activity of the heat resistant proteolytic enzymes in UHT-treated milks. It should be noted that the chemistry of ‘age gelation’ could be occurring in pasteurized milk products, especially as this gelation can occur at 2ºC (Kocak & Zadow, 1985). However, at this temperature the reaction rate is likely to be very slow and to be eclipsed by the saccharolytic activity of bacteria resulting in a pH-mediated gel (increase in viscosity) being produced first.

Kocak and Zadow as early as 1985 showed, not unexpectedly, that the gelation rate was greater at 25ºC than at 4ºC. This supports the earlier statement that the advantages conferred by UHT treatment, such as room temperature storage, is the parameter that allows age-gelation to manifest itself, especially given the long-shelf life associated with UHT products.

1.4.2 Overall effect of ‘age-gelation’ on the commercial aspects of the dairy industry

UHT milks have been promoted to consumers and manufacturers as a safe product with a long shelf-life (unopened) that can be stored at room temperature. The change in
rheological properties (let alone any associated odour or flavour changes) produced during the approved shelf-life would be very ‘off-putting’ for the consumers or manufacturers and a breach either of the stated claims or raw material specification contract, respectively. The flow on effect is clear: a loss of consumer or manufacturer’s confidence in the product and or the supplier followed by a loss of sales etc.

That being said, the question is how the following can be predicted (a) Whether the raw milk is suitable for UHT processing in that it will result in a UHT product that has the required shelf-life at room temperature and (b) the shelf-life of UHT treated milk, reliably. Both of these questions are industrially significant as they go towards protecting the manufacturer of UHT dairy products in terms of their reputation and business viability.

A significant number of studies have focused on measuring or evaluating the free peptides produced during storage (Manji & Kakuda, 1988; Datta, Hillbrick & Deeth, 1999). This approach, although scientifically very valuable in terms of understanding the process of gelation in UHT treated milk, is not directly helpful to industry. This is because with this test, the expense of processing would have already been incurred, the product made and possibly distributed to customers only to find out that the UHT treated milk is not acceptable as it is likely to gel within the stated shelf-life. It brings to mind the saying, ‘closing the stable doors after the horse has bolted’.

So, what is needed is a meaningful parameter that can be measured in the raw milk before consigning the milk for UHT processing and setting a limit for that parameter which can ensure that the milk if UHT processed is able to produce the required shelf-life. Which parameter is meaningful and what limit should be set? The meaningful parameter is the easier of the two to identify; it is the proteolytic (heat-resistant) enzymes, both bacterial and native proteinases (plasmin and its precursor) present in the raw milk just before UHT processing. The justification for selecting this parameter is simple; the lower concentration of these heat-resistant proteolytic enzymes in the raw milk will equate to a lower concentration in the UHT treated milks, and as a result the production of less product (from their activity) i.e. less free peptide groups available to form a gel. This translates to a longer shelf-life before age-gelation occurs.

The method to measure the proteolytic enzyme concentration in the raw milk and thereby set a limit (maximum) is far from simple. The reason being that the enzyme level present in the raw (or UHT treated) milk is extremely low; in the ng/mL (Merck, 1999). The current methods used are far from satisfactory. As shown by Button, Roginski, Deeth and Craven (2011) using the most promising method, FITC-casein, found that up to 14 days incubation was needed to detect low levels of the enzyme (bacterial in this case). Clearly, this
is totally impractical as the level of enzymes in the raw milk would have increased as well as the quality of the milk would have deteriorated significantly in that time. Unfortunately, we are not able to do a PCR type test where we can increase the concentration of enzyme during the test; enzymes do not ‘duplicate’ as chromosomes can. It is beyond the scope of this master’s thesis to propose and examine possible alternative methods other than to suggest that what is required is a test that is as sensitive as the antigen-antibody test associated with the enzyme-linked immunosorbent assay (ELISA) test. However, the ELISA test would need to include the detection of a range of heat resistant proteolytic enzymes produced by bacteria associated with raw milk as well as the indigenous proteinases (plasmin and its precursor).

1.4.3 Involvement of psychrotrophic microorganisms in ‘age-gelation’

Earlier it was stated that the ‘culprit’ for age-gelation was proteolytic enzymes present or produced in the milk during storage prior to UHT processing (De Koning & Kaper, 1985). The enzymes mentioned were the heat-resistant enzymes produced by bacteria or native enzyme (plasmin and its precursor plasminogen). With respect to the bacterial sources of proteolytic enzymes, several studies have investigated extracellular proteinases from various organisms typically found in milk (De Koning & Kaper, 1985). Of these, the most common organism associated with age-gelation is the Gram negative non-sporeforming rod Pseudomonas fluorescens (Ps. fluorescens) (Tran, Datta, Lewis & Deeth 2008). Strains of Ps. fluorescens have been found to be able to produce extra-cellular proteolytic enzymes capable of causing age-gelation in UHT treated milk (Law, Andrew & Sharpe, 1977; Fairbairn & Law 1985).

Given that the organism, Ps. fluorescens is not heat resistant and will, therefore, not survive the UHT process, we are not interested in the organism per se – or are we? It is of interest to know the source of the organism, its growth conditions and most importantly at what stage can extra cellular proteolytic enzymes be produced, detected and associated with the problem of age-gelation.

This organism is classified as a psychrotroph (also called psychrotolerant) which means that it can grow at low temperatures of around 0-4ºC albeit slowly as it exhibits optimum growth at 20ºC (Fairbairn & Law, 1985; Gounot, 1986). However, unlike a psychrophilic organism which ‘likes’ cold temperatures, psychrotrophic organisms do not like the cold but they can cope (survive and grow). This means that if GMP practices are implemented, the organism will be growing but at a very slow rate. Where GMP involves following the industry requirement for storage and transportation of milk at refrigeration
temperatures of 4-7ºC (Azcona, Martin, Asensio, Hernandes & Sans, 1987; Sorhaug & Stepaniak, 1997). In terms of sources of these organisms, there is a problem as, unlike psychrophiles, psychrotrophs are not restricted to permanently cold habitats, they, in fact, are widespread in natural environments.

What about the production of the proteolytic enzymes? Law, Andrew & Sharpe, (1977) mentioned that the production of proteases by psychrotrophic bacteria occurs at the early stationary phase. To reach early stationary phase, the psychrotrophic count would have reached or exceeded 10⁶ cfu/mL of milk (O’Connor, Ewings, Hayward & O’Rourke, 1986). This count is only one log from deeming the milk to be spoilt largely because at a bacterial count of 10⁷ cfu/mL, the milk would most likely smell ‘off’. The significance of these results is that provided good manufacturing practices (GMP including low temperature storage) are in place, there should not be any bacterial proteinases present in the raw milk and therefore the corresponding UHT treated milk and consequently no or a reduced ‘age-gelation’ problem (Mahari & Gashe, 1989).

So, what is happening; is the industry practicing GMP including low temperature storage or not? The answer, in general, is in the affirmative. However the issues are (a) time before processing and or (b) a misconstruing of the information. There could be sufficient time for the psychrotrophs to grow if there are delays in collection, transportation and silo holding times (Azcona, Martin, Asensio, Hernandes & Sans, 1987; Sorhaug & Stepaniak, 1997). The misunderstanding is related to the interpretation of the association between bacterial growth and the detection of the proteolytic enzymes. The fact that the proteolytic enzymes were not detected until the early stationary phase for Ps. fluorescens, does not mean that the enzymes were not being produced earlier during growth. Given that their function is to hydrolyse the proteins in the growth medium (milk) in preparation for absorption of the products of proteolysis, these enzymes have to be present throughout growth; from the lag phase through growth to the stationary phase. How else could the organism survive and grow. So, in fact, we return to the issue of methods; the methods of measuring very low concentrations of these proteolytic enzymes is wanting.

What is particularly ‘odd’ is that an organism, Ps. fluorescens with an optimum temperature of 25ºC, that is not itself heat resistant is capable of producing heat-resistant extra-cellular proteolytic enzymes which are able to cause gelation in UHT processed milks. As mentioned, it is understood that the extra-cellular proteolytic enzymes are needed by the bacteria as a means of hydrolysing proteins before absorption of the amino acids etc. into the bacterial cell. But why are the enzymes heat resistant? Although, unexplainable, the evidence is undeniable; as the bacterial extra-cellular proteolytic enzymes are heat resistant (Malik &
Mathur, 1983; Azcona et al., 1987; Sorhaug & Stepaniak, 1997) at temperatures (and times) used for UHT processing as shown in Table 1.2.

Some strains of *Ps. fluorescens* produced proteolytic enzymes that were able to retain 90% of their activity after exposure to UHT processing. In general, it appears that as the processing temperature and times increase the residual activity of the enzymes decrease. This trend needs to be confirmed as this data is not based on comparative experiments. However, the key points that can be gleaned are that the proteolytic enzymes are still active after UHT processing (with one exception) and that the retention level is dependent on the strain. This pattern is shown by the data for 135°C/2sec where the residual activity ranged from 0 to 95% and at 145°C/30sec where the residual activity ranged from 10 to 60%. Furthermore, the dependence of the residual activity on the specific strain, suggests that the structural nature of the proteolytic enzymes produced is highly variable and therefore, increases the difficulty associated with measuring the enzyme level meaningfully, especially if a very precise test, like the ELISA, is to be used.

Table 1.2 The heat stabilities of proteolytic enzymes extracted from *Pseudomonas fluorescens* isolated from raw milk (Adapted from Datta & Deeth, 2001).

<table>
<thead>
<tr>
<th>Strain of <em>Ps. fluorescens</em></th>
<th>Heating Temp/time, °C/sec</th>
<th>Residual activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM41</td>
<td>135/2</td>
<td>95.8</td>
</tr>
<tr>
<td>OM2</td>
<td>135/2</td>
<td>91.3</td>
</tr>
<tr>
<td>OM82</td>
<td>135/2</td>
<td>89.7</td>
</tr>
<tr>
<td>OM186</td>
<td>135/2</td>
<td>76.3</td>
</tr>
<tr>
<td>OM228</td>
<td>135/2</td>
<td>62.2</td>
</tr>
<tr>
<td>OM227</td>
<td>135/2</td>
<td>0</td>
</tr>
<tr>
<td>MC60</td>
<td>149/4</td>
<td>&gt;90</td>
</tr>
<tr>
<td>NCDO-2085</td>
<td>140/4</td>
<td>70</td>
</tr>
<tr>
<td>B59</td>
<td>149/30</td>
<td>60</td>
</tr>
<tr>
<td>189</td>
<td>149/30</td>
<td>56</td>
</tr>
<tr>
<td>53</td>
<td>149/30</td>
<td>35</td>
</tr>
<tr>
<td>B12</td>
<td>149/30</td>
<td>27</td>
</tr>
<tr>
<td>51</td>
<td>149/30</td>
<td>10</td>
</tr>
<tr>
<td>P1</td>
<td>150/0.8</td>
<td>10</td>
</tr>
<tr>
<td>AH-70</td>
<td>150/0.6</td>
<td>10</td>
</tr>
</tbody>
</table>

So, in the next section, the issue of what can the industry do to reduce the risk of age-gelation occurring in their UHT treated milks will be discussed.
1.4.4 Commercial practices that can be used to moderate the problem of ‘age-gelation’

Industry, at this point in time, has no specification that can be used for deeming a batch of raw milk as suitable or otherwise for UHT processing i.e. the UHT product will not gel within the required shelf-life. This is because, as explained earlier, the extremely low proteolytic enzymes levels that are capable of causing age-gelation cannot be measured in a meaningful way in the raw milk before UHT processing. So, what can the industry do to reduce the risk of producing a product with an ‘age-gelation’ problem?

The ‘culprit’ in age-gelation is clearly the proteolytic enzymes. So, one needs to ask where is the connection between these proteolytic enzymes and the handling of milk as practised commercially. The commercial factors that are important for controlling or limiting age-gelation are those that contribute in some way to increasing the level of proteolytic enzymes in the raw milk or activity in the UHT-treated product. Unfortunately, that list of factors is quite extensive (Deeth & Datta, 2000), ranging from the microbial species and strains present in the raw milk, microbial load in the milk at the time of processing, milk composition (fat, casein micelle and β-lactoglobulin) including plasmin (naturally occurring proteolytic enzymes) levels, temperature history: pre-processing as well as UHT conditions (Kocak & Zadow, 1985; Champagne, Liang, Roy & Mafu, 1994; Datta & Deeth, 2001). With the exception of fat content, the lower the parameter level, the longer the time before there is any change in milk quality (viscosity and flavour) and the longer the shelf-life of the UHT milk.

Of the factors, listed above, the key factors would have to be (a) the temperature and time of storage on the farm, during transportation and in the silo at the manufacturing site and consequently (b) the initial microbial load at the time of UHT processing (Table 1.3).
Table 1.3 Effect of the growth of psychrotrophs in raw milk before heat treatment on the quality of dairy products (Adapted from Sorhaug & Stepaniak, 1997)

<table>
<thead>
<tr>
<th>Psychrotrophic count in raw milk (Log cfu/mL)</th>
<th>Product type</th>
<th>Effects on quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0-8.0</td>
<td>Pasteurised milk</td>
<td>Shorter shelf-life, increase fouling in heat exchanger</td>
</tr>
<tr>
<td>7.6-7.8</td>
<td>Yoghurt</td>
<td>Bitter, unclean or fruity flavours, depending on the specific micro flora.</td>
</tr>
<tr>
<td>7.5-8.3</td>
<td>Hard Cheese</td>
<td>Various flavour defects predominantly rancidity and soapy taste as well as reduced cheese yield</td>
</tr>
<tr>
<td>6.9-7.2</td>
<td>UHT milk</td>
<td>Gelation after 2-10 weeks, gradual development of lack of freshness, slightly stale, unclean, bitter flavour.</td>
</tr>
<tr>
<td>6.5-7.5</td>
<td>Hard Cheese</td>
<td>Rancidity</td>
</tr>
<tr>
<td>6.3-7.0</td>
<td>Milk powder, freeze dried milk</td>
<td>Reduced heat stability, increased foaming capacity of reconstituted milk.</td>
</tr>
<tr>
<td>5.9</td>
<td>UHT milk</td>
<td>Gelation &gt; 20 weeks</td>
</tr>
<tr>
<td>5.5</td>
<td>Pasteurised milk</td>
<td>Inferior flavour compared with that of pasteurised milk produced from raw milk</td>
</tr>
<tr>
<td>5.0-7.8</td>
<td>Cottage cheese</td>
<td>Significant correlation between psychrotrophic count in raw milk and bitter taste</td>
</tr>
<tr>
<td>Not determined</td>
<td>Butter</td>
<td>Faster development of rancidity in butter made from cold-stored milk than in that made from fresh milk, lipases from pseudomonas was active in frozen butter.</td>
</tr>
</tbody>
</table>
The basic point is that milk as stated earlier is very nutritious for us and also any bacterial contaminant. The initial microbial load in the raw milk would be controlled by the standard operating procedures for personal hygiene and plant cleaning and sanitation as implemented at the farm, for transportation and storage silos at the factory site; for specifically, the operators, the equipment used for milking, pumping the milk to and from the holding vats as well as the tankers and silos used. All of these come into direct contact with the milk and as a result have an opportunity to introduce bacteria.

It has been stated by O’Connor, Ewings, Hayward and O’Rourke (1986) that bacterial population in raw milk is important as extracellular enzymes have been shown to be produced when the cell count exceeds about $10^6$ cfu/mL. The point being made is that even when the initial microbial load is low, if the storage temperature or time is allowed to increase, this will result in bacterial growth (Malik & Mathur, 1983; Martins, Pinto, Rocha, de Araujo & Vanetti, 2006) during which these extra cellular proteolytic enzymes can be produced.

The choice of milk can also affect the likelihood of age-gelation occurring. An increase in levels of the native proteases (plasmin and its precursor plasminogen) have been associated with an increase in rate of age-gelation. These native proteolytic enzymes are associated with the somatic cells, the membranes of fat globules and tend to reach elevated levels in the case of mastitis or late lactation milk especially from older cows (Bastian, Brown & Ernstrom 1991). So, clearly these milks are not suitable for UHT treatment, if an extended shelf-life is required.

It has been identified that the composition of the raw milk is also a factor in age gelation. Lopez-Fandino, Olano, Corzo and Ramos (1993) indicated that the UHT products made using skim milk were more susceptible to age-gelation than those made from full cream milk. They went on to explain this observation in terms of increased availability of the substrate ($\beta$-lactoglobulin bound to casein) to the enzyme in the case of skim milk. That is, the presence of fat globules interferes with enzyme access to the substrate. It is as if the fat is acting like the ‘doctoring’ agents used to prevent sugar crystallization in confectionery products.

With respect to the UHT process, it has been shown (McKellar, Froehlich, Butler, Cholette & Campbell, 1984) that the direct UHT method results in a shorter shelf-life than UHT milk produced by the indirect method. As can be seen in Table 1.4, in general, age-gelation occurs in < 200 days (6.6mths) for UHT milk produced by the direct method whereas it takes >300 days (9.9mths) for age-gelation to occur in UHT milk produced by the indirect method.
Table 1.4 Effect of UHT processing type (Direct or Indirect) on age-gelation (Adapted from Datta & Deeth, 2001).

<table>
<thead>
<tr>
<th>UHT treatment</th>
<th>Processing conditions</th>
<th>Storage Temperature (°C)</th>
<th>Gelation time (Days)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indirect heat</td>
<td>138°C/ 2-5 sec</td>
<td>4-30</td>
<td>390, irrespective of temperature</td>
<td>Samel, Weaver &amp; Gammack, 1971.</td>
</tr>
<tr>
<td>treatment</td>
<td>139°C/ 1.5 sec</td>
<td>4</td>
<td>570</td>
<td>Andrews, Brooker &amp; Hobbs, 1977.</td>
</tr>
<tr>
<td></td>
<td>139°C/ 1.5sec</td>
<td>30-35</td>
<td>Not gelled in 840</td>
<td>Manji, Kakuda &amp; Arnott, 1986.</td>
</tr>
<tr>
<td></td>
<td>145°C/ 3sec</td>
<td>4, 22, 25, 37</td>
<td>Not gelled in 182</td>
<td></td>
</tr>
<tr>
<td>treatment</td>
<td>140°C/ 4sec</td>
<td>20</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

The explanation generally given for this observation is that the direct method requires a longer processing time (Figures 1.2 and 1.3) in particular the ‘come-up’ and ‘come-down’ times. It is in this additional time that more extensive bonding in terms of complexity or strength between the β-lactoglobulin and the casein micelle can occur. This additional change in bonding makes the casein-β-lactoglobulin less susceptible to proteolytic attack and thereby reducing the amount of βκ-complex released and available for the subsequent changes that lead to gelation. An alternative explanation for the difference in age-gelation time caused by indirect /direct heating is that the residual enzyme activity is reduced during the extra processing time associated with the indirect method. However, as the data on come-up and come-down processing times are not usually reported, this postulate cannot be substantiated. Manji and Kakuda (1988) explained the difference in terms of increased denaturation of whey proteins in the case of the indirect UHT treatment which ultimately reduces the formation of βκ-complexes.

In summary, until the mechanism(s) of age gelation is understood sufficiently to be able to set and efficiently measure a specification for the maximum level of heat-resistant proteolytic enzymes in raw milk (or other meaningful parameter), manufacturers are limited to the following strategies to reduce the risk of the problem of ‘age-gelation’ occurring within the specified shelf-life of their products (Jelen, 1983; Gaucher et al., 2008):

- Enforcing GMP of low storage temperatures and in particular short holding times on the farm and in the silos,
- Using full cream milk (if possible),
- Using indirect UHT processing,
- Processing at the higher temperatures and times,
- Increasing the come-up time (or come down time).
1.5 Fortification and production of dairy-based products

1.5.1 What is fortification?

Food fortification simply refers to the deliberate addition of extra nutrients to the food in order to improve its nutritional quality. According to the Codex Alimentarius Commission General Principles for the Addition of Essential Nutrients to Foods (1991), “fortification” is defined as “the addition of one or more essential nutrients to a food whether or not it is normally contained in the food, for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups”.

Food fortification has been used for almost ten decades to address micronutrient malnutrition such as vitamins A, D, several B vitamins, iodine and iron. It is regarded as a sustainable and effective approach to improve public health since it provides the required nutrients to a human body without transforming the eating or purchasing patterns of consumers (Crane et al., 1995). Food fortification can be categorised into three types, mass food fortification, targeted fortification and market-driven fortification. The differences are that mass fortification is nearly always mandatory whereas targeted fortification can be either mandatory or voluntary. On the other hand, market-driven fortification is always voluntary but is controlled by regulatory agencies (Allen et al., 2006; Rowe & Dodson, 2012).

By contrast, functional foods are foods that go beyond simple nutrition and have specific targeted actions. Various strategies have been implemented to develop functional foods, such as addition of probiotics, prebiotics, synbiotics (a mixture of probiotics and prebiotics) and ingredients that are very specific and have a very targeted action e.g. conjugated linoleic acids or polyunsaturated fatty acids (Gerdes, 2000).

1.5.2 Commercially available fortified dairy-based food products

Over the past years, there has been a growing demand for fortified dairy products, especially products with high calcium, vitamins and protein content. Food fortification was originally implemented to reduce the prevalence of nutrient deficiency diseases, but the focus has shifted to achieving optimal health and dietary intake (Turner, 2003). Dairy-based products are considered in Australia to be appropriate vehicles for fortification since they are consumed by most of the Australian population. Moreover, milk provides a significant amount of daily nutritional requirements (El-Kholy, Osman, Gouda & Ghareeb, 2011). Dairy-
based products represent a complex food matrix and consequently any fortification could affect the quality characteristics of the product, such as the physicochemical and sensory attributes. Moreover, fortification could also affect the financial feasibility of the final formulation in commercial terms (Münchbach & Gerstner, 2010).

There is a wide range of fortified dairy-based food products currently available in the market for specific target groups. Among all fortified dairy-based food products, the products that are enriched with calcium, iron and vitamins are the most common in many countries (Boccio et al., 1997). Flavoured milk, yogurts and drinking yoghurts are examples of functional dairy products that are commercially available. Products such as dairy desserts enriched with D₃ and B-group vitamins and calcium; flavoured dairy beverages with omega-3 fatty acids and protein, as well as breakfast cereals with added dietary fibre are well-received by consumers.

Functional dairy products have the potential to reduce the risks associated with cardiovascular disease such as high blood pressure and high blood cholesterols. For example, plant sterols/stanols are known to reduce the assimilation of cholesterols whereas omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can contribute to better functioning of the cardiovascular system (Hennessy, Ross, Stanton & Murphy, 2003). Kroger Active Lifestyle™ milk and Lifetime Low Fat Cheese are examples of dairy products with plant sterols inclusion (www.kroger.com). Furthermore, Kemps Plus Healthy Lifestyle milk (www.kemps.com), Farmland Dairies Special Request 1% Plus milk (www.farlandddairies.com), and Horizon Organic DHA Omega-3 milk (www.horizonorganic.com) are examples of dairy products that contain omega-3 fatty acids.

Improving the health of the digestion system (Digestive health) is positioned to become the largest segment of the functional foods market worldwide (Sloan, 2007). In Europe and Asia and more recently in the USA, probiotics and prebiotics are being added to an increasing number of foods, particularly dairy foods, to improve digestive (gastrointestinal) health and provide other health benefits. Examples are Dannon’s Activia low-fat yogurt containing *Bifidus regularis* (scientifically known as *Bifidus animalis* DN-173 010), with the claim that it “helps regulate the digestive system when eaten daily for two weeks” (www.activia.com); Yoplait’s Yo-Plus, a lowfat yogurt with a unique blend of probiotic bacteria (*Bifidobacterium lactis* BB-12) and a prebiotic (i.e. inulin) to “help naturally regulate digestive health” (www.Yo-Plus.com); Kraft LiveActive natural cheese snacks, cubes, and sticks with probiotic culture, *Bifidobacterium lactis*, and cottage cheese with 3g of the prebiotic fibre (inulin) in each 4-oz (113g) cup for digestive health (www.LiveActiveFoods.com).
1.5.3 Ingredients for fortification of dairy-based food products with protein and dietary fibre

Protein sources for the fortification of dairy-based food products can be divided into plant-based and animal-based proteins. Soy protein is the most commonly used plant-based protein for fortification (Hirpara, Jana & Patel, 2011). Although plant products contribute about 65% to the world supply of edible protein, none of the plant proteins provide optimal levels of all essential amino acids as do animal-based protein such as whey proteins (Young & Pellet, 1994). Protein quality, such as amino acid profile, digestibility and presence of inhibiting or biologically active components in the structure of the protein, is one of the main factors that ‘drive’ the novel formulation concept (Hoppe et al., 2008).

Milk proteins, the common animal-based proteins used for fortification, can be categorised into two groups, caseins and whey proteins. Commercially available milk protein products are full fat and non-fat milk powders, whey powder (whey protein concentrate and isolate), milk protein concentrate, and other milk protein products such as caseinates, rennet and acidic casein powders (Etzel, 2004). Whey protein is an excellent protein source not only because of its high protein quality (as defined previously) but also because of its high content of Branched Chain Amino Acids (BCAAs). Whey protein is separated from the casein using the physical method of microfiltration (thus, free from chemical reagents). Whey protein (acid and sweet) is obtained from whey which is a liquid by-product produced during cheese manufacturing. Acid and sweet whey are both by-products of cheese manufacturing. However, the source of acid whey is acid-coagulated cheese processing and the source of sweet whey is the manufacture of rennet-coagulated and hard cheeses (Walzem, Dillard & German, 2002).

Commercially whey protein sources are available in the forms of whey protein isolate (WPI), whey protein concentrate (WPC), hydrolysed whey concentrate and undenatured whey protein. Protein concentrations in those products vary with WPI having an average of 90-95% protein whereas WPC will have between 30 to 90% protein (Marshall, 2004). Whey proteins consist of several proteins including β-lactoglobulin (50%), α-lactalbumin (19%), bovine serum albumin (5%), immunoglobulin (13%), lactoferrin (3%) and others (11%). These proteins contain a full spectrum of essential amino acids including the BCAAs (leucine, isoleucine and valine) which possess specific biological activities (Cayot & Lorient, 1997). Generally, BCAAs are essential for tissue growth and repair, and leucine in particular plays a key-role in the translation-initiation of protein synthesis. Whereas, the sulphur-containing
amino acids, cysteine and methionine, contribute to the enhancement of immune function (Marshall, 2004). Whey proteins have a wide range of applications, e.g. in soft drinks, special dietary foods, infant foods, bakery products, confectionary, frozen desserts, food gels (yogurts, pudding), whipped products (topping and filling), and as a water-binding agent in meat products.

Caseins are high quality proteins that contains all the essential amino acids in high proportions with the exception of cysteine. Caseins with either sodium or calcium can form a water-soluble particulate known as caseinate. The principle of caseinate manufacturing is based on the neutralisation of the acid casein curd or dry acid casein with dilute alkali such as sodium, calcium, potassium and ammonium hydroxide (Augustin, Oliver & Hemar, 2011).

Caseinates are used in food formulations to modify and improve the physical properties of the final product as well as provide nutrition (Mulvihill, 1989). The caseinates have a substantial water absorption capability that has been increasingly valued in food manufacturing for modifying or/and improving the texture of dough or bakery, cheese products and soups. In addition, caseinates are widely used in whipping and foaming applications, as well as in emulsions due to their ability to form films (Kenny, Wehrle, Auty & Arendt, 2001).

Dietary fibre (DF) is defined as polysaccharides which can be found in plants but which be digested by the human gut. Based on the water solubility, DF can be categorised as soluble dietary fibre and insoluble dietary fibre (Malone, 2005). DFs play an important role in the consistency of food products, in which they perform such functions as thickening and gelling aqueous solutions, stabilising foams, emulsifying and suspending, slowing and completely preventing the crystallization of water and sugar (Dickenson, 1993; Phillips, 2000; Ohr, 2004). Therefore, DF can be incorporated into dairy beverages, processed foods, cultured dairy products and frozen dairy desserts. DF is currently in great demand with its multiple benefits of providing appealing textures, increasing calcium absorption and promoting intestinal bacteria growth, cardiovascular health, cancer prevention and weight management (Kaur & Gupta, 2002).

1.5.4 Quality characteristics of dairy beverages and desserts

Food quality is the quality characteristics of food that is acceptable to consumers. This includes external factors such as appearance (size, shape, colour, gloss and consistency), texture, and flavour; and factors such as the Federal grade standards (e.g. of eggs) and internal (chemical, physical, microbial) characteristics. Food quality is an important manufacturing
requirement, because consumers are sensitive to any form of contamination that may have occurred during the manufacturing process. Many consumers also rely on manufacturing and processing standards, particularly want to know what ingredients are present, as it is related to dietary, nutritional requirements (kosher, halal, vegetarian) or a medical condition (e.g., diabetes or allergies).

Colour of the product is recognized by basic human vision and significantly affects the consumer perception of quality. If the colour is unacceptable, the other two major quality factors, such as flavour and texture, are not likely to be judged at all (Francis, 1995). Size of the product can determine the loss of material during processing and thus, the final product yield. The parameter of size includes the weight, volume, diameter, area, surface area, perimeter, length, skeleton length and width of a product, whereas shape generally refers to the profile or physical structure of the object’s geometry. Texture can be correlated to the sensory properties of food products and also be used to determine the chemical or physical properties of food products and thus provide more information about the product than colour and size does. The importance of texture in the overall acceptability of foods is certainly unquestionable and considered to have a significant contribution to the overall quality of cereal and dairy-based products, bakery, confectionary etc. The textural properties of food are a group of physical characteristics arising from the structural elements of food that are sensed primarily by the feelings of touch. Furthermore, textural properties are related to the deformation, disintegration and flow of the food under a force, which is measured objectively using the functions of mass, time and distance (Bourne, 2002).

