# MAPPING THE THERMO-TOLERANT PROTEASES IN ULTRA HIGH TEMPERATURE (UHT) TREATED MILK USING MOLECULAR APPROACHES

By

Nuwan Ruwani Sulthanagoda Vithanage

B.Sc. (Hons) Microbiology, Chemistry


College of Health and Biomedicine

Victoria University

Melbourne, Australia

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*Dedicated to my beloved parents, for making me who I am today,*
*& my dearest husband Jamith, two lovely daughters Janmi & Osini, for*
*their constant support and unconditional love all the way.*
*I love you all dearly.*

“Science is but a perversion of itself unless it has as its ultimate goal the betterment of humanity”.

- Nikola Tesla, 1919.

# ABSTRACT

Refrigerated storage of raw milk selects for psychrotrophic bacterial genera such as *Pseudomonas* (mainly *Pseudomonas fluorescens*), *Bacillus*, *Acinetobacter*, *Hafnia*, *Klebsiella*, *Rahnella, Stenotrophomonas* and *Aeromonas*, *Serratia*, which are disseminated by means of feed, faeces, bedding material, soil, air, water and milking and processing equipment at the farming and processing environments. These bacteria produce a wide range of extracellular proteases that are resistant to the current heating regimes used in Ultra High Temperature (UHT) processing, typically 135-150 °C for 2-10 s. The residual bacterial proteases can lead to the development of quality defects in UHT milk, including bitterness, increased viscosity, sedimentation and age gelation, all of which, cause spoilage and shelf-life deterioration during ambient storage and transportation of UHT milk and dairy products.

Controlling these problematic microorganisms in raw milk requires a rapid and reliable screening method that can be implemented as soon as the milk is received at the processing plant. The present project involves the isolation of a wide range of psychrotrophic microorganisms (n = 160) with different colony morphologies from raw milk, supplied from a commercial processor. The extracellular proteolytic activities and reference strains of these bacteria were determined by skim milk agar assays and extracellular proteases of potential proteolytic bacteria (n = 75) were produced in UHT milk under refrigerated conditions. These crude bacterial proteases were screened for heat-stability at 150 °C for 20 s, using a milk coagulation assay. Fifty-five of the isolates were able to produce heat-stable bacterial proteases. These isolates were identified using 16S rRNA gene sequencing (molecular), which is considered the gold standard in bacterial identification, as well as four commercial analytical systems, namely API, Microbact (biochemical), Biolog (metabolism) and Matrix Assisted Laser Desorption Ionisation-Time-of-Flight Mass Spectrometry (MALDI-TOF MS). These methods

enabled the identification of Gram-negative isolates to the species level at the rates of 100.0%, 86.8%, 63.2%, 60.5% and 57.9%, respectively. The identification rates of Gram-positive isolates by 16S rRNA gene sequencing, Biolog, MALDI-TOF MS and API at the species level were 100.0%, 85.0%, 95.0% and 90.0%, respectively. These methods showed a significant difference in the discrimination power, as expressed by Simpson's index of diversity. Phylogenetic analysis based on 16S rRNA gene sequencing further discriminated *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Hafnia alvei*, *Bacillus cereus sensu lato strains*, *Bacillus pumilus* and *Bacillus licheniformis* into subspecies. The identification rates of the Biolog, MALDI-TOF MS, Analytical Profile Index (API) and Microbact (MB) systems were affected by limited reference profiles in the corresponding databases. Both MALDI-TOF MS and Biolog demonstrated remarkable rapidity, followed by 16S rRNA gene sequencing, Microbact and API, while reproducibility was higher in 16S rRNA gene sequencing, followed by MALDI-TOF MS, Biolog, API and Microbact. These data indicate that both 16S rRNA gene sequencing and MALDI-TOF MS are better suited for the screening of spoilage and some potentially pathogenic microorganisms in raw milk in terms of rapidity, reproducibility and reliability, than other biochemical and metabolic approaches.

Bacterial ribosomal RNA genes are constitutively expressed, regardless of the culture condition used; in contrast, protein expression can be affected by media composition, incubation temperature and time, which may affect MALDI-TOF MS identification. Thus, we observed a significant effect of culture conditions on the identification of spoilage and pathogenic bacteria by MALDI-TOF MS. Additionally, sample preparation methods also affected MALDI-TOF MS identification. In order to minimise those effects, the spectral profiles generated for psychrotrophic bacteria were used to develop consensus peak profile with the help of SPECLUST (an on-line spectral analysis tool), followed by the identification of characteristic peaks, using the MASCOT database. This approach revealed that the majority of observed peaks were derived from ribosomal proteins and they were constitutively expressed under all conditions tested.

Thus, we developed a ribosomal protein database using the amino acid sequences of corresponding typed strains available from the NCBI database. Further, the molecular weight of each peptide was determined using the Expasy database. Then, the consensus peak lists, derived from the MALDI-TOF MS-proteomic (experimental) approach, were compared with theoretical data derived from the bioinformatics approach. Some of the species and genus specific peaks were expressed constitutively, regardless of the culture conditions and preparation methods used. This, in turn, resulted in a significant increase in the accurate assignment of psychrotrophic bacteria to the species level, where clustering patterns in the dendogram were significantly similar to the phylogenetic tree derived from 16S rRNA gene sequences. Thus, this combined proteomics-bioinformatics-based approach would be helpful in assigning unknown bacteria isolated from food and environmental samples, thereby improving the MALDI-TOF MS analysis, and allowing for rapid, reliable and large-scale identification of bacteria of dairy origin, as well as tracking their sources at the farming and processing environments.

Both 16S rRNA gene sequencing and MALDI-TOF MS were used for profiling of psychrotrophic microbiota in raw milk stored under different refrigeration conditions (2-10 °C) to mimic the conditions that are likely to be present in the farm bulk tank, insulated tanks and commercial silos. Additionally, the effect of seasonal changes on the microbial diversity of raw milk was investigated. The predominant microbiota isolated in raw milk included *Pseudomonas* (19.9%), *Bacillus* (13.3%), *Microbacterium* (5.3%), *Lactococcus* (8.6%), *Acinetobacter* (4.9%) and *Hafnia* (2.8%). We also observed a large number of hitherto unknown species and genera in raw milk. However, the biodiversity of the indigenous microbial population varied significantly, depending on the temperature, time and season. Furthermore, the bacteria isolated from raw milk varied significantly in their heat-stable enzymatic characteristics and the incidence of bacterial proteases (BPs) production by these bacteria also varied significantly. Among the proteolytic bacteria, 53% of *Pseudomonas* spp. and 30% of

*Bacillus* spp. showed strong heat-stable protease activity. In addition, the high heat resistant endospores produced by majority of *Bacillus* spp. such as *B. cereus, B. sporothermodurans, G. stearothermophilus* and *P. lactis* have shown to be able to withstand UHT heating process (135-150 °C/2-10 s) that may germinate during the ambient storage, and subsequent production of enzymes and potential toxins that can cause both spoilage and food-borne intoxication.

In order to control the BPs in UHT milk, it is important to screen raw milk for bacteria with higher proteolytic potential. However, screening for each and every species with higher proteolytic activity in raw milk is extremely difficult in the processing environment. Therefore, the psychrotrophic proteolytic bacterial count (PPrBC) (represented mainly by *Pseudomonas* spp.) is more informative with respect to milk spoilage. Thus, it is important to accommodate PPrBC as a major quality criterion in the screening of raw milk. Regardless of the temperature conditions used, there was a significant increase in protease activity and proteolysis, when the PPrBC reached 5 × $10^4$ cfu/mL. Thus, the time for PPrBC to reach this level was defined as the storage life of milk. Additionally, the thermoduric psychrotrophs (TDPC), represented mainly by *B. cereus*, increased with the rise in temperature above 6 °C. Due to the fact that the spores of *B. cereus* can withstand UHT heating and produce emetic and diarrhoeal type toxins, when reaching 1 × $10^4$ cfu/mL, TDPC counts reaching this level were defined as storage life of raw milk, with respect to food safety. However, deep cooling (at 2 °C) and high temperature short time (HTST) pasteurisation (75 °C for 15 s) resulted in significant reduction in the PPrBC and TDPC counts ($P < 0.05$); however the latter resulted only slight reduction in TDPC counts ($P > 0.05$) resulting in the decimal reduction time (time required to reduce the bacterial counts by $10^9$ cfu/mL) in the range of 1.2 to 75.0 min, with z value of 10.23 °C (with B* 0.67-1.38) in raw milk. The 16S rRNA and MALDI-TOF MS identification showed that PPrBC counts were represented by *Pseudomonas* spp. (93%), while TDPC counts were represented by *B. cereus* (85%), which cause spoilage and food borne illnesses, respectively.

Therefore, we used counts of *Pseudomonas* spp. and *B. cereus* for predicting the storage life of raw milk with respect to quality and food safety, respectively. To do this, we used several primary, secondary and tertiary mathematical models. Initially, the temperature dependence of these two bacterial counts was evaluated using the primary models of Baranyi as well as the modified Gompertz equations using DMFit3_5 software. It was then shown that these counts fitted well with the equations. Further, the data derived from these two models, such as lag phase duration and maximum specific growth rate, fitted well with the secondary model. Finally, the data derived from primary and secondary models were used for prediction of storage life using an exponential model (tertiary model). All of the mathematical models were characterised by high $R^2$ values, small root mean square error values and nearly ideal values of $B_f$ and $A_f$, further suggesting that these models can be used for predicting the storage life of raw milk by describing the growth of *Pseudomonas* spp. and *B. cereus* under various temperature conditions.

Besides the proteolytic activity, the extracellular proteases produced by these bacteria showed significantly higher heat-stabilities, which varied depending on the bacterial species or strains. Thus, the heat-stability of selected BPs was determined to be at 55-160 °C in full cream, skimmed milk and simulated milk ultrafiltrate (SMUF) buffer. Then, the inactivation kinetics and thermodynamic properties of each BP was determined under each condition used. The results of the study indicated that BPs were subject to significant heat-induced inactivation, especially ≥ 145 °C. This indicated that the new temperature and time parameters may be required to be defined for UHT processing, especially at 145-180 °C with shorter period such as 0.2-0.8 s. However, this may require calculations of $F_0$ values and evaluation of their effects on milk constituent and nutrients. Additionally, we observed low temperature inactivation (LTI) in SMUF buffer, while fat content and milk proteins showed a protective effect on BPs under the same conditions. This phenomenon could be used for inactivation of BPs along with ultrafiltration to remove the milk constituents prior to the LTI at 55-65 °C. Overall, the

current project provides useful information to improve the quality and shelf-life of UHT milk, allowing for large scale production and exportation to different geographical locations.

# CERTIFICATE

**Prof. Todor Vasiljevic, PhD (*UAlberta*)**

Professor of Food Science

Leader, Advanced Food Systems Research Unit

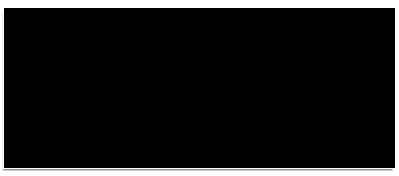
College of Health and Biomedicine

Victoria University, Werribee campus

Victoria

Australia

This is to certify that the Dissertation entitled **"Mapping the Thermo-Tolerant Proteases in Ultra High Temperature (UHT) Treated Milk Using Molecular Approaches"** is a bonafide record of independent research work conducted by **Nuwan Ruwani Sulthanagoda Vithanage (Student No: 3858056)** under my personal guidance and supervision and submitted to Victoria University in partial fulfilment of the requirement for the award of the **Doctor of Philosophy** with specialization in Food Science and Technology, which has not previously submitted to this university or to any other university for the award of any degree, diploma or other similar title.



Professor Todor Vasiljevic

(Principal Supervisor)

Date: 10.03.2017

# DECLARATION

"I, Nuwan Ruwani Sulthanagoda Vithanage, declare that the PhD thesis by Publication entitled **Mapping the Thermo-Tolerant Proteases in Ultra High Temperature (UHT) Treated Milk Using Molecular Approaches** is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

**Nuwan Ruwani Sulthanagoda Vithanage**

College of Health and Biomedicine

Victoria University, Werribee Campus, VIC 3030, Australia

Date: 24.03.2017

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VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

**PART A: DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION**

| **Item/ Chapter No.** | **Paper Title** | **Authors** | **Publication Status (**e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted ) | **Publication Title and Details** (e.g. date published, impact factor etc.) |
|---|---|---|---|---|
| 3 | Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk | Nuwan R. Vithanage, Thomas R. Yeager, Snehal R. Jadhav, Enzo A. Palombo, and Nivedita Datta | Published | International Journal of Food Microbiology; SJR Q1 |
| 4 | Species Level Discrimination of Psychrotrophic Pathogenic and Spoilage Gram-negative Raw Milk Isolates using a Combined MALDI-TOF MS Proteomics-Bioinformatics Based Approach | Nuwan R. Vithanage, Jeevana Bhongir, Snehal R. Jadhav, Chaminda S. Ranadheera , Enzo A. Palombo, Thomas R. Yeager, Nivedita Datta | Published | Journal of Proteome Research; SJR Q1 |
| 5 | Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential | Nuwan R. Vithanage, Muditha Dissanayake, Greg Bolge, Enzo A. Palombo, Thomas R. Yeager, Nivedita Datta | Published | International Dairy Journal; SJR Q1 |
| 6 | Microbiological Quality of Raw Milk Attributable to Prolonged Refrigeration Conditions | Nuwan R. Vithanage, Muditha Dissanayake, Greg Bolge, Enzo A. Palombo, Thomas R. Yeager and Nivedita Datta | Published | Journal of Dairy Research; SJR Q1 |

**Declaration by** (candidate Name)**:** Nuwan Ruwani Sulthanagoda Vithanage **Signature:** [redacted] **Date:** 24-03-2017

# CONFERENCE PROCEEDINGS

## Oral Presentation

Nuwan R. Vithanage, Thomas R. Yeager, Snehal R. Jadhav, Enzo A. Palombo, Nivedita Datta. Comparison of four commercial systems with 16S rRNA gene sequencing for identification of psychrotrophic bacteria in raw bovine milk. (2014). Annual post graduate research student conference, for the College of Health & Biomedicine, Victoria University, St. Albans, Victoria, Australia.