Nowadays, among the extensive array of commercially available dairy-based foods, dairy desserts and dairy beverages are very popular with the consumers as consumers associate them with a wide range of nutritional benefits and they also have pleasant sensory characteristics (Depypere, Verbeken, Torres & Dewettinck, 2008). The specific texture of the dairy-based product depends on the ingredients and their interactions within the food systems as well as the processing conditions applied during the manufacturing process of the product (Ta’rrega, Dura´n & Costell, 2005).

Modern technologies for food production include using various additives to improve the sensory (including textural) and other technological characteristics of products (Phillips, 2000). The term "hydrocolloids" includes polysaccharides and proteins that are widely used in dairy-based products and are associated with thickening or gelling functions in these products. Blends are used widely and most commercial preparations are a mixture of stabilising compounds (unless it is declared otherwise). The objective of blending these compounds together is to achieve a specific function or, in the majority of cases, to overcome one of the
limiting properties associated with a specific compound. For example, a single stabilising compound may be appropriate for the manufacture of fruit/flavoured yoghurt, but it may not be suitable for the production of frozen or dried yoghurt. Properties of some main hydrocolloids used in the dairy industry are presented in Table 1.4. In this study, chemically modified starch and carrageenan have been used to develop novel formulations for protein-enriched desserts and dairy beverages. Therefore, the chemical structure, applications of these hydrocolloids are discussed below.

**Starch** is a biopolymer naturally available as granules in plant tissues. It is a prime ingredient in many food formulations. Its use is on the increase because of the innumerable food applications which can result in a wide range of characteristics such as thickening, gelling, emulsifying, sweetener etc. (Voragen, 1998; Ahmad, Williams, Doublier, Durand, & Buleon 1999). Literature revealed that mainly starch in dairy desserts formulations is responsible for the body and mouth-feel of the product (Ranieri, 1979; Depypere et al., 2008).

Structure of the starch molecules is based on glucose units joined by glycosidic bonds. Starch is a mixture of two polymers; namely amylose and amylopectin. In amylose, glucose molecules are linked end to end by (1→4) glycosidic bonds and exist with a linear structure, whereas amylopectin is a highly branched molecule which consists of short chains of (1→4)-linked α-D-glucose with (1→6)-α-linked branches (Figure 1.5) (Jobling, 2004). However, the composition and structure of native starch granules varies and is dependent on the plant variety and storage conditions.

Nowadays, there is an excessive potential to be able to modify starch to obtain novel functionalities for industrial uses (Ahmad, Williams, Doublier, Durand & Buleon, 1999; Hermansson & Svegmark, 1996). Starch modification is the alteration of the native structure of starch by physical, enzymatic or chemical methods to enhance performance of the starch to provide increasing stability against high temperatures, shear, time, cooling and conditions below 0ºC resulting in the retention of the required physicochemical characteristics of the food matrix (Light, 1989). During the modification of starch, some of the hydrogen atoms in the hydroxyl groups of the starch molecules will be replaced with functional groups. Maltodextrin, di starch phosphate, hydroxypropylated starch and cross-linked maize starchers such as hydroxypropyl distarch phosphate (HPDP) can be found extensively used in the food industry.
Carrageenan is a colloidal sulphated polysaccharide obtained from sea weeds and mainly composed of repeating dimers of α (1→4)-linked D galactopyranose or 3,6 anhydro D galactopyranose and β (1→3)-linked D galactopyranose units. Based on the degree of sulfation, carrageenan has been categorized into three main groups namely κ-carrageenan, τ-carrageenan and λ-carrageenan (Figure 1.6). These sulphate groups are covalently bound via ester linkages on carbon atoms and consequently result in highly negative charged biopolymers (de Ruiter & Rudolf, 1997). The sulphate ester groups, the 3, 6-anhydrogalactose rings and their helix formation are essential for rheological properties of the respective carrageenans (Viebke, Borgstrom & Piculell, 1995).

Through the years, carrageenans, in particular κ-carrageenan, have been intensively investigated in fundamental research studies with regard to applications in dairy products. Specifically, the gelling properties and interactions between casein micelles and κ-carrageenan at different temperatures have been studied (Langendorff, Cuvelier, Launay & Parker, 1997). Thus, these studies revealed that the electrostatic interactions of negatively charged sulphate groups in κ-carrageenan with milk proteins (positively charged regions of casein micells) lead to a great variety of textures for the dairy-based products (Verbeken, Thas & Dewettinck, 2003).

Moreover, the broad quality characteristics of carrageenan such as thickening, gelling, texture enhancing, stabilizing etc., allow for its frequent use in dairy products to tailor the
Research indicated that the gel strength of dairy products mainly depends on the concentration of carrageenan (Verbeken et al., 2003).

**Figure 1.6** Schematic representations of different ideal structures of carrageenan (de Ruiter & Rudolf, 1997)
**Table 1.5** Properties of the main hydrocolloids used in the dairy industry (Adapted from Rapaille & Vanhemelrijck, 1998).

<table>
<thead>
<tr>
<th>Property</th>
<th>Alginate</th>
<th>Cellulose gum CMC</th>
<th>Locust bean gum</th>
<th>Xanthan gum</th>
<th>Gelatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility in milk</td>
<td>Insoluble Na(^+) swells in boiling milk; sol. with sequestering agents</td>
<td>Insoluble</td>
<td>Soluble above 85°C</td>
<td>Soluble Cold and hot</td>
<td>Soluble Above 40°C</td>
</tr>
<tr>
<td>Solution viscosity</td>
<td>Low above pH 5.5, high below pH 5.5</td>
<td>High</td>
<td>High up to 85°C</td>
<td>High below 100°C</td>
<td>Low</td>
</tr>
<tr>
<td>Optimum pH range</td>
<td>2.8-10.0</td>
<td>3.0-10.0</td>
<td>4.0-10.0</td>
<td>1.0-13.0</td>
<td>4.5-10.0</td>
</tr>
<tr>
<td>Effect on milk at neutral pH</td>
<td>Non insoluble</td>
<td>Precipitation</td>
<td>Separation</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Effects on milk and other proteins at acid pH</td>
<td>None</td>
<td>Adsorption to casein particles below pH 4.6</td>
<td>None</td>
<td>Precipitation below Iso-pH</td>
<td>None</td>
</tr>
<tr>
<td>Gelation conditions</td>
<td>pH below 4 or presence of Ca(^{2+}) 20-70 mg per g alginate.</td>
<td>Non-gelling</td>
<td>Non-gelling</td>
<td>Presence of LBG, tara gum, cassia gum, temperature below setting temperature</td>
<td>Temperature below setting temperature</td>
</tr>
<tr>
<td>Gel characteristics:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Texture</td>
<td>Calcium gels strong, brittle; thermo-irreversible</td>
<td>Non-gelling</td>
<td>Non-gelling</td>
<td>Cohesive, gummy, thermo-reversible; guar makes texture of xanthan LBG gel more brittle</td>
<td>Soft to strong, cohesive, gummy; thermo reversible</td>
</tr>
<tr>
<td>Setting temperature</td>
<td>Non-existent</td>
<td>Non-gelling</td>
<td>Non-gelling</td>
<td>Constant</td>
<td>Increase with increasing MW and maturing temperature</td>
</tr>
<tr>
<td>Gel strength</td>
<td>Increases with increasing Ca(^{2+}) concentration &amp; decreasing pH down to 3.6</td>
<td>Non-gelling</td>
<td>Non-gelling</td>
<td>Increase with increasing concentration</td>
<td>Increases with increasing concentration and decreasing salt</td>
</tr>
</tbody>
</table>
1.5.5 Methods for the production of fortified dairy-based food products

Processing stages of dairy-based food products include mixing, homogenisation and heat treatment before filling. As discussed earlier, heat treatment is a significant processing step during the manufacturing process of dairy-based products as it is the main stage at which microorganisms are inactivated. In particular, pathogenic and spoilage microorganisms in raw milk are reduced to a level at which they do not constitute a significant health hazard or reduce the required shelf-life, respectively. Examples of heat treatment commonly employed in the food industry are pasteurization and sterilization. Figure 1.7 depicts the flow diagram for the manufacturing of dairy-based food products.

Pasteurisation aims to reduce the number of viable pathogens so they are unlikely to cause disease (assuming the pasteurized product was stored as required and was consumed before its expiration date). As discussed earlier, there are two types of pasteurisation techniques used in the food industry; there is the low temperature long time pasteurisation (LTLT) or the high temperature short time pasteurisation (HTST) with temperature-time combinations of 63°C / 30 min and 72°C / 15 sec, respectively (Ledenbach & Marshal, 2009). By contrast, sterilisation aims to eliminate all forms of microbial life that is capable of growth in the product to create a commercially sterile product. This can be achieved by applying one or a combination of the following: heat (under pressure to reach >100°C), chemicals, irradiation and high pressure. To produce dairy-based products with extended shelf-life, the sterilisation techniques are commonly coupled with aseptic filling or packaging.
Figure 1.7 General flow diagram for the production of dairy-based food products
1.6 References


CHAPTER 2
MATERIALS & METHODS

2.1 Materials

Materials used in the shelf-life study of UHT-treated milk (Chapter 3) and in the investigations on the development of protein-enriched dairy beverages and dairy desserts (Chapters 4 and 5) are summarised in Tables 2.1 and 2.2, respectively.

Table 2.1 Materials used for the shelf-life study of UHT-treated milk

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier information</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure culture of <em>Pseudomonas fluorescens</em></td>
<td>Culture collection, RMIT microbiology laboratory.</td>
<td>Extraction of crude protease</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>Oxoid (CM0067)</td>
<td>Growth of <em>Pseudomonas fluorescens</em></td>
</tr>
<tr>
<td>Nutrient Agar</td>
<td>Oxoid (CM0003)</td>
<td>Enumeration of bacteria cells and agar diffusion method</td>
</tr>
<tr>
<td>UHT skim milk</td>
<td>Devondale</td>
<td>Agar diffusion method</td>
</tr>
<tr>
<td>Unpasteurised skim milk</td>
<td>Parmalat Victoria (Pauls Limited).</td>
<td>Raw milk for the UHT treatment</td>
</tr>
<tr>
<td>Type III FITC-Casein</td>
<td>Sigma (C0528)</td>
<td>FITC-casein assay</td>
</tr>
<tr>
<td>FITC isomer 1</td>
<td>Sigma (F7250)</td>
<td></td>
</tr>
<tr>
<td>Trichloro acetic acid (TCA)</td>
<td>Sigma (T6399)</td>
<td></td>
</tr>
<tr>
<td>Fluorescamine</td>
<td>Sigma (F9015)</td>
<td>Fluorescamine method</td>
</tr>
<tr>
<td>Trichloro acetic acid (TCA)</td>
<td>Sigma (T6399)</td>
<td></td>
</tr>
<tr>
<td>Leu-Gly dipeptide</td>
<td>Sigma (L9625)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 Materials used for the development of protein-enriched dairy beverages and dairy desserts

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Supplier information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocoa powder</td>
<td>Maltra Foods, Australia</td>
</tr>
<tr>
<td>Flavour (Natural vanilla extract)</td>
<td>Queens Fine food, Australia</td>
</tr>
<tr>
<td>Chocolate brown colour</td>
<td>CHR Hanson, Australia (CA-50,000D-WS)</td>
</tr>
<tr>
<td>Cherry Pink colour</td>
<td>Queen Food Colouring Company, Australia</td>
</tr>
<tr>
<td>Lecithin</td>
<td>Langdon, Australia (Centrolex G, 44235)</td>
</tr>
<tr>
<td>Inulin</td>
<td>Orafti, Belgium</td>
</tr>
<tr>
<td>Whey protein concentrate</td>
<td>Fonterra, New Zealand (Alacen™ 392)</td>
</tr>
<tr>
<td>Sodium caseinate</td>
<td>Fonterra, New Zealand (Alanate™ 180)</td>
</tr>
<tr>
<td>Skim milk</td>
<td>Pauls, Australia</td>
</tr>
<tr>
<td>Full cream milk</td>
<td>Pauls, Australia</td>
</tr>
<tr>
<td>Caster sugar</td>
<td>RMIT, Food Science lab</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>FMC, USA (Gelcarin GP379)</td>
</tr>
<tr>
<td>Hydroxy propyl di –starch phosphate (HPDP)-Modified Maize Starch</td>
<td>National Starch, Australia</td>
</tr>
<tr>
<td>Hydrolysed gelatin</td>
<td>Gelita, Australia (Gelita sol D)</td>
</tr>
<tr>
<td>Skim milk powder high heat</td>
<td>Fonterra, New Zealand</td>
</tr>
<tr>
<td>Whey protein isolate (WPI)</td>
<td>Fonterra, New Zealand (894)</td>
</tr>
<tr>
<td>Whey protein concentrate</td>
<td>Arla Foods Ingredient, Denmark (Lacprodan, DI-7017)</td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>CP Kelco, USA (Keltrol)</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Gelita Australia (High Bloom 25F)</td>
</tr>
<tr>
<td>Vegetable shortening</td>
<td>BASF chemical company, Australia (Cegepal TG 186)</td>
</tr>
<tr>
<td>Essential Flavours (Banana flavour)</td>
<td>Industrial sponsor</td>
</tr>
<tr>
<td>Colour</td>
<td>CHR Hansen, Australia (Vegex Lutein WSC50)</td>
</tr>
<tr>
<td>Calcium caseinate</td>
<td>Fonterra, New Zealand (Alanate™ 385)</td>
</tr>
</tbody>
</table>

2.2 Microbiological methodology used in the extraction of enzyme from *Pseudomonas fluorescens*

2.2.1 Culture Storage

To ensure the availability of viable culture at any stage of the experiment the cell storage is very important in any microbiological work. Nowadays, sophisticated methods such as freeze drying is available for bacterial culture storage, but agar slants are preferred for
medium term storage (Winters & Winn, 2010). The base material used in this study was a pure culture of *Pseudomonas fluorescens*. After a purity check, the culture was stored on nutrient agar slopes at 4°C and subcultured every month to maintain a viable culture. Medium to long term storage poses nutrient depletion problems in agar caused by organisms utilising the nutrients during their multiplication. This can stress the organism and lead to death. Therefore, it is important to subculture the cells before they are stressed.

### 2.2.2 Cell Growth

In general, individual bacterial cells grow larger only to divide into new individuals. Thus, the approach in a study of microbial growth requires defining bacterial cells not in terms of cell size but in terms of the increase in the number of cells which occurs by cell division. *Pseudomonas fluorescens* cells are classified as Gram negative aerobic rods (Meera & Balabaskar, 2012; Wong, Levi, Baddal, Turton & Boswell, 2011). Nutrient requirements for *Pseudomonas fluorescens* are similar to those required for general bacterial cell growth, namely: a source of carbon, nitrogen, water and appropriate atmospheric conditions (Battu & Reddy, 2009). In this study the cells of *Pseudomonas fluorescens* were transferred from an agar plate (Nutrient Agar, NA) into a broth (Nutrient Broth, NB) to enhance cell growth. The NB used in this study was comparable to meat infusion which contained nutrients such as nitrogen compounds, carbon compounds, vitamins and also some trace ingredients all necessary for the growth of bacteria.

The typical growth curve for microorganisms such as bacteria is illustrated in Figure 2.1. The lag phase occurs when the bacteria are transferred into a new medium with a different chemical environment and possibly under different temperature and atmosphere conditions than those the organism experienced in the previous medium. During this stage, there is no change in the cell numbers. This lag in division is associated with the cells’ physiological adaptation to the new environment, prior to their division. For each bacterial culture, the period of the lag phase depends on how the culture ‘copes’ with the differences in nutritional and atmosphere of the two growth media (the original versus the new medium). The change from lag to the exponential (log) phase is defined by the increase in cell numbers due to cell division in which the population doubles for every generation. The rate of doubling depends on the nutrient supply, atmosphere and temperature. Stationary phase refers to the stage where some cells can no longer multiply due to a growth-limiting factor such as nutrient depletion, and/or the formation of inhibitory products (e.g. organic acids). This phase is a
steady-state equilibrium where the rate of cell growth of some cells is exactly balanced by the rate of cell death by other cells. Stationary phase is followed by the death phase where the cells are unable to continue growing and multiplying and at this point the growth curve would show a downward slope (Al-Qadiri, Al-Alami, Lin, Al-Holy, Cavinato & Rasco, 2008).

![Graph of bacterial growth curve](image)

**Figure 2.1** Typical growth curve for Bacteria

### 2.2.3 Cell enumeration

Determination of cell numbers can be accomplished by a number of direct or indirect methods. The conventional methods for the enumeration of bacteria in food industry are colony count methods, either the spread-plate method or pour plate method (Jasson, Jacxsens, Luning, Rajkovic & Uyttendaele, 2010). In this study, the spread plate method was used to enumerate bacteria on NA plates after incubation. The common problem associated with the enumeration process is that the bacterial cells cannot be seen with the naked eye. However, when the bacterial cells multiply on a semi solid nutrient medium such as NA, the cells grow, due to limited mobility, in a restricted area referred to as a colony. This colony becomes visible to the naked eye when the cell count in the colony is \(>10^6\). Each colony, in reality, represents one cell in the broth being sampled. Therefore, by counting the colonies, in fact the cells are being counted and as a result the total number of viable cells is usually reported as colony-forming units (CFUs) rather than cell numbers.

According to the U.S. Food & Drug Administration 2001, the recommended number of colonies in individual NA plate is 25-250. The lower number is based the problem of errors
in small numbers having a significant effect whereas the upper limit is based on the fact that an agar plate of 9 cm in diameter will not support the growth of more than 250 CFU. Therefore in such situations, serial dilutions are required to avoid the underestimation of the actual cell number. A common diluent used in serial dilutions is saline (NaCl, 0.85% w/v) due to its isotonic nature. To obtain an accurate count of the cell number during enumeration, the dilution and plating processes are limited to a 20 min time period in this way cell multiplication and death during plating is avoid. After incubation, the colonies are counted, thus, the original number of viable cells can be calculated by taking into account the respective dilution plated.

The main aim of this microbiology study is the extraction of proteolytic enzymes from *Pseudomonas fluorescens*. For this purpose, pseudomonas cells, specifically *Pseudomonas fluorescens* cells, were grown in a NB culture and it was anticipated that during growth extracellular enzymes would be released into the NB. Although the optimum growth temperature for *Pseudomonas fluorescens* is 20ºC, it has been shown that most strains of *Pseudomonas fluorescens* could grow at low temperatures such as 4ºC (Griffiths, Phillips & Muir, 1981). Enzymatic activities, such as extracellular proteases and lipases, could vary if the bacterial cells are grown at different temperatures (Guillou & Guespin-Michel, 1996). Further, the enzymatic activities are dependent on the growth conditions such as the composition of the medium and the growth phase. However in this study, pseudomonas cells were grown at 4ºC to mimic the silo temperature used in the dairy industry. Because of the low temperature conditions used a question of ‘time’ was raised - how long should this culture be incubated before detectable proteases at 4ºC would be produced? Therefore, to address this question, the agar diffusion method was used periodically during the growth study to check for the production of proteases in the broth.

### 2.2.4 Agar Diffusion Method (ADM)

Agar diffusion method is commonly used in the study of antimicrobial activities (Valgas, de Souza, Smania & Smania, 2007) to determine the protein digestion activities of bacterial enzymes (Braun, Fehlhaber, Klug & Kopp, 1999). This technique is a simple assay for detecting the presence of extracellular proteases produced by microorganisms on agar plates. In this study, it was used to detect the proteolytic activity of the protease which could produce a visible clear area around the wells when they digested the substrate. The principle of this method is the movement of molecules through a matrix that is formed by the gelling of
agar. Substrate incorporated in the agar could vary according to the objective of the experiment. In this study, the agar plates were prepared by adding NA to UHT skim (1% v/v) milk (Chitra, Saravanan, Radhakrishnan & Balagurunathan, 2011) as the proteins in the UHT will form the substrate for any relevant proteolytic enzymes produced by the bacterial culture being tested.

To obtain the extract of crude protease in a cell-free broth, centrifugation which is based on the principle of relative centrifugal force (RCF) x g was used to sediment the bacterial cells. In general, the centrifugation can be measured in terms of revolution per minute (rpm) or relative centrifugal force (g). The conversion factor between rpm and g depends on the radius of the centrifuge rotor. The required g value is in turn dependent on factors such as particle diameter, density and viscosity of the medium. The centrifugation of the broth culture was carried out using a micro centrifuge (Sigma 1-14K, Figure 2.2) at ca. 6000 × g for 10 minutes to ensure the sedimentation of the growth of Pseudomonas fluorescens cells and to obtain a cell-free broth still containing any enzymes produced (Koka & Weimer, 2000). After introducing the enzyme extract into the wells of the agar plates, the inverted plates were incubated for 96 h at 20 ± 1°C. The clearing around the wells as illustrated in Figure 2.3 indicated proteolytic activity.

![Figure 2.2](image1.png)

**Figure 2.2** Micro centrifuge (Sigma 1-14K)

![Figure 2.3](image2.png)

**Figure 2.3** Agar diffusion plate; clear area around the well.
2.2.5 Extraction of protease from *Pseudomonas fluorescens*

To obtain a crude extract of protease produced by *Pseudomonas fluorescens* cells, the culture was introduced into nutrient broth (NB) and incubated at optimum growth temperature for 48 h to allow for meaningful cell growth (Koka & Weimer, 2000). Afterwards, centrifugation was carried out as described in section 2.1.3 to obtain a cells free broth. The washing of the precipitated cells was carried out using an isotonic diluent such as NaCl (0.85 % w/v), and this procedure was repeated several times to obtain cells free of broth. Subsequently, the bacterial cells (i.e. cells-free of NB) were introduced into a UHT skim milk sample and incubated at ca. 4°C (to mimic the silo temperature in industry) for 12 days (based on the results obtained from the growth of *Pseudomonas fluorescens* at 4°C in this study) to allow enzyme production. After incubation, the milk sample (with a bacterial culture) was centrifuged and sterilised using a Millipore filter sterilisation unit with a 220 nm membrane filter to ensure the removal of bacterial cells. The remaining filtrate could now be introduced into raw skim milk for the UHT storage trial as it now contained the crude protease (and other enzymes) but was free of any bacterial cells.

2.3 Ultra high temperature (UHT) treatment.

2.3.1 Preparation of milk samples with added crude protease

To determine the relationship between the level of proteolytic enzymes in the raw milk and the visible onset of age-mediated gelation in the UHT–treated milk, the enzyme (a crude protease extract from *Pseudomonas fluorescens*) was inoculated into raw skim milk (5L). Aliquots of the crude enzyme extract ranging from 0 (control) to 0.005% were used to produce 5 levels of enzyme concentrations designated as 0-5 in Table 3.1 of Chapter 3.

2.3.2 UHT treatment of raw milk with/without added crude protease

Ultra high temperature (UHT) processing involves sterilizing foods at high temperatures for short time, resulting in commercially sterile products under aseptic environmental conditions. Milk samples (with/without added protease) in this project were subjected to UHT treatment, and as a prerequisite, milk was pre-heated to 55 - 60°C and held at that temperature for 30 - 60 seconds to maximize the efficiency of homogenisation.
Homogenisation in dairy industry is an essential step to reduce the trend of fat separation during storage (Hayes & Kelly, 2003). It is a physical treatment used when emulsions require a high degree of dispersion. The process of homogenisation causes a reduction of the fat globule size (average size < 1µm) and spreads them uniformly, making the product homogeneous (Chandan, 2008). The principle of the homogenizer is that the product is passed through a narrow orifice under high pressure where the fat globules are effectively ‘broken open’, and reduced in dimensions thereby being dispersed as equal and smaller particles. In this study, a five-stage homogenization was carried out at 50 bars per stage using a FT9 homogeniser (Figure 2.4) after the pre-heating step.

Following homogenisation, the milk samples were processed with indirect UHT/HTST heat exchanger processing unit (Figure 2.5) at 135°C for 2 seconds. This unit is capable of processing samples at UHT flow rates of 10-20 L/h and possesses a high-speed mode, which is primarily used for the clean-in-place (CIP) operation. The high-speed mode can also be used for products that are processed at lower processing temperatures.

There are three main sections in the plate heat exchanger which are the preheating, heating and cooling sections. In indirect plate heat exchangers, there is no direct contact between the product and the heating medium. To conserve energy, the regeneration section uses the heat from the hot product (after processing) to preheat the cold incoming product. The product is brought up to the desired operating temperature in the heating section using pressurised hot water as the heating medium. In the cooling section, the pre-cooled product from the regeneration section is cooled with water. The size of the holding tube is important since it affects the holding time of the product at processing temperature. Generally, product
in this machine may be held at the processing temperature between 2 to 15 seconds depending on the flow rate. Minimum time-temperature requirements for the sterilisation of most resistant bacteria presented in milk are presented in Figure 2.6.

**Figure 2.5** UHT/HTST processing unit (FT74X, Armfield)

**Figure 2.6** Bacteriological killing effects in heat-treated milk (Kessler, 1981)
2.4 Analytical methods used to monitor protease level and activity during the shelf-life study of UHT milk samples

Fluorescein isothiocyanate-Casein assay and fluorescamine method are utilised to measure the proteolytic activity of added crude enzyme in skim milk (before and after UHT treatment) and the concentration of free amino groups presented in the milk samples during storage, respectively.

2.4.1 Fluorescein isothiocyanate (FITC)-Casein assay

Sutherland (1993) stated that the use of fluorogenic substrates for protease assays is an effective and efficient method in terms of sensitivity, accuracy and ease of handling in research and industrial application. Twining (1984) introduced fluorescein isothiocyanate (FITC) labelled casein for an assay of proteases which was based on trypsin and chymotrypsin activity. Later, Christen and Senica (1987) evaluated FITC-casein as a suitable substrate for the detection of the activity of bacterial proteases in milk. In this Thesis, the proteolytic enzymes were produced by *Pseudomonas fluorescens* which were introduced into raw skim milk. The activity of crude enzyme extract was measured using FITC-casein assay before and after UHT treatment.

The principle behind the FITC-casein assay is the quantitative determination of the activity of proteases presented in the sample. FITC labelled casein is used in an effort to mimic the natural substrate of the protease, which in this study the natural substrate for the proteases was a milk protein. In the intact substrate, casein is heavily labelled with FITC, resulting in significant fluorescence. The protease present will then act on this substrate leading to the hydrolysis of the peptide bonds and yielding fluorescent dye-labelled short peptides (Twining, 1984) (Figure 2.7).

![Figure 2.7 Schematic diagram of cleavage of peptide fragments in FITC-casein assay](image-url)
Accordingly, after introducing the enzyme (i.e. the milk samples with added crude protease being tested) to the substrate (FITC-casein), the mixture was incubated in the dark at 37°C for a minimum of 30 min (Christen & Senica, 1987). The sensitivity of the method can be improved with a longer incubation time, i.e. up to 24 h, however, this is not advised by the supplier as FITC stability is questionable for this period of time. This can result in the lack of detection of free peptides and therefore, an inaccurate estimation of the level of free peptides, anyway. Trichloroacetic acid (TCA) was used to precipitate the unreacted substrate and large peptides. After acidification, the reaction mixture was incubated for another 30 min (in the dark) to allow precipitation. Following that, the reaction mixture was centrifuged to separate the precipitated protein (Thompson, Saldana, Cong & Goll, 2000). In this study, the centrifugation was carried out at 13,000 × g for 5 min to ensure the precipitation of the remaining FITC-casein, large peptides, proteins present in the sample and to obtain a supernatant containing the acid soluble small peptides with FITC attached (Christen & Senica, 1987).

The supernatant was neutralized with a phosphate buffer and the fluorescence intensity of the FITC-labeled fragments was measured using a luminescence spectrometer (LS50B Perkin-Elmer, Figure 2.8). This spectrometer is a computer controlled luminescence spectrometer with the capability of measuring fluorescence, phosphorescence, chemiluminescence and bioluminescence. Fluorescence occurs when a molecule absorbs light photons from the UV-visible light spectrum and transitions into a high-energy state, known as excitation, and then rapidly emits light photons as it returns to its ground state. Based on that, fluorescence compounds can be identified and quantified by the intensity of their emission properties. In this study, fluorescence intensity was measured with an excitation wavelength at 490 nm and monitoring the emission wavelength at 515 nm to determine the activity of protease present. Control sample contained only the substrate and buffer in place of the enzyme (in this study it was milk sample containing crude protease of *Pseudomonas fluorescens*). The increase in intensity of the relative fluorescence is proportional to the degree of proteolytic activity of enzyme (Homer & Beighton, 1990).
In this investigation, various concentrations of the FITC isomer 1 were used to construct a standard curve. Fluorescein isothiocyanate (FITC) is widely used to attach a fluorescent label to proteins. The isothiocyanate group in the dye reacts with the amino terminal and primary amines in proteins to produce fluorescent label substrates (Manikwar, Zimmerman, Blanco, Williams & Siahaan, 2011). The degree of proteolysis was calculated by relating the intensity of fluorescence of each milk sample with the emission fluorescence intensity of FITC isomer 1.

2.4.2 Fluorescamine method

In this Thesis, Fluorescamine method was used to determine the concentration of free amino groups in UHT-treated milk with/without added crude protease during storage. Similar to the FITC-casein assay, this method is a fluorescence-based assay. However, the substrate in the Fluorescamine method is the free peptides already produced, as compared with the amount of protease present in the case of the FITC-casein assay. During storage of UHT milk with added crude protease, proteolytic enzymes act on the milk protein components and produce free amino groups. Concentration of the free amino groups varies according to exposure time and enzyme concentration. The principle behind this method is the rapid reaction of fluorescamine with the free amino groups present in the sample (Le, Datta & Deeth, 2006) (Figure 2.9).