Nuwan R. Vithanage, Jeevana Bhongir, Snehal R. Jadhav, Gregory Boldge, Enzo A. Palombo, Thomas R. Yeager, Nivedita Datta. Identification of Psychrotrophic Milk Spoilage Bacteria by MALDI-TOF MS. Australian Institute of Food Science and Technology (AIFST) Food Science summer school. January 28-30, 2015. Royal Melbourne Institute of Technology (RMIT) University, Melbourne, Australia.

Nuwan R. Vithanage, Jeevana Bhongir, Snehal R. Jadhav, Gregory Boldge, Enzo A. Palombo, Thomas R. Yeager, Nivedita Datta. Identification of Milk Spoilage Bacteria Using MALDI-TOF-MS Finger Printing. (2015). 2$^{nd}$ annual post graduate research student conference, for the College of Health & Biomedicine, Victoria University, St. Albans, Victoria, Australia.

## Poster Presentation

Nuwan R. Vithanage, Thomas R. Yeager, Snehal R. Jadhav, Enzo A. Palombo, Nivedita Datta (2014). Comparison of Five Different Systems to Identify Spoilage Bacteria in Raw Milk. Australian Society for Microbiology Annual Scientific Meeting. July 6-9, 2014, Pullman and Mercure Melbourne Albert Park, Victoria, Australia.

Nuwan R. Vithanage, Jeevana Bhongir, Snehal R. Jadhav, Gregory Boldge, Enzo A. Palombo, Thomas R. Yeager, Nivedita Datta. Proteomic Characterisation of Heat-Stable Protease Producing *Pseudomonas fluorescens* Grown in Various Media Using MALDI-TOF Mass Spectrometry. 13th Student Symposium. The Melbourne Protein Group**,** July, 2014, Bio21 Institute, Parkville, Victoria, Australia.

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| | | |
|---|---|---|
| $A_f$ | = | Accuracy factor |
| %AI | = | Percentage identification accuracy |
| %D | = | Percentage discrepancy |
| $(M+H)^+$ | = | Monoisotopic peaks |
| ˚C | = | Degree Celsius |
| µg | = | Microgram |
| µunits | = | Microunits |
| 2D-zymography | = | Two dimensional zymography |
| ACN | = | Acetonitrile |
| AHL | = | *N*-acyl homoserine lactone |
| ANOVA | = | Analysis of variance |
| AOAC | = | Association of official agriculture chemists |
| API | = | Analytical profile index |
| ATCC | = | American type culture collection |
| ATP | = | Adenosine triphosphate |
| BBC | = | Brilliance™ *Bacillus cereus* agar supplemented with polymyxin B supplement |
| BC | = | Bacterial counts |
| $B_f$ | = | Bias factor |
| BHIA | = | Brain heart Infusion agar |
| BLAST | = | Basic local alignment search tool |
| BPs | = | Bacteria proteases |
| B-value | = | A reduction of spore count by $10^9$ per unit |
| CAGR | = | Healthy compound annual growth rate |
| CCI | = | Correlation coefficient index |
| CCPs | = | Critical control points |
| CFC agar | = | *Pseudomonas* agar base containing cetrimide, fucidin and cephaloridine as selective agents |
| CFU/cfu | = | Colony forming units |
| CHCA | = | A-cyano-4 hydroxycinnamic acid |
| C-value | = | Disruption of 3% of thiamine |
| d | = | Days |
| DH | = | Degree of hydrolysis |
| DHB | = | 2, 5-dihydroxy benzoic acid |
| DI water | = | Deionised water |
| DMSO | = | Dimethyl sulfoxide |
| DNA | = | Deoxyribonucleic acid |
| DPA | = | Dipicolinic acid |
| D-value | = | Decimal reduction time |
| EDTA | = | Ethylenediaminetetraacetic acid |
| EGTA | = | Ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid |
| ELISA | = | Enzyme-linked immunosorbent |
| EPS | = | Extracellular polymeric substance |
| EU | = | European Union |
| $F_0$-value | = | Combined effect of time and temperature on the inactivation of bacterial spores and/or enzymes |
| FDA | = | U S Food and drug administration |
| FITC-casein | = | Fluorescein isothiocyanate casein |
| Fluorescamine | = | 4-phenylspiro [furan-2(3H), 1-phthalan]-3, 3-dione |

| | | |
|---|---|---|
| FSANZ | = | Food safety Australia New Zealand |
| FTIR | = | Fourier transform infrared spectroscopy |
| GNB | = | Gram-negative bacilli |
| GNP | = | Gram-negative psychrotrophs |
| GPB | = | Gram-positive bacilli |
| GPC | = | Gram-positive cocci |
| h | = | Hours |
| H | = | Shannon's Index of diversity |
| HCA | = | Hierarchical cluster analysis |
| HPH | = | High pressure homogenisation |
| HTST | = | High-temperature short time pasteurisation |
| ISI | = | Innovative steam injection |
| K | = | Kelvin |
| kDa | = | Kilodalton |
| L/l | = | Litre |
| LAB | = | Lactic acid bacteria |
| LPM | = | Low-pressure mercury |
| LPM | = | Low-pressure mercury lamps |
| LTI | = | Low temperature inactivation |
| LTLT | = | Low-temperature long time pasteurisation |
| *m/z* | = | Mass to charge ratio |
| $M_9$ | = | Minimal media |
| MAC | = | MacConkey agar |
| MALDI-TOF | = | Matrix assisted laser desorption ionisation time-of-flight |
| MS | | Mass spectrometry |
| MANOVA | = | Multivariate analysis of variance |
| MB | = | Microbact |
| MCL | = | Maximum composite likelihood |
| MF | = | Microfiltration |
| MF | = | Microfiltration |
| mg | = | Milligram |
| mL/ml | = | Millilitre |
| MPM | = | Medium-pressure mercury |
| Mw | = | Molecular weight |
| n | = | Number of observations |
| N, y(t) | = | Logarithm of microbial counts ($\log_{10}$ cfu /ml) |
| $N_{0,}$ y(0) | = | Logarithm of initial cell count at time t = 0($\log_{10}$ cfu/ml) |
| NA | = | Nutrient agar |
| NB | = | Nutrient broth |
| NCBI | = | National centre for biotechnology information |
| NCBInr | = | National centre for biotechnology Information non-redundant protein |
| NEM | = | N-ethylmaleimide |
| NF-GNB | = | Non-fermenter Gram-negative bacilli |
| ng | = | Nanogram |
| Nm/nmoL | = | Nanomoles |
| $N_{max,}$ $y_{max}$ | = | Maximum cell number increase at the stationary phase in logarithm ($\log_{10}$ cfu/ml) |
| NS | = | Not specified |
| obs | = | Observed value of counts |
| OPA | = | *O*-phthaldialdehyde |
| PA | = | Protease activity |
| PBC | = | Psychrotrophic bacteria counts |

| | | |
|---|---|---|
| PCM | = | Plate count agar |
| PCR | = | Polymerase chain reaction |
| PEF | = | Pulsed electric field |
| pH | = | Hydrogen ion concentration |
| p*I* | = | Isoelectric point |
| pkat | = | Picokatal, catalytic activity of enzymes |
| PL | = | Proteolysis |
| PMF | = | Peptide mass fingerprint |
| PMO | = | Pasteurised milk ordinance |
| PMSF | = | Phenylmethane sulfonyl fluoride |
| PPrBC | = | Psychrotrophic proteolytic count |
| pred | = | Predicted value |
| $Q_{10}$ | = | Temperature dependence of a reaction |
| qPCR | = | Quantitative real time PCR |
| Q-value | = | Average free energy for the transfer of amino acid chains from ethanol to water |
| $R^2$, CC | = | Correlation coefficient |
| RFU | = | Relative fluorescence units |
| RL | = | Prokaryotic ribosomal larger subunit proteins |
| RMSE | = | Root mean squares error |
| RNA | = | Ribonucleic acid |
| RPG | = | Ribosomal protein gene |
| RP-HPLC | = | Reversed-phase high-performance liquid chromatography |
| rRNA | = | Ribosomal RNA |
| RS | = | Prokaryotic ribosomal smaller subunit proteins |
| s | = | Seconds |
| SAPs | = | Small Acid-soluble proteins |
| SARAMIS | = | Spectral Archive and Microbial Identification system |
| SBA | = | Sheep blood agar |
| SDS-PAGE | = | Sodium dodecyl sulphate gel electrophoresis |
| SE | = | Standard error of the mean |
| SH | = | Sulfhydryl group |
| SHMP | = | Hexametaphosphate |
| SID | = | Simpson’s Index of diversity |
| SID | = | Simpson's Index of diversity |
| SIM | = | Similarity index |
| $SL_{obs}$ | = | Observed storage life |
| $SL_{pred}$ | = | Predicted storage life |
| $S_{LQ}$ | = | Storage life for quality aspect: time required to reach *Pseudomonas*/PPrBC counts of $5 \times 10^4$ cfu/mL |
| $S_{LS}$ | = | Storage life for safety aspect: time required to reach *Bacillus cereus*/TDPC counts of $1 \times 10^4$ cfu/mL |
| SMA | = | Skim milk agar |
| SMUF | = | Simulated milk ultra filtrate |
| SNP | = | Single nucleotide polymorphism |
| SPECLUST | = | A web tool for hierarchical clustering of peptide mass spectra |
| Subsp. | = | Subspecies |
| T | = | Temperature |
| t | = | Time |
| TCA | = | Trichloroacetic acid |
| TDC | = | Thermoduric count |

# CHAPTER 1: INTRODUCTION

## 1.1 Background

Ultra High Temperature (UHT) treatment of milk and milk products has gained more consumer attention in the recent years due to the long shelf-life (6-12 months) that it imparts to milk and milk products, which are destined for export markets such as south-east Asia and China (Chavan et al. 2011). Furthermore, the UHT milk can be stored under ambient conditions, which significantly reduces the cost for storage and transportation under refrigerated condition (Chavan et al. 2011). Alternatively, this results as such in the reduction of greenhouse gas emission, which is associated with global warming (Tomasula et al. 2014).

The process typically involves heating of raw milk at 135-150 °C for a holding period of 2-10 s, followed by aseptic filling and packaging into sterile containers, which renders milk free from pathogens, some spores and other spoilage microorganisms and maintains its stability at ambient storage, thus making it safe for human consumption (Champagne et al. 1994, Chavan et al. 2011). Nevertheless, the heating regimes of UHT processing are not severe enough to inactivate heat-stable enzymes and highly heat-resistant spores (Champagne et al. 1994).

Psychrotrophic bacteria are a group of microorganism that can proliferate under refrigeration condition regardless of their optimum growth conditions (Champagne et al. 1994). They secrete heat resistant exoenzymes e.g. proteases, lipases, phospholipase and amylases during refrigerated storage in farm bulk tanks and commercial silos, prior to processing (Vithanage et al. 2016). Additionally, the bacterial proteases can activate indigenous plasminogen (resulting from the cattle inflammatory disease, mastitis) that can be present in raw milk and is also known to be extremely heat-stable (Chavan et al. 2011). However, the incidence of bacterial proteases (BPs) and their effect is more frequent compared to the

other exoenzymes such as lipases, phospholipase and amylases in commercial milk processing (Button et al. 2011).

Bacterial genera responsible for production of bacterial proteases include *Pseudomonas, Acinetobacter, Serratia, Klebsiella, Hafnia, Erwinia* and *Bacillus,* which are frequently found in raw milk and dairy products (Samarzija et al. 2012, Vithanage et al. 2016).

*P. fluorescens* is often found to dominate in raw milk bacterial communities, and has accounted for 50-84 % of the total raw milk microbiota (Marchand et al. 2009, Vithanage et al. 2016). *Bacillus* sp., which is associated with raw milk contamination, includes *B. cereus* sensu lato species complex, *B. subtilis*, *B. licheniformis*, *B. pumilus*, *P. lactis* and *B. sporothermodurans* (Scheldeman et al. 2006).

The psychrotrophic bacteria produce three major types of heat-stable BPs that are particularly important in milk spoilage (Dufour et al. 2008, Häse and Finkelstein, 1993, Marchand et al. 2009). The Gram-negative psychrotrophs (GNPs) produce an extracellular alkaline zinc metalloprotease, designated Aprx, which belongs to the serralysin super family (Dufour et al. 2008). *Bacillus* spp. produce heat-stable neutral zinc metalloproteases known as thermolysin and substilisin, which belong to the serine super family (Häse and Finkelstein, 1993). These BPs are encoded by *aprx, npr* and *apre* genes, respectively (Dufour et al. 2008, Häse & Finkelstein 1993, Tran et al. 1991).