Fluorescamine is a reagent used for the quantitative determination of proteolysis in the picomole range (Funk, Hunt, Epps, & Brown, 1986). Study by Garesse, Castell, Vallejo and Marco (1979) stated that fluorescamine-based assays could measure the initial step(s) of enzyme reactions, which facilitate the examination of the rate limiting parameters that control proteolytic activity and/or the detection of the activity of specific proteinases.
In this study, the initial acid precipitation was carried out using TCA to separate free amino groups present in the sample from the large peptides and proteins which in this case have been precipitated by TCA. After the filtration of the precipitate, the filtrate with acid soluble small peptides was neutralized with phosphate buffer and then mixed with fluorescamine solution. Fluorescamine itself is a non-fluorescent compound but when it reacts with primary amines in proteins, such as the terminal amino group of peptides (Figure 2.9), it forms highly fluorescent moieties (Funk, Hunt, Epps, & Brown, 1986).

\[
\text{Florescamine} \quad \rightarrow \quad \text{Fluorophor}
\]

**Figure 2.9 Reaction of fluorescamine and amino group (Held, 2006)**

So, the critical step is the TCA precipitation as any remaining compounds that have terminal amino group will react with the fluorescamine and give false-positive results. Similarly, if the TCA solution is excessive (concentration or exposure time), peptides could be precipitated resulting in a reduced response. Nevertheless, the simple addition of one reagent within a short incubation time allows for rapid determination of free amino groups present in the samples. Fluorescence of the solution was measured after 15 min of incubation using luminescence spectrometer with excitation at 390 nm and monitoring the intensity at the emission wave length of 475 nm. The concentration of free amino group shows a direct correlation with fluorescence intensity. The relative fluorescence units were observed from the luminescence spectrometer and converted to peptide concentration with reference to a standard curve prepared with Leu-Gly dipeptide.
2.5  Formulation principles and preparation of dairy-based food products

2.5.1  Formulation principles

Product development is a basic activity in the food industry. The strategy for product development requires decisions on the type of product to develop and the viability of the project for the company as follows:

- Will the product satisfy consumer needs and wants?
- Will the product be safe to consume?
- Is the product legal?
- What is the market and can the company service this market?
- Can the present market channels be used, or are new ones needed?
- Are the raw materials available?
- Will the production be accommodated in the present equipment, or is new equipment needed?
- What are the costs of further development, commercial production and marketing?
- Can the present storage and transport methods be used, or is a new method needed?
- What are the estimated profits and probabilities of success?

The typical outcomes of successful formulation engineering are the “new, improved” products. These novel formulation concepts can be advantageous from the economical prospect and completed within the relatively short development time that is required to accomplish the goals, such as replacement or removal of ingredients (Fuller, 2005).

Practically, the formulation design needs to incorporate the following aspects:

- Improvements in physical and sensory characteristics of a product to match the competitive products or to stay within the market trend.
- Enrichment with novel ingredients that have nutritional value.
- To overcome raw material problems in terms of either unavailability of raw materials or/and high raw material cost.
- Application of new technologies to improve processing.
- To meet the legal requirements for the product ingredients or additive.
- To create a new market niche for existing products (for example, custard products with lower calories) (Earle, Earle & Anderson, 2001).

For the last decades, requirements for the development of products enriched with protein and dietary fibre have gained considerable recognition in the food industry due to their
versatile features. Fortification of dairy products with protein and dietary fibre is well known in the food industry. Dietary fibre includes insoluble fibre (lignin, cellulose and hemicelluloses) and soluble fibre (pectins, β-glucans, galactomanan gums, and a large range of nondigestible oligosaccharides including inulin). Although soluble fibre is less common in foods than insoluble fibre, it is believed that they have important effects in the digestive and absorptive processes (Yangilar, 2013).

The sources of dairy proteins are classified into two main groups: casein-based ingredients (skim milk powder, sodium caseinate and calcium caseinate), and whey-based ingredients (whey protein isolate and why protein concentrates). Despite the nutritional benefits to food products which are enriched with protein and dietary fibre, the type and amount of the ingredients have been considered as a technological challenge – the challenge is to obtain the desirable textural and sensory profile for the final product.

2.5.2 Sample preparation of dairy beverages and desserts

Based on the aim of improving the nutritional value, dairy products were enriched with protein and dietary fibre to create various formulations for dairy beverages and dairy desserts which included whey protein concentrate and isolate, sodium and calcium caseinates, inulin and carrageenan. Sample preparation of both dairy beverages and desserts were carried out in two stages. In the first stage, the samples from various formulations were produced and analysed at the RMIT research laboratory. In the second stage, the formulation(s) that displayed similar textural behaviour to that of the commercial product were further scaled up in the RMIT pilot plant utilising different heat treatment techniques.

**Sample preparation at RMIT Food Science research laboratory.** Dry and milk ingredients were weighed separately and mixed together with a magnetic stirrer for 20 min at room temperature. Temperature of the systems was increased to 50°C and held for 6 min to ensure proper dissolving of the ingredients, followed by “pasteurisation” at 85°C for 5 min. The samples were cooled to 55°C, filled into plastic containers and stored at appropriate temperatures (4°C and -20°C for the dairy beverages and desserts, respectively).

**Sample preparation at RMIT Food Science pilot plant.** Based on the physicochemical and sensory characterisation of the enriched dairy beverages and dairy desserts prepared at the RMIT research laboratory, the selected formulation were scaled up using processing facilities available at the RMIT pilot plant which were similar to those used in the manufacturing process for the production of the commercial product. Dry ingredients were added into milk
ingredients and mixed with an electric mixer (Kitchen Aid, USA) for 20 min at room temperature. Temperature of the product was raised up to 50°C to ensure proper dissolving of the protein and fat in the formulation. After preheating, four-stage homogenization was carried out at 70 bars in each stage using FT9 homogeniser (Figure 2.3). The following heat treatment techniques were utilised for the production of dairy beverages and dairy desserts:

(i) Indirect UHT processing with plate-heat exchanger system (FT74X, Armfield, UK) at 140°C for 2 to 5 s (Figure 2.5).
(ii) Heat treatment at 85°C for 5 min using the Stephan Kettle (Figure 2.10)

The beverages or dairy desserts were filled into sterilized 250 mL glass containers in an aseptic environment and stored at the required temperature (4°C and -20°C for the dairy beverages and desserts, respectively) prior to physicochemical analysis and sensory evaluation.

![Stephan Kettle](image)

**Figure 2.10** Stephan Kettle

### 2.6 Rheology

#### 2.6.1 Principles of rheology

Rheology is the study of the deformation and flow of matter. In terms of food, “rheology is the study of the deformation and flow of the raw materials, the intermediate products, and the final products of the food industry” (Bourne, 2002). Rheological properties are determined in relation to the stress applied on a material and the subsequent deformation as a function of time. Key parameters involved in rheological measurements are stress and strain. Stress is the force applied per unit area and the type of stress is determined by the
direction of the force on the surface which normally can be either an extension or compression force. Stress is expressed in units of Pa or N.m\(^{-2}\) and conventionally denoted by \(\sigma\). On the other hand, the extent of deformation is represented by the strain (Tabilo-Munizaga & Barbosa-Ca’novas, 2005).

Based on their mechanical behaviour, materials can be divided into two groups, namely: solids and liquids. For ideal solids, the resistance is independent of the rate of deformation, but increases linearly with the extent of deformation. The ratio of stress/strain is known as the ‘modulus’ \((G = \text{stress/strain})\). For ideal liquids (Newtonian liquids) viscosity is independent of shear rate and the constant ratio of shear stress to shear rate is known as the steady shear viscosity \((\eta = \text{shear stress/shear rate})\). These responses from materials are extreme and only occur for an ideal solid or liquid. In reality, most of the materials demonstrate combined solid-like (elastic) and liquid-like (viscous) behaviour, giving rise to ‘viscoelasticity’. According to Dorbraszczyk and Morgenstern (2003), small deformation dynamic oscillation is one of rheological techniques that can be used to characterise the viscoelastic properties of materials as a function of time, temperature, strain and frequency.

### 2.6.2 Small deformation dynamic oscillation

Knowledge of the desired textural properties of food is essential for formulation engineering since the textural properties are created by the structural characteristics of the key elements (e.g., gelation, melting) of the product. Moreover, it is relevant to obtain information about the viscoelastic properties of the developed system and compare it to the required profile (Vliet, 1999). Rheological characterisation of viscoelastic properties includes small deformation dynamic oscillation and large deformation techniques. In large scale deformation, the geometry applies continuous unidirectional shear to the sample, thus, it will damage the structure of the sample. By contrast, the geometry occurring in dynamic oscillation causing small scale deformation moves in two directions (bi-directional) and only applies small strain to the sample thus preserving the network structure of the material.

In this Thesis, the degree of viscoelastic characteristics of dairy desserts and dairy beverages was quantified by the technique of dynamic small-scale deformation using ARG-2 from TA Instruments (Figure 2.11). As described by the manufacturer, ARG-2 is a controlled strain rheometer with combined motor and transducer equipment requiring smaller sample volumes and good resolution at low frequencies. Furthermore, this rheometer is capable of
delivering accurate rotational motion over a wide range of angular displacements and speeds (TA instruments, n.d-b).

![Advanced Rheometer Generation 2 (ARG-2)](image)

**Figure 2.11** Advanced Rheometer Generation 2 (ARG-2).

For pure elastic samples, stress is in phase with the strain, whereas for Newtonian liquids stress is 90° out of phase with the strain. Most of the food systems with viscoelastic properties have both elastic (solid) and viscous (liquid) components where the shear stress lies between 0° and 90°. In such systems the stress component which is in-phase with shear strain is responsible for the elastic component and is defined as the storage modulus (elastic modulus, $G'$). The magnitude of the storage modulus is proportional to the number of permanent interactions and the strength of the interactions present in the sample. Thus, $G'$ is a measure of the structure of the sample. On the other hand, the stress factor which is 90° out of phase represents the viscous component where the shear strain is defined as the loss modulus (viscous modulus, $G''$) and measures the flow properties of the sample (Bourne, 2002; Vliet, 1999).

One of the important parameters derived from dynamic oscillatory experiments is the complex modulus ($G^*$) which refers to the overall resistance of the sample to the applied deformation and is defined as $G^* = (G'^2 + G''^2)^{1/2}$. The phase angle or $\tan \delta$ is a parameter that is associated with the degree of viscoelasticity of the sample, $\tan \delta = (G''/G')$. Thus, viscous samples are characterised with $\delta$ being close to 90°.
In this study, strain sweep was implemented using a strain from 0.1 to 100% to determine the extent of the linear viscoelastic range of the network. Within the region of viscoelasticity, the magnitudes of stress and strain are linear, producing constant values of storage and loss modulus for a range of strains. Once the linear viscoelastic region (LVR) was established, frequency sweep test were carried out to understand the physicochemical properties of our samples as a function of frequency at a constant strain and temperature. Derived from this test, parameters such as complex viscosity (η*) and tan δ provide evidences for the nature of samples (dilute solution, entangled solution, weak or strong gels). The above mentioned principles in dynamic oscillation in shear will be used extensively to elucidate the viscoelastic behaviour of dairy dessert in this thesis.

### Table 2.3 Standard rheological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Symbol</th>
<th>Units (SI)</th>
</tr>
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<tbody>
<tr>
<td>Shear stress</td>
<td>Force per unit area</td>
<td>σ</td>
<td>Pa</td>
</tr>
<tr>
<td>Shear strain</td>
<td>Relative deformation in shear</td>
<td>γ</td>
<td>-</td>
</tr>
<tr>
<td>Shear rate</td>
<td>Change of shear strain per unit time</td>
<td>γ̇</td>
<td>s⁻¹</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Resistance to flow</td>
<td>η</td>
<td>Pa.s</td>
</tr>
<tr>
<td>Shear storage modulus</td>
<td>Measure of material elasticity</td>
<td>G’</td>
<td>Pa</td>
</tr>
<tr>
<td>Shear loss modulus</td>
<td>Ability of the material to dissipate energy</td>
<td>G”</td>
<td>Pa</td>
</tr>
<tr>
<td>Complex viscosity</td>
<td>Resistance to flow of the sample in the structured</td>
<td>η*</td>
<td>Pa.s</td>
</tr>
<tr>
<td></td>
<td>state, originating as viscous or elastic flow</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>resistance to the oscillating movement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynamic viscosity</td>
<td>Internal friction of liquid</td>
<td>η</td>
<td>Pa.s</td>
</tr>
<tr>
<td>Phase angle</td>
<td>Degree of viscoelasticity</td>
<td>tan δ</td>
<td>-</td>
</tr>
</tbody>
</table>

#### 2.6.3 Texture profile analysis (TPA)

Texture is a sensory property encompassing many attributes of a product which are based on the molecular structure of the food. Generally the evaluation of texture is based on the judgement of sensory panels. However, using an instrument such as texture profile analyser (TPA) provides a fast and cost effective way to measure the texture under well-defined and control conditions. (Szczesniak, 2002).

The concept of texture profile analysis was initially introduced by General Foods in the mid-1960s and later it was adapted by Instron Universal Testing Machine (IUTM)
(Meullenet, Lyon, Carpenter & Lyon, 1998). TPA is a simple and popular method for determining the fracture properties of foods using uniaxial compression that relates to its sensory properties as detected by humans and imitates the mastication action of the human mouth (Rosenthal, 2010). Testing procedure for TPA is based on the concept of large scale deformation and is used to measure the mechanical characteristics of either solid or semi-solid food. In this study, TA.XTplus texture profile analyser was implemented to evaluate the texture of the dairy desserts (Figure 2.12).

![Figure 2.12 Texture profile analyser (TA.XTplus)](image)

Testing conditions or parameters that affect the results of the compression test are shape and size of the sample (in this study, height and diameter of the sample), diameter of the probe, degree of deformation and speed of the compression (Breene, 1975). In the present work, the sample was placed on a base plate and compressed to 80% at a speed of 1 mm/s. The developed force required for the compression is recorded as a function of distance (deformation) and time. Mechanical parameters, such as hardness, firmness, softness, crispiness that are on a scale of resistance to the applied compressive forces, were interpreted and quantified from the resulting force deformation curve (Figure 2.13). Parameters that could be identified by using the TPA are listed in Table 2.4.
To identify the texture of the dessert in this study, we considered TPA parameters such as hardness and adhesiveness. Hardness shows the strength of the network which is related to the force required to compress food between the tongue and the palate (for soft samples, like yoghurt) and molars (for hard samples). By contrast, adhesiveness is a parameter that describes the consistency of product that measures the work needed to pull the probe out of the sample which can be closely related to the force required for removing the material that adheres to the mouth during eating.

In this experiment, a cylindrical aluminium probe of 25 mm was driven into a larger ring (40 mm diameter) to achieve the comprehensive deformation of 80% during the first compression cycle. In many instances hardness correlates with the break of gel strength and expressed in units of force (N). However, in this case, the stress goes through its maximum value and is calculated by dividing the force with the surface area of the probe as shown in equation (2.1).
Table 2.4 Parameters measured by Texture Profile Analysis (Rosenthal, 1999).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensorial definition</th>
<th>Instrumental definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness</td>
<td>Force required to compress a food between the molars</td>
<td><img src="image" alt="Hardness graph" /></td>
</tr>
<tr>
<td>Elasticity</td>
<td>The extent to which a compressed food returns to its original size when the load is removed</td>
<td><img src="image" alt="Elasticity graph" /></td>
</tr>
<tr>
<td>Adhesiveness</td>
<td>The work required to pull the food away from a surface</td>
<td><img src="image" alt="Adhesiveness graph" /></td>
</tr>
<tr>
<td>Cohesiveness</td>
<td>The strength of the internal bonds making up the food</td>
<td><img src="image" alt="Cohesiveness graph" /></td>
</tr>
<tr>
<td>Brittleness</td>
<td>The force at which the material fractures. Brittle foods are never adhesive</td>
<td><img src="image" alt="Brittleness graph" /></td>
</tr>
<tr>
<td>Chewiness</td>
<td>The energy required to chew a solid food until it is ready for swallowing</td>
<td>$= \text{Hardness} \times \text{Cohesiveness} \times \text{Elasticity}$</td>
</tr>
<tr>
<td>Gumminess</td>
<td>The energy required to disintegrate a semisolid food so that it is ready for swallowing.</td>
<td>$= \text{Hardness} \times \text{Cohesiveness}$</td>
</tr>
</tbody>
</table>

Stress (Pa) = Force (N) / Area (m) \hspace{1cm} (2.1)

The mechanical behaviour (hardness) was evaluated by expressing data in stress (kPa) versus strain. Percentage of strain can be calculated as follows:

\[
\% \text{ Strain} = \left(\frac{l}{L}\right) \times 100 \hspace{1cm} (2.2)
\]
Where $L$ is the initial height of the sample, and $l$ is the distance travelled by the probe into sample and is calculated by multiplying the experimental time with the compression speed (equation 2.2).

Following the compression cycle, the force was removed from the sample as the machine's crosshead moved back to its original position. Since dairy desserts are adhesive, the value of force becomes negative. The area of this negative peak was taken as a measure of the adhesiveness of the sample. There are no real units for this parameter, which is expressed in the internal integrator units of the computer.

2.6.4 Viscosity determination using Brookfield viscometer

Viscosity is a measure of a fluid’s resistance to flow which is dependent on the physicochemical characteristics of liquid and the temperature of measurements. Brookfield viscometer is an instrument that employs the principle of rotational viscometry based on large deformation testing procedure (Brookfield Engineering Laboratories, Figure 2.14). In this work, the viscosity of the sample was monitored during shearing of the product at a constant shear rate. During the testing procedure, when the spindle is immersed in the liquid, the viscometer provides information on the force required to rotate the spindle in a fluid which is expressed as torque percentage and indicates the viscosity of the sample in cP (1cP = 1mPa.s). Viscosity of the sample can be measured by utilising a series of rotation speeds (0.3, 0.6, 1.5, 3, 6, 12, 30, 60 and 100 RPM) and various spindle combinations (Bourne, 2002). Afterwards, the viscosity of the sample is expressed as a function of shear rate which is calculated using the equations 2.3 to 2.5 as follows:

\[
\text{Shear rate} = \frac{(2\omega R_c^2 R_b^2)}{(x^2)} (R_c^2 - R_b^2)
\]  

(2.3)

where,

\[
\omega = \left(\frac{2\pi}{60}\right) \text{N},
\]

(2.4)

and,

\[
x = \frac{(R_c - R_b)}{2},
\]

(2.5)

where N is RPM, Rb is the radius of spindle (cm), and Rc is the radius of beaker (cm).

According to the manufacturer’s information, Brookfield viscometer has an accuracy scale of ±1% for any spindle/speed rotation when the torque is between 10 - 100%. If the torque is higher than 100%, the machine will display “EEE”, and if it is less than 10%, the cP
and torque displays will start blinking and the viscosity value for the negative torque will be displayed as “---”. Once the viscosity reading reaches its maximum value, it will stabilise for few seconds and then starts decreasing. It is always recommended to record the maximum stabilised reading of viscosity. To perform appropriate Brookfield measurements, for a particular sample a correct spindle and speed combination should be chosen. In general, it is recommended to apply large spindles for low viscous samples, and small spindles at low speed are utilized for high viscous products. It is essential to maintain the proper sample conditions during the experiment, such as performing measurements at a constant and uniform temperature.

Figure 2.14 Brookfield DV-I PRIME viscometer

2.7 Colour evaluation in food

In food products colour is an important quality aspect, since most likely consumers assess the initial quality of the product by its appearance and colour. Generally, any object will produce colour by absorbing light energy and only the unabsorbed light can be seen as a transmitted wavelength. The perceived colour of any object is depended on various factors such as its structure and chemical composition, spectral composition of the light source, illumination environment and sensitivity of the viewer’s eye(s) (Wu & Sun, 2013). Determination of colour by the human is the individual response to the visual signals generated by light from the product. As a result, objectively communicating a particular
colour to someone without some type of standard is difficult. The solution was a measuring instrument that explicitly identifies a colour as a numeric value. Today, the most commonly used instruments for measuring colour are spectrophotometers. Spectro technology measures reflected or transmitted light at many points on the visual spectrum, which results in a curve. Since the curve of each colour is as unique as a signature or fingerprint, the curve is an excellent tool for identifying, specifying and matching colour (McLaren, 1986).

Colour is typically described with various descriptors, such as hue or hue angle which is the actual colour of the object that consumers refer to, luminosity/brightness, intensity, and chroma (saturation, purity). By describing a colour using these attributes, we can accurately identify a particular colour and distinguish it from any other. An international standard for colour measurement developed by the Commission Internationale de l’éclairage (CIE) in 1976 included initially chromaticity diagram (Figure 2.15) based on xyz axis which later was modified to a colour space of \( L^*, a^*, b^* \) as colour parameters (Figure 2.16). This colour space, which is very close to colour identification by a person, has a uniform distribution of colours and allows quantification of visual differences that are perceived by a standard human (Wu & Sun, 2013).

![Figure 2.15 CIE Chromaticity diagram (MacDougall, 2002).](image)

In this system, coordinates are designated as \( L^*, a^* \) and \( b^* \) and a colour is completely determined by the three coordinates: \( L^*, a^* \) and \( b^* \) or \( L^*, C^* \) and \( H^* \) (McLaren, 1986). \( L^* \) is the vertical coordinate of a three-dimensional system of colours, which has values from 0 (black) to 100 (for shiny). \( a^* \) is the horizontal coordinate the values of which range from –60
(green) to +60 (red). $b^*$ is the horizontal coordinate the values of which range from –60 (blue) to +60 (yellow) (Leo´n, Mery, Pedreschi & Leo´n, 2006).

![Figure 2.16 CIELAB uniform colour diagram representing relationship of red/green ($a^*+/-$) and yellow/blue ($b^*+/-$) opponent co-ordinates to lightness $L^*$, chroma $C^*$ and hue angle $h^*$ (MacDougall, 2002).](image)

In this study, the reflected colour of dairy beverages was determined using the Minolta Chromo-meter (CR-400); a tristimulus colour analyser (Konica Minolta Sensing, Inc., Sakai, Osaka, Japan) (Figure 2.17). As discussed, this colorimeter measures reflected light that is passing through a red, green and blue glass filters to displays that indicate the intensity of the
signals. The instrument was calibrated using a standard white tile with the values of colour scale as follow: \( L \) of 93.13, \( a \) of 0.3138, \( b \) of 0.3199. Based on the obtained \( L^* \), \( a^* \) and \( b^* \) values, the quantitative colour parameters (\( \Delta E; \Delta H; H \)) of formulated dairy beverage enriched with protein and dietary fibre and commercial dairy beverage as a reference were calculated. Colour difference (\( \Delta E \)) of the sample was evaluated with the following equation:

\[
\Delta E^*_{ab} = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}
\]  

(2.6)

Calculating the maximum possible colour difference (\( \Delta E^*_{\text{max}} \)) using maximum values for \( L^* \), \( a^* \) and \( b^* \) referring to their colour scale we can determine the percent of colour difference between two samples. Thus, \( \Delta E^*_{\text{max}} \) is 196.98. Statistical interpretation of data shows the small difference between two samples occurs if the subtraction between \( \Delta E^*_{\text{max}} \) and \( \Delta E \) is no more than 0.5%, whereas a difference of more than 5% between \( \Delta E^*_{\text{max}} \) and \( \Delta E \) will indicate a significant difference between the samples:

\[
\% \text{ of } \Delta E^*_{\text{max}} = (\Delta E^* \times 100) / \Delta E_{\text{max}}
\]  

(2.7)

However, there are two limitations in \( \Delta E^*_{ab} \). First, although it gives the value of difference, \( \Delta E^*_{ab} \) does not indicate in what way the direction of the difference. To address this issue, \( \Delta H^* \) which describes the Euclidian difference between the colour of the two samples can be calculated using the following equation:

\[
\Delta H^* = ((\Delta E^*_{ab})^2 - (\Delta L^*)^2 - (\Delta C^*)^2)^{1/2}
\]  

(2.8)

The second limitation of \( \Delta E^*_{ab} \) is that the human eye cannot differentiate the colour within narrow neighbouring regions, even if they are different. A new equation for calculating colour difference to address these problems has been proposed, but it has not being accepted by the international standard body as yet not used extensively in the literature and as a result was not used in this Thesis.

Colour of the novel formulations of dairy beverages was also determined by calculating the chroma (\( C^* \)) and hue angle (\( h_{ab} \)) as indicators of colour deviation using the following equations (Iserliyska, Chinnan & Resurreccion, 2012):

\[
C^*_{ab} = ((a^*)^2 + (b^*)^2)^{1/2}
\]  

(2.9)
\[ h_{ab} = \tan^{-1} \left( \frac{b^*}{a^*} \right) \]  \hspace{1cm} (2.10)

The instrumental measurement of colour is widely used in the food industry, but still there are some practical issues including discontinuity, bubbles and translucence associated with this technique that could contribute errors to the instrumental readings.

2.8 Optical microscopy

Optical microscopy (light microscopy) is a well-established and conventional technique used to analyse the microstructure of materials. Optical microscopes generally consist of an eye piece, lens system, focus knobs, stage (to hold the sample), light source, diaphragm and condenser. The principle behind the optical microscope is that visible light rays are reflected from the viewed object and pass through a series of lenses to form an enlarged image of the object with the appropriate resolution.

In this study, samples of the desserts were spread on top of a glass slide, ensuring that the desserts’ structure was not destroyed and that the dessert was not applied too thickly or thinly onto the slide. To obtain a flat imaging plane, a glass coverslip was placed over the sample and a little pressure applied to it. Afterwards, the typical light source used in light microscope which is a low voltage halogen bulb was focused and used to illuminate the sample via the objective lens to create a magnified image. Illumination makes a difference to a resolution and quality of the image. The most common illumination method used in research requiring an optical microscope is the Köhler illumination (Figure 2.18), where a condenser is placed in front of the lamp guaranteeing even illumination of the specimen.

Generally, images are captured by normal light sensitive cameras to develop micrographs. However, in modern technology, for optical microscopes, the digital camera is attached to the computer to show the resulting image directly on a computer screen without the need for eyepieces. In this study, images were collected with a Leica DM 2500 M microscope (Figure 2.19) with an attached Leica DFC400 digital camera at x100 magnification.
Sensory evaluation of food

Sensory evaluation is a method used to measure, analyze and interpret the characteristics of food products in terms of its appearance, texture, flavor, taste, aroma as they are perceived by human senses. Sensory perception of a product is a decision making activity
that can be incorporated into various aspects of the undertakings in food production. Very often food companies use sensory evaluation to compare their product with competitors as well as for improving the product qualities, such as nutritional composition, shelf life etc. Sensory evaluation is an important activity when the goal is to ensure the quality of a product.

There are several important factors that should be considered as part of the implementation of taste panels to ensure the accuracy of results. The crucial issue that is a preliminary step for sensory evaluation is to choose the right sensory test that could answer the desired question. To accomplish this, the reasons for undertaking the sensory trial and the associated critical objectives have to be defined. There are three kinds of sensory evaluation methods, namely: discrimination tests (to identify the existence or otherwise of overall differences between the products), descriptive analysis (to quantify the perceived intensities of sensory characteristics of the product), and preference or acceptance tests (to identify the level of consumer liking or disliking) (Lawless & Heymann, 2010). In any test panel, the general requirements for setting up the panel involves the preparation of samples and facilities, panellists’ selection, legal information about the project, location, layout (design of taste panel area and booth), and environmental conditions (e.g. odour, light, and general comfort) (Resurreccion, 1998).

Consumer testing is an essential step in product development activities that has been used as a guide for developers to screen the products in every stage of development by assessing a response from current and potential customers for the product and/or its specific characteristics. Thus, hedonic sensory panel has been used in food industry for many years. This test is used to determine the overall acceptance of food providing information on how much the consumer likes or dislikes the product (Chollet, Gille, Schmid, Walther & Piccinali, 2013). Hedonic test consists of a number scale from extremely like to extremely dislike in which the scale can be divided into 5, 7, 9 or 11 points. The disadvantage of a 5-point scale is that there is little choice in product evaluation and is only applicable if the products are very different. On the other hand, 11-point scale can confuse the panellists with so many levels to sort out. Generally, 7 and 9- point scales are the most common to be utilised in hedonic sensory panels, and out of these two, the 9-point scale is the most appropriate one. Besides the overall acceptance of the product, the hedonic taste panel can characterise the level of liking for specific product parameters, such as texture, sweetness, and flavour.

To evaluate the overall acceptance of dairy desserts and dairy beverages, hedonic taste panels for preference testing were carried out in this study. Panellists were asked to rank how much they liked the samples on a hedonic scale ranging from extremely dislike (1) to
extremely like (9). Before undertaking the taste panel trial, it is important to inform the panellists about the product which they are going to taste to make sure that they do not have any allergies or any other problem with tasting this kind of product. Also it is important to provide the plain language statement in simple language that explains the nature and content of the trials and the consent forms which they will be asked to sign. After answering any queries and if the panellists are in agreement, it is essential to inform them about the testing procedures before they sign the consent form (if they are in agreement). Generally samples are identified by triad numbered sample codes which are selected from a random number table, thus, neither the panellists nor the presenter know the product codes. The next important thing is to cleanse their palate before tasting and between each sample. Afterwards, panellists will be asked to identify the intensity of the particular parameters of the samples, overall how much they liked the samples, the specific parameters on a hedonic scale ranging from extremely dislike (1) to extremely like (9). However, high variability of individual preferences can arise from this type of test. This issue can be overcome with increased number of panelists to ensure the accuracy of the results. Another drawback of the hedonic test is that panelists only express their level of liking but they do not mention the reason for this.
2.10 References


CHAPTER 3
CHARACTERISATION OF RAW MATERIAL QUALITY THROUGH DETERMINATION OF PROTEOLYTIC ACTIVITY IN UHT-TREATED MILK

3.1 ABSTRACT

The advent of UHT technology commercially in the 1950s provided significant benefits to the producers and eventually consumers once consumers accepted the fact that UHT-treated products do not require refrigeration. Although the UHT-treated milk has a long shelf-life (6-12 months) in comparison to pasteurised milk (5-10 days), that long shelf-life can be limited by deterioration in the quality of milk. That deterioration became known as age-gelation. It is now known that one of the key factors in age-mediated gelation is the presence of heat-resistant proteolytic enzymes which have been produced by psychrotrophic bacteria during raw milk storage. However, what is not known is the actual relationship between the level of these proteolytic enzymes in the raw milk and the visible onset of age-mediated gelation in the UHT–treated milk.

The current study focused on the determination of protease activity in UHT- treated raw milk, in relation to age-gelation over a 10 week storage period at 20°C. This project involved extraction of proteases from a pure culture of *Pseudomonas fluorescens*, an organism known to produce heat stable proteolytic enzymes. The enzymes were isolated as a crude extract from this organism during the late exponential phase of the growth at which time the bacterial load was *ca.* $10^9$ cfu/mL. Levels (5) ranging from no addition (as the control) to 0.005% (v/v) of the bacterial cell-free extract of the crude enzyme extract were added to batches (5 L) of raw skim milk before UHT treatment (135°C/2s). The UHT-treated batches were then held at room temperature (20°C) and analysed for bacterial count, pH, viscosity, free amino group (using the fluorescamine assay) and enzyme activity (using the FITC-casein assay) at regular intervals.

The relationship between the concentration of added enzyme (crude extract) and the proteolytic activity as measured by FITC-casein assay was found to be natural log in which y
(level of enzyme activity as measured by the FITC-casein assay) = 38.7 ln(x) + 139.36 and x is the concentration (v/v) of added enzyme extract with an R² of 0.98. The effect of UHT treatment was to reduce both the bacterial count to < 1 cfu/mL and the enzyme activity by 0.23% to 9.97% (depending on the enzyme level added to the batch) but there was no change in pH or viscosity after UHT treatment.

During 20°C storage, the pH and bacterial count of all the samples remained the same as that recorded immediately after UHT-treatment indicating that any gelation is not due to acidification of the milks. The control samples did not show any change in viscosity or fluorescamine levels within the 10 week trial. However, for the milks with added crude enzyme, there was an increase in both of these parameters. Significantly, the relationship between the concentration (v/v) of added enzyme extract and level of peptides produced as measured by the fluorescamine assay was shown to be linear.

At the time gelation was identifiable visually, these peptides (free amino groups) ranged from 30-36 μM, and produced a viscosity which ranged from 15-21 mPa.s, irrespective of the concentration of added crude protein extract. What is dependent on the proteolytic enzyme (concentration or activity) is the shelf-life of the UHT-treated milk. The association between the concentration of added crude extract or more importantly the proteolytic activity of the extract and the time taken to gel, i.e. the shelf-life was found to require a negative power equation, in which parameters are: y (shelf-life in weeks) = 9.6301 x^{-2.12} with an R² of 0.9943 and x the enzyme activity in terms of the concentration of FITC-isomer 1 (x 10^{-4} nM). The threshold for a number of possible storage periods for UHT–treated milk was estimated from this empirically derived equation. For example, for a shelf-life of 40 weeks (9.3 months) at 20°C, the threshold of proteolytic enzyme maximum would be < 0.464 x 10^{-4} nM FITC isomer 1 in the raw milk (allowing for a 10% loss during heating).
3.2 INTRODUCTION

The dairy industry has for a long time had the challenge of preserving raw milk given its very nutritious nature (Fox, 2009) and consequently very short shelf-life (days under refrigeration). There is no natural mechanism, such as low water activity or low pH, in milk itself to help extend its shelf-life. Consequently, technology in the form of heat has been used initially to produce pasteurized milk. This produced a safer product but with still a limited shelf-life. The process of extending the heat to produce ultra-high temperatures (UHT) and associated UHT-treated products that required very short processing times (seconds) was ‘born’ fortuitously into an age of increased technology tempered with a concern for sustainability and energy demand.

Milk that has been UHT-treated and for that matter other products have a number of obvious advantages over their previous counterparts (pasteurized products). These advantages include: energy savings in the regeneration stages of the UHT process, further energy savings because of the lack of a requirement for refrigeration in the warehousing, transportation and on the supermarket shelves and at home. As well as this, the product (whilst unopened) can be held for a considerable period of time (months) at room temperature (Gedam, Prasad & Vijay, 2007). This allows for production efficiencies, and consumer convenience in reducing the frequency of purchasing and waste, respectively.

However, these same advantages, room temperature storage, long shelf-life also ‘work’ against these UHT-treated products. In other words, the advantages turn into disadvantages, in a way. This is shown by the fact there can be changes in colour, aroma, flavour but in particular an increase in viscosity followed by gel formation during long term storage of these products; an undesirable textural change (Celestino, Iyer & Roginski, 1997). The problem of the formation of a gel during storage is called age-gelation or age-mediated gelation. This is to distinguish it from gelation in milk that is caused by acidification of the milk as a result of bacterial multiplication (growth). These age-mediated problems are commercially of great concern. This is because the shelf-life of the UHT-treated product ‘falls’ well short of the expected shelf-life of 6-12 months (Marchand, Coudijzer, Heyndrickx, Dewettinck & de Block, 2007). This has a ‘flow on’ in that the customer, be they another food manufacturer or from the general public will lose ‘faith’ in both the product and the specific manufacturer. The food industry relies on repeat business; a loss of trust is not
likely to enhance repeat purchasing. This is on top of the environmental and economic issues of wasting precious resources.

In terms of what is known about the factors that cause and effect age-mediated gelation, there is an excellent review article by Datta and Deeth (2001) on this issue. Basically, it is known that the bacterial population in raw milk do not survive the UHT-treatment. But it is the activities of these same bacteria before UHT-treatment that are responsible for age-mediated gelation experienced by the UHT-treated milk long after the organisms have been eliminated. These organisms are capable of producing extracellular enzymes that have proteolytic and lipolytic activity. It is clear that the function of these enzymes, from the point of view of the bacteria, is to aid in the supply of food, specifically, the hydrolysing of proteins and fats in their surrounding medium for ‘easy’ transmembrane transfer. What is most interesting is the fact that these enzymes are produced by psychrotrophic bacteria which are growing at refrigeration temperatures (0-4°C) but these same proteases and lipases are reported as being very heat resistant capable of withstanding UHT-temperatures (Dupont, Lugand, Rolet-Repecaud & Degelaen, 2007). It was established some time ago (Adams, Barach & Speck, 1975; Mitchell, Ewings & Bartley, 1986) that these extracellular proteases produced by some strains of Pseudomonas fluorescens retain as much as 90% of their proteolytic activity after UHT treatment at 135°C for 2s and 149°C for 4s.

An additional fact that is known about these enzymes is that they do not target casein itself but the cross linked casein - β-lactoglobulin that is formed during heating. The real cause of age-mediated gelation in UHT-treated milks is associated with the cross-linking of the peptides released by the activity of these proteases. In other words, the mechanism of age-mediated gelation requires some four (4) main steps, namely: production of extracellular heat resistant proteases, binding of casein with β-lactoglobulin, hydrolysis resulting in the release of a βκ-complex, a small peptide that is capable of cross-linking with itself and other peptides to form a gel (Datta & Deeth, 2001).

What is not known or understood about this mechanism is a number of relationships. Accordingly, this chapter will undertake a preliminary study involving the addition of a crude extract of proteolytic enzyme to raw milk before UHT treatment to determine the relationships between: (a) the concentration of added enzyme and the proteolytic activity of the extract, (b) the added crude extract and concentration of peptides produced in the UHT-treated milk (c) the change in viscosity and peptide production during a 10 week storage trial of UHT-treated milk stored at 20°C, (d) the viscosity and associated peptide level at the onset
of gelation and (e) an estimation of the relationship between the level of proteolytic activity in the raw milk and the shelf-life of the UHT treated milk.

3.3 EXPERIMENT PROTOCOL

3.3.1 Materials

A pure culture of *Pseudomonas fluorescens* 79 used during this study, for the extraction of crude proteinases, was kindly provided by RMIT microbiology laboratory from their culture collection.

Nutrient Broth (CM0067) was purchased from Oxoid Microbiology Products Australia. According to the manufacturer’s data sheet it includes ‘Lab-Lemo’ powder, peptone, and sodium chloride (5g/L) with a pH of 7.5 ± 0.2. The dehydrated powder must be free flowing and like the broth is described as being straw-coloured.

Nutrient Agar (CM0003) is a basic culture medium used to subculture microorganisms and was supplied by Oxoid Microbiology Products Australia. According to the product specification, it contains ‘Lab-Lemo’ powder, yeast extract, peptone, sodium chloride (5g/L) and agar with a pH of 7.4 ± 0.2.

Type III Fluorescein iso thiocyanate casein (FITC-Casein) (C0528) is a salt-free, lyophilized powder which has been labelled with 50-100 μg FITC/mg (w/w) solid. The supplier was Sigma Aldrich, Australia.

FITC isomer 1 (F7250) was purchased from Sigma Aldrich Australia and is a yellow colour powder, which is used as a fluorophore to label proteins via amine groups and as a result create a fluorescence product.

Fluorescamine (F9015) was purchased from Sigma Aldrich Australia and is a non-fluorescent reagent that reacts readily with primary amines in amino acids and peptides to form stable highly fluorescent compounds. It is off-white to yellow colour powder, which is soluble in acetone to yield a clear solution.

Leu-Gly dipeptide (L9625) is a white powder purchased from Sigma Aldrich Australia. According to the product specification, storage temperature of this product is -20°C.
Unpasteurised skim milk used in this study was kindly provided by Parmalat Victoria (Pauls Limited). The raw milk was delivered to Parmalat from the farms associated with the company. Milk was brought in 3 L plastic containers to RMIT Food Science pilot plant on the morning of the same day. The milk was kept chilled (4 ± 1°C) until required for subsequent experimentation, which included ± enzyme addition followed by UHT treatment within the next 24 h.

3.3.2 Enzyme & sample preparation

a.) Sterility check on bacterial culture - *Pseudomonas fluorescens*

The centre of a well isolated colony from a culture of *Pseudomonas fluorescens* provided was stabbed with a straight wire and used to streak out for single colonies. The inoculated nutrient agar (NA) plate was incubated at 30°C for 48 h. Following incubation, the same procedure was repeated and then the purity and the nature of the culture was confirmed by microscope examination using a Gram stain. *Pseudomonas fluorescens* is a Gram negative non-sporing aerobic rod (Rajasree & Suman, 2012).

b.) *Pseudomonas fluorescens* growth curve at 4 ± 1°C

Three colonies (3) of the microorganism were transferred from nutrient agar (NA) into nutrient broth (250 mL). The inoculated nutrient broth (NB) was stored at 4 ± 1°C for 35 days. The culture was enumerated daily by transferring an aliquot (1 mL) into a saline diluent (9 mL, 0.85% w/w) which was further serially diluted (10⁰ to 10⁻⁸). The serial dilutions were plated (0.1 mL or 1 mL) using the spread plate method onto duplicate NA plates. The NA plates were incubated at 30 ± 1°C for 48 h and then plates with 20 – 250 colonies were counted as colony forming units (cfu/mL). The results were reported as an average of two samples.

c.) Preparation of medium-free *Pseudomonas fluorescens* cells and crude proteinase from those *Pseudomonas fluorescens* cells

The procedure of Richardson (1981) was followed as a guide for the extraction of crude protease from *Pseudomonas fluorescens*. To maximise enzyme production, colonies (3)
of *Pseudomonas fluorescens* from NA were inoculated into nutrient broth (100 mL), which was incubated at 25 ± 1°C for 48 h. An aliquot (10 mL) of the inoculated NB was centrifuged (Allegra 64R, Beckman coulter) at 6000 x g for 10 min. The filtrate was discarded and the precipitate (bacterial cells) was resuspended in sterile isotonic saline solution (0.85% w/v, 1 mL). The washing (centrifugation and resuspending) procedure was repeated three times to obtain nutrient broth-free cells and subsequently, these cells were resuspended in saline solution (0.85% w/v, 1 mL) before being introduced into UHT skim milk (99 mL, 0.1% fat). After incubation for 12 days at 4 ± 1°C, an aliquot (10 mL) of incubated UHT milk was centrifuged at 24,000 x g for 10 min at 4 ± 1°C. The resulting supernatant was filter sterilized using a 220 nm (pore size) membrane filter (with a Millipore stericup vacuum filtration unit) to remove any bacterial cells. The filtrate produced was taken as the crude protease extract to be used in subsequent experiments. The crude enzyme extract was stored at 2 ± 1°C until required (usually within 24 h) for the experimental studies outlined in section 3.3.2d.

d.) **Addition of crude enzyme extract to raw skim milk**

Crude protease extract (extracted as described in section 3.3.2c) from *Pseudomonas fluorescens* was inoculated into raw skim milk (5 L). The aliquots ranged from 0 (control) to 0.005% to produce 5 levels of enzyme concentrations which were labeled as 0-5 (table 3.1), where level 0 was the control (no added crude enzyme extracts). The prepared milks were stored at 1- 4°C until required for homogenization (section 3.3.2d), which was within two hours of inoculation.

e.) **Ultra High Temperature (UHT) Treatment**

Raw milk with crude enzyme including control was preheated on the stove until the milk temperature reached to 55-60°C and held at same temperature for 30-60 s while stirring at all times. After preheating, a five-stage homogenization was carried out at 50 bars in each stage using a FT9 homogeniser (Armfield, UK).

Following homogenization, all batches of raw skim milk were processed using an indirect HTST/UHT heat exchanger processing unit (FT74X, Armfield, UK). The control batch (no added enzyme) was processed first followed by batches with increasing enzyme concentrations (starting with the batch with the lowest amount of added enzyme). The conditions and parameters used during the UHT treatment were as follows:
Pressure – 3 Bar
Inlet flow – 19 L/h
Inlet temperature – 28.5°C
Pre-heat temperature – 115°C
UHT conditions - 135°C for 2 s
Outlet temperature – 18.1°C

Treated milk was aseptically filled into pre-sterilized (121°C/15 min) containers (250 mL) which were hermetically sealed. After filling, containers were then stored in an incubator set at 20 ± 2°C throughout the duration of the storage trial.

### 3.3.3 Assessment methods

The quality of the raw skim milk was assessed within two hours of the milk arriving at RMIT Food Science pilot plant in terms of pH, viscosity, total plate count and proteolytic activity. All the methods used are described in the section below.

**Total plate count** of the UHT skim milk samples stored at 20 ± 2°C with and without added crude protease was determined by the spread plate method. The medium used was NA and the microbial load was enumerated by transferring an aliquot (1 mL) of milk into a saline diluent (9 mL, 0.85% w/w) which was further serially diluted (10^0 to 10^-3). Aliquots (0.1 mL or 1 mL) of the serial dilutions were spread onto NA plates in duplicate. NA plates were incubated at 30 ± 1°C for 48 h and plates with 20 - 250 colonies were counted as colony forming units (cfu/mL). The results were reported as an average of two samples.

**pH** of the milk samples before, and after, UHT treatment were measured in triplicate at room temperature (20 ± 2°C) using a laboratory pH meter (Hanna instruments 8520). The precision of the calibrated pH meter was ±0.02.

**Viscosity** of the skim milk samples before, and after, UHT treatment were measured by a Brookfield viscometer (Brookfield DV-1+) at room temperature (20 ± 2°C) as an average of two (mPa.s). The spindles number 61 and 62 and a speed 100 rpm used in this study.

**Agar diffusion method (ADM)** was carried out to identify the proteolytic activity of the enzymes produced by a pure culture of *Pseudomonas fluorescens* during its growth at 4 ± 1°C in nutrient broth. The method used was based on Christen and Marshall (1984) with some modifications as follows: Sterile NA was poured into petri dishes in two equal layers of 15 mL each. The bottom layer contained NA only which after setting was covered with another
layer of NA to which UHT skim milk (1% v/v) had been added. Each plate was divided into four equal parts and wells (diameter = 5 mm) were punched into the top layer of the agar using a sterilized (121°C, 15 min) disposable glass Pasteur pipettes. Each plate contained two control wells (which contained NB only) and two sample wells.

An aliquot (1 mL) of the NB (which was incubated at 4°C containing Pseudomonas fluorescens pure culture) was centrifuged using a micro centrifuge (Sigma 1-14K) at 6000 x g for 10 min to precipitate the bacterial cells and then an aliquot (10 µL) of supernatant was pipetted into each of two sample wells. The plates were incubated inverted for 96 h at 20 ± 1°C and proteolytic activity was identified by the presence or absence of any clearing around the wells.

**FITC-casein assay** was used to measure the proteolytic activity of each milk sample (before and after UHT treatment) as described by Button, Roginski, Deeth and Craven (2011). FITC-casein type III (10 mg) was dissolved in a sodium phosphate buffer (10 mM, pH 8, 4 mL) to produce 0.25% w/v FITC-casein assay mixture. Milk sample (17 µL) was added to the assay mixture (69 µL) with gentle shaking and incubated in the dark at 37 ± 1°C for 2 h. Trichloro acetic acid (TCA) (306 mM, 14 µL) was added to the incubated mixture and held at room temperature in the dark for 30 min. To separate the precipitated protein, the assay mixture was centrifuged using a micro centrifuge (Sigma 1-14K) at 13,000 x g for 5 min and an aliquot (50 µL) of the supernatant was mixed with sodium phosphate buffer (300 mM, pH 8.5, 1.2 mL). Fluorescence was measured at 490 nm (excitation) and 515 nm (emission) using a luminescence spectrometer (LS50B, Perkin Elmer). Degree of proteolysis was calculated by relating the intensity of fluorescence of each milk sample with the fluorescence intensity of FITC isomer 1. The different concentrations of the FITC isomer 1 at 5.2, 26, 52, 78, and 104 x 10^{-4} nM were used to construct the standard curve (Figure 3.1a) for the FITC-casein assay.

**Fluorescamine method** was used to determine the concentration of free amino groups present in the milk samples as described by Button et al. (2011). All the samples were analyzed in duplicate. The analysis was as follows: equal volume (0.5 mL) of milk sample and TCA (1.67 M) solution were added into small tube and held at room temperature for 20 min. The resulting mixture was filtered through No - 41 Whatman filter paper. An aliquot (12.5 µL) of filtrate was mixed (by gently shaking) with sodium phosphate buffer (100 mM, pH 8, 1 mL) and fluorescamine in acetone (719 µM, 500 µL). Fluorescence of the solution was measured after 15 min using a luminescence spectrometer (LS50B, Perkin Elmer) at an excitation of 390 nm and an emission of 475 nm. Free amino group concentrations of the samples were calculated by relating the fluorescence intensity of the samples with respect to
the different Leu-Gly dipeptide concentrations. The concentrations of Leu-Gly dipeptide 1, 5, 10, 20, 40 ppm were used to construct the standard curve (Figure 3.1b) for the fluorescamine method.

3.4 RESULTS AND DISCUSSION

3.4.1 The growth of *Pseudomonas fluorescens* and production of extracellular proteases

To examine some parameters involved with the science behind the gelation of UHT milk associated with proteolytic enzymes, one of the first tasks was to grow an organism that is known to produce extracellular proteolytic enzymes. In effect, the experimental design for this chapter is based on a modification of Koch’s postulate (Marshall, Amstrong, McGechie & Glancy, 1985) for a causal relationship in which the suspected organism is isolated from the product (in this case, raw milk that resulted in age-mediated gelation of UHT milk and in Koch’s case a person with an illness) and reintroduced to recreate the effect; in this case gelation (in Koch’s case, it was the specific symptoms). As stated in the introduction, several papers (Clements, Wyatt, Symons & Ewings, 1990; Law, Andrew & Sharpe, 1977) have mentioned that the proteolytic enzymes from *Pseudomonas fluorescens* (Ps. fluorescens) are capable of causing age-gelation in UHT-treated milk. So a pure culture of *Ps. fluorescens* was inoculated into skim milk (UHT).

The incubation temperature was set at 4 ± 1°C, this is similar to that stated in the literature (Adams et al., 1975) in which the incubation condition for bacterial growth ranged from 4°C. The justification for selecting an incubation temperature of 4°C to grow the *Ps. fluorescens* culture is the fact that the silos in which raw milk is stored before processing are usually set at 4°C (Perin, Moraes, Almeida & Nero, 2012) and therefore a temperature of 4°C was used to ‘mimic’ the recommended industrial practice (Dairy Food Safety Victoria, 2013).

According to the growth curve (Figure 3.2), the bacterial cell count increased some 6 logs from an initial load of $10^4$ to $10^{10}$ cfu/mL to reach the stationary phase after 18 days at 4°C. This represents a cell doubling on an average every 21.8 h. It is comparable to the doubling time of 26.4 h (at 0°C) stated by Herbert and Sutherland (2000), for the same organism. At the late exponential phase, the cell count was *ca.* $10^9$ cfu/mL (from $10^5$ cfu/mL) which for the exponential phase is a generation time of 14.5 h at 4°C. This growth rate is close to that reported by Gibb, Martin, Davidson, Walker and Murphy (1995) who found that *Ps. fluorescens*’s count doubled every 14.4 h in a blood (very nutritious) medium at 4°C.
What is important is whether these cells can produce extracellular proteolytic enzymes capable of causing gelation. In this study, from daily ADM tests, clear zones around the wells indicating proteolytic activity was observed only when the cell count reached $10^9$ cfu/mL on day 15 (at 4°C), which was in the late exponential phase of growth (Figure 3.2). This result supports the findings of Grieve and Kitchen (1985) who reported that protease activity was first observed in the late exponential phase. However, they recorded the microbial load at that time as $10^8$ cfu/mL, one log lower than in this study. The difference in the methods used to monitor proteolytic activity may explain the disparity in the minimum microbial load result ($10^8$ versus $10^9$ cfu/mL): ADM in this study, and SDS-polyacrylamide gel electrophoresis and fluorescamine measurement in Grieve and Kitchen’s study. Conceivably, the method used by Grieve et al. (1985) was more sensitive than the ADM method and therefore able to detect lower levels of protease activity produced by lower bacterial loads. Hockney and Cousin (1985) have stated that the ADM is not sensitive enough to detect low levels of proteases. In essence, the ADM method was used in this study because it is a simple, easy and quick test that is meaningful in terms of showing that a culture is able (or not) to produce extracellular proteases, and it is also possible by measuring the diameter of the clearing to refer to a relative protease level, if required.

One other issue arises from the rate of growth of *Ps. fluorescens* at 4°C and the presence of extracellular proteases. It is generally stated that the problem of age-mediated gelation is associated with raw milk that was not handled at optimum conditions such as silo temperature increases, poor microbiological quality of milk, mastitis milk, etc. (Tondo, Lakus, Oliveria & Brandelli, 2004). It was found that the optimum temperature for protease activity (based on both a plating and azocasein method) in milk was 17.5°C (Gugi et al., 1991); this is not to be confused with optimum temperature for gelation (Kocak & Zadow, 1985). It has been mentioned that in the dairy industry, the milk temperature fluctuated between 4°C to 12°C due to poor refrigeration conditions and the long distance separating collection centres and farms (Ksontini, Kachouri & Hamdi, 2011). In addition, Marchand et al., (2007) suggested that the regular addition of fresh milk to the silo can lead to silo temperature increases.

It could be said that the results from this study are in agreement with Tondo et al. (2004) because it was not until the bacterial count increased by 5 logs ($10^4$ to $10^9$) was there any evidence of protease activity on the ADM plates. A five log increase took some 15 days at 4°C. Given that the silo milk is most likely to be used well within this timeframe (15 days),
age-mediated gelation in the subsequently produced UHT milk is not an issue if the raw milk was held at 4°C.

However, this may not be the case. This study so far has only determined the presence of protease activity from a bacterial culture. This constitutes the first stage of age-mediated gelation. The crucial questions are still to be addressed; questions such as, can extracellular heat-resistant proteases at levels (not detected by ADM or other methods cited above) produce age-mediated gelation in UHT milks. That is: are protease levels below the detection level, referred to in this thesis as ‘sub-detection’ level, capable of causing age-mediated gelling or is there a minimum level required? An associated question of importance is whether these enzymes at the ‘sub-detection’ level can cause age-gelation within the required shelf-life for UHT milks i.e. will the shelf-life be shortened to an unacceptable level? Thus, it is presumptive, at this stage in the thesis, to state that good quality milk held at 4°C would not lead to age-gelation in UHT milks.

To establish the relationship between protease activity and age-mediated gelation in UHT milks as well as overcoming the question of whether the low level of protease in good quality milk can or cannot produce age-gelation in UHT-treated milk, known volumes of a crude extract of extracellular bacterial enzymes will be added to the raw milk before UHT treatment. However, before this preliminary study can be carried out, the next task is to establish the quality of the raw milk used in this study.

### 3.4.2 Quality of skim milk before UHT treatment

The quality of the raw milk used for the UHT trials is reported in Table 3.2. Comparing the bacterial results obtained with that specified by the Australia New Zealand Food Standards, code (1.6.1) it can be stated that the skim milk used in this study was of an acceptable quality for human consumption. From the regulation, a bacterial count of 400 cfu/mL is only 1.6% of the stated value for ‘m’ of < 2.5 x10^4 cfu/mL where ‘m’ was set as the ideal bacterial count for raw milk.

Significantly, the bacterial count in this study and in the ANZFA’s regulation was based on plates incubated at 30°C. This temperature allows for the optimum growth of psychrotrophic organisms such as *Ps. fluorescens* (Herbert & Sutherland, 2000) which according to Todar (2012) will grow as low as 0°C and shows optimum growth at 15-30°C.

The other parameters tested (pH, viscosity and protease activity) were compared with those stated in the literature where possible (Table 3.2). The pH was within the expected level
for raw milk, but the same could not be said for viscosity and protease activity as the literature was wanting with respect to these parameters. However, it should be noted that the importance of the values obtained for these parameters lies not in their explicit values but the relative change that could occur as a result of UHT treatment and/or storage time i.e. they are the baseline.

One issue of concern is the fact that the change in added enzyme (activity) is not proportionally reflected in the FITC-isomer 1 reading. For example in Figure 3.3, a tenfold increase in added crude enzyme concentration from 0.0005% to 0.005% resulted in an increase of only 10% in protease activity, as shown by an increase in FITC isomer 1 concentration from 20 to 22 x 10^4 nM. Figure 3.4 shows the relationship between the amount of added crude enzyme extract and the measured enzyme activity (FITC isomer 1), after UHT treatment. This plot with a R^2 of 0.9802 shows a log relationship with:

\[ y = 38.723 \ln(x) + 139.36 \]  

(3.1)

Where y is the FITC-isomer 1 concentration and x is the concentration in percentage (v/v) of the crude extract added to the raw milk. With an R^2 of 0.98, this log relationship can be taken as being acceptable. However, the initial thought was that a problem with the enzyme activity method may be limiting the correlation recorded. In the FITC-casein method, the substrate FITC-casein is made available for the enzyme present in the sample to hydrolyse. The fluorescence of the peptide produced is measured. The major issue was the incubation time for the enzyme (crude extract) to react with the substrate (FITC-casein). According to Cupp-Enyard (2009), the incubation time should be 1 h but not extended beyond 24 h as FITC-casein is deemed to deteriorate after this. In this study, 2 h incubation was used and in a study by Button et al. (2011) an incubation period of several hours, 1 d, 2 d, up to 14 days was used. The data from Button et al. was plotted (Figures 3.5a and b) to show the relationship between the level of fluorescein (standard curve used in that study) and the level of enzyme added as a result of different incubation periods used in the assay. The plot of the data after 1 day incubation (Figure 3.5a) showed a linear relationship with:

\[ y = 17.5x - 2.5 \]  

(3.2)

where, y is the fluorescein concentration and x is the volume of crude extract added to the raw milk. Similarly, after 14 days incubation (Figure 3.5b), the data showed a linear relationship with:
$y = 76.5x - 0.5$ \text{(3.3)}

Although the relationship between fluorescein and the level of enzyme added is linear, the $R^2$ values were 0.9459 and 0.9614 for equation 3.2 and 3.3, respectively. It should be noted that both studies (this and Button et al., 2011) used the FITC-casein assay for evaluating enzyme activity with the difference being the standard curve used. Button \textit{et al.} used fluorescein whereas this study used FITC isomer 1 (which contains fluorescein) for the standard curves.

Thus, increasing the incubation from 2 h for the FITC-casein test may improve the correlation between the enzyme present (in the milk or extract) and the FITC- Isomer 1 result obtained as was the case for the fluorescein test reported by Button \textit{et al.} (2011). However, the selection of the incubation time for the test must include the practical issue of obtaining a result in a meaningful time, particularly, if the test were to become an industry practice.

\subsection*{3.4.3 The initial effect of UHT-treatment on both milk quality and the activity of added crude enzyme extract}

Milk after UHT treatment had a bacterial count of $< 10$ cfu/mL, and no meaningful change in pH and viscosity, as compared to the raw skim milk (Table 3.2). These results including the low microbial count after treating the milk at $135°C$ is to be expected. However, what is required in this study is the absence of any bacterial growth so that the age-mediated gelling of the UHT milk is due to the activity of heat-resistant proteolytic enzymes not milk acidification based on bacterial growth. Therefore, bacterial activity via bacterial numbers and pH of the UHT milk were monitored throughout the trial (data not shown).