These heat-stable BPs preferentially attack κ-casein, β-casein and $\alpha_{s1}$-casein of casein micelles (Fairbairn & Law 1986), leading to the destabilization of UHT milk and development of undesirable quality attributes such as bitterness, increased viscosity, sedimentation and age-gelation during the storage of UHT milk under ambient conditions (Bagliniere et al. 2012, Datta & Deeth 2001). Many studies observed that even a trace quantity of bacterial proteases (as little as 1 ng /mL) can reduce the shelf-life of UHT milk from the typical 9 months to 3 months (Datta & Deeth 2001). Thus, the ability of trace amounts of BPs to cause spoilage as well as the diversity that they possess brings major challenges in terms of dealing with the spoilage problem in UHT milk. Thus, the milk producers receiving a small quantity of raw milk containing trace level of BPs may find quality defects in an entire UHT

milk batch. Since there is an increased demand for UHT milk and milk products in distant markets (e.g. China and South-East Asia), challenges with gelation and other deterioration issues occur quite frequently during the storage and transportation, and are becoming increasingly prominent (especially at high ambient temperature during shipping). This may consequently lead to product recalls, significant financial loss and a negative brand image to the commercial UHT milk processor. However, the mechanisms responsible for gelation/phase separation of UHT milk are not clearly understood, which is why control strategies for the improvement in the quality of UHT milk during storage and transportation have not been established yet (Figure 1.1).

It was observed that the control or elimination of heat-stable enzymes can be achieved by various thermal and non-thermal methodologies (Datta & Deeth 2007). Enzymes may be partially or completely inactivated by heating them at low temperature (Barach et al. 1976). In addition to thermal technologies, non-thermal technologies such as high pressure homogenization (Hayes & Kelly 2003, Pereda et al. 2007) and ultrasonication (Anese et al. 2013, Ganesan et al. 2015) are promising inactivation strategies for some heat-stable enzymes in food. The synergistic effects of combined technologies may be able to control or completely eliminate the heat-stable enzyme activity.

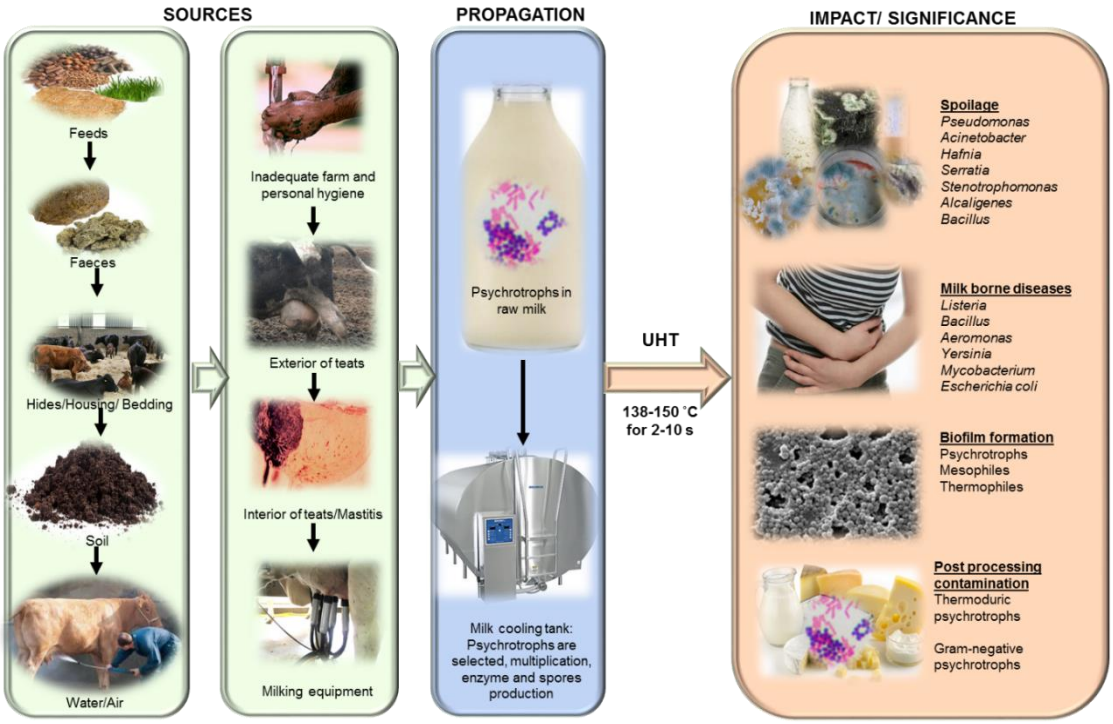


**Figure 1.1** Schematic representation of dissemination and propagation of psychrotrophs in raw milk, and their impact/significance in UHT milk processing.

## 1.2 Research Objectives

Thus, the current research is specifically aimed at isolating, characterizing, assessing the heat stability and analysing the expression heat-stable BPs, which have the potential to create quality defects in UHT milk. The project will involve,

1. Isolation and identification of protease-producing psychrotrophic bacteria from raw milk.
2. Optimisation of rapid and reliable identification system for further research.
3. Determination of the effect of different storage conditions on microbial counts, diversity and proteolytic activity.
4. Evaluation of the importance of psychrotrophic proteolytic bacteria and thermoduric psychrotrophs for screening of raw milk, when producing UHT milk.
5. Prediction of storage life of raw milk stored under different temperature conditions prior to the processing.
6. Determination of the inactivation kinetics and thermodynamic properties of selected BPs.

## 1.3 Thesis Outline

Chapter 1 provides the background, research objectives and outline of the Thesis. Chapter 2 presents a literature review, explaining the importance of psychrotrophic bacteria in UHT milk processing in detail, while also focussing on UHT milk processing, occurrence of psychrotrophs in raw milk, diversity and associated factors, their sources, spoilage potential, biochemical and molecular characteristics of different bacterial proteases, their heat-stability, specificity, impact, detection and finally, methods to control them using a variety of innovative process techniques. The isolation and characterisation of psychrotrophic bacteria using polyphasic approach is revealed in Chapter 3. Chapter 4 reports the optimisation of MALDI-TOF MS for identification of milk-spoiling bacteria. Chapter 5 describes the factors affecting psychrotrophic microbiota in raw milk, while Chapter 6 underlines the importance of psychrotrophic proteolytic bacteria (PPrBC) and thermoduric psychrotrophs (TDPC) as

quality and safety criteria in raw milk and their threshold values for UHT processing. Prediction of storage life of raw milk to produce high quality UHT milk based on PPrBC and TDPC by using mathematical modelling equations is discussed in chapter 7. Chapter 8 explains the heat-stability and thermodynamic parameters of selected BPs and effects of thermal treatment on controlling them in UHT milk. The conclusions of the overall study and the scope for the future work are delivered in Chapter 9.

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# CHAPTER 2: LITERATURE REVIEW

## 2.1 Ultra High Temperature (UHT) Milk Processing

UHT processing involves heating of raw milk at a high temperature for a short period time to inactivate microorganisms and some of their spores, while causing minimal thermal destruction to milk constituents (e.g. vitamin and whey proteins) (Chavan et al. 2011, Rauh et al. 2014b). The heating process is followed by aseptic filling into sterile containers to achieve commercial sterility of the product, which can be stored at room temperature for prolonged periods (Datta & Deeth 2007).

Depending on the medium used for heating the milk to the UHT, the UHT technologies can be classified as direct and indirect systems (Manji et al. 1986). Direct heating systems involve injection of steam into milk (steam injection) or milk into steam (steam infusion); thus leading the temperature of the product to increase almost instantly due to the latent heat of vaporization (Chavan et al. 2011). This method uses steam, hot water and electricity as the mode of heat-treatment (Datta & Deeth 2007). Since the product is being mixed with the heating system, the culinary steam must be of high quality and must not impart any off-flavours to the milk product (Chavan et al. 2011). Although the steam can dilute the milk, its subsequent cooling and condensation in the vacuum chamber can separate the introduced water from milk (Datta & Deeth 2007). In contrast, the indirect heating methods, such as plate or tubular heat exchangers, incorporate a stainless steel barrier to separate the heating system from the product (Chavan et al. 2011).

## 2.2 History of UHT Milk Processing

UHT Milk processing system originally manufactured in 1893 was an indirect heating technique that was characterized by continuous-flow heating (125 ºC for 6 min) (Datta & Deeth 2007). In 1912, a patented, continuous-flow, direct steam injection technology was introduced to achieve temperatures between 130 ºC and 140 ºC. The aseptic filling process was introduced in 1953 (Chavan et al. 2011).

## 2.3 Market Status of UHT Milk

UHT milk is currently produced by more than 60 countries around the world (Chavan et al. 2011). Increasing urbanisation, the ability of UHT milk to be stored at room temperature for longer times, limited refrigerated distribution systems in developing countries, increasing consumption of milk, the recent concept of consuming milk, while on the go and distribution of milk in schools are all key growth drivers for expansion of the global UHT milk market (Anon, 2014b). Additionally, significant reduction in the refrigerated storage also reduce the emission of green-house gases that are involve in global warming (Tomasula et al.2014).

The global revenue of the UHT milk market was worth US$ 60.8 billion in 2012, and is expected to increase up to US$ 137.7 billion by the end of 2019. Over the forecast period 2013-2029, the global UHT milk market is predicted to expand at a healthy compound annual growth rate (CAGR) of 12.8%. In 2009, the market share for UHT milk as a percentage of total milk consumption in different countries varied; in Australia, for instance, UHT milk accounted for 9%, whereas in France, it was 88%, 83% in Spain, 63% in Germany, 55% in Italy, and about 5% to 13% in the United Kingdom (Harrington 2009). The Chinese UHT import market was worth US$76 million in 2012 and the value topped US$ 85 million in 2013. However, China was the major market on a global scale in 2014 with 60% of the market share, and it is further expected to increase during the 2013-2029 period (Astley 2013).

UHT milk sales by volume garnered 53% of market share in South Africa in 2013, outstripping demand for chilled milk for the first time (Ntuli 2015, Anon, 2016). African flavoured milk consumption is also set to increase overall by 7.1%, reaching as high as 12.4% in North Africa, between 2013 and 2016, according to Tetra Pak's Dairy Index 2014. India is estimated to witness a growth rate of 20%, and is expected to grow more than threefold during 2010-11 and 2016-17 (Anon, 2011). Brazil and a few other countries in Latin America are also expected to witness a significant growth over the forecast period (Anon,

2014a). Australia will also emerge as a lucrative market for UHT milk, which was only 10% in 2012 (Anon, 2016).

However, consumer concerns about reduced nutritional value, the presence of butyric acid and sour aromatic flavour, loss of colour and lack of freshness are major challenges for the market growth of the UHT milk (Anon, 2011). The heating regime of UHT processing can cause denaturation of whey protein in milk whereas other important nutrients such as riboflavin and thiamine can be lost altogether during the processing and storage of UHT milk (Figure 2.1). The major defects encountered in UHT milk include adverse effect on taste (bitterness), colour, odour, gelation, sedimentation, separation, and viscosity. These factors can be mainly affected by the microbiological quality of raw milk, pre-treatment process, process type, homogenization pressure, deaeration, post process contamination and package barriers (Champagne et al. 1994).

## 2.4 Principles of UHT Milk Processing

The production processes of UHT milk are designed to ensure the commercial sterility (free of microorganisms that can grow under non-refrigerated conditions) and acceptable sensory quality attributes of the milk throughout its extended shelf-life. The severity of the heat-treatments are designed to achieve maximum disruption of bacterial spores and heat-stable enzymes, with minimal effect on milk proteins and nutrients (Figure 2.1). These effects can be calculated using the following mathematical formulas (Chavan et al. 2011, Van Boekel 2008).

### 2.4.1 $Q_{10}$ value

The parameter $Q_{10}$ describes the temperature dependence of a reaction that can be increased by a certain factor, when the temperature is increased by 10 °C. This can be calculated using following formula:

$$Q_{10} = \left(\frac{R2}{R1}\right)^{\left[\frac{10}{T2-T1}\right]} \quad (1)$$

Where $R$ is the rate, and $T$ is the temperature in ºC or K.

The $Q_{10}$ value for flavour changes is around 2 to 3, which means changing in flavour attributes is 2 or 3 times higher if the temperature raised by 10 ºC. The $Q_{10}$ for bacterial killing effect is often 8-30 times, which varies due to the distinct temperature dependence of different bacterial spores.

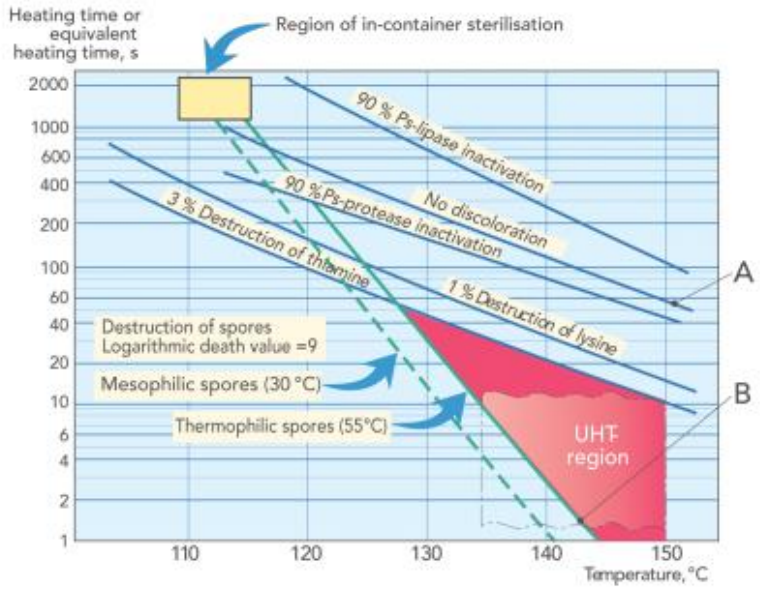


**Figure 2.1** The effect of UHT heating on microbiological, chemical and sensory quality of milk. The values within the brackets (30 ºC and 55 ºC) are expressed on the basis of the optimal growth condition of mesophilic and thermophilic spore-formers (Source: Kessler & Horak 1981).

### 2.4.2 Z-value

Z-value expresses the increase in temperature necessary to obtain the same lethal action in 1/10$^{th}$ of the time. The Z-value for *Bacillus stearothermophilus* inactivation is 10.5, colour changes is 29.0, losses of vitamin B1 31.2 and losses of lysine 30.9. This value can be obtained using the following equation or graph (Figure 2.2A).