With respect to enzyme activity, there appears (Figure 3.3) to be a decrease in the average FITC-isomer 1 level in the control milk samples (i.e. no added crude enzyme extract) which indicates a loss of proteolytic enzymes as a result of UHT treatment. This is predictable as the raw milk would contain both heat resistant and heat sensitive proteolytic enzymes. Therefore, it can be postulated that the 3.6\% decrease in FITC-isomer 1(from 14.67 to 14.14 $\times 10^{-4}$ nM) is due to the denaturing of heat sensitive proteolytic enzymes during UHT treatment. It should be noted that the proteolytic activity of plasmin is not included in the FITC-isomer 1 results. This is because the peptides produced by plasmin activity are large and as a result are precipitated by the trichloroacetic acid step in the FITC-casein method (Andrews & Alichanidis, 1983).
A similar result, namely a reduction after UHT treatment in the average FITC-isomer 1 level as a measure of proteolytic enzyme activity, was recorded for all levels of added-enzyme (Figure 3.3). This loss, ranging from about 0.2% to 10.0%, in enzyme activity as a result of treatment of 135°C (for 2 s) is likely as the crude enzyme extract (as well as the milk) would contain a range of extracellular proteases that are either heat sensitive or heat resistant. The former would be denatured during UHT treatment accounting for the decrease in enzyme activity. The level of reduction in this study can be compared to the 40-60% reduction in pseudomonad protease activity after treatment at 140°C for 5 s as reported by Griffiths, Phillips and Muir (1981). The results from this study are well within the range reported by Mitchell et al. (1986) with reductions in enzyme activity of 4.2%, 7.7%, 10.3%, 23.7%, 48% and 100% for 5 strains of *Ps. fluorescens* after heating at 135°C for 2 s.

It should be noted that some authors (Petersen & Gunderson, 1960) do in fact acknowledge that *Ps. fluorescens* can produce a range of proteolytic enzymes. However, this issue is not resolved, as other authors (Nemeckova, Pechacova, & Roubal, 2009; Richardson, 1981) state that the relationship is 1:1 i.e. one strain of *Ps. fluorescens* produces one proteolytic enzyme.

### 3.4.4 Effect of storage time and enzyme (protease) concentration on the viscosity and free amino group concentration of UHT-treated skim milk

The control batch (no added crude enzyme extract) of UHT-treated milk did not show any significant changes in the viscosity and free amino groups (concentration equivalent to Leu-Gly/μM) over the period of the trial; some 10 weeks at 20°C (Figures 3.6a and b). This suggests that the initial level of heat resistant proteolytic enzyme, at 14.1 x 10⁻⁴ nM based on FITC-isomer 1 results, present in the UHT-treated milk was not enough to initiate age-mediated gelation within this time period. As mentioned earlier, however, heat resistant proteolytic enzyme at a level of 14.1 x 10⁻⁴ nM may be enough to initiate age-gelation if the milk had been stored at 20°C for a longer period; closer to the expected shelf-life for UHT milk.

For the batches of UHT-treated milk with added crude enzyme extract, both the viscosity and the level of free amino groups increased with storage time (Figures 3.6a and b). The overall trend in the viscosity and the level of free amino groups were similar in that the milks with the higher enzyme loads (50 x 10⁻⁴ % and 10 x 10⁻⁴ %) showed a similarly steep rise in both parameters as did the lower enzyme levels (1 x 10⁻⁴ % and 5 x 10⁻⁴ %). It should
be noted that neither the bacterial count nor the pH varied from that measured immediately after UHT processing (Table 3.2). So, the gelation observed in these batches could not be attributed to acidification due to bacterial growth but rather to age-mediated gelation.

Given that the chemistry behind age-mediated gelation in UHT milks is based on the separation from $\beta\kappa$-complexes of $\beta$-lactoglobulin and cesin, which then crosslink with each other (and with other small peptides) to produce a gel (Datta & Deeth, 2001), the relationship between the viscosity and free amino group results is of interest. This is particularly so, as the fluorescamine test measures the concentration of free amino groups presented in $\beta\kappa$-complexes. $\beta\kappa$-complexes are the precursors needed for gelling, and are in fact the peptides that have been released as a result of the activity of the proteolytic enzyme. Significantly, the viscosity, a measure of the degree of gelation, can be seen as the product of the cross-linking of the $\beta\kappa$-complexes.

From Figures 3.7a and b, it can be seen that there is an exponential relationship between the cause (free amino groups) and the effect (increase in viscosity) of age-mediated gelation. Furthermore, the amount of free amino groups (as measured by the fluorescamine method) present at the time that a change in viscosity becomes visible appears to be independent of the amount of crude enzyme extract present in the milk. For the lowest (1 x $10^{-4}$ %) level of proteolytic enzyme trialled, at the time that the viscosity was observed to change, the free amino level had reached 30.7 $\mu$M (based on the fluorescamine test). Similarly, for the highest level of proteolytic enzyme trialled, the level of free amino groups needed to cause an increase in viscosity was around 31.4 $\mu$M. This lack of dependence on the amount of added crude proteolytic enzyme extract can be seen more clearly in Figure 3.8a and b, which shows that the viscosity ranged from 15-21 mPa.s at the time visual gelation was observed whilst the associated level of free amino group concentration ranged from 30-37 $\mu$M, across the crude enzyme levels trialled. Although both the viscosity and the free amino group concentration appear at the onset of gelation to be independent of the level of crude enzyme extract present in the UHT-treated milk, the time taken to reach these levels of free amino groups was dependent on the amount of crude enzyme added (Figure 3.6b).

It is in fact the time taken to create a gel in the UHT-treated milk that is one of the crucial issues from a commercial point of view; in effect the shelf-life of the UHT-treated milk. More specifically, the relationship between the proteolytic enzyme in the raw milk and the time to produce age-mediated gelation in the UHT-treated milk is of interest to ultimately determine the threshold activity of proteolytic enzymes needed for a specific shelf-life.
From Figure 3.9a of enzyme activity (as measured by FITC-casein assay) versus time taken for a visual change in viscosity (onset of gelation), a line of best fit provides the following relationship between these two parameters:

\[ \text{Gelation time (weeks)} = y = 14.611x^{-1.708} \]  \hspace{1cm} (3.4)

where \( y \) = time to gel in weeks and \( x \) = proteolytic activity as \( 10^{-4} \) nM as measured by FITC-casein. Unfortunately, the \( R^2 \) is only 0.778 which is unacceptably low. It is easy to see from Figure 3.9a why the correlation is so poor. If the data for batch 2 (0.0005%) is removed, the results are significantly improved with an \( R^2 \) of 0.9943 (Figure 3.9b) providing the following relationship:

\[ \text{Gelation time (weeks)} = y = 9.6301x^{-2.12} \]  \hspace{1cm} (3.5a)

\[ \text{Gelation time (months)} = y = 2.2396x^{-2.057} \]  \hspace{1cm} (3.5b)

There is some literature (Merck, 1999; Mitchell & Ewing, 1985) where the relationship between time for gelation and some form of proteinase content was determined. For example, the company Merck (1999) stated that the relationship could be described by the following equation:

\[ \text{Gelation time (months)} = 2.3916 \times 0.6449 \]  \hspace{1cm} (3.6)

where \( x \) is the proteinase activity in ng/mL as measured using Merck’s test kit. An earlier equation was reported Mitchell & Ewing, (1985) in which the proposed relationship was as follows:

\[ \text{Gelation time (weeks)} = 5.623 - 8.45 \log \text{(activity in U/mL)} \]  \hspace{1cm} (3.7)

where equation (3.7) was based on an empirically derived regression line.

The next step would be to discuss these equations. However, the equations are based on different approaches and as a result are rather difficult to compare. Moreover, in most cases, derivation of the equations uses different amounts of enzyme. The reason why this is a problem is that the enzyme amount may or may not reflect the level of activity of the enzyme present. In this study, FITC-casein approach evaluates the effectiveness of the enzyme to hydrolyse the \( \beta\kappa \)- complexes rather than the amount of enzyme present – which nevertheless is still of interest.
Using the empirical equation 3.5a, Table 3.3 records the relationship between the time to gel in UHT-treated milk and threshold enzyme activity in the raw milk before UHT treatment. Thus, for a shelf-life at 20°C of 40 weeks (9.3 months), the raw milk used to produce the UHT-treated milk should have < 0.0000464 nM of proteolytic enzyme (based on the FITC-casein method). The literature (Merck, 1999; Mitchell & Ewing, 1985; Richardson & Te Whaiti 1978) state a threshold of 0.3 to 2 ng/mL for a shelf life at ca. 25°C for 3-4 months (Table 3.4). From this study, a 3 month (9.9 weeks) shelf-life would require a threshold of < 0.897 x10^-4 nM in raw milk (allowing for a 10% reduction due to UHT treatment). To compare the results from this study, the literature equations and stated threshold, an experimental design involving a parallel study of several methods for evaluating activity and amount of proteolytic enzymes would need to be carried out. That was beyond the scope of this study.
3.5 CONCLUSION

The standards for milk quality centred on microbial load and pH are of limited value in establishing the suitability of raw milk for UHT-treatment. This is based on the fact that the threshold values in the raw milk for proteolytic enzymes are very low, at e.g. ca. 0.00009 nM of FITC-isomer 1 for a three month shelf-life at 20°C, according to the results from this study. This level of enzyme activity, it is postulated, is most likely to be produced when the bacterial count was low, i.e. the milk is of acceptable quality for human consumption (<2.5 x 10^4 cfu/mL) but not for UHT processing. This raises the spectre of appropriate methodology for estimating proteolytic enzyme activity; which method is sensitive enough to detect very low levels of proteolytic enzyme activity within a practical timeframe? This, as well as the issue of the threshold for proteolytic enzymes from the mixed flora (rather than a single isolate) of raw milk on the shelf-life needs to be determined. This is particularly so, if this approach (estimating proteolytic activity) is to have any commercial value for setting a threshold specification for raw milk that is to be used for UHT processing. Work in this chapter provides guidance in food safety considerations for the utilisation of thermally processed milk as the basis for the development of dairy-based products with novel techno-and nutritional functionality, in subsequent experimental chapters of this thesis.
3.6 REFERENCES


Table 3.1 Concentration of crude protease* added to raw skim milk samples for UHT treatment.

<table>
<thead>
<tr>
<th>Designated enzyme level</th>
<th>Concentration of enzyme (%)</th>
<th>Enzyme volume added **(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0000</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.0001</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>0.0005</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>0.0010</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>0.0050</td>
<td>250</td>
</tr>
</tbody>
</table>

* Crude protease extracted from pure culture of *Pseudomonas fluorescens*.

**Enzyme amount added to 5 L of raw skim milk
Table 3.2 Skim milk quality

<table>
<thead>
<tr>
<th>Sample</th>
<th>Milk quality (average*) in terms of</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>
|                                             | Total plate count (cfu/mL)         | Viscosity (mPa.s) at 20 ± 2°C | pH at 20 ± 2°C   | Degree of proteolysis (x10^nM)***
| Raw skim milk                               | 3.95 ± 0.5 x 10^2                 | 4.26             | 6.70             | 14.67            |
| Raw milk quality as stated in literature**  | 2.50x10^4                         | NR               | 6.79±0.05        | NR               |
| Processed (UHT) milk on day1                | < 10                               | 4.26             | 6.67             | 14.14            |

* Average of two samples with a range of zero unless stated as ± range

** Total plate count (Australia New Zealand Food Standards code 1.6.1, 2012) n = 5, c = 1, m = 2.5x10^4(cfu/ml), M = 2.5x10^5 (cfu/ml) at 30°C; pH = 6.7 (Regnault, et al., 2004)

Total plate count (cfu/mL) = number of colony forming units in milk on NA plates at 30°C

***Degree of proteolysis based on [FITC isomer1]

NR = Not reported
Table 3.3 Estimated relationship between time for UHT-treated milk to gel and the activity of proteolytic enzymes present in the raw milk before UHT treatment.

<table>
<thead>
<tr>
<th>Required UHT milk shelf-life (weeks) at 20°C</th>
<th>Maximum level* of proteolytic activity (FITC isomer 1 x10^-4nM) in raw milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.9 (3mth)</td>
<td>0.897</td>
</tr>
<tr>
<td>12</td>
<td>0.819</td>
</tr>
<tr>
<td>24</td>
<td>0.591</td>
</tr>
<tr>
<td>40</td>
<td>0.464</td>
</tr>
<tr>
<td>52</td>
<td>0.410</td>
</tr>
</tbody>
</table>

*Derived from the empirical equation of gelation time (weeks) = 9.6301x^{2.12} (equation 3.5a) and allowing for a 10% reduction in proteolytic activity due to UHT treatment
Table 3.4 Threshold proteolytic enzyme values for UHT-treated milk for age-mediated gelation from the literature.

<table>
<thead>
<tr>
<th>Recommended maximum (threshold levels)</th>
<th>For an estimated shelf-life of</th>
<th>Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1-2ng/mL (crude proteinase)</td>
<td>3mths</td>
<td>25°C</td>
<td>Richardson &amp; Te Whaiti (1978)</td>
</tr>
<tr>
<td>&lt;1ng/mL (based on Alcalase R equivalence)</td>
<td>3mths</td>
<td>25°C</td>
<td>Merck (1999)</td>
</tr>
<tr>
<td>ca.0.3ng/mL</td>
<td>4mths</td>
<td>23°C</td>
<td>Mitchell &amp; Ewing (1985)</td>
</tr>
</tbody>
</table>
Figure 3.1 Standard curves for FITC isomer1 for the fluorescein isothiocyanate-casein (FITC-casein) assay (a) and Leu-Gly dipeptide for the fluorescamine assay (b).
Figure 3.2 Growth of *Pseudomonas fluorescens* in NB at 4 ± 1°C. Each point is an average of two samples counted on NA plates incubated at 30 ± 1°C.
Figure 3.3 Effect of UHT treatment on the activity of proteolytic enzyme in skim milk samples with added crude protease. Enzyme concentration ranged from 0%, (for the control sample) to 0.005% (% v/v). Where (■) is before UHT treatment and (□) is after UHT treatment. n=2 and the vertical bars on the histograms represent the range in the data obtained.
**Figure 3.4** Relationship between the enzyme concentration (%, v/v in raw skim milk) and the proteolytic activity (equivalent to FITC isomer 1 concentration in the FITC-casein assay) in UHT-treated milk. The FITC-casein assay involved an incubation period of 2h. The solid line represents the line of best fit.
Figure 3.5 Relationship between the enzyme concentration (% v/v in raw skim milk) and the proteolytic activity (equivalent to fluorescein concentration in the FITC-casein assay) in UHT-treated milk. The FITC-casein assay involved an incubation period of 1 day (a) and 14 days (b) (Button et al., 2011). The solid line represents the line of best fit.

\[
\begin{align*}
\text{a} & & \text{b} \\
\text{Concentration of Fluorescein (nM)} & & \text{Concentration of Fluorescein (nM)} \\
\text{Enzyme concentration (% v/v)} & & \text{Enzyme concentration (% v/v)} \\
\text{y} &= 17.5x - 2.5 & \text{y} &= 76.5x - 0.5 \\
R^2 &= 0.9459 & R^2 &= 0.9614
\end{align*}
\]
Figure 3.6 Effect of storage time (at 20°C) on viscosity (a) and proteolysis of UHT–treated skim milk (b) with added crude protease enzyme (% v/v) from Pseudomonas fluorescens: 0%, (control sample (♦)), 0.0001% (■), 0.0005% (▲), 0.001% (●), 0.005% (+).
Figure 3.7 Relationship between the concentration of free amino groups and the viscosity (at 20°C) in UHT-treated skim milk samples with added crude protease enzyme (% v/v) of *Pseudomonas fluorescens*, where (a) was the lowest added enzyme concentration (0.0001%), and (b) was the highest added enzyme concentration (0.005%). The solid line represents the line of best fit.
Figure 3.8 The viscosity at the onset of gelation (a) and the corresponding free amino group concentration (b) of the UHT-treated skim milk stored at 20°C, irrespective of the added crude enzyme concentration.
Figure 3.9 Relationship between the proteolytic activity and the onset of gelation (weeks at 20°C) of UHT–treated milk samples with added crude enzyme concentrations (% v/v), namely: for 0.0001, 0.0005, 0.001, 0.005 (a) and for 0.0001, 0.001, 0.005 (b). The solid line represents line of best fit.
CHAPTER 4
DEVELOPMENT OF SHELF-STABLE DAIRY BEVERAGES ENRICHED WITH PROTEIN AND DIETARY FIBRE

4.1 ABSTRACT

It is well-known that dairy proteins and dietary fibre display a wide range of nutritional benefits for various market segments including sports nutrition and the specific diets of the 65+ population. In this chapter of the Thesis, the purpose of the work undertake was to develop shelf-stable formulations of dairy beverages enriched with protein and dietary fibre by characterising their sensory and physicochemical properties to determine consumer acceptance for these novel formulations. Moreover, this study targeted UHT processing for the manufacture of dairy beverages as it has been successfully implemented in the preceding Chapter. The latter showed reduction in microbial growth and enzymatic activity following application of UHT thus obtaining finished products of high quality.

Initially, reverse formulation engineering of selected commercial products has been performed via the investigation of the flow behaviour of a model dairy beverage made at the RMIT pilot plant. Once the textural similarity of the commercial product and model formulation produced at RMIT was confirmed, a series of enriched formulations of dairy beverages have been developed. Formulation that resulted in acceptable sensory characteristics and rheological profile in relation to the commercial product was chosen for further scaling up using UHT and pasteurisation techniques available in-house. The data from experimental work indicated that UHT processing is advantageous for the development of enriched dairy beverages, as compared to the pasteurisation technique as it results in a product with improved microbial quality. Rheological and colour observations for the commercial product and model dairy beverage with 6.1% protein and 2% dietary fibre indicated that both products are similar and equally acceptable. This was further confirmed with sensory evaluation by 40 panellists in relation to organoleptic attributes and overall acceptability of the commercial and developed products.
4.2 INTRODUCTION

Milk is a wholesome food, which naturally contains a vast range of nutrients and delivers unique benefits for people who are engaged in physical activities and for the general population at large (Haug, Hostmark & Harstad, 2007). On the average, milk solids comprise approximately 4.8% lactose, 3.7% fat and 3.4% protein; however the actual composition of milk depends on the breed of caw, stage of lactation, intervals between milking, etc. (O’Brien, Ryan, Meaney, McDonagh & Kelly, 2002). Among dairy beverages, chocolate milk shows a wide market share with its flavour, colour and potential health benefits such as antioxidant properties (Thompson et al., 2004; Bayoumi, Mohamed, Sheikh, Farrag & Eissa, 2011). Moreover, an interest in the application of chocolate dairy beverages in sport nutrition (Karp et al., 2006) and beyond has also been observed due to competitive advantages of ready-to-serve beverages and good nutrition.

Recently, researchers compared the protein requirements across several market segments, and specially focused on the needs for sport nutrition suggesting that the intake of nutrient-enriched foods is a healthy foundation for athletes (Paul, 2009). Studies revealed that the recommended daily allowance of protein for healthy individuals with age above 19 years is 0.8 g per kg of body weight, whereas for those performing sports activities the required level of protein will be greater depending on energy requirements (Tarnopolsky et al., 1992). Furthermore, growing popularity of protein-rich diets within consumers relates back to weight management programme considerations (Phillips, 2006; Noakes, 2008; Soenen & Westerterp-Plantenga, 2008).

Protein is a macronutrient that plays a major role in various anabolic processes in the body (Hoffman & Falvo, 2004). It was shown that the intake of both carbohydrates and proteins after resistance exercise will positively affect the muscle net protein balance due to providing fuel for biological activities. Therefore, it was suggested that addition of extra protein and carbohydrates in any formulation will be advantageous to address the balance of protein synthesis for people who are highly involved in physical activities (Børsheim et al., 2004). In this context, dairy proteins (casein and whey) are a superior protein source that provides a large proportion of Branch Chain Amino Acids (Beucler, Drake & Foegeding, 2005) and plays an integral role in muscle metabolism and protein synthesis (Roy, 2008).

Besides protein, there is a wide range of scientific literature that relates to the extensive functional properties and potential health benefits of soluble dietary fibre (Anderson et al., 2009; Whelton et al., 2005). Currently, the food industry is turning more often on
soluble dietary fibres than starchy polysaccharide derivatives in novel formulation engineering for their unique functionality and excellent nutritional properties (Koh, Jiang, Kasapis & Foo, 2010).

In product development, ingredients and processing conditions are the key factors that finalise the overall quality of the product, its safety and shelf life. As we discussed in the previous chapter of the Thesis, current demand for UHT-treated dairy based products is set to increase further over the years due to safety and convenience in their distribution and storage (Koffi, Shewfelt & Wicker, 2005).

To meet the market demand, manufacturers have been attempting to produce protein and dietary fibre enriched products from various animal and plant sources (Paul, 2009). It is well known to them that both protein and dietary fibre change in their structural and functional characteristics following application of heat treatment, thus providing various textures to the final product. It is important to optimise the temperature treatment regimes in order to obtain food systems with highly functional properties incorporating proteins and dietary fibre (Alting, Hamer, Kruif & Visschers, 2000). In this study, we utilised sodium caseinate and inulin in the area of product development, since those ingredients provide various nutritional and textural attributes to the final product and afford relatively easy conditions of processing, e.g. fail to gel during heat treatment (Guzma´n-Gonza´lez, Morais, & Amigo, 2000).

In the previous Chapter of Thesis, we utilised indirect UHT facilities with a plate-heat exchanger system to observe changes occurred in treated milk during subsequent storage. Current investigation builds on the results obtained previously in order to develop highly nutritious dairy beverage under UHT conditions via the observation of structural properties using rheological techniques. Understanding of rheological properties in dairy beverages is important in relation to texture and stability of food products over time (Othman, Asteriadou & Fryer, 2009). Also, the experimental method observes in some detail the effect of ingredients in the formulation and chosen processing conditions.

The objective of the current study is to formulate first and then develop a shelf-stable ready-to-drink dairy beverage enriched with protein and dietary fibre for athletes and active individuals. Investigations on textural properties and colour evaluation were performed in order to confirm the similarity of the product with currently available products in the market, which, however, do not have additional protein and dietary fibre in the formulation. Consumer acceptability for the novel nutritious product was assessed using a sensory hedonic taste panel for the range of parameters used in this work.
4.3 EXPERIMENTAL PROTOCOL

4.3.1 Materials

Whey Protein Concentrate Alacen™ 392 was from Fonterra, New Zealand, and had 80% whey protein, 6.5% fat, 4.4% moisture and 3% ash. According to the manufacturer’s quality control procedures, the product has good emulsifying and nutritional qualities, and was tested for chemical, sensory and microbial parameters using internationally recognized procedures.

Sodium caseinate Alanate™ 180 was from Fonterra, New Zealand. Based on the certificate of analysis provided by the supplier, the product is a readily soluble powder, had good emulsifying properties and passed the microbiological testing under the food grade standards. The typical composition was 93% protein, 3.9% moisture, 1.2% fat, 0.1% lactose, 4.3% ash, 1.2% sodium and 0.02% calcium.

Carrageenan was from FMC, USA. It was in the form of a light brown powder and was recommended for food applications.

Lecithin Centrolex G was from Langdon, Australia. According to the product specification, it contains primary phospholipids in the ratio that occur naturally in soya beans. The maximum moisture content is 1%.

Inulin was from Orafi, Belgium. The product was a standard powder from the chicory inulin that consists of a mixture of oligo- and polysaccharides.

Cocoa powder was from Maltra Foods, Australia. The product was food grade in the form of alkalised pure Dutch cocoa powder.

Food colours chocolate brown (CA-50,000D-WS) was supplied by CHR Hanson Australia; cherry pink was from Queen Food Colouring Company, Australia, and consists of water, alcohol (about 22%), glycerine and cochineal extract (E 120).

Food flavours natural vanilla extract from Queens Fine Food, Australia. According to the product specification, it has almost no alcohol and is recommended to use in products subjected to high temperatures.
Skim milk and full cream milk were supplied by Parmalat Australia. Based on nutritional information, the average composition of total fat, protein and carbohydrates in the full cream milk and skim milk were 3.8%, 3.2%, 4.9% and 1.3%, 3.4%, 4.9%, respectively.

### 4.3.2 Sample preparation

Based on the concept of improving nutrition by enriching with protein and dietary fibre, different formulations of dairy beverages were created with whey protein concentrate, inulin and sodium caseinate. Sample preparation was carried out in two stages. First, samples were prepared with various formulations and evaluated at the RMIT research laboratory. Second, formulations that produced product which displayed similar textural behaviour to that exhibited by the commercial product were further scaled up at RMIT pilot plant utilising different heat treatment techniques.

**Sample preparation at RMIT Food Science research laboratory.** Samples of dairy beverages at a 100 g batch were produced according to the formulations in Table 4.1. Dry and wet ingredients were weighed separately, then combined together and mixed with a magnetic stirrer for 20 min at room temperature. Temperature of the systems was increased up to 50°C and held there for 6 min to ensure proper dissolution of the ingredients followed by “pasteurisation” at 85°C for 5 min. The samples were cooled down to 55°C, filled into 120 ml plastic containers and stored at 4°C.

**Sample preparation at RMIT Food Science pilot plant.** Based on the physicochemical and sensory properties of the dairy beverages enriched with protein and dietary fibre prepared at the RMIT research laboratory, formulation 3 in Table 4.1 was scaled up using processing parameters available at the RMIT pilot plant that match the manufacturing process for the production of the commercial product. For this, dry ingredients were added to milk ingredients and mixed with an electric mixer (Kitchen Aid, USA) for 20 min at room temperature. Temperature of the product was raised up to 50°C to ensure proper dissolution of the protein and fat in the formulation followed by a four-stage homogenization at 70 Bar using FT9 homogeniser (Armfield, UK). The following heat treatments were performed for dairy beverage production:

1. **Indirect UHT processing with plate-heat exchanger system (FT74X, Armfield, UK).** The method was carried out with parameters as indicated below:
   
   - Pressure – 4.62 Bar
   - Inlet flow – 12.6 L/h
Inlet temperature – 25.5°C
Pre-heat temperature – 107°C
UHT conditions - 140°C for 2 to 5 s
Outlet temperature – 16.8°C

(iv) Heat treatment at 85°C for 5 min using Stephan Kettle.

The beverages were filled into sterilized 250 mL glass containers in aseptic environment and refrigerated at 4°C for 24.0 ± 1.0 h before physicochemical analysis and sensory testing.

4.3.3 Methods

Samples were taken from the refrigerator just before the physicochemical measurements and sensory analysis.

**Steady shear viscosity** was carried out on a controlled strain rheometer (AR-G2 from TA Instruments, New Castle, DE, USA) with a 40 mm diameter parallel-plate geometry and 1 mm gap at 4°C. The viscosity of the samples was analysed as a function of shear rate ranging from 0.1 to 100 s⁻¹.

**Sterility** of the dairy beverages enriched with protein and fiber and stored at 4°C was analyzed using a spread plate method. The medium used was nutrient agar (NA) and the microbial load was enumerated by taking an aliquot (1 mL) of dairy beverage sample and serially diluting it with isotonic saline solution (0.85% w/v, 10⁰ to 10⁻⁴). Nutrient agar plates were incubated at 37°C for 48 h and the colonies counted. The results reported as an average of three replicates.

**Colour evaluation** of the dairy beverages enriched with protein and dietary fibre, as compared to the commercial sample, was carried out on the Minolta Chromometer (CR-400, Japan). Different colour scales are represented in \( L^* \), \( +a^* \), \( -a^* \), \( +b^* \), \( -b^* \) as the degree of lightness, redness, greenness, yellowness or blueness, respectively (Jafarpour, Sherkat, Leonard & Gorczyca, 2008). Initially, the instrument was calibrated using a white tile with the values of colour scale being \( Y = 93.13 \), \( x = 0.3138 \), \( y = 0.3199 \). Forty ml of the dairy beverage samples were poured into small plastic containers and the colour attributes were recorded for each sample in triplicate. Colour of the novel formulations of dairy beverages compared to the commercial sample was determined by calculating the degree of lightness, chroma \( (C^*) \), hue
angle ($h_{ab}$) and $\Delta E$ as colour deviation using the following equations (Iserliyska, Chinnan & Resurreccion, 2012):

$$\Delta E_{*ab} = ((\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2)^{1/2}$$  \hspace{1cm} (4.1)

$$\% \text{ of } \Delta E_{max}^* = (\Delta E* \times 100) / \Delta E_{max}$$  \hspace{1cm} (4.2)

where, $\Delta E_{max}$ is a standard value ($\Delta E_{max} = 196.98$).

$$C_{*ab} = ((a*)^2 + (b*)^2)^{1/2}$$  \hspace{1cm} (4.3)

$$h_{ab} = \tan^{-1} (b*/a*)$$  \hspace{1cm} (4.4)

$$\Delta H = ((\Delta E_{*ab})^2 - (\Delta L*^2 - (\Delta C*)^2)^{1/2}$$  \hspace{1cm} (4.5)

Generally, $\Delta E$ tells us the difference of two products but not in what way (whether it is due to L, a, b singly or in their combinations). Therefore, from the hue angle ($h_{ab}$) we know about the absolute colour differences, whereas $\Delta H$ describes the euclidian difference between the colour of the two samples.

**Sensory analysis** taste panel trial including a hedonic taste panel of preference test was carried out with panellists (n = 40) from RMIT students and staff (aged 18-65). Commercial dairy beverage with low protein and dietary fibre levels versus dairy beverage enriched with protein and dietary fibre made at RMIT pilot plant were trialled by the panellists.

Panellists were asked to identify the intensity of the particular parameters of the dairy beverages, overall how much they liked the samples, and their specific parameters on a hedonic scale ranging from extremely dislike (1) to extremely like (9) (Appendix 4.III). Before undertaking the trial, each panellist was asked to read a plain statement (Appendix4.I) that explained the nature and content of the trials. After answering any queries and if the panellist was in agreement, they were asked to read a consent form (Appendix 4.II). Following that, the panellists signed the form and only then were the samples and the taste panel form brought out to them. The sampling codes (Appendix 4.IV) used were selected from a random design number table, and the form and plates were numbered so that neither the panellists nor the presenter knew the product codes. The panellists were instructed to
cleanse their palette before tasting with lemon juice followed by water and to do so in between each sample, and to taste the samples from left to right.