$Z = 10/log\ Q_{10}$ (**2**)

### 2.4.3 D-value

D-value refers to the decimal reduction time which explains the time required to reduce 90% of bacteria (1-log) under a given condition. This value is calculated using the thermal death time (TDT) curve in Figure 2.2B.

### 2.4.4 $F_0$ value

The $F_0$ value refers to the combined effect of time and temperature on the inactivation of bacterial spores and enzymes. Herein, $F_0$ = 1 after the product is heated at 121.1 ºC for one minute.

$F_0 = 10^{(T-121.1\,°C)/Z}.t/60$ (**3**)

Where:

t = processing time (s)

T = processing temperature (°C)

Z= Z-value (°C)

$F_0$ = 1 after the product is heated at 121.1 °C for 1 min.

To practically obtain commercially sterile milk from good quality raw milk, UHT plants are designed to achieve a minimum $F_0$ value of 5-6 min. According to legislation in some countries, a minimal $F_0$ value of 3 min is required (correspondent to a 12 log reduction of *Clostridium botulinum* spores).

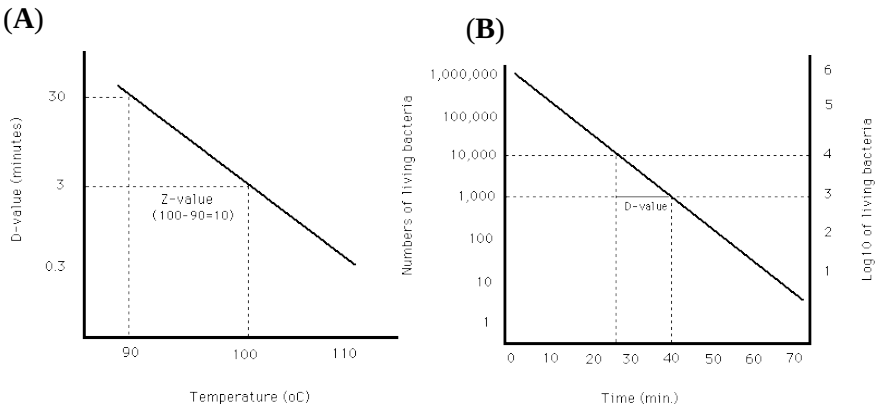


**Figure 2.2** Schematic representation of (A) interpretation of *Z*-value, and (B) calculating D-value using TDT curve. Adapted from (Goff 2005).

### 2.4.5 B* and C* values

B* is calculated based on the assumption of commercial sterility by heating the raw milk at 135 ºC for 10.1 s, with a corresponding Z-value of 10.5 ºC. This reference process is given a B* value of 1.0, which represents a reduction of spore count by $10^9$ per unit.

$B^* = 10^{(T-135)/10.5}.t/10.1$ (**4**)

Where

T = heating temperature (°C)

t = heating time (s)

C* value is calculated based on the assumption of achieving higher thiamine content (nutrient) at 135 ºC for 30.5 s with a *z*-value of 31.4 ºC. The reference process is given a C* value of 1, when disrupting only 3% of thiamine.

$$C^* = 10^{(T-135)/31.4}.t/30.5 \quad (\mathbf{5})$$

t = heating time in s

T = heating temperature, °C

Thus, for high quality UHT milk conditions of B* > 1 and C* < 1 should be maintained.

## 2.5 Factors Affecting Shelf-Life of UHT Milk

There are several factors affecting the shelf-life of UHT milk and they include: age of cow; stage of lactation; the presence of mastitis; and, microbiological quality. The first three factors are often associated with indigenous plasmin-induced quality defects, which are out of the scope of the present review. Thus, this review will focus on the effect of the microbiological quality of raw milk for the production of UHT milk and associated dairy products.

## 2.6 Microbiological Quality of Raw Milk

In order to reduce the frequency of milk collection, refrigerated storage of raw milk in farm bulk tanks requires an extension of 2-5 days prior to the heat-treatment, depending on the season and milk supply (Oliveira et al. 2015). This practice selectively inhibits the growth of mesophilic microbiota with a concomitant increase in the psychrotrophic population.

Adaptation of bacterial cells to low temperature can affect bacterial growth rates, conformation of cellular macromolecules, and their intracellular enzymatic reactions that are crucial for cell viability (Chattopadhyay 2006). Temperature adaptation also affects the phospholipid and natural lipid composition, leading to an increased production of unsaturated fatty acids, which involves reduction in the melting point that may serve to

maintain the fluidity in the cells (Beales 2004). This results in the continued functionality of bacterial metabolism, solute transportation, and secretion of extracellular substances. For example, expression of 266 genes (about 5% of the genome) was observed in *Pseudomonas putida* at 4 ºC, compared to 30 ºC. Those genes were involved in increasing the stability of DNA/RNA, membrane fluidity and growth rate with reduction of protein misfolding under refrigerated conditions (Fonseca et al. 2011).

### 2.6.1 Gram-negative psychrotrophs (GNP)

The GNP genera, including *Pseudomonas*, *Acinetobacter*, *Hafnia*, *Rahnella*, *Klebsiella*, *Serratia*, *Enterobacter*, *Stenotrophomonas*, *Aeromonas*, *Acromobacter*, *Alcaligenes*, *Chryseobacterium*, *Escherichia*, *Yersinia*, *Salmonella*, *Chromobacterium* and *Flavobacterium*, may account for 44-85% of the total microbiota in raw milk (Figure 2.3) (Machado et al. 2017, Vithanage et al. 2016). Among these genera, *Pseudomonas,* comprising of both fluorescent and non-fluorescent pseudomonads, are typically the most predominant isolates in refrigerated raw milk. The majority of these isolates produce heat-stable extracellular enzymes, mainly proteases and lipases that are associated with flavour and quality defects in their associated final products. Of Particular, *P. fluorescens* is the major cause of milk spoilage. These isolates are ubiquitous in nature and have a wide range of metabolic activities. For example, Dogan and Boor (2003) observed the differences in the proteolytic activities in different subspecies of *P. fluorescens* group associated with milk and dairy products. This is consistent with the study conducted in our laboratory, in which a variety of proteolytic activities in *P. fluorescens*, *P. poae*, *P. proteolytica*, *P. gessardii*, *P. salomonii*, *P. azotoformans* and *P. gingeri*, collectively known as *P. fluorescens* group (Vithanage et al. 2016), was observed. In the same study, slightly lower proteolytic activities in *P. fragii*, *P. lundensis*, *P. psychrophila*, *P. stutzeri*, *P. putida* and *P. aeruginosa* were noted. These bacteria appear to possess different hydrolytic potential, possibly related to the specificities of their corresponding proteases (Unpublished data). However, *Pseudomonas* spp. demonstrated efficient adaptation to cold temperatures due the presence of higher levels (59-72%) of unsaturated lipids in the cell membrane, which is believed to maintain

efficient membrane functionality, specifically in the solute as well as in the transport and secretion of enzymes into extracellular milieu at refrigeration temperatures (Fonseca et al. 2011, Oliveira et al. 2015). Milk contains low concentrations of free iron in the form of lactoferrin that can be sequestered by *Pseudomonas* spp. by means of the diffusible pigment pyoverdine, acting as siderophore (Oliveira et al. 2015). Besides *Pseudomonas* spp., other genera belonging to Gammaproteobacteria and *Flavobacteria* appear to contain distinct heat-stable enzymatic characteristics (Hantsis-Zacharov & Halpern 2007, Vithanage et al. 2016, von Neubeck et al. 2015).

### 2.6.2 Thermoduric psychrotrophs (TDP)

Psychrotrophic bacteria capable of surviving pasteurisation conditions and growing at psychrotrophic temperatures are known as thermoduric psychrotrophs. They include Gram-positive spore-formers (i.e. *Bacillus*, *Aneurinibacillus, Brevibacillus, Geobacillus, Paenibacillus, Ureibacillus, Virgibacillus* and *Clostridium*) and Gram-positive vegetative bacteria such as *Arthrobacter*, *Microbacterium*, *Streptococcus* and *Corynebacterium* (Figure 2.3) (Vithanage et al. 2016, Scheldeman et al. 2006). Gram-positive spore-formers encompass more than 200 species that can withstand harsh environmental conditions such as heat or cold, pressure, drought, starvation, biocides, and UV irradiation. This is possible due to the formation of dormant and reproductive structures called endospores (Moeller et al. 2008). These differentiated cells consist of an innermost core containing DNA, ribosomes, large amounts of dipicolinic acid (DPA) and small acid-soluble proteins (SAPs) surrounded by a cortical membrane and a cortex with peptidoglycan.

Sporulation is a complicated process triggered by starvation, high cell density, DNA damage, or the presence of minerals in the dairy environment (Burgess et al. 2010). Bacterial endospores exhibit extreme heat-resistance and can therefore may withstand UHT heating regimes (at 135-150 °C for 2-10 s) (Table 2.1). The spore inactivation experiments conducted in our lab also revealed that some thermoduric spore forming psychtrophs can withstand heating 150 °C for 20 s heating (Table 2.1) with B* 0.87-1.38 (data not shown). This is due to the lower water activity, presence of minerals, mainly calcium dipicolinate,

small acid-soluble proteins and membrane impermeability of spores (Burgess et al. 2010, Gopal et al. 2015). Conversion of spores into vegetative cells is referred to as germination, and comprises of activation, germination and outgrowth.

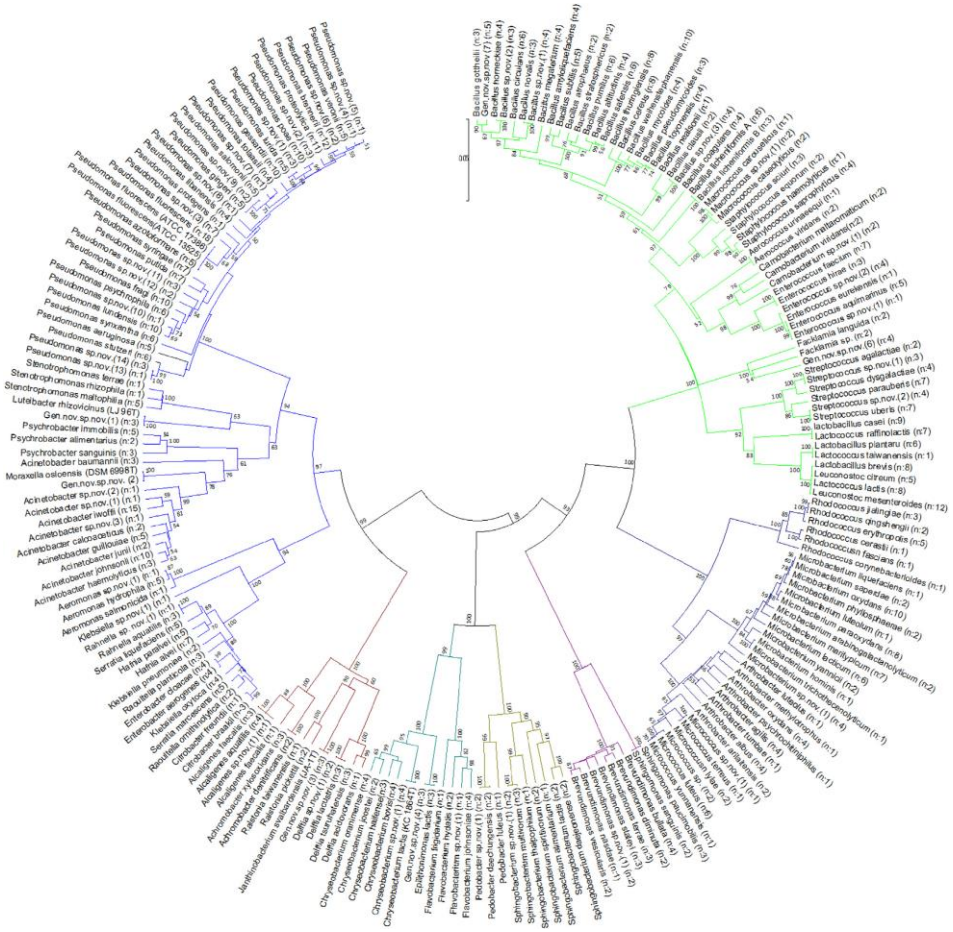

**Figure 2.3** Diversity of microbiota in raw milk (Source: Vithanage et al. 2016).

Heating is more likely to trigger the activation of the spores in the dairy industry, which subsequently germinate to produce vegetative cells during storage (Burgess et al. 2010, Gopal et al. 2015).

## 2.7 Factors Affecting Microbial Diversity in Raw Milk

The occurrence and diversity of microbiota in raw milk is significantly affected by the initial microbiota profile (or quality of raw milk). For example, experiments conducted in our laboratory observed that poor quality raw milk with higher bacterial counts was associated with a greater range of bacteria and higher viable counts after refrigerated storage,

compared to medium quality and high quality raw milk (Machado et al. 2017, Vithanage et al. 2016, Vithanage et al. 2017). This appears to correlate with the various management systems established in different dairy farms, and the selection and propagation of bacteria under those conditions (Cempírkova 2007).