4.4 RESULTS AND DISCUSSION

4.4.1 Characterisation of the flow behaviour in the commercial beverage and the prototype beverage made at the research laboratory

To introduce novel formulations in relation to highly nutritious dairy beverages, reverse formulation engineering of the commercial product has been performed. Composition of the commercial sample of chocolate dairy beverage according to the ingredient label was skim milk, milk, sugar, water, cocoa powder (min 0.3%), flavours, vegetable gum (407). Nutritional information per 100 g was stated as: protein – 3.3 g, fat – 3.4 g, carbohydrates – 9.6 g, including sugars – 9.3 g. As an approximation to the above formulation, we engineered a commercial prototype of dairy beverage indicated in Table 4.1. In order to investigate whether the processing parameters available at RMIT research laboratory reproduce the commercial product, the reverse engineered sample of dairy beverage was made in the research laboratory with the parameters described in section 4.3.2. Products were analysed and compared for textural behaviour using measurements on steady shear viscosity and basic sensory tests.

The trend of steady-shear viscosity in Figure 4.1 declined dramatically for both materials with increasing shear rate from 0.1 (\( \eta \sim 55 \text{ mPa s} \)) to 100 s\(^{-1}\) (\( \eta \sim 21 \text{ mPa s} \)) thus demonstrating the shear thinning nature of the two matrices. The rheological profile for the two samples indicated that the commercial prototype developed at RMIT research laboratory can “mimic” the flow of the commercial sample by displaying near overlapping traces. Basic sensory test performed among 5 RMIT staff showed that there was no considerable difference in taste between the two dairy beverages. Thus, the processing parameters utilised for the production of the commercial prototype in our laboratory are suitable for further experimental work.
4.4.2 Characterisation of flow behaviour in dairy beverages prepared at the research laboratory based on the concept of protein and dietary fibre enrichment

Different formulations of the dairy beverage were created and evaluated containing whey protein concentrate, sodium caseinate, inulin and carrageenan (2 and 3 in Table 4.1). We also show formulation (1) of the commercial prototype, which was made at the research laboratory, containing a total of 3.3% dairy protein but no inulin.

Novel formulations of dairy beverages enriched with protein and dietary fibre produced a congruent trend in the steady-shear dependence of viscosity to that recorded earlier for the commercial product and prototype (Figure 4.2). This rheological profile indicated that there is an effect of the addition of extra protein and dietary fibre on the flow behaviour of dairy beverages showing higher viscosity values, as compared to the commercial product and prototype, due to the differences in solids content. Moreover, sodium caseinate, whey protein concentrate and inulin have a high water holding capacity and create intermolecular interactions during heat processing that affect the flow characteristics of the enriched products.

Basic sensory evaluation was performed among 5 RMIT staff indicating that the formulation of dairy beverage with sodium caseinate is more acceptable than the preparation with whey protein concentrate. Moreover, the choice of the dairy beverage with sodium caseinate is accompanied by a greater nutritional value comprising a total of 6.1% protein, which is almost double the amount of protein in the commercial product. That formulation was proposed for further scaling up to 4 kg of product using processing parameters available at the RMIT pilot plant.

4.4.3 Characterisation of flow behaviour in dairy beverages enriched with protein and dietary fibre prepared at the pilot plant

The preceding work provides insights into the development of dairy beverages enriched with protein (sodium caseinate) and dietary fibre (inulin). To develop a model industrial processing environment, a formulation with 3.5% sodium caseinate, 0.1% lecithin, 0.01% carrageenan and 2% inulin was scaled up at the pilot plant utilising indirect ultra-high temperature treatment with a heat plate exchanger system, or by pasteurising at 85°C using a Stephan Kettle and holding there for 5 min.
Rheological properties were evaluated for both samples, in comparison to the commercial dairy beverage, as depicted in Figure 4.3 reproducing the trend in viscosity variation as a function of experimentally accessible shear rate. Patterns of shear thinning profiles are similar for all materials. For all practical purposes, viscosities were similar for beverages made under UHT conditions and high-temperature pasteurisation being about $\eta \sim 66$ mPa s (0.1 s$^{-1}$) and $\eta \sim 27$ mPa s (100 s$^{-1}$).

During UHT processing, the product was treated at a very high temperature (140°C) with a short holding time at the heating section of the heat plate exchanger system (2-5 s). Following this, the product immediately reached room temperature (~ 20°C) at the cooling section of the heat plate exchanger system. In the treatment with Stephan Kettle, the product was heated up to 85°C, held there for 5 min with no immediate cooling process. Utilised heat treatment regimens may affect sensory attributes of products for colour, odour and taste. UHT processing is preferable for the development of shelf-stable food products, and this statement was further examined by analysing physical changes including colour alteration and food safety of the UHT and pasteurised counterparts.

### 4.4.4 Microbiological examination of protein and dietary fibre enriched beverages

Total plate count of the UHT-treated dairy beverage straight after production was < 10 cfu/mL. This represents a significant effect of the UHT treatment on the bacterial count of the sample being about $8.4 \times 10^4$ cfu/mL before the thermal treatment. Table 4.2 shows that the total plate count during 6 days of storage of the UHT-treated dairy beverage was similar to that of the day of production (< 10 cfu/mL). The increase in total microbial count up to $2.5 \times 10^3$ cfu/mL was noted on the eighth day of storage. The microbial population in the first week of storage indicates the lag phase of the microorganism’s growth by adapting to growth conditions due to the differences in the storage temperature of the beverages (4°C) and their optimum temperature of growth (25-30°C). Our results were in agreement with previous studies on the reduction of total plate count in dairy beverages during the first week of storage at 4°C (Hassan, Abdalla & Nour, 2009). They indicate the highly sterile conditions that can be achieved in a properly operated UHT setting in an industrial context.

Table 4.2 indicates that pasteurisation of the dairy beverages using Stephan Kettle had a very low effect on the total microbial count, which is maintained at about $1.75 \times 10^4$ cfu/mL following processing, hence being comparable to the initial microbial load of the sample ($8.4 \times 10^4$ cfu/mL). It is reasonable to expect these results, since the processing parameters utilised
at Stephan Kettle did not reach the sterilisation conditions required for microbial safety. Moreover, there is recontamination of the product following pasteurisation due to inability to achieve a fully contained aseptic environment, which is better achieved under UHT settings with, for example, an adjoining aseptic laminar-flow cabinet. Pasteurised dairy beverages, maintained at the same storage conditions as for the UHT-treated products (4°C), displayed an exponential growth phase that started on the third day, with values of the total microbial count being about $1.8 \times 10^4 \text{cfu/mL}$, and on the sixth day of storage, the number of bacterial colonies was high at $1.1 \times 10^6 \text{cfu/mL}$.

### 4.4.5 Investigation on colour differences of commercial and model dairy beverages

Over the years, it has been recognised that the scales for $L^*$, $a^*$, $b^*$ show the most appropriate variations in colour within the dark region that is relevant to the chocolate beverages (Bayoumi et al., 2011). Usually, “$L$-values” are used to indicate differences in lightness whereas the values in “$a$” and “$b$” relate to redness and yellowness of preparations, respectively (Lanier, 1992). As shown in Table 4.1, chocolate brown colour was introduced in our prototype formulations for comparison with the relevant commercial product in Table 4.3.

Colour differences in Table 4.3 indicate that dairy beverage enriched with protein (total of 6.1%) and dietary fibre (total of 2%) is lighter than the commercial sample as the $L^*$ value of the former is greater. However, a difference of 5 units in $L^*$ values between the commercial product and our sample does not convey a detectable difference in visual lightness. Individual colour parameters of $a^*$ and $b^*$ show positive values for both products; the values of redness and yellowness are higher in the pilot plant preparation, as compared to the commercial product. As for lightness, differences of 1 or 2 units in $a^*$ and $b^*$ values are not significant in the visual observation of both materials.

Overall, the colour difference ($\Delta E^{*}_{ab}$) between the commercial product and the protein/dietary fibre preparation was $5.43 \pm 0.53$, whereas the maximum possible colour difference ($\Delta E^{*}_{\text{max}}$) between the two materials was 2.75%, which is neither small (< 0.5%) nor large (5%). $C$ values in Table 4.3 that depict the colour strength of the commercial product and the RMIT sample are 13.49 ± 0.16 and 15.56 ± 0.04, respectively. This indicates that the product made at RMIT pilot plant is 15.34% brighter than the commercial dairy beverage [(15.56 - 13.49) / 13.49]. The actual colour of the dairy beverages was expressed as hue angle ($h_{ab}$) in Table 4.3 to show a slight difference in the measured values. The dairy beverage enriched with protein and dietary fibre was closer to “$+b^*$” ($57.66^0 \pm 0.06$), which is pale red.
with a tinge of yellow colour, whereas the commercial product \((55.58^0 \pm 0.04)\) has a smaller yellow tint. The recorded hue difference of the two dairy beverages is rather small \((\Delta H = 0.53 + 0.03)\).

It appears that both dairy beverages (commercial product and RMIT pilot plant made) are similar embodiments in terms of their colour attributes and flow behaviour, as indicated in the viscosity profiles in Figure 4.1, an outcome which encourages us to examine the sensory perception of the two systems in some detail.

### 4.4.6 Reporting sensory evaluation data for the individual beverages in this investigation

In the taste panel of this work, commercial dairy beverage versus dairy beverage enriched with protein and dietary fibre made at the RMIT pilot plant under UHT conditions were trialled by 40 panellists. In this trial, he panellists were asked to identify the intensity of a particular parameter for the dairy beverages ranging from extremely less (1) to extremely high (9) intensities, and how much they liked the sample on a hedonic scale ranging from extremely dislike (1) to extremely like (9) (Appendix 4.III).

According to the visual observations for colour in the dairy beverages, 47.5% and 10% of the panellists assessed the colour of the commercial dairy beverage and the product enriched with protein and dietary fibre as “dark chocolate”. Other panellists, i.e. 52.5% and 90%, scored 5 (neither dark nor light) for the commercial product and the RMIT made enriched dairy beverage, respectively. Out of 40 panellists, 57.5% had no preference and 45% scored 7 (liked) for the colour of both products. Among other panellists, 12.5% and 17.5% gave levels of liking to the colour with a score of 8 (liked very much) for the dairy beverage enriched with protein and dietary fibre and the commercial product, respectively. Score of 6 (slightly liked) for the colour assessment was given to the commercial product and RMIT made enriched product by 25% and 27.5% of the panellists, respectively. Score of 5 (neither like nor dislike) was obtained by 12.5% and 7.5% of the panellists for the colour of the commercial product and dairy beverage enriched with protein and dietary fibre. Score of 4 (slightly dislike) for the colour assessment was given to the protein and dietary fibre enriched product by 7.5% of the panellists. The average scores (± SD) for the colour preference were 6.68 (± 0.92) and 6.53 (± 0.98) for the commercial product and the protein/dietary fibre enriched beverage, respectively.
Regarding smoothness, results reveal that 50% and 40% of the panellists assessed as “neither grainy nor smooth” the commercial dairy beverage and the product made at RMIT, respectively. Both dairy beverages were evaluated as “very smooth” by 50% and 60% of the panellists. Among 40 panellists, 50% had no preference in smoothness of the products. A panellist group (17.5%) scored 9 (extremely liked), 8 (liked very much) and 6 (slightly liked) the smoothness of the enriched product, whereas the same number of panellists gave a score of 6 (slightly liked) for the smoothness of the commercial product. Another panellist group (7.5%) scored 9 (extremely liked) and 5 (neither like nor dislike) for the smoothness of the commercial product, while 12.5% of the panellists scored 8 (liked very much) for the same material. A score of 7 (liked) was given by 50% and 32.5% of the panellists for the smoothness of the commercial and the protein-enriched formulation, respectively. The smoothness of the enriched product was scored with 5 (neither like nor dislike) by 5% of the panellists, whereas the same percentage of the panellists assessed the smoothness of the commercial dairy beverage with 4 (slightly disliked). Smoothness of the dairy beverage enriched with protein and dietary fibre was scored with 4 (slightly disliked) by 10% of the panellists. The average preference scores for smoothness (± SD) were 6.80 (± 1.15) and 6.95 (± 1.48) for the commercial product and the enriched formulation, respectively.

Regarding the sweetness of the products, 2.5% of the panellists assessed the commercial product as “not sweet”, and the same material was evaluated as “sweet” and “very sweet” by 52.5% and 45% of the panellists. In comparison, the enriched with protein and dietary fibre dairy beverage was assessed as “not sweet” by 17.5% of the panellists, while scores of “sweet” and “very sweet” were given to the product by 67.5% and 15% of the panellists. According to the hedonic scale for sweetness, 12.5% of the panellists had no preference in sweetness between the two samples. A panellist group (17.5%) scored 8 (liked very much) and 4 (slightly disliked) for the commercial beverage, whereas the same percentage of panellists scored 5 (neither liked nor dislike) for the enriched preparation. The sweetness of the novel formulation was assessed as 8 (liked very much), 7 (liked), 6 (slightly liked) and 4 (slightly disliked) by 7.5%, 30%, 35% and 10% of the panellists, respectively. The panellists (22.5% and 42.5%, respectively) scored the sweetness of the commercial product with 7 (liked) and 6 (slightly liked). The average preference scores for sweetness were 6.23 (± 1.27) and 6.08 (± 1.09) for the commercial product and novel preparation.

Consumer perception for flavour in the two types of products: Fifteen percent of panellists observed “no chocolate flavour” in the commercial dairy beverage. Among others, 47.5% and 37.5% assessed the flavour for the commercial product as “moderate chocolate”
and “strong chocolate”, respectively. For the flavour of the enriched product, 30% of the panellists perceived that the product had “no chocolate flavour”, 65% confirmed its flavour as “moderate chocolate”, and 5% of the panellists found it to be “strong chocolate”. A panellist group (15%) had no preference between the two products, but the flavour of the commercial product was scored as 8 (liked very much) and 4 (slightly disliked) by 12.5% of the panellists. Other panellists (7.5%) gave scores of 8 (liked very much) and 6 (slightly liked) to the flavour of the RMIT made beverage. The commercial product and enriched preparation were scored with 7 (liked) by 47.5% and 32.5% of the panellists, respectively, while scores of 6 (slightly liked) and 5 (neither liked nor disliked) were given to the commercial product by 15% and 10% of the panellists. The flavour of the dairy beverage enriched with protein and dietary fibre was scored with 5 (neither liked nor disliked) and 4 (slightly disliked) by 17.5% and 30% of the panellists, respectively. Other panellists (2.5%) gave scores of 3 (dislike) for commercial dairy beverage while 5% of panellists gave score of 1 (extremely dislike) to the flavour of the RMIT made beverage. Average preference scores for flavour of the commercial product and enriched formulation were 6.30 (± 1.32) and 5.45 (± 1.73), respectively.

Besides the preference test for particular parameters (colour, smoothness, sweetness and flavour) of the two products discussed in the preceding paragraphs, overall acceptance has also been determined (Appendix 4.III). Out of 40 panellists, 15% had no preference (same score for both samples tested) and 7.5% scored 9 (extremely liked) for the commercial product. Among other panellists, 27.5% and 12.5% gave levels of liking with a score of 8 (liked very much) for our preparation and the commercial system, respectively. A panellist group (45%) scored 7 (liked) for the commercial beverage, whereas 30% of panellists indicated the same preference level for the novel formulation. Scores of 6 (slightly liked) and 5 (neither liked nor disliked) were given to the commercial dairy beverage by 15% of the panellists, but the novel product was assessed with a score of 6 (slightly liked) by 30% of panellists. The commercial product and dairy beverage enriched with protein and dietary fibre were scored with 4 (slightly disliked) by 5% and 10% of the panellists, respectively. Finally, 2.5% of the panellists scored with 2 (very much disliked) for the enriched product. Average scores (± SD) for overall acceptance of the commercial dairy beverage and the product enriched with protein and dietary fibre were 6.67 (± 1.25) and 6.55 (± 1.39), respectively.
4.4.7 Relating intensity to preference from the sensory evaluation data of the beverages in this investigation

The above results of the sensory panel have been summarised in Figure 4.4 (a-d) that makes the comparison between commercial product and dairy beverage enriched with protein and dietary fibre prepared at the RMIT pilot plant. The figure indicates the average outcome in the overall preference test, which relates to the assessment of important intensity parameters in these systems. This relationship justifies the present protocol including readings for both parameter intensity and preference. It is summarised, as follows:

Figure 4.4a shows the results of preference and intensity tests for the particular parameters in the commercial dairy beverage. We may assume from the data that the preference scores for sweetness and flavour, i.e. 6.23 (± 1.27) and 6.30 (± 1.32) (slightly liked), relate to the average intensity perception of “slightly sweet” (6.03 ± 1.46) and “slight chocolate flavour” (5.93 ± 1.67), respectively. Smoothness intensity was “smooth” (6.95 ± 1.66) that was “liked” in the preference test by the panellists with an average score of 6.80 (± 1.15). Product colour was assessed in preference as “liked” (6.68 ± 0.92) and the actual intensity was scored “slightly dark” (3.78 ± 0.46).

In the sensory evaluation of the RMIT made beverage (Figure 4.4b), we may consider that the preference in sweetness was “slightly liked” (6.08 ± 1.09) due to its intensity assessment of “neither not sweet nor sweet” (5.40 ± 1.60). Similarly, the flavour of the product has been preferred as “slightly liked” by the consumers (5.45 ± 1.73) since the perceived intensity was “neither no chocolate flavour nor chocolate flavour” (4.70 ± 1.54). The smoothness and colour of the dairy beverage enriched with protein and dietary fibre were preferred as “liked” by the panellists (6.95 ± 1.48 and 6.53 ± 0.98, respectively) that relate to the intensity measurements of “smooth” texture (6.73 ± 1.48) and “neither dark nor light” colour (5.08 ± 1.44).

Further comparisons in Figure 4.4(c-d) argue that our preparation belongs to the same statistical population for sweetness, colour and smoothness with the commercial product (p > 0.05). According to ANOVA statistical test (one way), there was no significant (p > 0.05) difference in the preference level of the two beverages (overall acceptance of the commercial and prototype formulations were 6.67 and 6.55, respectively), which further argues that both dairy beverage are acceptable by the consumer with a score of “liked”. Therefore, addition of 6.1% protein and 2% dietary fibre in the UHT treated prototype creates an acceptable
mouthfeel that is assessed similarly to the commercial product with only 3.1% protein and no dietary fibre in the formulation.
4.5 CONCLUSIONS

Research in this chapter of the Thesis dealt with the reformulation of a commercial chocolate beverage. This was carried out by enriching existing formulations with relatively high levels of protein and dietary fibre (for a beverage) using thermal processing available at the research laboratory and pilot plant. The aim was to improve considerably the nutritional profile and maintain acceptable mouthfeel in the novel formulation. Following several trials, UHT was chosen as the thermal processing of choice, which is also preferred in industrial production due to efficient microbial kill and enhanced shelf-life of products. Dietary fibre and protein enriched formulations exhibited similar shear thinning behaviour of viscosity to the commercial counterparts, which encouraged us to assess the sensory profiles of the two systems. Sensory evaluation among forty panellists evaluated first the intensity of product quality parameters of colour, flavour, sweetness and smoothness, which allowed further comparisons in terms of a preference test. It was documented that the commercial dairy beverage with about 3.1% protein and no dietary fibre, and our preparation with 6.1% protein and 2% dietary fibre are equally acceptable and liked by the panellists. Future recommendations include a large consumer trial to determine preference levels and scaling up of the formulation with enhanced nutritional profile in the factory of the industrial sponsor for subsequent product launching in the market.
4.6 REFERENCES


Table 4.1 Formulations of dairy beverages enriched with protein and dietary fibre made at the RMIT research laboratory and pilot plant

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>(1) Commercial prototype</th>
<th>(2) 3.5% Alacen (whey protein concentrate), 2% inulin, 0.1% lecithin, 0.01% carrageenan</th>
<th>(3) 3.5% Alanate 180 (sodium caseinate), 2% inulin 0.1% lecithin, 0.01% carrageenan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td>7.21</td>
<td>6.21</td>
<td>6.21</td>
</tr>
<tr>
<td>Full cream milk</td>
<td>87.56</td>
<td>82.34</td>
<td>82.34</td>
</tr>
<tr>
<td>Caster sugar</td>
<td>4.58</td>
<td>5.37</td>
<td>5.37</td>
</tr>
<tr>
<td>Cocoa powder</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Flavour (Natural vanilla extract)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Chocolate brown colour</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Cherry Pink colour</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>0.30</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Lecithin (Centrollex G)</td>
<td>0.00</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Inulin (Orafti)</td>
<td>0.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Alacen 392 (whey protein concentrate)</td>
<td>0.00</td>
<td>3.50</td>
<td>0.00</td>
</tr>
<tr>
<td>Alenate 180 (sodium caseinate)</td>
<td>0.00</td>
<td>0.00</td>
<td>3.5</td>
</tr>
<tr>
<td>Product %</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Protein, %</td>
<td>3.10</td>
<td>5.67</td>
<td>6.11</td>
</tr>
<tr>
<td>Fat, %</td>
<td>3.34</td>
<td>3.29</td>
<td>3.21</td>
</tr>
<tr>
<td>Carbohydrates, %</td>
<td>9.60</td>
<td>11.99</td>
<td>11.80</td>
</tr>
<tr>
<td>dietary fibre, %</td>
<td>0.30</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>sugar, %</td>
<td>9.30</td>
<td>9.99</td>
<td>9.80</td>
</tr>
<tr>
<td>Sodium, %</td>
<td>0.04</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>Total solids, %</td>
<td>16.20</td>
<td>21.09</td>
<td>21.30</td>
</tr>
</tbody>
</table>
### Table 4.2 Effect of processing parameters on total aerobic bacterial count of the dairy beverage enriched with protein and dietary fibre made at the RMIT pilot plant

<table>
<thead>
<tr>
<th>Processing procedure for the enriched dairy beverage</th>
<th>Total aerobic bacterial count (cfu/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before heat treatment</td>
</tr>
<tr>
<td>UHT</td>
<td>8.40x10⁴</td>
</tr>
<tr>
<td>Pasteurisation at high temperature using the Stephan kettle</td>
<td>8.40x10⁵</td>
</tr>
</tbody>
</table>

*Average of n=3; cfu/mL = colony forming unit per millilitre,
** = after heat treatment
NC= not counted
Table 4.3 Colour parameters of the commercial dairy beverage and product enriched with protein and dietary fibre made at the RMIT Pilot plant

<table>
<thead>
<tr>
<th>Sample</th>
<th>Visual assessment of colour</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>C</th>
<th>h_ab</th>
<th>ΔE*_{ab}</th>
<th>ΔH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial sample</td>
<td>Chocolate brown colour</td>
<td>54.36±0.40</td>
<td>7.63±0.09</td>
<td>11.14±0.12</td>
<td>13.49±0.16</td>
<td>55.58±0.04</td>
<td>5.43±0.53</td>
<td>0.53±0.03</td>
</tr>
<tr>
<td>Dairy beverage enriched with protein and dietary fibre</td>
<td>Chocolate brown colour</td>
<td>59.35±0.13</td>
<td>8.33±0.03</td>
<td>13.15±0.03</td>
<td>15.56±0.04</td>
<td>57.66±0.06</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = 3; ± = SD; L* = lightness axis (0 is black, 100 is white); a* = red - green axis (“+” values are red, “-” values are green, 0 is neutral); b* = blue – yellow axis (“+” values are yellow, “-” values are blue, 0 is neutral); C = chroma; h_ab = hue angle; ΔE*_{ab} = total colour difference; ΔH = hue difference
Table 4.4 Taste panel data (%) for (A) description of 4 sensory parameters on a 9 point scale for two products, namely: Commercial beverage (C), and protein & fibre-enriched formulation (EF).

<table>
<thead>
<tr>
<th>A</th>
<th>Panellists (%) assessment of Description (A) for the following sensory parameters:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colour</td>
</tr>
<tr>
<td></td>
<td>Description</td>
</tr>
<tr>
<td>Dark chocolate.</td>
<td>Dark chocolate</td>
</tr>
<tr>
<td>Neither dark nor light</td>
<td>Neither sweet nor not sweet</td>
</tr>
<tr>
<td>Light chocolate</td>
<td>Light chocolate</td>
</tr>
<tr>
<td>Total</td>
<td>Total</td>
</tr>
</tbody>
</table>

138
Table 4.5 Taste panel data (%) for (B) Product acceptability based on a hedonic scale (ranging from extremely like (9) to extremely dislike (1) for two products, namely: Commercial beverage (C), and protein & fibre-enriched formulation (EF)

<table>
<thead>
<tr>
<th>Acceptability Scores</th>
<th>Colour (%) for:</th>
<th>Smoothness (%) for:</th>
<th>Sweetness (%) for:</th>
<th>Flavour (%) for:</th>
<th>Overall (%) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>EF</td>
<td>C</td>
<td>EF</td>
<td>C</td>
</tr>
<tr>
<td>9 Extremely liked</td>
<td>0</td>
<td>0</td>
<td>7.5</td>
<td>7.5</td>
<td>0</td>
</tr>
<tr>
<td>8Liked very much</td>
<td>17.5</td>
<td>12.5</td>
<td>12.5</td>
<td>17.5</td>
<td>17.5</td>
</tr>
<tr>
<td>7 Liked</td>
<td>45.0</td>
<td>45.0</td>
<td>50.0</td>
<td>32.5</td>
<td>22.5</td>
</tr>
<tr>
<td>6 Slightly liked</td>
<td>25.0</td>
<td>27.5</td>
<td>17.5</td>
<td>17.5</td>
<td>42.5</td>
</tr>
<tr>
<td>5 Neither liked nor disliked</td>
<td>12.5</td>
<td>7.5</td>
<td>7.5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>4 Slightly disliked</td>
<td>0</td>
<td>7.5</td>
<td>5</td>
<td>10</td>
<td>17.5</td>
</tr>
<tr>
<td>3 Disliked</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 Very much disliked</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
**Figure 4.1** Steady shear viscosity for the commercial dairy beverage (▲), and dairy beverage made at RMIT research laboratory according to the commercial formulation (■) at 4°C.
Figure 4.2 Steady shear viscosity for the commercial dairy beverage (▲), dairy beverage made at RMIT research laboratory according to the commercial formulation (■), and dairy beverages enriched with protein and dietary fibre produced at RMIT research laboratory with 3.5% Alacen 392 (whey protein concentrate), 2% inulin, 0.1% lecithin and 0.01% carrageenan (●), and 3.5% Alanate 180 (sodium caseinate), 0.1% lecithin, 0.01% carageenan, 2% inulin (♦) at 4°C.
Figure 4.3 Steady shear viscosity for the commercial dairy beverage (▲), and dairy beverages enriched with protein and dietary fibre containing 3.5% Alanate 180 (sodium caseinate), 0.1% Lecithin, 0.01% carageenan, 2% inulin manufactured at RMIT pilot plant with UHT treatment (■), and high temperature pasteurisation using Stephan kettle (●) at 4°C.
Figure 4.4 Sensory evaluation at hedonic scale (solid line) and assessment of intensity for the parameters of the dairy beverages (dash line): for the commercial dairy beverage (a); for the dairy beverage enriched with protein and dietary fibre (b); for the two beverages at preference scale (c); and for the two beverages assessing the intensity of the parameters (d). The commercial product is indicated as a red circles, whereas the novel formulation is presented as green diamonds.
INVITATION TO PARTICIPATE IN A RESEARCH PROJECT

PARTICIPANT INFORMATION

Project title:

Characterisation of proteolytic activity and physicochemical changes in UHT milk and dairy-based food products

Investigators:
Professor Stefan Kasapis:
Senior Project Supervisor: Food Chemistry Group, School of Applied Sciences (Food Science and Technology, RMIT University), stefan.kasapis@rmit.edu.au

Mrs. Elizabeth Gorczyca
Senior lecturer: Food Chemistry Group, School of Applied Sciences (Food Science and Technology, RMIT University), elizabeth.gorczyca@rmit.edu.au

Dr. Anna Bannikova,
Research Fellow, Food Chemistry Research Group, School of Applied Sciences (Food Science and Technology, RMIT University), anna.bannikova@rmit.edu.au

Baddegamage Anusha Lakmini Buthgamuwa
Master student, program MRO45 (Food Chemistry Research Group, School of Applied Sciences (Food Science and Technology, RMIT University), s3352958@student.rmit.edu.au

Dear Participant,

You are invited to participate in a Masters by Research project being conducted by Baddegamage Anusha Lakmini Buthgamuwa at RMIT University, Food Science discipline. This information sheet describes the project in straight forward language, or ‘plain English’.

Please read the sheet carefully and be confident that you understand its contents before deciding whether to participate.

If you have any questions about the project, please feel free to ask any one of the investigator listed above.

Who is involved in the project and why it is important?

This is a project undertaken by the Food Chemistry Research group under the direction of Professor Kasapis.
The project is evaluating the dairy beverages with increased desirable protein and dietary fibre levels in relation to nutritional requirements while maintaining an acceptable organoleptic profile.

Another reason why this project is important is to have a thorough and all encompassing approach to the development of products that are commercially useful. All encompassing means that a range of meaningful techniques to evaluate a product can be employed, such as those involved in rheological analysis to determine textural characteristics as well as evaluation of the sensory characteristics through taste panel trials of the products developed.

The sensory trials will provide information for potentials in collaboration with industry. This may lead to a major industrial contract for Food Sciences RMIT, so your assistance is very much appreciated.

**Why have you been approached?**

You have been approached because you represent typical consumers in the age group of 25-65 years.

**What is project about?**

Keeping the bone, muscle and cognitive health is very important throughout life. Protein is a very important macronutrient for humans by playing numerous roles on body functioning. It is widely accepted by the scientific community that protein requirements well depend progressively from age and physical requirements, and protein origin or quality are important considerations for individuals. Thus, a case study was undertaken to incorporate desirable protein and dietary fibre levels to commercial product formulations in relation to nutritional and age requirements while maintaining an acceptable organoleptic profile.