**Table 2.1** Inactivation kinetics of spores of *Bacillus* spp. of dairy origin.

| **Species**[a] | **Temp/time (°C/s)** | **Inactivation (log cfu/mL)** |
|---|---|---|
| *G. stearothermophilus* ATCC 7953[a] | 121*/42 | 9 |
| *G. stearothermophilus* ATCC 7953[a] | 121*/7.3 | 9 |
| *G. stearothermophilus*[‡] | 150/20 | 3.2-4.3 |
| *Geobacillus* spp. [a] | 143/4 | 6.4–7.5 |
| *Geobacillus* sp. [a] | 143/4 | 9 |
| *A. flavithermus*[a] | 143/4 | 9.5–12 |
| *B. licheniformis* NCTC 6346[a] | 120*/3 | 9 |
| *B. licheniformis*[a] | 125*/4 | 8.5 |
| *B. licheniformis* ATCC 14580[‡] | 150/20 | 12.5 |
| *B. licheniformis*[‡] | 150/20 | 7.8-10.2 |
| *B. licheniformis*[a] | 135/10 | NS |
| *B. cereus*[a] | 120*/300-600 | 0.13-0.81 |
| *B. cereus* ATCC 10876[‡] | 150/20 | 6.7 |
| *B. cereus* ATCC 10987[‡] | 150/20 | 5.3 |
| *B. cereus* ATCC 14579[‡] | 150/20 | 8.4 |
| *B. cereus*[‡] | 150/20 | 6.5-8.9 |
| *B. thuringiensis*[‡] | 150/20 | 7.6-9.2 |
| *B. weihenstephensis*[‡] | 150/20 | 5.4-7.4 |
| *B. subtilis*[a] | 120*/4 | 7–10.5 |
| *B. subtilis*[a] | 135/10 | NS |
| *B. subtilis* ATCC 6633[‡] | 150/20 | 16.3 |
| *B. subtilis*[‡] | 150/20 | 12.3-14.8 |
| *B. pumilus*[a] | 135/10 | NS |
| *B. pumilus*[‡] | 150/20 | 34.3-43.2 |
| *B. safensis*[‡] | 150/20 | 23.3-48.7 |
| *B. altitudinis*[‡] | 150/20 | 25.3-34.1 |
| *B. sporothermodurans*[a] | 125*/780 | 9 |
| *B. sporothermodurans*[a] | 125*/120 | 9 |
| *B. sporothermodurans*[‡] | 150/20 | 5.3-8.2 |
| *P. lactis*[‡] | 150/20 | 4.5-5.6 |

NS: not specified

[a](Burgess et al. 2010, Dogan et al. 2009, Janštová & Lukášová 2001, Scheldeman et al. 2006).

[‡] Unpublished data.

*Although the temperature values are not representing the UHT heating regime the values can be used to calculate the lethal effect of the UHT heating using the $F_0$ value (equation 3).

Raw milk microbiota also showed significant variation, depending on different time and refrigeration conditions. For example, temperatures ≤ 4 ºC were shown to increase the population of the *Pseudomonas* spp., while simultaneously reducing certain enteric isolates (e.g. *Hafnia*, *Rahnella*, *Klebsiella*, *Enterobactor*, and *Serratia*); however, the increase in temperature above 6 ºC resulted in increased numbers of mesophilic bacteria such as *Acinetobacter*, *Serratia*, *Klebsiella*, *Escherichia*, *Stenotrophomonas* and *Bacillus* (Vithanage et al. 2016). The extended storage of raw milk at 4 ºC also resulted in an increase in both psychrotrophic counts and their diversity, enabling the growth of certain potentially pathogenic isolates (e.g. *Aeromonas* and *Stenotrophomonas*). Furthermore, the occurrences of psychrotrophic bacterial genera such as *Pseudomonas*, *Acinetobacter*, *Psychrobacter* and some *Bacillus* were higher in winter months, while those of *Hafnia*, *Serratia*, *Stenotrophomonas*, *Klebsiella*, *Enterobactor*, *Acinetobactor*, some *Pseudomonas*, *Aeromonas, Brevundimonas* and *Pseudoalteromonas* were higher in spring and autumn (Vithanage et al. 2016). Mesophilic *Bacillus* species and some enteric bacteria were shown to be higher in summer months. Isolates belonging to *Alphaproteobacteria*, *Betaproteobacteria*, *Flavobacteria* and *Sphingobacteria* were detected throughout the year, but at lower levels (Vithanage et al. 2016).

## 2.8 Potential Contamination Sources of Raw Milk

Bacteria are ubiquitous in nature, and are commonly found in soil, air and water; however, some bacteria are even natural colonizers of the gastrointestinal tract of warm-blooded animals. Microbes that disseminate into raw milk originate from the exterior of the teats, interior of teats, milking equipment and milk handlers; these sources are soiled by contaminated feed, manure, bedding, soil and water (Figure 2.4) (Vissers & Driehuis 2009). *Bacillus* spp. are often disseminated via soil and feeds (e.g. silage), whereas *Pseudomonas* spp. can be disseminated into raw milk via water sources. Enteric isolates can enter raw milk via cross contamination from faeces, while some pathogenic strains such as *Streptococcus*

spp., *Corynebacterium* spp., *S. aureus, Escherichia coli* and *Klebsiella* spp. can be disseminated into raw milk through infected teats, as a result of inflammatory diseases in cattle known as mastitis (Vissers & Driehuis 2009). Furthermore, raw milk contamination can also originate from milking equipment that is inadequately cleaned and sanitised (Vissers & Driehuis 2009). The microbial load can increase in the farm bulk tank due to the multiplication of psychrotrophs during refrigerated storage (Champagne et al. 1994).

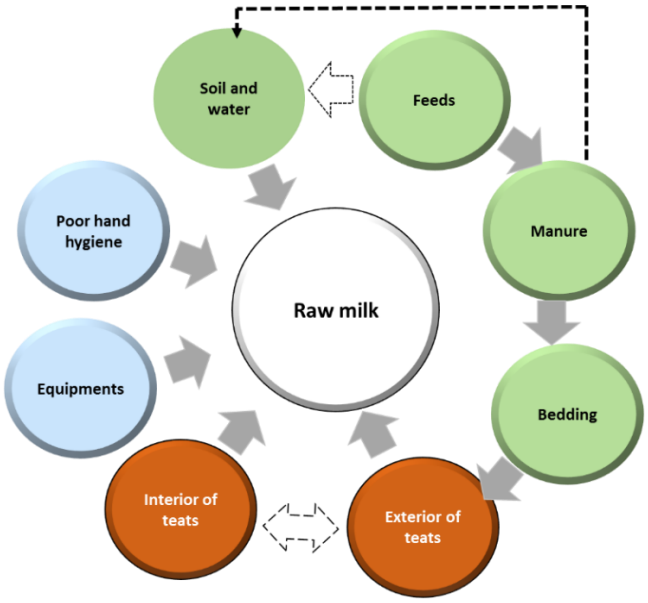


**Figure 2.4** Contamination routes of psychrotrophic bacteria in raw milk.

## 2.9 Spoilage Potential of Psychrotrophic Bacteria

Besides the ability to grow under refrigeration conditions, Gram-negative and some thermoduric psychrotrophic bacteria including *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Hafnia, Klebsiella* and *Stenotrophomonas* are shown to be associated with the production of heat-stable extracellular proteases, lipases, phospholipase C and α-amylase during extended storage at refrigeration conditions (Vithanage et al. 2016, Hantsis-Zacharov & Halpern 2007).

However, the expression of these enzymes is found to vary according to different species and strains due to their genetic heterogeneity, environmental factors and biofilm formation (Burger et al. 2000, Vithanage et al. 2016, Teh et al. 2012). These enzymes exhibited significant heat-stability, with 50-75% residual activity after heating to 142 °C for 4 s (Vithanage et al. 2016). The residual enzymes can result in the development of undesirable

sensory and quality defects (Champagne et al. 1994). However, such poor quality attributes are often encountered in UHT milk and dairy products due to the heat-stable proteolytic enzymes produced by psychrotrophs during the ambient storage (Champagne et al. 1994, Chavan et al. 2011, Machado et al. 2017). These attributes include bitterness, increased viscosity, gelation and fat separation (Button et al. 2011, Champagne et al. 1994, Chavan et al. 2011, Datta & Deeth 2001, Glück et al. 2016, Harwalkar 1992, McKenna & Singh 1991, Nieuwenhuijse & van Boekel 2003, Stoeckel et al. 2016a, unpublished data). In addition, some bacteria can cause sweet curdling, bitty cream, unclean, soapy flavour, thinning, rancidity, “cardboardy” flavour, oxidation, metallic, fruity or fishy tastes, fouling on heat exchangers, shorter or longer coagulating time, gas production, acidity and alkalinity, acid proteolysis and ropiness in milk and dairy products (Champagne et al. 1994, Samarzija et al. 2012). Moreover, *Rahnella aquatilis* has been shown to produce guaiacol that could produce a smoky/phenolic odour in milk stored at refrigeration temperatures (Jensen et al. 2001). However, the presence of some spore-formers such as *Bacillus sporothermodurans* do not cause noticeable spoilage, except for the sterility issues associated with excessive bacterial counts beyond the regulatory limits (Chavan et al. 2011). Nevertheless, the quality defects in UHT milk and dairy products are often encountered due to the heat-stable proteolytic enzymes produced by psychrotrophs during storage.

## 2.10 Bacterial Extracellular Metalloproteases of Dairy Origin

The term bacterial extracellular metalloproteases used in the present review describes the proteases secreted by bacteria into the extracellular milieu that catalyses the hydrolysis of internal peptide bonds of milk protein (endopeptidases) and requires metal ions for functionality (Häse & Finkelstein 1993). Both Gram-negative and Gram-positive bacteria (aerobic or anaerobic) produce zinc-containing metalloproteases. Within the family of bacterial zinc-metalloprotease, there exist two superfamilies viz. serralysin (EC 3.4.24.40), and serine (EC 3.4.21). Particularly, Aprx (Apra: (EC 3.4.24.40) from the serralysin super family, and substilisin (EC 3.4.21.62) and thermolysin (EC 3.4.24.27) from the serine super

family are regarded to be significant in the dairy processing industry (Chen et al. 2003). Regardless of their sources, these enzymes exhibit significant amino acid homology in the primary structure, due to their necessity for zinc for catalytic functions (Figure 2.5) (Häse & Finkelstein 1993).

### 2.10.1 Serralysin superfamily proteases

Among the serralysin subfamily of proteases, the alkaline metalloproteases such as Aprx, Apra, Apr, Ser, SerA and Prtc, produced by GNPs, are predominantly involved in the spoilage of dairy products. *Pseudomonas* spp., including the predominant *P. fluorescens*, produce a key heat-stable, extracellular, alkaline metalloprotease known as Aprx (or Aprx-like) (Andreani et al. 2016, Dufour et al. 2008, Vithanage et al. 2016). The Aprx protease of *P. fluorescens* displays 50-90% amino acid sequence homology with proteases produced by *Pseudomonas aeruginosa (*Apra*)*, *Acinetobacter* spp. (Ser), *A. hydrophila* (Sera), *S. marsecens* (Apr), and *Erwinia chrysanthemi* (e.g. Prtc, Prtb, and Prta)(Baumann et al.1993, Liao & McCallus 1998).

The gene encoding Aprx protease is known as *aprx* (also known as *apra*) and is located in a polycistronic operon (*aprx-lipa*) with a single open reading frame that consists of 1449 nucleotides (14 Kb) encoding 482 amino acids (Kumura et al. 1999, Woods et al. 2001). The operon also contains several other genes such as protease inhibitor (*inh*), ABC-type secretion apparatus (*aprdef*) and lipase (*lipa*). Thus, the secretion of this enzyme could be attributed to the expression of *aprdef* genes (Dufour et al. 2008); however, the absence of any of these genes in bacteria can result in loss or relatively low rate of proteolytic activity (Liao & McCallus 1998). The expression of *aprx* is tightly regulated by temperature (low temperature-dependent), $Fe^{3+}$, NaCl and the *ompr–envz two-component system, N*-acyl homoserine lactone (AHL), and some regulatory genes (*degu*, *degq, hpr, abrb* and*, sinr*) (Blumer et al. 1999, Heeb & Haas 2001, Liu et al. 2007, McCarthy et al. 2004, Reimmann et al. 1997, Valbuzzi et al. 1999). Additionally, the production of this protease is known to be associated with biofilm formation, possibly as an adaptive response to the different environmental conditions (especially cold condition), or nutrient starvation (lack of free iron due the presence of lactoferrin in milk) (Teh et al. 2011).

**2.10.2 Serine super family proteases**

In contrast to GNP isolates, the Gram-positive bacilli produce heat-stable neutral extracellular proteases such as substilisin and thermolysin, or their related proteases. Typically, the metalloproteases produced by different *Bacillus* spp., such as *B. thermoproteolyticus* (thermolysin), *B. stearothermophilus* (NprM, NprS, NprT), *B. subtilis* (NprE/subtilisin), *B. cereus*, *B. brevis*, *B. polymyxa*, *B. caldolyticus*, *B. megaterium*, *B. amyloliquefaciens* and *B. mesentericus*, share a high degree of amino acid sequence homology (84-92%) with each other (Hase & Finkelstein 1993, Tran et al. 1991). The genes that encode for substilisin and thermolysin are known as *apre* and *npr*, respectively. The *apre* gene encoding substilisin is located adjacent to a sigma A (sigma 43) promoter, and expression of this gene is regulated by a cascade of other genes such as *spooa, spoo, prtr, sacq, sacu, sacv, hpr*, *sin* and *sen* that are involved in early sporulation processes (Park et al. 1989). The *trp* operon accommodates both ORF X and *npr* genes that are involved in the

expression of thermolysin (Večerek & Venema 2000). *Bacillus* spp. show different enzymatic characteristics under different temperature conditions but with a significantly lower rate (4.6%) compared to *Pseudomonas* exoprotease at 7 ºC storage (Montanhini et al. 2013). However, the possibility of producing proteases by psychrophilic *Bacillus* spp. under refrigeration conditions is also described (Hanamant & Bansilal 2012, Meer et al. 1991).

All of these BPs are secreted into the extracellular milieu as a proenzyme and become activated after secretion, possibly due to the autolysis or release of signal peptides (only in thermolysin and substilisin) (Hase & Finkelstein 1993, Kumura et al. 1999, Woods et al. 2001).