**What is the question being addressed?**

The purpose of the taste panel trials is to answer the following questions:

- What is the level of ‘liking’ (on a 9 point like – not like scale) of the samples (2) of dairy beverage samples provided?

**What is the risks associate with this project?**

Please note that the ingredients used to prepare the dairy desserts are commonly used in a range of manufactured foods and are regarded as safe. All risks have been reduced significantly if not eliminated by asking the panellist to spit out the samples being evaluated. That is, you are instructed NOT TO SWALLOW.

However

(i) Panellist who are allergic to dairy or dairy-based products should NOT participate in this taste panel trials

We thank anyone who finds themselves in this position for their time and consideration
Although the products were prepared in sanitized utensils and time and temperature of handing were strictly controlled, the potential risk to food safety has been further reduced by the instruction NOT TO SWALLOW the samples being trialled.

**What is the advantages associate with this project?**
The advantages of this project include:

(i) Alternative to current formulations of dairy beverages with increased protein and dietary fibre levels while maintaining acceptable organoleptic profile storage.

(ii) A research group at RMIT University, The Food Research Chemistry group, which has the capability to encompass the full gambit of technical skills including sensory skills needed to develop and critically analyse these novel food products.

**What is the benefits associate with participation?**
There are no direct benefits to you as the taste panellist.

However, you should have the satisfaction of knowing that you have participated and assisted with a project that will help food manufacturers produce products that are of benefit to the consumer.

**What will happen to the information I provide?**
Data will remain confidential. Anonymity will be provided by using a coded system, in which the products and taste panel forms are identified by codes selected by the investigators. The record sheet containing both the panellist name and the codes (product and taste panel form) will be handed over to an investigator for analysis.

From this point onwards the only information communicated from the taste panel trial will be the aggregated results and associated statistical analysis.

The taste panel forms completed by the participants will only be seen by the investigators stated above. The taste panel forms will be locked in a filing cabinet and kept securely at RMIT for a period of 5 years after the trial, before being destroyed.

Any information that you provide can be disclosed only if

(i) It is to protect you or others from harm,

(ii) A court order is produced, or

(iii) You provide the researchers with written permission.

**How will the results be disseminated?**
The data will be presented at meetings or reported as an aggregate only, and the individual result will not be identifiable in any form.

This data (as an aggregate) will be included as part of a preliminary report to industry

**What are any rights as a participant?**
As a participant you have the rights:

(i) To withdraw from participation at any time.
(ii) To have any unprocessed data withdrawn and destroyed, provided it can be reliably identified, and provided that so doing does not increase the risk for the participant.
(iii) To have any questions answered at any time.

**Whom should I contact if I have any questions?**

**Principal investigator:**
Professor Stefan Kasapis
Editor of Food Hydrocolloids

Food Chemistry Group
School of Applied Sciences
RMIT University
City Campus, 459-469 Swanston St.
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Melbourne, Vic 3001
AUSTRALIA

Tel: +61 (0) 3 9925 5244
Fax: +61 (0) 3 9925 5241
Email: stefan.kasapis@rmit.edu.au

**Other Investigators:**
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anna.bannikova@rmit.edu.au

Mrs. Elizabeth Gorczyca
Senior lecturer: Food Chemistry Group, School of Applied Sciences (Food Science and Technology, RMIT University), elizabeth.gorczyca@rmit.edu.au

Baddegamage Anusha Lakmini Buthgamuwa
Master student, program MRO45 (Food Chemistry Research Group, School of Applied Sciences (Food Science and Technology, RMIT University), s3352958@student.rmit.edu.au

**Yours sincerely,**
Professor Stefan Kasapis

Dr. Anna Bannikova, PhD

Mrs. Elizabeth Gorczyca

Baddegamage Anusha Lakmini Buthgamuwa

20th June 2013
PARTICIPANT’S CONSENT

1. I have had the project explained to me, and I have read the information sheet
2. I agree to participate in the research project as described
3. I agree to volunteer to be a panellist for the sensory trials.
4. I acknowledge that:

   (a) I understand that my participation is voluntary and that I am free to withdraw from the project at any time and to withdraw any unprocessed data previously supplied (unless follow-up is needed for safety).

   (b) The project is for the purpose of research. It may not be of direct benefit to me.

   (c) The privacy of the personal information I provide will be safeguarded and only disclosed where I have consented to the disclosure or as required by law.

   (d) The security of the research data will be protected during and after completion of the study. The data collected during the study may be published, and a report of the project outcomes will be provided to the investigators. Any information which will identify me will not be used.

Participation Consent

Participant: ___________________________ Date: ________________

(Signature)

Any complaints about your participation in this project may be directed to the Ethics Officer, RMIT Human Research Ethics Committee, Research & Innovation, RMIT, GPO Box 2476V, Melbourne, 3001. The telephone number is (03) 9925 2251.

Details of the complaints procedure are available on the Complaints with respect to participation in research at RMIT page.
RECORDING SHEET

Date: .................... Products: Dairy beverage

Taster Name (print)......................................................................................................................

Instructions: Please read and follow these instructions

(i) **Most importantly**, let us know if you have allergies or any problems with eating this product.

(ii) Rinse your mouth **before** tasting our samples.

(iii) Rinse your mouth **between** samples.

(iv) Spit the sample out (do not swallow)

(v) Take a ‘reasonable’ amount of sample for testing.

(vi) Take the samples and taste them from left to right.

You can re-sample if you wish but use the same order

(vii) Please complete all aspects of the record sheet

(viii) If you have any queries please feel free to ask.

The test:

(a) Rinse your mouth with lemon water and followed by water

(b) First taste sample no 695 and then spit it out

(c) Complete the tables – **tick** the most appropriate box for each sample

(d) Repeat (a), (b) and (c) for the next sample - no.213

**Test for sample no 695:**

(a) Rinse your mouth with lemon water and followed by water

(b) Take note of the parameters we would like evaluated: sweetness, chocolate flavour, smoothness

(c) Then taste sample no 695 and then spit it out

(d) Complete the tables below

<table>
<thead>
<tr>
<th>695</th>
<th>Not sweet at all</th>
<th>Neither not sweet nor sweet</th>
<th></th>
<th>Extremely sweet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweetness</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
When you have completed this product, can you please evaluate the product on the next page?

Test for sample no 213:
(a) Rinse your mouth with lemon water and followed by water
(b) Take note of the parameters we would like evaluated: sweetness, chocolate flavour, smoothness
(c) Then taste sample no 213 and then spit it out
(d) Complete the tables below
Thank you for your participation in our taste panel trial.
Your contribution is very much appreciated.

<table>
<thead>
<tr>
<th>695</th>
<th>Not sweet at all</th>
<th>Neither not sweet nor sweet</th>
<th>Extremely sweet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweetness</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>695</th>
<th>No chocolate flavour</th>
<th>Neither no chocolate flavour nor chocolate flavour</th>
<th>Extremely strong chocolate flavour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocolate Flavour</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>695</th>
<th>Dark</th>
<th>Neither dark nor light</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
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<th>695</th>
<th>Grainy</th>
<th>Neither grainy nor smooth</th>
<th>Very smooth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoothness</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
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<tr>
<th>Sample 695</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters to evaluate</td>
<td>Extremely disliked</td>
<td>Very much disliked</td>
<td>Disliked</td>
<td>Slightly disliked</td>
<td>Neither liked nor disliked</td>
<td>Slightly liked</td>
<td>Liked</td>
<td>Liked very much</td>
<td>Extremely liked</td>
</tr>
<tr>
<td>Colour</td>
<td></td>
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<td>Sweetness</td>
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<td>Chocolate flavour</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

152
Appendix 4. IV

Plate arrangement for the sensory trials:

Commercial dairy beverage *versus*
Dairy beverage made at the RMIT pilot plant with 6.1% protein and 2% inulin;

<table>
<thead>
<tr>
<th>Plate no*</th>
<th>Sample codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>695</td>
<td>213</td>
</tr>
<tr>
<td>1</td>
<td>1**</td>
</tr>
<tr>
<td>2</td>
<td>2**</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Also panellist number
** In the taste panel

1 = The commercial dairy beverage
2 = Dairy beverage made at RMIT pilot plant with 6.1% protein and 2% inulin
CHAPTER 5

PROTEIN ENRICHMENT IN THE
DEVELOPMENT OF HIGHLY NUTRITIOUS
DAIRY DESSERTS FOR SENIORS

5.1 ABSTRACT

As touched upon in the previous chapter of the Thesis, development of a range of protein-enriched dairy products meets consumer nutritional expectations. Furthermore, successful marketing of such nutritional or meal supplements will deliver commercial benefits to the food industry. Following our earlier work on novel beverage products, the present investigation deals with the development of protein-enriched dairy desserts that utilize whey protein and/or casein to create formulations which incorporate up to 11.5% protein plus 1.2% leucine. Such products will offer real benefits for the nutrition of the senior members of our community providing, among other things, a high level of leucine. This is important as it has been established that leucine is a key amino acids associated with the efficient synthesis of muscle protein in the elderly.

The development was carried out in two stages. First, a selected commercial product and dairy desserts were prepared at the RMIT’s research laboratory and pilot plant. This was done by using reverse engineering of the commercial product. The physicochemical properties of these products were investigated to yield a database of the structure-function relationships. Drawing confidence from the above, desserts with the aforementioned levels of dairy protein and leucine were designed and prepared at a laboratory (lab) scale. Physicochemical behaviour using steady shear viscosity, small deformation dynamic oscillatory in shear and compressive textural testing were carried out. Second, formulations that produced product that showed similar rheological profile to that of the commercial product were scaled up using processing parameters that mimic thermal treatment used industrially. Rheological evaluation and microscope images strongly support the conclusion that the structural characteristics of the protein-fortified formulations are comparable to the commercial counterpart. Preference tests from sensory analysis indicate that the novel formulations are acceptable to the panellists who rated the products with an average score of “like”.

154
5.2 INTRODUCTION

Protein is one of the major constituents in the biological cell and hence it plays an important role in the life processes of the human body. As a macronutrient, it is built from smaller units called amino acids, which are either synthesized in the human body or must be supplied in the diet (essential amino acids; Hoffman & Falvo, 2004). In general, an increase in protein intake up to 25-30 g per meal is a requirement to obtain the necessary amount of essential amino acids to maintain or improve adult health (Heaney & Layman, 2008; Paddon-Jones & Rasmussen, 2009). By contrast, the current recommendation for protein intake for an average healthy adult is 0.8 g.Kg\(^{-1}\).d\(^{-1}\) (60g/day or 20g protein/ meal for 75Kg person if distributed evenly across three main meals (Paddon-Jones & Rasmussen, 2009).

Although, there is an increasing consumer awareness of the importance of a healthy diet, almost fifty percent of aged people in developed countries have been suffering from malnutrition (Valerio, Antona & Nisoli, 2011). Even though, shifting food consumption habits with age has led to an intake of natural and nutritious ingredients, this, unfortunately, is well below recommended protein levels. Research further reveals that almost forty percent of people at the age of seventy-plus do not meet their daily recommended allowance of protein. This lack of protein could accelerate the decline of skeletal muscle metabolism during aging (Burton & Sumukadas, 2010). It is known that inadequate protein intake by aged individuals causes degenerative loss of their muscle mass and strength; a condition called Sarcopenia (Rom, Kaisari, Aizenbud, & Reznick, 2012; Volpi, Kobayashi, Sheffield-Moore, Mittendorfer & Wolfe, 2003).

As the world population continues to grow older (Loenneke & Pujol, 2011), there is an increase in studies examining the dietary criteria for the elderly in particular for the prevention and treatment of insufficient intake of protein. Another outcome of the changing age of the population is an increased interest in the market for the production of protein-enriched products.

Quality and quantity of the protein are key considerations, since by consuming the right type of protein at the right time healthy elderly can achieve consistent physiological responses and optimization of their protein intake. Among the amino acids, mainly the branched chain amino acids (leucine, isoleucine and valine) have the ability to stimulate muscle protein synthesis (Kimball & Jefferson, 2001; Kobayashi, Kato, Hirabayashi, Murakami & Suzuki, 2006). It appears that leucine is the most effective for elderly individuals due to its specific action on the production of ribosomes; ribosomes are the site of
protein manufacturing in the cell (Fujita & Volpi, 2006). Therefore, it was suggested that the addition of extra leucine to products will be advantageous for improving muscle protein synthesis in the elderly (Katsanos, Kobayashi, Sheffield-Moore, Aarsland & Wolfe, 2006). In this context, dairy products are a superior protein source delivering high levels of branched chain amino acids, and it is available in flexible formats that are easy to digest and incorporate into a diet (Newton, Yemm, Abel & Menhinick, 1993).

Growing consumer interest in foods that are readily available and meet nutritional requirements has led to the manufacture of various products with specific nutritional targets. In particular, manufacturers have attempted to produce protein-enriched products from various sources; with the predominant sources being whey protein and caseinates (Remeuf, Mohammed, Sodini, & Tissier, 2003). In the protein-enriched beverages developed earlier in the Thesis, we utilised sodium caseinate. In the development of a dairy dessert, whey proteins are also considered so that the range of textural characteristics is broadened and to increase the amount of leucine available in preparations (de Wit, 1998; Smithers, 2008; MacLean, Graham & Saltin, 1994; Hulmi, Lockwood & Stout, 2010).

It is well known that the texture and sensory properties of dairy products are highly dependent on the composition and type of protein in the system (Saint-eve, Juteau, Atlan, Martin, & Souchon, 2006). Therefore, it is essential to understand what the ingredients and processing methods used bring to the products being developed as the aim is to achieve the required functional and textural characteristics in the protein-enriched products developed. (Purwantia, Goota, Boom, & Vereijkena, 2010).

The dairy industry utilises efficiently a wide range of thermal treatments to facilitate manufacturing of dairy products with a wide range of properties (Lucey, Tamehana, Singh & Munro, 1998). It is well known that heat treatment alters the structure of proteins and carbohydrates, and induces interactions between the various components of the food matrix, thus promoting considerable changes in texture (Guzma´n-Gonzalez, Morais, & Amigo, 2000). Rheological properties are an important determinant of product quality, since they provide information on the effect of processing conditions and ingredients involved in food manufacture. Moreover, these properties govern consumer acceptance of the final product (Mullineux & Simmons, 2008).

In consultation with the industrial sponsor, the objective of the present study is to develop protein-enriched dairy desserts with more than 10% protein in the formulations. These were subjected to quantitative analysis to identify whether they meet nutritional targets for the elderly. Tables of amino acid profiles of formulations were generated, which includes
the amount of leucine present. Physicochemical properties of the novel desserts are further investigated with steady shear viscosity, small-scale deformation oscillation in shear and light microscopy to compare textural aspects particularly with currently available commercial products containing low protein content. To confirm consumer acceptability of the developed protein-enriched desserts, sensory tests were carried out using a hedonic taste panel.
5.3 EXPERIMENTAL PROTOCOL

5.3.1 Materials

Whey Protein Concentrate Lacprodan DI-7017 was supplied by Arla Foods Ingredient Group (Denmark). According to the manufacturer’s specification, it is a whey protein concentrate (73%) developed for clinical nutrition drinks and is heat stable under UHT treated conditions. The amount of moisture, ash, fat and lactose in the product were 5.5%, 3%, 16.5% and 2%, respectively. Lacprodan DI-7017 has passed microbiological testing and all other quality control procedures under the food grade standards.

Whey Protein Concentrate Alacen™ 392 was from Fonterra, New Zealand, and had 80% whey protein, 6.5% fat, 4.4% moisture and 3% ash. According to the manufacturer’s quality control procedures, the product has good emulsifying and nutritional qualities, and was tested and found to be acceptable for chemical, sensory and microbial parameters using internationally recognized procedures.

Calcium caseinate Alanate™ 385 was from Fonterra, New Zealand. According to the manufacturer’s specification, it is a spray dried milk protein produced directly from fresh skim milk and recommended for flavour sensitive formulations. The composition of the sample was 93% protein, 3.8% moisture, 1.2% fat, 0.1% lactose, 4.3% ash, 0.01% sodium and 1.4% calcium. Typical microbial estimates show that the product is acceptable according to food grade standards.

Sodium caseinate Alanate™ 180 was from Fonterra, New Zealand. Based on the certificate of analysis provided by the supplier, the product is a readily soluble powder, has good emulsification properties and passed the microbiological testing under the food grade standards. Typical composition is 93% protein, 3.9% moisture, 1.2% fat, 0.1% lactose, 4.3% ash, 1.2% sodium and 0.02% calcium.

Whey protein isolate was supplied by Fonterra, New Zealand. It is a protein isolate commercially available as WPI Instantised 894. The production method involved cross flow micro-filtration and ultra-filtration, which minimise protein denaturation. According to the manufacture’s nutritional analysis, the amount of protein in the product was \( \frac{N \times 6.38}{100} \) 90.4% (w/w; dry basis). Fat, ash and moisture contents were 1%, 3%, and 4.7% (w/w), respectively, with the pH being around 6.9.
Hydrolysed gelatin (Peptiplus XB) was from Gelita, Australia. It is a pure collagen protein with an extremely low mineral content and is free of fat, cholesterol, carbohydrates, and dietary fibre. The chemical composition includes 96% protein and less than 1% ash.

Carrageenan (Gelcarin GP379) was from FMC, USA. It was in the form of a light tan powder and recommended for food applications.

Xanthan gum (Keltrol) was from CP Kelco, USA. The sample was suitable for food applications.

Hydroxy propyl distarch phosphate (HPDP), Thermo Flow (modified maize starch), was from National Starch and assessed as food grade.

Gelatin High Bloom 25F. The material was a halal edible gelatin with 220 Bloom and supplied by Gelita Australia Pty Ltd, Australia.

Vegetable shortening Cegepal TG 186 was purchased from BASF chemical company (Australia). The chemical composition was stated as being 70% fat, 14% protein and 4% moisture. The product, a powder form, contained sunflower oil, glucose syrup solids, milk protein, an emulsifier and an antioxidant. According to the supplier’s specification, Cegepal TG 186 meets the general and specific requirements of purity of the WHO/FAO standards and the EU directives.

Skim milk and full cream milk were supplied by Parmalat Australia. Based on nutritional information, the average composition of total fat, protein and carbohydrates in the full cream milk and skim milk were 3.8%, 3.2%, 4.9% and 1.3%, 3.4%, 4.9% respectively.

5.3.2 Sample preparation

In developing protein-enriched desserts, different formulations for dairy desserts were created using whey protein isolate, whey protein concentrate, Lacprodan, sodium caseinate and calcium caseinate as sources of proteins. Sample preparation was carried out at two scales which used different processing methods. First, on a small scale (100g), desserts were prepared and evaluated in the RMIT research laboratory, and those formulations that produced products which displayed similar textural behaviour to those of the commercial product were proposed for further larger scale studies in the RMIT pilot plant.
Sample preparation at lab scale. Desserts (100 g) were prepared according to the formulations in Table 5.1. Dry ingredients and skim/whole milk were weighed separately, and then mixed together using a magnetic stirrer (speed adjusted to ensure dissolving of the components) for 20 min at room temperature. A water bath was used to increase the temperature of the mix to 75°C. The mix was held there for 6 min to ensure proper dissolving of the ingredients after which the mix was pasteurized at 85°C for 5 min. The mix was cooled to 55°C, before being filled into 120 mL plastic containers (single serving) and stored in a blast freezer set at -20°C to produce the frozen desserts required for this investigation.

Sample preparation in the pilot plant. The results of the evaluation at ambient temperature (22°C) of the formulations prepared on the lab scale led to scaling up of selected formulations in the pilot plant. These selected formulations are presented in Table 5.2; selection was based on the fact that formulations produced products that matched the commercial product. Scale up involved preparing a 4 kg batch by adding the dry ingredients to the skim/whole milk and mixing these with an electric mixer (KitchenAid, USA) for 20 min. The temperature of the mix was raised to 50°C in a water bath; this ensured proper dissolving of the protein and fat in the formulation. After a four-stage homogenization at 70 Bar using a FT9 homogeniser (Armfield, USA), the mix was heated at 85°C for 5 min in a Stephan kettle. The mix was cooled to 55°C, filled into 120 mL plastic containers and stored in a blast freezer at -20°C, which is the storage temperature of the commercial product.

5.3.3 Methods

The dairy desserts produced at the research laboratory or pilot plant were thawed in a refrigerator at 4°C for 24.0 ± 1.0 h before physicochemical analysis and sensory testing.

Small-deformation dynamic-oscillation measurements in shear were made using a controlled strain rheometer (AR-G2 from TA Instruments, New Castle, DE, USA) with a 40 mm diameter parallel-plate geometry and 1 mm gap at 22°C. The viscosity of the samples was analysed as a function of shear rate from 0.1 to 100 s⁻¹, and small deformation dynamic oscillatory studies were conducted at 0.1% strain and a frequency range of 0.1 to 100 rad/s.

Hardness and adhesiveness of the samples were determined using TA.XT2 Texture Analyser (Stable Micro Systems, UK) with a load cell of 5 kg. The measuring geometry consisted of a cylindrical aluminium probe (25 mm diameter), which was driven into a larger ring (40 mm diameter) containing the sample to be compressed. Tests were carried out at 1
mm/s with a trigger force of 10 g and a compressive deformation of 80% of the original height of the sample. The mechanical behaviour (hardness) was evaluated by expressing data in stress (kPa) versus strain and the maximum stress was taken as the hardness of the sample. Following the compression cycle, force was removed from the sample as the machine's crosshead moved back to its original position. Since dairy desserts are adhesive, the force becomes negative. The area of this negative peak was taken as a measure of the adhesiveness of the sample. There are no specific units for this parameter, which is expressed in the internal integrator units of the computer. Each experiment was carried out three times and an average result was calculated. All experiments were conducted at room temperature (22 ± 1°C) and under aerobic conditions.

**Light microscopy.** Images were collected with a Leica DM 2500 M Microscope (Germany) with an attached Digital Camera Leica DFC400 at x100 magnification. Desserts were prepared for the microscope by gently spreading a sample on a glass slide to minimise destroying the desserts’ structure. To obtain a sample that was not too thick or thin and to obtain a flat imaging plane, a glass coverslip was placed over the sample and a little pressure applied to it.

**Sensory analysis** in the form of hedonic taste panels was carried out. Untrained panels were engaged and the panellists (n = 20) were RMIT students and staff (aged 18-65). In the first sensory panel, the commercial dairy dessert with 6.7% protein versus the dairy dessert made at RMIT with 11.5% of protein were trialled. The second sensory test was conducted by trialling two protein-enriched products made at RMIT, namely: i) 11.5% protein consisting of sodium caseinate and Lacprodan, and ii) consisting of 10.8% protein containing sodium caseinate and calcium caseinate.

In these trials, panellists were asked to identify how much they liked the samples on a hedonic scale (Appendix 5.III) ranging from extremely dislike (1) to extremely like (9). Before undertaking the trial, each panellist was asked to read a plain language statement (Appendix 5.I) that explained the nature and content of the trials. After answering any queries and if the panellist was in agreement, they were asked to read a consent form (Appendix 5.II). Following that, the panellists signed the form (if they agreed) and only then were the taste panel form and samples brought out to them. The sampling codes (Appendix 5.IV) were selected from a random design number table, and the form and plates were numbered so that neither the panellists nor the presenter knew the product codes. The panellists were instructed to cleanse their palate before tasting and to do so in between each sample, and to taste the samples from left to right.
In addition to the above hedonic taste panel, preliminary taste panel trials involving 5 panellists were carried out. These trials also used the hedonic scale.

5.4 RESULTS AND DISCUSSION

5.4.1 Rheological characterisation of the commercial dairy dessert

To investigate whether the processing parameters were achievable using the processing equipment in the RMIT research laboratory and the pilot plant and to determine whether products that duplicate the rheological profile associated with the commercial product could be created, desserts were prepared and compared with the actual commercial counterpart. Work was carried out using the parameters described in the Materials and Methods section so all samples were analysed for flow and textural behaviour.

Figure 5.1a represents the flow characteristics of all three samples (based on commercial formulation prepared in the lab, in the pilot plant and actual commercial product) with increasing shear rate from 0.1 to 100 s\(^{-1}\). The trend in steady shear viscosity readings yields a linear and descending profile indicative of shear thinning behaviour. Results show that attempts to reproduce the flow characteristics of the commercial product were successful, in that the viscosity values for all products trialled ranged from about 100 to 1 Pa.s at the beginning and end of the experimental routine, respectively.

Besides the flow measurements, small scale deformation (dynamic oscillation) at rest was also employed to probe the viscoelasticity of the three products trialled. Figure 5.1b illustrates the mechanical spectra obtained at ambient temperature. The elastic component of the network (\(G'\)) remains well above the viscous component (\(G''\)) over the experimental frequency range of 0.1 to 100 rad s\(^{-1}\), with the value of tan \(\delta\) (\(G''/G'\)) being about 0.71. The magnitude of readings and their trend with increasing frequency of oscillation indicate that all products display the typical behaviour of weak gels.

Compression testing complements the aforementioned small and large deformation analysis in shear. The focus for the compression testing was on the attributes of hardness and adhesiveness. Although, the hardness of the product prepared in the research lab was not significantly (p>0.05) different from that of the commercial product, the product produced in the pilot plant was significantly (p<0.05) softer (Figure 5.2a). This outcome correlates with the pattern of viscoelasticity results observed in Figure 5.1b. The reason for this trend lies in the differences in the severity of the processing procedure used. It is not possible to be more
specific as the specific details of the commercial operation are not known to the researcher. However, for adhesiveness, the similar responses shown in the data (Figure 5.2b) describe all three products as having a ‘rich’ texture that “sticks in the palate during chewing”.

5.4.2 Rheological characterisation of dairy desserts prepared in the research laboratory

Once, a product similar to the commercial; product was produced (as shown in the previous section), development of protein-enriched formulations using whey protein isolate (WPI) and whey protein concentrate (Alacen 392 or Lacprodan) could proceed. The aim is to produce a product that has improved nutritional characteristics alongside maintaining an acceptable organoleptic profile. To do this, the commercial product containing 6.7% protein and less than 0.5% leucine was prepared once more (Table 5.1) as a control. In addition four formulations (also reproduced in Table 5.1) for the dairy dessert with about 12% protein and 1.2% leucine using whey protein were prepared.

All formulations (the commercial prototype and the four novel formulations with a high protein content) showed a similar pattern for shear thinning behaviour with comparable viscosity values (Figure 5.3). Variation in storage (elastic) and loss (viscous) moduli over the experimentally accessible range of oscillatory frequency was also determined (Figure 5.4). This parameter is important as dynamic moduli denotes the microstructure of the intact gel network, which in turn relates to the nature and the strength of the molecular bonds supporting the three dimensional structure (Vliet, Dijk, Zoon & Walstra, 1991). Slightly positive gradient of $G^\prime$ traces with values just above 100 Pa indicates weak gel behaviour for the products trialled.

The three formulations containing 2.6 to 3% WPI and 7 to 7.8% WPC (Lacprodan or Alacen 392) show similar values in hardness (around 0.9 kPa) as documented in Figure 5.5a. However, the gels of these preparations (formula 1, 2 & 3 in Table 5.1) are relatively weaker than the fourth novel formulation, 3.2% WPI and 7.5% WPC in the form of Lacprodan, which had a hardness of around 1.7 kPa. These findings are associated with the increasing strength of the continuous network. Matumoto-Pintro, Rabiey, Robitaille & Britten (2011) suggested that a higher level of protein solids would be able to form supporting tertiary structures and aggregates that affect gel firmness. In this case, the explanation for the hardness in formula 4 as compared with the other products tested is more likely related to the nature of the protein rather than the amount of protein or total solids. Variation in adhesiveness for the five
compressed products is illustrated in Figure 5.5b. The products exhibit comparable and considerable adhesiveness ranging in values from about -0.02 to -0.03 units; an outcome that supports the description of products that are creamy and full body mouth feel.

Once the similarity of the dairy desserts to the commercial product was established in terms of their flow characteristics, structure and textural properties, a basic sensory test by 5 RMIT staff was performed confirming product similarity (data not shown). Consequently, the next step is to scaling up (4 kg batch) preparation and to do so using the pilot plant equipment, and then repeat physicochemical and sensory evaluation of the products produced.

### 5.4.3 Physicochemical characterisation of dairy desserts prepared in the pilot plant

Table 5.2 identifies various attempts to scale up preparations in the pilot plant using the Stephan kettle. These include formulation (1) containing 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin plus 0.5% HPDP, and formulation (2) with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carrageenan plus 0.6% HPDP. It was found that parameters used currently in industrial production, in particular heating to 85°C and holding for 5 min, are not suitable for the manufacture of protein-enriched products. This these conditions lead to the denaturation of whey protein resulting in the formation of lumpy matrices. Thus, processing parameters were altered to improve the textural consistency of the WPI/Lacprodan samples. Specifically, the processing temperature was varied from 70 to 80°C and the holding time kept at 10, 15 or 20 min. Visual observations and preliminary sensory tests with 5 RMIT staff showed that heating at 75°C for a holding period of 10 min yields desserts with the required gel firmness (data not shown) without lumping.

However, it is known that industry prefers to maintain their current processing procedures. So, alternative formulations were proposed so that product with the required textural consistency can be produced using the industrial processing regime. According to the supplier’s specification, Lacprodan is stable at temperatures approaching the UHT range. For this reason Lacprodan was used at relatively high levels in formulation (3) recorded in Table 5.2, i.e. 11% Lacprodan or 14.28 g of the ingredient providing 1.41 g leucine per 120 g of serving. A four kg batch of this formulation was prepared by heating to 85°C (and holding for 5 min). Following freezing (at -20°C for 24 h) and thawing at ambient temperature, the Lacprodan dessert (formulation 3) displayed a homogeneous and shiny body. However, a preliminary sensory test (with 5 RMIT staff) revealed that this product had a residual after
taste; described as ‘like raw bananas’ (astringency). An after taste not detected in the commercial product.