Thus, understanding the types of proteases, species involved, and the expression and regulation of relevant genes would enable alterations in certain processing criteria and storage conditions of raw milk, depending on the microbial diversity of raw milk, to improve the quality of the product. Alternatively, the genetic information can be used to develop more rapid and reliable molecular based approaches for rapid and reliable identification, which will be discussed in a different section.

### 2.10.3 Production of proteases and relationship with the bacterial counts

Maximum production of these proteases is observed during the late exponential or stationary phase of psychrotrophic (GNP and TDP) growth, and this is where the sporulation occurs in TDPs (Dufour et al. 2008, Park et al. 1989). Several studies observed the highest proteolytic activity of psychrotrophic bacteria, when their counts reached $10^6$-$10^7$ cfu/mL (Brar et al. 2007, Cousin 1982, Griffiths et al. 1987, Haryani et al. 2003, Kent et al. 2012, O'Connell et al. 2016). More recently, we observed a significant protease activity and proteolysis in raw milk by psychrotrophic bacteria, comprising of proteolytic GNP and TDP, when the counts reached $5 \times 10^4$ cfu/mL or above at different refrigeration temperatures (Vithanage et al. 2017). This is similar to the previous descriptions of increased proteolytic activity, proteolysis and bitter peptide production with raw milk microbiota reaching greater than $10^4$ cfu/mL (Gillis et al. 1985, Kent et al. 2012, Vyletelova et al. 2000a). Due to the fact that UHT milk is more prone to proteolysis during the extended storage, it can be speculated that there is a

possibility that $10^4$ cfu/mL of psychrotrophic proteolytic bacterial count (PPrBC) and thermoduric psychrotrophic count (TDPC) can be used as threshold limits for screening of raw milk quality in the aspects of quality and safety, when producing high quality UHT milk and UHT treated dairy products (Silveira et al. 1999; Vithanage et al. 2017, Vyletelova et al. 2000b). Alternatively, knowledge of PPrBC counts under different storage and refrigeration conditions can be used to predict the storage life of raw milk and also in defining the shelf-life of UHT milk and UHT-treated products.

**2.10.4 Structure of bacterial proteases**

The primary structure of Aprx consists of an N-terminal active site (proteolytic domain) with a zinc binding motif (HEXXHXUGUXH; X: arbitrary amino acid; U: bulky hydrophobic amino acid residues, often leucine), while substilisin and thermolysin comprise a HEXXH zinc binding motif with glutamate that has a catalytic function (Kumeta et al. 1999, Weaver et al. 1977), which is known to have evolved and is conserved in all bacterial zinc metalloproteases (Hase & Finkelstein 1993). This substrate binding cleft comprises of a globular protein consisting of three parallel ß-sheets and four α-helices, which form two-folded lobes accommodating zinc ligands in a cleft that can possibly prevent the catalytic domain from heat-induced denaturation and higher flexibility, but restricts substrate specificity (Figure 2.5) (Baumann et al. 1993).

The C-terminal domain of the Aprx comprises seven fully occupied and one partially occupied $Ca^{2+}$ ion (Baumann et al. 1993), while both thermolysin and substilisin contain only four calcium ions, which are known to be associated with thermal stabilities of these enzymes (Rao et al. 1998). Thus, lower calcium ions are likely to reduce the thermal stabilities of *Bacillus* proteases, as compared to Aprx. Genetic engineering of bacteria to Increase in $Ca^{2+}$ ions in proteases would enable for its commercial uses in the food and dairy industry. In contrast, the information would be a foundation for possible inactivation strategies of heat stable protease causing gelation of UHT milk. However, these $Ca^{2+}$ ions are buried in the central region of the parallel ß-barrel comprising multiple glycine rich repeats (GGXGXD: X, hydrophobic residue), which are significantly hydrophobic (Figure 2.5)

(Baumann et al. 1993). This phenomenon may be involved in the refolding of these enzymes, allowing for conformational changes during enzyme activation and denaturation, thus increasing the heat-stability due to hydrophobic and ionic interactions and hydrogen bonding (Baumann et al. 1993). Additionally, the zwitterionic nature of glycine allows for their functionality in hydrophilic or hydrophobic environments. However, all three enzymes contain zero cysteine residues and, hence, no thiol groups or disulphide bridges (Kumeta et al. 1999, Rao et al. 1998), which are known increase the heat-stability of other enzymes (Wetzel et al. 1988).

**2.10.5 Heat stability of bacterial proteases**

The extracellular BPs produced by psychrotrophic bacteria have been shown to be remarkably

heat-stable and can even withstand UHT processing that involves heating at 135-150 ºC for 2-10 s. Adams et al. (1975), observed that heat treatment at 149 ºC/4 s was sufficient for the complete inactivation of spores from *G. stearothermophilus* (PA 3679). In contrast, *Pseudomonas* proteases (MC 60) were inactivated by only 10%. Thus, it was estimated to be 400 times more heat resistant than bacterial spores. Many studies demonstrated heat-induced partial inactivation of BPs with different time-temperature combinations (Table 2). The highest heat resistance was reported for protease of *P. fluorescens* (strain Rm12), which showed 9% residual activity even after heating at 160 ºC for 0.3 min (Mu et al. 2009). Studies conducted in our laboratory also observed 0.7-3.3% residual activities of AprX, thermolysin and substilisin like proteases produced by a wide range of bacteria (55 isolates) after 150 ºC/0.3 min heating (Unpublished data). Furthermore, 50-75% residual activities of proteolytic enzymes, produced by *Pseudomonas*, *Bacillus* and *Microbacterium* isolates after heating to 142 °C for 4 s, were observed (Vithanage et al. 2016). However, some bacteria showed no activity or significantly lower activity (0.3-1%) after UHT heating (Table 2.2).

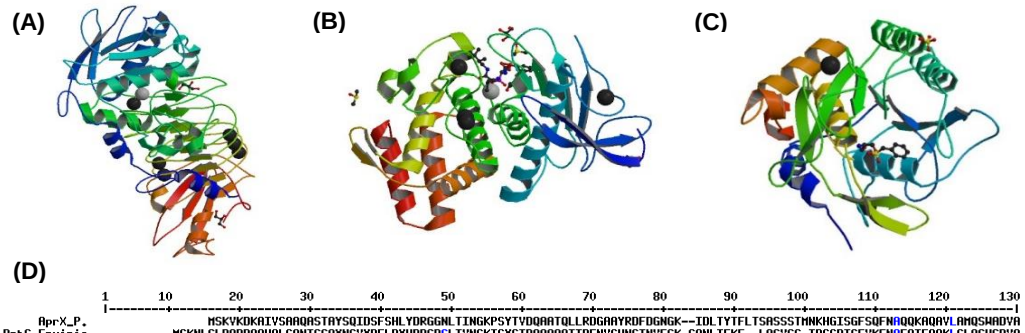

**Figure 2.5** Comparison of the crystal structures of (A) Aprx (B) thermolysin (C) substilisin. All three proteases contain α/β fold proteins with one catalytic $Zn^{2+}$ coordinated in the active site, harbouring several $Ca^{2+}$ ions that are important for structural stabilization and C-terminal extension composing of β-sheets is shown in orange. (D) Multiple sequence alignments of Aprx of *P. fluorescens*, Prtc form *E. chrysanthemi*, Ser of *S. marcescens*, Ser of *A. hydrophila*, Ser of *A. goulligue*, AprA of *P. aeruginosa*, Substilisin of *B. subtilis* and thermolysin of *B. cereus*; the conserved sequences are highlighted in blue. Green boxed residues are thought to the catalytic domain, while red boxed residues thought to participate in calcium binding.

**Table 2.2** Inactivation kinetics of heat-stable BPs.

| Source of enzyme | Heating medium | Heating system | Temperature/time (ºC/min) | Residual activity (%) | Source |
|---|---|---|---|---|---|
| *Pseudomonas* spp. (MC 60) | SMUF | Capillary tubes | 149/1.5 | 10 | (Adams et al. 1975) |
| *P. fluorescens* (AFT36, other strain) | Synthetic milk salts buffer | Capillary tubes | 130/1.2<br>140/1<br>150/0.15 | 50<br>10<br>10 | (Stepaniak & Fox 1983) |
| *P. fluorescens* | Skim milk | Stainless steel tubes | 140/2.2 | 10 | (Kroll & Klostermeyer 1984) |
| *Pseudomonas* spp. (AFT21) | SMUF<br>Phosphate buffer | Capillary tubes | 130/2.0<br>130/1.3 | 10 | (Stepaniak & Fox 1985) |
| Seven *P. fluorescens* strains (OM2, OM41, OM82, OM186, OM227, OM228) | SMUF | Capillary tubes | 140/0.1-5.0 | 10 | (Mitchell et al. 1986) |
| *Pseudomonas* spp. AFT21; Proteinase III | Tris-HCl buffer | Capillary tubes | 140[b]/2.6 | 10 | (Baral et al. 1995) |
| *P. fluorescens* 22F | DI water | Stainless steel tubes | 100[b]/69.2 | 10 | (Schokker & van Boekel 1997) |
| *P. fluorescens* NCDO2085 | Whey (pH 6.8) | Thermoresistometer | 127/3.0 | 10 | (Vercet et al. 1997) |
| *Pseudomonas* spp. W15a and *P. proteolytica* 691 | Whole milk | screw-capped steel tubes | 120/6.3-10.7<br>130/4.0-4.9<br>140/1.3-1.8 | 10<br>10<br>10 | (Stoeckel et al. 2016) |
| *Three Pseudomonas* spp. (WS4992, WS 4993, WS 4672) | SMUF<br>Whole milk | Test tubes | 138/0.3 | 13-36<br>46-59 | (Glück et al. 2016) |
| *P. fluorescens* Rm12 | buffer at pH 7.5 | capillary tubes | 100/0.3<br>120/0.3<br>140/0.3<br>160/0.3 | 88<br>60<br>40<br>9 | (Mu et al. 2009) |
| *Pseudomonas panacis* | SMUF Milk | stainless steel tubes | 138/0.3 | 37.1 88.0 | (Baur et al. 2015) |
| Seven species of *Bacillus* spp. including *G.stearotheromophilus* (am, bm, cm), *B. licheniformis* (Dm, F/G, $F^{ta}$) and *B. subtilis* | C-medium | glass ampoules | 90/10 | 3-64 | (Chen et al. 2004) |
| *P. fluorescens* T16 | Trypticase soy broth with 1-2% skim milk | glass ampoules | 120/7.6 | 50 | (Patel et al. 1983) |
| *P. fluorescens* BJ-10 | plastic tubes | Culture media | 100/3 | 94.6 | (Zhang & Lv 2014) |
| 55 of psychrotrophic bacterial genera (*Pseudomonas, Hafnia, Serratia, Acinetobacter, Stenotrophomonas, Rahnella, Klebsiella, Bacillus*) | UHT skim milk | screw-capped Pyrex tubes | 150/0.3 | 0.7-3.3 | (Own data, unpublished) |

The heat stability of bacterial proteolytic enzymes is likely to be related to their specific structure, or structural modifications like conformational changes, which likely occur during the heating process (Kask et al. 2004, Sawle & Ghosh 2011). Additionally, heat stability can be related to the presence of metal ions like $Ca^{2+}$ in the structure and also in the surrounding medium (especially in $Ca^{2+}$ rich medium like milk) that can form ionic bonds or electrostatic interactions. Additionally, milk proteins and fat globules can interact with the BPs to form structures which can prevent BPs from autolysis and heat-induced inactivation (Chavan et al. 2011, Nielsen et al. 2003, Patel & Bartlett 1988).

The heat-induced inactivation of BPs follows first-order kinetics' parameters, but uses a three-state model (Glück et al. 2016). In the first step, the enzyme can undergo reversible denaturation by transforming into an inactive unfolded state at denaturation temperature ($T_d$), resulting in a mixture of folded (50%) and unfolded (50%) molecules. This may be followed by irreversible denaturation of unfolded enzymes above $T_d$ due to reshuffling of disulphide bonds, destruction of amino acid residues, aggregation, formation of incorrect structures or irreversible covalent modifications (deamination), thus leading to conformational changes in the native structure (Ahern & Klibanov 1988). Finally, the unfolded molecules can be hydrolysed by indigenous proteases in milk at temperatures in the range of $T_d$ (Ahern & Klibanov 1988, Glück et al. 2016). Alternatively, proteolytic enzymes showed significant inactivation at low temperature conditions (55-60 ºC), possibly due to the autoproteolysis or formation of enzyme casein aggregates (Barach et al. 1976, Chavan et al. 2011, Glück et al. 2016).

Thus, it is important to determine the inactivation parameters required for the total inactivation of these BPs. However, the comparison of those parameters described by different studies is extremely difficult due to various factors, including expression variation of proteases in different bacteria and under different experimental conditions, the use of different heating media and systems and the use of different detection methods for residual activity. Thus, UHT heating systems may not be severe enough to eliminate proteolytic activity of some psychrotrophic bacteria in milk and an increase in the temperature higher

than current regimes would be detrimental to the functionality of the milk protein. As a result, a proportion of BPs present in milk is unlikely to be destroyed by the heat treatments applied in the commercial milk processing and may be active in the final product, causing significant spoilage during extended storage (Muir, 1996). Therefore, the recommending required shelf-life of UHT milk may depend on the residual protease activity in milk, which may require a rapid and more sensitive screening method than the one being used currently. Alternatively, extension of shelf-life can be achieved by means of other thermal and non-thermal technologies.