To improve the sensory characteristics, maintain the required protein level, as specified by the industrial partner to around 11% protein and also allow for the manufacturing constraints to produce an acceptable dairy dessert, the following formulations were trialled. Formulation (4) containing 2.2% sodium caseinate and 5.5% calcium caseinate, i.e. 12.91 g total protein including gelatin and providing 1.13 g leucine per 120 g of the dessert package, and formulation (5) with 6.5% sodium caseinate and 2.5% Lacprodan, i.e. 13.80 g total protein including gelatin providing 1.45 g leucine per 120 g of dessert serving as recorded in Table 5.2 were produced and evaluated. The pilot plant equipment and procedure, namely: the Stephan kettle at 85°C for a 5 min holding time was used for the production of these products. The two products produced were evaluated using a preliminary sensory test (5 RMIT staff) after the products have been frozen for 24h and then thawed for testing. The preliminary testing indicated that the products had desirable organoleptic properties.

The above product development work paved the way for further characterisation of products produced by the novel formulations (4 and 5) in Table 5.2. The shear rate dependence of viscosity follows the same descending patterns to that discussed for the previous preparations outlined in Table 5.1. This outcome supports the argument for the long range flow of the high protein samples being compatible to that of the commercial product with only 6.7% total protein (Figure 5.6). This observation is further supported by the mechanical spectra in Figure 5.7 where the traces, for example, of storage (elastic) modulus define upper and lower bounds around the values of the commercial product, with all preparations exhibiting weak gel behaviour.

As before, the compression testing attributes of hardness and adhesiveness were examined for the products produced from the protein-enriched formulations and the commercial product (Figures 5.8a and 5.8b). The former products exhibited a consistent structural property as shown by a hardness values of around 1.4 kPa, and adhesiveness of -0.027 which indicates a rich chewable structure. By comparison, the commercial product exhibited hardness and adhesiveness levels of 1.1 kPa and -0.030, respectively, with the lower hardness being largely attributable to the lower dairy protein content in the commercial formulation (Table 5.2).

Six representative micrographs (Figure 5.9) of the protein-enriched dairy desserts and commercial product have been taken at ambient temperature to further facilitate discussion on the structural aspects of these food matrices. The commercial dairy dessert can be described
as forming a homogeneous system with a delicate gel-like property (Figure 5.9a). Protein-enriched samples with caseinate and/or whey protein showed similar properties to those of the commercial counterpart producing a micro phase-separated network, often referred to as the outcome of the concept of “thermodynamic incompatibility” between macromolecules (Figure 5.9b-f). Results provide tangible evidence of a protein network that is evenly dispersed within the three-dimensional matrix. Further, they indicate, in agreement with the rheological work, that high levels of protein (11 to 12% in Table 5.2) impart a smooth textural consistency to the novel products.

5.4.4 Sensory evaluation of the dairy desserts

A sensory panel was established to directly compare the commercial prototype which had a low protein content and the novel protein-enriched formulations (formulation 4 & 5 from Table 5.2) and establish which protein-enriched formulation was more acceptable. In the first taste panel, the commercial dairy dessert versus formulation (5) outlined in Table 5.2 were trialled by 20 panellists. They were asked to identify how much they liked the samples using a hedonic scale ranging from extremely dislike (1) to extremely like (9) (Appendix 5.III).

Out of 20 panellists, 15% had no preference returning the same score for both products tested. With respect to how much they liked the products, 95% and 70% panellists rated the commercial product and formulation 5, respectively as being acceptable where acceptable means that the product was extremely liked to slightly liked (scores of 9, 8, 7 or 6) by these panellists (Table 5.3). The number of panellists that were neutral (neither liked nor disliked) about the products trialled was low at 5% for the commercial product and 10% for formulation 5. However, some 20% rated formulation 5 below a score of 5. Nevertheless, the average scores (± SD) were 7 (± 0.85) and 6.05 (± 1.7) for the commercial product and the protein-enriched dairy dessert (formulation 5) trialled, respectively, indicating consumer acceptance for both products.

In a second sensory panel, two protein-enriched dairy desserts with 11.5% protein (formulation 5 Table 5.2) which consisted of Lacprodan (2.5%), sodium caseinate (6.5%), gelatin (0.3%) and HPDP (2%), and the dessert with 10.76% protein (formulation 4) containing sodium caseinate (2.2%), calcium caseinate (5.5%), gelatin (0.3%) and HPDP (2.5%) but no Lacprodan, were evaluated (formulations 5 and 4 in Table 5.2). Twenty
panellists were asked to identify how much they liked the samples with comparable total protein content on a hedonic scale ranging from extremely dislike (1) to extremely like (9).

Out of 20 panellists, 20% had no preference providing the same score for both products tested. With respect to how much the panellists liked the product, the scores have slightly improved over those given in taste panel 1(Table 5.3). Some 90% and 85% panellists rated the formulation 4 and 5, respectively as being acceptable where acceptable means that the product was extremely liked to slightly liked (scores of 9, 8, 7 or 6) by these panellists. There were panellists that were neutral about the products as they rated them with a score of 5 (neither liked nor disliked). This was the case for 10% and 15% of panellists for the product with 10.76% (formulation 4) and 11.5% (formulation 5) protein, respectively. Significantly, no panellists rated the products as unacceptable (score of <5). The average scores (± SD) were 6.75 (± 1.16) and 6.6 (± 0.88) for the dairy dessert with 10.76% and 11.5% protein, respectively. According to ANOVA statistical testing (one way), there was no significant (p > 0.05) difference in the preference level for the two dairy desserts, with both products being liked by panellists. Thus, formulations (4) and (5) in Table 5.2 were graded as organoleptically acceptable by the panellists as well as maintained the high nutritional requirement to contribute to preventing sarcopenia in the elderly population.
5.5 CONCLUSIONS

Protein-enriched desserts were designed at a laboratory scale and produced in a pilot plant in collaboration with the industrial partner. The search for optimum techno- and nutritional functionality prompted the trial of several formulations and processing conditions within the desired industrial context. Long range flow properties, structural characteristics of the intact gel networks and textural attributes from compression testing show that the high protein samples and the low protein commercial counterparts are similar. Results were further confirmed with optical microscopy, which provides tangible evidence of the aqueous and proteinaceous matrix of the two types of products. Sensory evaluation of novel formulations containing 13 to 14 g total protein including 1.1 to 1.4 g of leucine per 120 g product serving were liked by the panellists. This is an encouraging result, since both products are made with processing parameters currently utilised by industry for the production of low protein frozen custards, i.e. heating at 85°C and holding there for 5 min. Further industrial engagement is required for appropriate scale up of the novel products with a view to launching into the market highly nutritious and yet tasty frozen desserts for the elderly.
5.6 REFERENCES


Table 5.1 Formulations (percentage) of protein-enriched dairy desserts made in the RMIT research laboratory

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Commercial prototype</th>
<th>(1) 3% WPI, 7.1% Alacen 392, 0.37% gelatin, 0.6% HPDP</th>
<th>(2) 3% WPI, 7% Alacen 392, 0.37% gelatin, 0.04% carrageenan, 0.6% HPDP</th>
<th>(3) 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP</th>
<th>(4) 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carrageenan, 0.6% HPDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy propyl di-starch phosphate (HPDP)-Modified Maize Starch</td>
<td>2.808</td>
<td>0.600</td>
<td>0.600</td>
<td>0.5000</td>
<td>0.6000</td>
</tr>
<tr>
<td>Hydrolysed gelatin (Gelita sol D)</td>
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<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Skim milk powder high heat</td>
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<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
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<td>3.000</td>
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<td>3.200</td>
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<td>7.100</td>
<td>7.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
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<td>0.000</td>
<td>0.000</td>
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<td>7.500</td>
</tr>
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<td>0.040</td>
<td>0.040</td>
<td>0.000</td>
<td>0.040</td>
</tr>
<tr>
<td>Xanthan gum</td>
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<td>0.000</td>
</tr>
<tr>
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<td>0.300</td>
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<td>28.000</td>
<td>28.000</td>
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<td>1.192</td>
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<td>27.130</td>
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<td>Ingredients</td>
<td>Commercial prototype</td>
<td>(1) 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP</td>
<td>(2) 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carrageenan 0.6% HPDP</td>
<td>(3) 11% Lacprodan, 0.3% gelatin, 0.5% HPDP</td>
<td>(4) 2.2% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP</td>
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<td>----------------------</td>
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<td>--------------------------------------------------------------------------</td>
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<td>Whey protein isolate</td>
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<td><strong>100.000</strong></td>
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Table 5.3 Percentage preference for taste panel one and taste panel two on hedonic scale score ranging from extremely like (9) to extremely dislike (1)

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<tr>
<th>Taste panel number</th>
<th>Score</th>
<th>Percentage preference for</th>
<th>2.2% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP</th>
<th>6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatin, 2% HPDP</th>
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<td>Formulation 5 (Table 5.2)</td>
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<td>1</td>
<td>9</td>
<td>Extremely liked</td>
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<td>0</td>
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<tr>
<td></td>
<td>8</td>
<td>Liked very much</td>
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<td>20</td>
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<tr>
<td></td>
<td>7</td>
<td>Liked</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Slightly liked</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Neither liked nor disliked</td>
<td>5</td>
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</tr>
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<td></td>
<td>4</td>
<td>Slightly disliked</td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<td>15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Very much disliked</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>1</td>
<td>Extremely disliked</td>
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<td>0</td>
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<td>2</td>
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<td></td>
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<td>Liked very much</td>
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<td>10</td>
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<td></td>
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<td>2</td>
<td>Very much disliked</td>
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<td></td>
<td>1</td>
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<td><strong>Total score (%) for taste panel 2</strong></td>
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<td>100</td>
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NA= Not applicable
Figure 5.1 Steady shear viscosity (a) and Elastic ($G'$, closed symbols) and viscous ($G''$, open symbols) moduli for the applied frequency range from 0.1 to 100 rad/s with 0.1% strain (b) for the commercial dairy dessert (♦), and dairy dessert made at the RMIT research laboratory (■) and pilot plant (●) using the commercial formulation at 22°C.
Figure 5.2 Hardness (a) and adhesiveness (b) for the commercial dairy dessert, and dairy dessert made at the RMIT research laboratory and pilot plant according to the commercial formulation at 22°C. n=2 and the vertical bars on the histograms represent the range in the data obtained.
Figure 5.3  Steady shear viscosity for the dairy dessert made at RMIT research laboratory according to the commercial formulation (●), and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (♦), with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carrageenan, 0.6% HPDP (○), with 3% WPI, 7.1% Alacen 392, 0.37% gelatin, 0.6% HPDP (■), and 3% WPI, 7% Alacen 392, 0.37% gelatin, 0.04 % carrageenan, 0.6 % HPDP (▲) at 22°C.
Figure 5.4 Elastic ($G'$, closed symbols) and viscous ($G''$, open symbols) moduli for the dairy desserts made at RMIT research laboratory according to the commercial formulation (■,□), and protein enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatine, 0.5% HPDP (♦,◊), with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carageenan, 0.6% HPDP (▲,∆), with 3% WPI, 7.1% Alacen 392, 0.37% gelatin, 0.6% HPDP (●,○), and 3% WPI, 7% Alacen 392, 0.37% gelatin, 0.04% carrageenan, 0.6% HPDP (- ,= ) for the applied frequency range from 0.1 to 100 rad/s with 0.1% strain at 22°C with 0.1% strain at 22°C.
Figure 5.5  Hardness (a) and adhesiveness (b) for the dairy desserts made in the RMIT research laboratory according to the commercial formulation, and protein-enriched formulations with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.04% carrageenan, 0.6% HPDP (formulation 4 Table 5.1), with 3% WPI, 7.1% Alacen 392, 0.37% gelatin, 0.6% HPDP (formulation 1), with 3% WPI, 7% Alacen 392, 0.37% gelatin, 0.04% carrageenan, 0.6% HPDP(formulation 2), and 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (formulation 3) at 22°C. n=2 and the vertical bars on the histograms represent the range in the data obtained.
Figure 5.6 Steady shear viscosity for the dairy dessert made at RMIT pilot plant according to the commercial formulation (●), and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (●), with 6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatine, 2% HPDP (■), with 11% Lacprodan, 0.3% gelatin, 0.5% HPDP (▲), with 2.2% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP (▲), and 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.6% HPDP and 0.04% carrageenan (+) at 22 °C.
Figure 5.7 Elastic ($G'$, closed symbols) and viscous ($G''$, open symbols) moduli for the dairy dessert made at RMIT pilot plant according to the commercial formulation (●, ○), and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (●, ○), with 6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatin, 2% HPDP (■, □), with 11% Lacprodan, 0.3% gelatin, 0.5% HPDP (■, □), with 2.5% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP (▲, ∆), and 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.6% HPDP and 0.04% carrageenan (▲, ∆) for the applied frequency range from 0.1 to 100 rad/s with 0.1% strain at 22 °C.
Figure 5.8 Hardness (a) and adhesiveness (b) for the dairy dessert made at the RMIT pilot plant using the commercial formulation, and protein-enriched formulations with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (formulation 1 Table 5.2), with 6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatin, 2% HPDP (formulation 5), with 2.2% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP (formulation 4), with 11% Lacprodan, 0.3% gelatin, 0.5% HPDP (formulation 3), and 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.6% HPDP and 0.04% carrageenan (formulation 2) at 22 °C. n=2 and the vertical bars on the histograms represent the range in the data obtained.
Figure 5.9  Micrographs of the commercial dairy dessert (a), and protein-enriched formulations with 2.2% sodium caseinate, 5.5% calcium caseinate, 0.3% gelatin, 2.5% HPDP (b), with 6.5% sodium caseinate, 2.5% Lacprodan, 0.3% gelatin, 2% HPDP (c), with 11% Lacprodan, 0.3% gelatin, 0.5% HPDP (d), with 2.6% WPI, 7.8% Lacprodan, 0.3% gelatin, 0.5% HPDP (e), and with 3.2% WPI, 7.5% Lacprodan, 0.3% gelatin, 0.6% HPDP and 0.04% carrageenan (f) at 22°C (magnification is x100).
INVITATION TO PARTICIPATE IN A RESEARCH PROJECT

PARTICIPANT INFORMATION

Project title:

Characterisation of proteolytic activity and physicochemical changes in UHT milk and dairy-based food products

Investigators:

Professor Stefan Kasapis:
Senior Project Supervisor: Food Chemistry Group, School of Applied Sciences (Food Science and Technology, RMIT University), stefan.kasapis@rmit.edu.au

Mrs. Elizabeth Gorczyca
Senior lecturer: Food Chemistry Group, School of Applied Sciences (Food Science and Technology, RMIT University), elizabeth.gorczyca@rmit.edu.au

Dr. Anna Bannikova,
Research Fellow, Food Chemistry Research Group, School of Applied Sciences (Food Science and Technology, RMIT University), anna.bannikova@rmit.edu.au

Baddegamage Anusha Lakmini Buthgamuwa
Master student, program MRO45 (Food Chemistry Research Group, School of Applied Sciences (Food Science and Technology, RMIT University), s3352958@student.rmit.edu.au

Dear Participant,

You are invited to participate in a Masters by Research project being conducted by Baddegamage Anusha Lakmini Buthgamuwa at RMIT University, Food Science discipline. This information sheet describes the project in straight forward language, or ‘plain English’.

Please read the sheet carefully and be confident that you understand its contents before deciding whether to participate.

If you have any questions about the project, please feel free to ask any one of the investigator listed above.

Who is involved in the project and why it is important?

This is a project undertaken by the Food Chemistry Research group under the direction of Professor Kasapis.

The project is evaluating the dairy desserts with increased desirable protein levels in relation to nutritional requirements while maintaining an acceptable organoleptic profile.

Another reason why this project is important is to have a thorough and all-encompassing approach to the development of products that are commercially useful. All-encompassing means that a range of meaningful techniques to evaluate a product can be employed, such as
those involved in rheological analysis to determine textural characteristics as well as evaluation of the sensory characteristics through taste panel trials of the products developed.

The sensory trials will provide information for potentials in collaboration with industry. This may lead to a major industrial contract for Food Sciences RMIT, so your assistance is very much appreciated.

**Why have you been approached?**

You have been approached because you represent typical consumers in the age group of 50-65 years.

**What is project about?**

Keeping the bone, muscle and cognitive health is very important throughout life. Protein is a very important macronutrient for humans by playing numerous roles on body functioning. It is widely accepted by the scientific community that protein requirements well depend progressively from age and physical requirements, and protein origin or quality are important considerations for individuals. Thus, a case study was undertaken to incorporate desirable protein levels to commercial product formulations in relation to nutritional and age requirements while maintaining an acceptable organoleptic profile.

**What is the question being addressed?**

The purpose of the taste panel trials is to answer the following questions:

- What is the level of ‘liking’ (on a 9 point like – not like scale) of the samples (2) of dairy dessert samples provided?

**What is the risks associate with this project?**

Please note that the ingredients used to prepare the dairy desserts are commonly used in a range of manufactured foods and are regarded as safe.

All risks have been reduced significantly if not eliminated by asking the panellist to spit out the samples being evaluated. That is, you are instructed NOT TO SWALLOW.

However

(i) Panellist who are allergic to dairy or dairy-based products should NOT participate in this taste panel trials

We thank anyone who finds themselves in this position for their time and consideration.

Although the products were prepared in sanitized utensils and time and temperature of handing were strictly controlled, the potential risk to food safety has been further reduced by the instruction NOT TO SWALLOW the samples being trialled.

**What is the advantages associate with this project?**

The advantages of this project include:

(i) Alternative to current formulations of dairy desserts with increased protein levels while maintaining an acceptable organoleptic profile storage.
(ii) A research group at RMIT University, The Food Research Chemistry group, which has the capability to encompass the full gambit of technical skills including sensory skills needed to develop and critically analyse these novel food products.

**What is the benefits associate with participation?**

There are no direct benefits to you as the taste panellist.

However, you should have the satisfaction of knowing that you have participated and assisted with a project that will help food manufacturers produce products that are of benefit to the consumer.

**What will happen to the information I provide?**

Data will remain confidential.

Anonymity will be provided by using a coded system, in which the products and taste panel forms are identified by codes selected by the investigators. The record sheet containing both the panellist name and the codes (product and taste panel form) will be handed over to an investigator for analysis.

From this point onwards the only information communicated from the taste panel trial will be the aggregated results and associated statistical analysis.

The taste panel forms completed by the participants will only be seen by the investigators stated above.

The taste panel forms will be locked in a filing cabinet and kept securely at RMIT for a period of 5 years after the trial, before being destroyed.

Any information that you provide can be disclosed only if

(i) it is to protect you or others from harm,

(ii) A court order is produced, or

(iii) You provide the researchers with written permission.

**How will the results be disseminated?**

The data will be presented at meetings or reported as an aggregate only, and the individual result will not be identifiable in any form.

This data (as an aggregate) will be included as part of a preliminary report to industry

**What are any rights as a participant?**

As a participant you have the rights:

(i) To withdraw from participation at any time.
(ii) To have any unprocessed data withdrawn and destroyed, provided it can be reliably identified, and provided that so doing does not increase the risk for the participant.

(iii) To have any questions answered at any time.

**Whom should I contact if I have any questions?**

**Principal investigator:**
Professor Stefan Kasapis  
Editor of Food Hydrocolloids

Food Chemistry Group  
School of Applied Sciences  
RMIT University  
City Campus, 459-469 Swanston St.  
Building 39, Level 4, Room 6  
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AUSTRALIA

Tel: +61 (0) 3 9925 5244  
Fax: +61 (0) 3 9925 5241  
Email: stefan.kasapis@rmit.edu.au

**Other Investigators:**

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Research Fellow  
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anna.bannikova@rmit.edu.au

Mrs. Elizabeth Gorczyca  
Senior lecturer: Food Chemistry Group, School of Applied Sciences (Food Science and Technology, RMIT University),  
elizabeth.gorczyca@rmit.edu.au

Baddegamage Anusha Lakmini Buthgamuwa  
Master student, program MRO45 (Food Chemistry Research Group, School of Applied Sciences (Food Science and Technology, RMIT University),  
s3352958@student.rmit.edu.au

**Yours sincerely,**

Professor Stefan Kasapis

Dr. Anna Bannikova, PhD

Mrs. Elizabeth Gorczyca

Baddegamage Anusha Lakmini Buthgamuwa

23rd April 2013
PARTICIPANT’S CONSENT

5. I have had the project explained to me, and I have read the information sheet

6. I agree to participate in the research project as described

7. I agree to volunteer to be a panellist for the sensory trials.

8. I acknowledge that:

   (c) I understand that my participation is voluntary and that I am free to withdraw from the project at any time and to withdraw any unprocessed data previously supplied (unless follow-up is needed for safety).

   (d) The project is for the purpose of research. It may not be of direct benefit to me.

   (c) The privacy of the personal information I provide will be safeguarded and only disclosed where I have consented to the disclosure or as required by law.

   (d) The security of the research data will be protected during and after completion of the study. The data collected during the study may be published, and a report of the project outcomes will be provided to the investigators. Any information which will identify me will not be used.

Participation Consent

Participant: ___________________________ Date: ___________________________

(Signature)

Any complaints about your participation in this project may be directed to the Ethics Officer, RMIT Human Research Ethics Committee, Research & Innovation, RMIT, GPO Box 2476V, Melbourne, 3001. The telephone number is (03) 9925 2251.

Details of the complaints procedure are available on the Complaints with respect to participation in research at RMIT page
RECORDING SHEET

Date: .................... Products: Dairy dessert
Taster Name (print)............................................................................................

Instructions: Please read and follow these instructions

(ix) Most importantly, let us know if you have allergies or any problems with eating this product.

(x) Rinse your mouth before tasting our samples.
(xi) Rinse your mouth between samples.
(xii) Spit the sample out (do not swallow)

(xiii) Take a ‘reasonable’ piece of sample for testing.
(xiv) Take the samples and taste them from left to right.

You can re-sample if you wish but use the same order

(xv) Please complete all aspects of the record sheet
(xvi) If you have any queries please feel free to ask.

The test:

(e) Rinse your mouth with lemon water and followed by water
(f) First taste sample no 695 and then spit it out
(g) Repeat (a) and (b) for the middle sample
(h) Repeat (a) and (b) for the last sample on the right

What is the Overall acceptability of the samples?

(Tick the most appropriate box for the 2 samples)

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<th>213</th>
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<td>Extremely liked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Liked very much</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Liked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Slightly liked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Neither liked nor disliked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Slightly disliked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Disliked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Very much disliked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Extremely disliked</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Thank you for your participation in our taste panel trial.
Your contribution is very much appreciated.
Plate arrangement for the sensory trials:
1. Commercial dairy dessert versus dairy dessert made at RMIT with 11.5% of protein;
2. Protein-enriched dairy dessert with 11.5% of protein containing sodium caseinate and Lacprodan versus 10.8% of protein containing sodium caseinate and calcium caseinate

<table>
<thead>
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<th>Plate number*</th>
<th>Sample codes</th>
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<td>695</td>
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<td>1</td>
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</table>

* Also panellist number

** In taste panel 1

1 = The commercial dairy dessert;

2 = Protein-enriched dairy dessert with 11.5% of protein containing sodium caseinate and Lacprodan

In taste panel 2

1= Protein-enriched dairy dessert with 11.5% of protein containing sodium caseinate and Lacprodan

2= Protein-enriched dairy dessert with 10.8% of protein containing sodium caseinate and calcium caseinate
CHAPTER 6
CONCLUSIONS AND FUTURE WORK

Ultra-high temperature (UHT) treatment in the food industry provides technical sufficiency in the production and transportation of products, their textural functionality and long shelf-life on top of convenience for consumers. However, UHT-treated products still retain some of the heat stable lipolytic and proteolytic enzymes, which eventually limit the shelf-life of food. This Thesis mixes concepts of advanced processing with aspects of food safety and food quality to illustrate the effect of thermal treatment on raw milk quality and utilise this as a basis for the development of dairy-based products with superior nutritional, textural and sensory characteristics. Thus, the key areas of focus from this work can be summarised as follows:

- Threshold determination of enzymatic activity for raw milk that is to be used in UHT processing
- Development of nutritious and desirable dairy-based beverages utilizing UHT-treatment
- Development of dairy-based desserts with superior nutrition and likelihood of acceptance by the consumer

The overall objective of the first experimental chapter of this Thesis is the shelf-life of UHT-treated milk in relation to quality deterioration, which is known as age-gelation and is primarily caused by bacterial proteases present in the raw material. It has been found in this work that the threshold values of proteolytic enzymes in raw milk should be very low in order to meet the requirements for stable UHT-treated milk at room temperature over a storage period of at least six months. This raises the practical issue of a method that is sensitive enough to detect proteolytic activity resulting in spoilage of UHT-treated milk during the expected shelf-life period. Dupont, Lugand, Rolet-Repecaud and Degelaen (2007) have proposed a monoclonal antibody ELISA technique that is applied directly to the raw material for the estimation of instability in UHT-treated milk. Future work based on results from this Thesis and the literature argues for optimisation of existing assays or even the design of new assays for proteolytic activity that could be utilised in the identification of a commercial threshold specification in raw milk aimed for subsequent UHT processing.

Work in this study is limited to the crude enzyme extract from Pseudomonas fluorescens. However, it is known that raw milk consists of mixed flora that is able to produce
a range of proteases (Matta & Punj, 2000). Additional studies on the extraction of enzymes from a diverse pool of common proteolytic bacterial flora present in raw milk are essential to determine the cut-off point of protease activity in raw milk for UHT processing. To achieve this, a wider sampling schedule of raw milk from various locations would be beneficial in future experimental work. Isolation and purification of those bacterial strains and their enzymes would be advantageous to determine the protease activity in raw and UHT-treated milk in shelf-life studies.

Apart from that, an extended effort would be required for the identification of structural characteristics and physicochemical interactions of peptide groups generated during proteolysis. It would be useful to elucidate the chemistry of proteolysis and mechanisms of age-gelation in UHT-treated milk. Consequently, the knowledge gained would be beneficial to enhance the existing assays and also to design sensitive, cost effective and accurate assays for the early detection of proteolysis in UHT-treated milk during storage at conditions of industrial interest.

Nevertheless, the main purpose of the food safety work in this Thesis is to prepare a bench mark of quality for subsequent experimentation on the utilisation of thermally processed milk in the development of dairy-based products with novel techno- and nutritional functionality. Dairy-based food products have been widely consumed locally and internationally, and work in this Thesis aims to develop added value materials for various market segments including sports nutrition and the 65+ population. Currently, dairy-based foods in Australia typically contain 3 to 4 percent dairy protein (Dairy Australia, 2012).

This work designed and developed dairy-based beverages with 6.1% (w/w) protein and 2% (w/w) dietary fibre, the flow properties and mouthfeel of which were similar to those of a commercial counterpart with about 3.1% (w/w) protein and no dietary fibre. Moreover, it was shown that application of UHT technologies in the production of dairy-based beverages is beneficial due to reduction in the microbial growth and enzymatic activity, thus obtaining an end product of high quality. Dairy beverage production utilised a UHT facility with a plate heat exchanger, which is suitable for liquid-like systems of relatively low solids (< 25% w/w) and viscosity. Tubular UHT systems are widely used in the industry and extension of the work to these thermal processors would be of interest. Eventual commercialisation of this product would, of course, require scaling up at factory production capacity and large consumer trials in Victoria that include discrimination and hedonic taste panels to determine consumer response for successful launching in the market.
Work on the development of protein-enriched dairy desserts in this Thesis was an effort to address specific dietary requirements in elderly. Promising formulations were created containing up to 14 g total protein (whey protein and/or casein) and 1.4 g leucine per 120 g product serving, as compared to commercial counterparts with up to 6% (w/w) protein and less than 0.5% (w/w) leucine. Experimental results obtained from sophisticated mechanical measurements, texture profile analysis and accompanying sensory evaluation argue for the development of protein enriched formulations with desirable organoleptic characteristics. These were made with relatively high levels of leucine, a branched chain amino acid that facilitates muscle-protein synthesis, and thermal processing that follows industrial production of the sponsoring company.

Best results were obtained using thermal treatment at 85°C for 5 min leading to the production of frozen desserts with improved nutritional profile in comparison to existing commercial products. However, it would be worth investigating the development of formulations using UHT facilities for a storage period of up to 12 months at ambient temperature. It is unlikely that a plate heat exchanger would be able to process these relatively high viscosity systems, and it should be replaced by a tubular UHT facility. The latter allows versatile parameterisation of product homogenisation prior or after UHT processing in order to optimize mouthfeel by avoiding product grittiness due to excessive whey protein denaturation (Rapaille & Vanhemelrijck, 1998).

Further work on the achievements of this Thesis may require addition of vitamins and minerals to the formulation according to government legislation and health programs (Holick & Chen, 2008). Thus, dietary guidelines for food and nutrition in Australia and New Zealand should be taken into account with existing nutritional surveys, observation on population, animal and human experimentation, which are required knowledge for product development in relation to age, life style and presence of chronic/deficiency diseases (NHMRC, 2005). Several documents could be referred to for the calculation of Recommended Daily Intake (RDI), but there is certain flexibility in this respect, since in FSANZ Standard 1.1.1, Issue 138, the RDI values are not specific on sex or age of the adults and there is no information for some important dietary constituents including protein, potassium, fluoride and choline.

Finally, specific interest for future development work in the category of “formulated meal replacement and formulated supplementary foods” can also be addressed based on FSANZ Standard 2.9.3. This addresses the targeted delivery of micronutrients and trace elements in processed food, for example, that of vitamin A, vitamin D and calcium to dairy desserts (also in FSANZ Standard 1.3.2, Clause 3). Dairy desserts for meal replacement can
be engineered in 150 g product serving provided that the total of the naturally occurring and added quantity of vitamin A, vitamin D and calcium is no more than 125 µg, 1.6 µg, and 320 mg per serving, respectively.
6.1 REFERENCES