### 2.10.6 Specificity of bacterial proteases on bovine casein micelles

The BPs preferentially hydrolyse peptide bonds containing hydrophobic amino-acids in bovine casein micelles, compared to whey protein (Gebre-Egziabher et al. 1980). The order of susceptibility of the different casein fractions to BPs is in the following order: $\kappa > \beta > \alpha_{s1} > \alpha_{s2}$ (Chavan et al. 2011). This is possibly related to the accessibility of different casein fractions arranged in the micellar structure or to the specificity of the different peptide bonds. The κ-caseins are often located in the outer layer and are most susceptible to proteolysis with 18 targeted peptide bonds (Table 2.3). Particularly, the hydrolysis of κ-casein in the peptide of $Phe_{105}$-$Met_{106}$ results in para-κ-casein and caseino macropeptide, both of which, are similar to rennet coagulation. There were 34 potential peptide bonds of β-casein shown to be hydrolysed by Aprx protease. The $\alpha_{s1}$ and $\alpha_{s2}$ were subjected to hydrolysis from 23 and 4 peptide bonds, respectively. These peptide bonds often contain glutamine, lysine, and tyrosine amino acid.

### 2.10.7 Biochemical characteristics

These BPs show various biochemical characteristics (Table 2.4). For example, the molecular weight (Mw) of Aprx is in the range of 45 kDa and 50 kDa, with an isoelectric point (pI) of 4 (Dufour et al. 2008, Kumeta et al.1999). Thermolysin and substilisin comprise of Mw of 34.5-37.5 kDa and 16.9-38.0 kDa and pI of 5.2 and 8.4, respectively. These enzymes also have different temperatures and pH optima. Divalent cationic chelators which has the affinity for $Zn^{2+}$ and $Ca^{2+}$ ions inhibit Aprx, possibly related to the reaction between chelates and

respective ions, which result in loss of $Zn^{2+}$ and $Ca^{2+}$ necessary for functionality of the corresponding enzymes.

**Table 2.3** Predicted peptides of different bovine casein fractions hydrolysed by BPs.

| Amino acid sequence | Peptides |
|---|---|
| **κ-casein** | |
| MMKSFFLVVT ILALTLPFLG A[1]QEQNQEQPI RCEKDERFFS DKIAKYIPIQ YVLSRYPSYG LNYYQQKPVA LINNQFLPYP YYAKPAAVRS PAQILQWQVL SNTVPMKSCQ AQPTTMARHP HPHLSFMAIP PKKNQDKTEI PTINTIASGE PTSTPTTEAV ESTVATLEDS PEVIESPPEI NTVQVTSTAV[169] | $Tyr_{25}$-$Ile_{26}$, $Tyr_{30}$-$Val_{31}$, $Tyr_{38}$-$Gly_{39}$,$Gly_{39}$-$Leu_{40}$,$Asn_{41}$-$Tyr_{42}$, $Tyr_{42}$-$Tyr_{43}$,$Tyr_{61}$-$Ala_{62}$,$Ala_{71}$-$Gln_{72}$, $Thr_{94}$-$Met_{95}$, $Arg_{97}$-$His_{98}$, $Leu_{103}$-$Ser_{104}$, $Ser_{104}$-$Phe_{105}$, $Phe_{105}$-$Met_{106}$, $Lys_{111}$-$Lys_{112}$, $Lys_{112}$-$Asn_{113}$, $Asn_{113}$-$Gln_{114}$, $Asn_{123}$-$Thr_{124}$, $Glu_{158}$-$Ile_{159}$. |
| **β-casein** | |
| MKVLILACLV ALALA[1]RELEE LNVPGEIVES LSSSEESITR INKKIEKFQS EEQQQTEDEL QDKIHPFAQT QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP PFLQPEVMGV SKVKEAMAPK HKEMPFPKYP VEPFTESQSL TLTDVENLHL PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL SQSKVLPVPQ KAVPYPQRDM PIQAFLLYQE PVLGPVRGPF PIIV[209] | $Asn_{7}$-$Val_{8}$, $Gly_{10}$-$Glu_{11}$, $Glu_{11}$-$Ile_{12}$, $Tyr_{24}$-$Arg_{25}$,$Lys_{28}$-$Lys_{29}$,$Lys_{29}$-$Ile_{30}$,$Ile_{30}$-$Glu_{31}$,$Gln_{40}$-$Thr_{41}$,$Asp_{43}$-$Glu_{44}$,$Pro_{51}$-$Phe_{52}$,$Phe_{52}$-$Ala_{53}$, $Ala_{53}$-$Gln_{54}$, $Gln_{54}$-$Thr_{55}$, $Thr_{55}$-$Gln_{56}$,$Ser_{57}$-$Leu_{58}$,$Leu_{58}$-$Val_{59}$, $Val_{59}$-$Tyr_{60}$,$Tyr_{60}$-$Pro_{61}$,$Phe_{62}$-$Pro_{63}$,$Ser_{67}$-$Leu_{68}$, $Pro_{68}$-$Asn_{69}$, $Asn_{69}$-$Ser_{70}$, $Thr_{78}$-$Glu_{79}$, $Val_{83}$-$Val_{84}$,$Val_{84}$-$Pro_{85}$,$Phe_{87}$-$Leu_{88}$,$Gln_{89}$-$Pro_{90}$, $Gly_{94}$-$Val_{95}$, $Val_{95}$-$Ser_{96}$,$Ser_{96}$-$Lys_{97}$,$Val_{98}$-$Lys_{99}$, $Lys_{99}$-$Glu_{100}$,$Ala_{101}$-$Met_{102}$,$Met_{102}$-$Ala_{103}$, $Lys_{107}$-$Glu_{108}$, $Glu_{108}$-$Met_{109}$, $Pro_{110}$-$Phe_{111}$, $Lys_{113}$-$Tyr_{114}$, $Thr_{120}$-$Glu_{121}$, $Ser_{122}$-$Gln_{123}$, $Ser_{124}$-$Leu_{125}$,$His_{134}$-$Leu_{135}$, Pro138-Leu139,$Leu_{140}$-$Glu_{141}$,$Trp_{143}$-$Met_{144}$, $Met_{144}$-$His_{145}$,$His_{145}$-$Gln_{146}$, $Pro_{147}$-$His_{148}$, $His_{148}$-$Gln_{149}$,$Met_{156}$-$Phe_{157}$,$Gln_{160}$-$Ser_{161}$, $Ser_{161}$-$Val_{162}$,$Leu_{163}$-$Ser_{164}$, $Ser_{164}$-$Leu_{165}$,$Leu_{165}$-$Ser_{166}$, $Ser_{166}$-$Gln_{167}$, $Gln_{167}$-$Ser_{168}$, $Lys_{169}$-$Val_{170}$, $Pro_{174}$-$Gln_{175}$, $Gln_{175}$-$Lys_{176}$, $Lys_{176}$-$Ala_{177}$, $Ala_{177}$-$Val_{178}$,$Val_{178}$-$Pro_{179}$,$Pro_{181}$-$Gln_{182}$, $Gln_{182}$-$Arg_{183}$,$Arg_{183}$ $Asp_{184}$, $Asp_{184}$-$Met_{185}$,$Gln_{188}$-$Ala_{189}$,$Ala_{189}$-$Phe_{190}$, $Phe_{190}$-$Leu_{191}$, $Leu_{191}$-$Leu_{192}$, $Tyr_{193}$-$Gln_{194}$, $Gln_{194}$-$Glu_{195}$, $Arg_{202}$-$Gly_{203}$, $Pro_{206}$-$Ile_{207}$ |

| | |
|---|---|
| **$\alpha_{s1}$-casein** | |
| MKLLILTCLV AVALA[1]RPKHP | $Lys_{7}$-$His_{8}$, $His_{8}$-$Gln_{9}$, $Gln_{9}$-$Gly_{10}$, $Gly_{10}$-$Leu_{11}$, $Pro_{12}$-$Gln_{13}$, $Glu_{14}$-$Val_{15}$, $Asn_{17}$-$Glu_{18}$, $Arg_{22}$-$Phe_{23}$, $Phe_{23}$-$Phe_{24}$, $Phe_{24}$-$Val_{25}$, $Lys_{36}$- |
| IKHQGLPQEV LNENLLRFFV | $Val_{37}$, $Lys_{79}$-$His_{80}$, $His_{80}$-$Ile_{81}$, $Lys_{83}$-$Glu_{84}$, $Tyr_{91}$-$Leu_{92}$, $Gly_{93}$-$Tyr_{94}$, $Leu_{95}$-$Glu_{96}$, $Arg_{100}$-$Leu_{101}$, $Lys_{102}$-$Lys_{103}$, $Lys_{105}$-$Val_{106}$, $Lys_{109}$- |
| APFPEVFGKE KVNELSKDIG | $Glu_{110}$, $His_{121}$-$Ser_{122}$, $Lys_{124}$-$Glu_{125}$, $His_{128}$-$Ala_{129}$, $Ala_{129}$-$Gln_{130}$, $Lys_{132}$-$Glu_{133}$, $Gly_{137}$-$Val_{138}$, $Ala_{143}$-$Tyr_{144}$, $Phe_{145}$-$Tyr_{146}$, $Arg_{151}$- |
| SESTEDQAME DIKQMEAESI | $Gln_{152}$, $Phe_{153}$-$Tyr_{154}$, $Tyr_{154}$-$Gln_{155}$, $Tyr_{165}$-$Tyr_{166}$, $Gly_{170}$-$Thr_{171}$, $Tyr_{173}$-$Thr_{174}$, $Gly_{187}$-$Ser_{188}$, $Lys_{193}$-$Thr_{194}$ |
| SSSEEIVPNS VEQKHIQKED | |
| VPSERYLGYL EQLLRLKKYK | |
| VPQLEIVPNS AEERLHSMKE | |
| GIHAQQKEPM IGVNQELAYF | |
| YPELFRQFYQ LDAYPSGAWY | |
| YVPLGTQYTD APSFSDIPNP | |
| IGSENSEKTT MPLW[199] | |
| **$\alpha_{s2}$-casein** | |
| MKFFIFTCLL AVALAKNTME | $Asn_{162}$-$Phe_{163}$, $Phe_{174}$-$Ala_{175}$, $Tyr_{179}$-$Leu_{180}$, $Ala_{189}$-$Met_{190}$ |
| HVSSSEESII SQETYKQEKN | |
| MAINPSKENL CSTFCKEVVR | |
| NANEEEYSIG SSSEESAEVA | |
| TEEVKITVDD KHYQKALNEI | |
| NQFYQKFPQY LQYLYQGPIV | |
| LNPWDQVKRN AVPITPTLNR | |
| EQLSTSEENS KKTVDMESTE | |
| VFTKKTKLTE EEKNRLNFLK | |
| KISQRYQKFA LPQYLKTVYQ | |
| HQKAMKPWIQ PKTKVIPYVR YL | |

The amino acid sequences for bovine (*Bos tratus*) casein fractions were obtained from NCBI database (https://www.ncbi.nlm.nih.gov/protein) and the molecular weight and p*I* was calculated by Expasy database (http://web.expasy.org/cgi-bin/compute_pi/pi_tool). The highlighted section indicate the signal sequence of prosequence of each bovine casein fraction. the peptides formed by AprX protease was obtained from (Matéos et al. 2015), the Amino acid nomenclature: cysteine: C (Cys); histidine: His (H); isoleucine: Ile (I); methionine: Met (M); serine: Ser (S); valine: Val (V); alanine: Ala (A); glycine: Gly (G); leucine: Leu (L); proline: Pro (P); threonine: Thr (T); phenylalanine; Phe (F); arginine: Arg (R); tyrosine: Tyr (Y); tryptophan: Trp (W); aspartic acid: Asp (D); asparagine; Asn (N); glutamic acid: Glu (E); glutamine: Gln (Q); lysine: Lys (K). S: phosphoserine residues.

These properties are important in characterising unknown proteases that are encountered in dairy processing as well as defining inactivation strategies and for the development of reliable identification techniques (e.g. spectrophotometry using specific substrates).

**Table 2.4** Comparison of biochemical characteristics of BPs.

| **Characteristics**[a] | **AprX (Apr)** | **Thermolysin** | **Substilisin** |
|---|---|---|---|
| Superfamily | Serralysin | Serine | Serine |
| pH condition | Alkaline | Neutral | Neutral |
| Mw (kDa) | 45-50 | 34.5-37.5 | 16.9-38.0 |
| Subunits | Monomer | Monomer | Monomer |
| pI | 4.0 | 5.2 | 8.4 |
| Temperature optima (ºC) | 35-45 | 25-65 | 37-60 |
| pH optima | 6.5-8.5 | 6.5-7.5 | 7.5-10.0 |
| Cofactors | $Ca^{2+}$, $Co^{2+}$ | $Ca^{2+}$, $Co2^{+}$, $K^{+}$, $Li^{+}$, $Na^{+}$, $Zn^{2+}$ | $Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$ |
| Potential inhibitors | EGTA[b], O-phenanthroline[c] , EDTA[d],chymostatin,dithiothreitol, Soybean trypsininhibitor, N-ethylmaleimide,Elastatinal | DMSO, EDTA[d], 3-methylaspirin 2-propanol, 1-butanol, dimethylformamide, hydroxamic acid inhibitors, oxyquinoline, etc. | EDTA[c], EGTA[b], PMSF,triton X-100, tween 20,chymostatin, N-ethylmaleimide,iodoacetamide |
| Substrates | Casein, azocasein, t-butyloxycarbonyl-Arg-Val-Arg-Arg-4-methylcoumaryl-7-amide etc. | Casein, bovine alpha-lactalbumin, bovine beta-lactoglobulin A , azocasein | Casein, D-Val-Leu-Lysp-nitroanilide,azocasein, |

[a]these characteristics can be slightly vary depending on the microorganism used for the production of those proteases (Dufour et al. 2008) and Brenda database (http://www.brenda-enzymes.org) , [b]$Ca^{2+}$ chelator, [c]$Zn^{2+}$ chelator, [d]divalent cation chelator. EDTA: Ethylenediaminetetraacetic acid; EGTA: ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid; DMSO: Dimethyl sulfoxide; PMSF: phenylmethane sulfonyl fluoride.

## 2.11 Impact of Psychrotrophic Bacteria on Milk and Dairy Products

Production of extracellular proteases by psychrotrophic bacteria can result in bitterness, increased viscosity and age gelation in UHT milk (Champagne et al. 1994, Samarzija et al. 2012). The hydrolysis of casein can be evident as a greyish colour, bitter taste and gelation of spoiled milk (Vyletělová & Hanuš 2000a). It was observed that the onset of different quality defects in UHT milk as a function of time at ambient condition proceeds in the order of bitterness<increase in particle size/viscosity < cream layer formation < sediment <

gelation with > 0.05 pkat/mL (pkat/mL: picokatal, measurement of catalytic activity of enzymes) (Stoeckel et al. 2016a). However, gelled UHT milk samples are bitter or would show signs of fat separation (Stoeckel et al. 2016a). The differences are depend on the enzymatic potential and enzymatic specificities of bacteria and their specificity (Stoeckel et al. 2016a). Particularly gelation and fat separation result from bacteria with both proteolytic and lipolytic potential (Champagne et al. 1994, Samarzija et al. 2012). The effects of proteolytic enzymes on gelation are listed in the Table 2.5.

**Table 2.5** Effect of proteolytic enzymes on shelf–life of UHT milk.

| Enzyme | Bacterial count (cfu/mL) | Protease activity/ proteolysis | Impact | Reference |
|---|---|---|---|---|
| *Pseudomonas* spp. (MC 60) | $10^3$ -$10^9$ | 18-8900 u/mL | Bitterness by < 3 d | (Adams et al. 1975) |
| *Pseudomonas* spp. | < $10^4$ | NS | κ-CN breakdown after 50 d | (Adams et al. 1976) |
| *P. fluorescens* (AR 11) | 8 x $10^6$ | 78% loss of caseins | Gelation by 56-70 d | (Law et al. 1977) |
| *P. fluorescens* (OM41) | NS | 60 u/mL | Gelation by 95-123 d | (Fairbairn & Law 1986) |
| *P. fluorescens* (OM 227) | NS | 29.7 u/mL or 3 ng/mL | Gelation by 144-151 d | (Fairbairn & Law 1986) |
| *P. fluorescens* (22F) | 5 x $10^5$ | NS | Bitterness and gelation by 49 d | (Torrie et al. 1983) |
| *P. fluorescens* (RM14), | x $10^8$ | NS | Bitterness after 7 d | (Fairbairn & Law 1986) |
| Psychrotrophs | NS | 30 x $10^{-3}$ u/mL | Gelation after 150 d | (Renner 1988) |
| Psychrotrophs | NS | < 6 x $10^3$ u/mL | Gelation > 90 d | (Griffiths et al. 1988) |
| Psychrotrophs | 2.5 x $10^7$ | NS | Gelation within 30 d | (Griffiths et al. 1988) |
| Psychrotrophs | NS | 6 x $10^2$ u/mL or 1-2 ng/mL | Gelation after 90 d | (Mitchell & Ewings 1985) |
| Psychrotrophs | $10^4$-$10^6$ | NS | Proteolysis and bitterness 90 d | (Gillis et al. 1985) |
| *P. fluorescens* | $10^6$ | NS | greyish colour, bitter taste and gelation after 20 d | (Vyletělová & Hanuš, 2000b) |
| Psychrotrophic proteolytic bacteria | 5.1-5.4 x $10^4$ | 1·0 × $10^2$-4·0 × $10^4$ RFU/ml or 3.5-11.9 ng/mL | Increased viscosity and particle size after 10 d | (Vithanage et al. 2017) |
| *Pseudomonas* spp. | 1 x $10^4$ | > 0.05 pkat/mL | Increased in particle size, cream layer formation, bitterness and gelation after 60 d | (Stoeckel et al. 2016a) |

NS: not specified; measurement of proteolytic activity was exhibited different units; u/mL: units per mL; RFU/mL: relative fluorescens units per mL; pkat/mL: picokatal, measurement of catalytic activity of enzymes per millilitre, d: days.

### 2.11.1 Bitterness

The proteolytic activity in milk can result in unmodified peptide fractions of casein that are potentially bitter in taste (Table 2.6) (Fiat & Jollès 1989, Lemieux & Simard 1992). The activities of neutral or alkaline proteases generally result in more bitterness as compared to acidic proteases (Lemieux & Simard 1992). There was a significant correlation of hydrophobicity with bitterness, using Q-value (average hydrophobicity of a peptide), which is calculated as the average free energy for the transfer of amino acid chains from ethanol to water using following equations (Ney 1979, Ney 1971).

$$\mathrm{Q} = \frac{\sum \Delta \mathrm{f}}{\mathrm{n}} \quad (\mathbf{6})$$

where Q = the average hydrophobicity of a peptide, ∑ ∆f = the sum of free energy for the transfer of amino acid side chains from ethanol to water in cal/mol for each residue (Tanford 1962), and n = the number of amino acid residues. Tanford's free energy calculation can be summarized by Eq. 7:

$$\sum \Delta \mathrm{f} = -T\Delta S_{conf} + \sum \Delta f_u \quad (\mathbf{7})$$

where -T∆Sconf is the change in conformational entropy of the polypeptide chain from ethanol to water and ∆fu is the change in free energy for the transfer of small component groups of the protein molecule from the native form to the unfolded form. Ney reported that peptides with molecular weight of ≤ 6 kDa and Q values ≥ 1,400 cal/mol are potentially bitter in taste compared to Q-values ≤ 1300 cal/moL. Any peptide of molecular weight > 6 kDa with Q values ≥ 1,400 cal/mol were also not bitter. This rule has been used to determine the bitterness of hydrolysates prepared from different submicelles of milk casein and found distinct variation in the Q-values of various casein submicelles (Bruce & Pawlett 1997, Nielsen et al. 2017). For example; κ-casein possesses a Q-value between 1300-1400 cal/moL, whereas hydrolysates of β, $\alpha s_1$, $\alpha s_2$ caseins resulted in bitter peptides with Q-value between 1400-2500 cal/moL (Bruce & Pawlett 1997, Nielsen et al. 2017). These proteins are known to be more hydrophobic than κ-casein and are located in the hydrophobic core of the

casein micelles, thus the bitterness can be more intense in β, $\alpha s_1$, $\alpha s_2$, than κ-casein (Bruce & Pawlett 1997, Nielsen et al. 2017). Bitterness is caused by the presence of hydrophobic amino acid side chains containing histidine, arginine, methionine, valine, isoleucine, phenyl alanine, tryptophan, leucine, proline and is intensified when the carboxyl groups of the amino acids are blocked (Lemieux & Simard 1992, Ney 1972). In addition, hydrophobicity of the amino acid side-chains and the length of a peptide can significantly affect the bitterness in milk (Ney 1979).

The onset of bitter taste was observed after 6 weeks of storage (Lemieux & Simard 1992). However, a recent study observed bitter flavour development in UHT milk after 4 weeks of storage with protease activity as low as 0.05 pkat/mL (Stoeckel et al. 2016a). Similarly, Gillis et al. (1985) observed a significant increase in proteolysis and bitter peptide production with raw milk microbiota > $10^4$ cfu/mL. A recent study described an increase in the levels of potentially bitter peptides (Q-value ≥ 1400 cal/moL) from 86 to 116 days of storage in lactose-hydrolysed UHT milk (Nielsen et al. 2017). *Bacillus* proteases also produce bitter peptides in sodium caseinate (Slattery & Fitzgerald 1998). Furthermore, McKellar et al. (1984) showed that UHT milk processed by direct method was more prone to bitter flavour production (at 20 °C for 20 weeks) compared to indirectly processed milk. This may be related to the greater inactivation of proteases by indirect heating due to the severity of the heating process compared to direct heating (Datta et al. 2002). Skimmed milk is more susceptible to development of bitterness due to lower fat content, which is known to reduce the protective effect of milk proteins by enzymatic attack.

### 2.11.2 Increase in viscosity and age gelation

Proteolysis of milk by BPs and its relationship to the age gelation has been extensively studied and reviewed (Button et al. 2011, Chavan et al. 2011, Datta & Deeth 2001, Harwalkar 1992, McKenna & Singh 1991, Nieuwenhuijse & van Boekel 2003). The majority of these studies were conducted either by adding BPs or allowing psychrotrophic bacteria to grow in milk prior to pasteurisation and UHT processing.

In particular, the UHT-treated milk and dairy products are prone to age gelation due to their extended storage for 9-12 months at ambient temperature. It was also determined that even very low concentrations of protease can cause gelation during storage.

**Table 2.6** Predicted bitter peptide fractions derived from BPs.

| Caseins | Bitter peptides[a] with Q value ≥1400 (cal/moL) |
|---|---|
| β-casein | f46-65, f46-67, f46-84, f53-79, f61-69, f 84-89, f99-109, f99-110, f103-105, f106-113, f106-125, f108-113, f108-125, f128-139, f167-175, f170-176, f176-182, f183-193,f190-192, f193-204, f193-207, f193-208, f193-209, f194-207, f194-209, f195-209, f202-209, f203-208, f203-209, f208-221, f208-222, f210-221, f214-222. |
| $\alpha_{s1}$-casein | f1-7, f1-8, f1-9, f1-24, f3-7, f21-23, f23-34, f23-36, f24-36, f25-36, f26-32, f26-33, f29-32, f40-52, f40-53, f41-48, f41-49, f41-50, f41-51, f41-52, f41-53, f91-100, f91-102, f91-103, f91-105, f92-99, f94-100, f95-99, f99-101, f100-105, f143-149, f145-150, f145-151, f167-179, f194-199, f198-199, f207-214 |
| $\alpha_{s2}$-casein | f171-181, f171-184, f174-181, f174-182, f174-188, f182-197, f182-207, f189-197, f189-199, f189-207, f198-205, f198-207, f200-207, f207-212, f198-202, f198-203, f198-204, f201-206 |

[a](Christensen et al. 2003, Lemieux & Simard 1992, Nielsen et al. 2017, Rauh et al. 2014a, Singh et al. 2005); [b]Q value was calculated according to Ney (1971) in cal/moL.

For example, Button et al. (2011) demonstrated that spiking of UHT milk with 0.0003% (v/v) of *Pseudomonas* protease produced in milk with a cell count of ~$10^6$ cells/mL resulted in gelation after 5 months of storage. Several attempts were made to correlate bacterial proteinase activity with the shelf-life as determined by the onset of gelation. For example, Mitchell and Ewings (1985) determined that a level of proteinase of as little as 0.3 ng/mL can result in a shelf-life of 4 months in UHT milk, whereas Richardson and Newstead (1979) suggested a level of 1-2 ng/mL for a storage life of at least 3 months. While these levels are a useful guide, it must be emphasized that the threshold limits will vary with the activity and the substrate specificity of different proteases. The effect of proteolytic enzymes on gelation is listed in the Table 2.6. The gelation time can be obtained from the following equation:

$$Gelation\ time\ (months) = 2.3916 \times X^{-0.6449} \quad (\mathbf{8})$$

Wherein *X* is the proteinase activity measured by the Merck proteinase test kit using dehydrogenase as the substrate and is relating the activity to the equivalent concentration of alcalase R (Novo Industrials, Denmark) in ng/mL (Mitchell & Ewings 1985). However, calculating gelation time require to use the corresponding proteinase kit and the concentration of protease activity is measured based on alcalase activity, which may be

different from the BPs used in current project. It was shown that BPs lead to the formation of a custard like gel with a tighter protein network containing thicker strands and more intact casein micelles and micelle aggregates (Harwalkar 1992). de Koning et al. (1985) observed that BPs mediated UHT skim milk gel formation with a thread-like structure containing partly deformed casein micelles that had not been degraded by proteolysis. Similar observations were found with the experiment conducted in our lab using UHT full cream and skimmed milk spiked using minimal amount of bacterial proteases with no growth after ambient storage (data not shown).

There have been several attempts at elucidating the mechanism of age gelation in sterilised milk. It is probably a combination of physicochemical and proteolytic processes that plays some part in the mechanism of gelation (McKenna and Singh, 1991). Typically, gelation occurs with the gradual increase in viscosity during the storage of up to ~10 mPa s (Chavan et al. 2011). In contrast, some studies have observed that there is a decrease in viscosity (a thinning effect) (de Koning et al. 1985), possibly related to the excessive digestion of milk proteins into smaller peptides by the proteases of different specificities. In any case, gelation is an irreversible process, which generally renders the product unacceptable.

According to McMahon, (1996), gelation is a two-stage process, where the BPs cleave the peptide bonds of *κ*-casein, facilitating the dissociation of *βκ*-complexes (β-lactoglobulin–κ-casein-complex) from casein micelles and, secondly, aggregation of *βκ*-complexes and formation of a 3D network of cross-linked proteins (Figure 2.6). In contrast, another study proposed a three-stage mechanism. Herein, the first stage is similar to the two-stage mechanism and is followed by enzyme mediated plastein formation from smaller released peptides and subsequent aggregation of the plastein peptides by non-covalent bonds and possibly hydrophobic interactions (Chavan et al. 2011). Although plastein formation was previously established, the gels formed by these components appear to be reversible, which is opposite to the irreversible enzyme mediated age gelation. Factors affecting gelation include severity of the heat-treatment, additives and storage temperature (Chavan et al. 2011).

### 2.11.3 Biofilm formation and biofouling

Inadequate cleaning and disinfection of equipment that is used for collection, transportation and storage of refrigerated raw milk, as well as for processing, can lead to retained milk residues or organic matter forming milk films on stainless steel surfaces (Marchand et al. 2012).

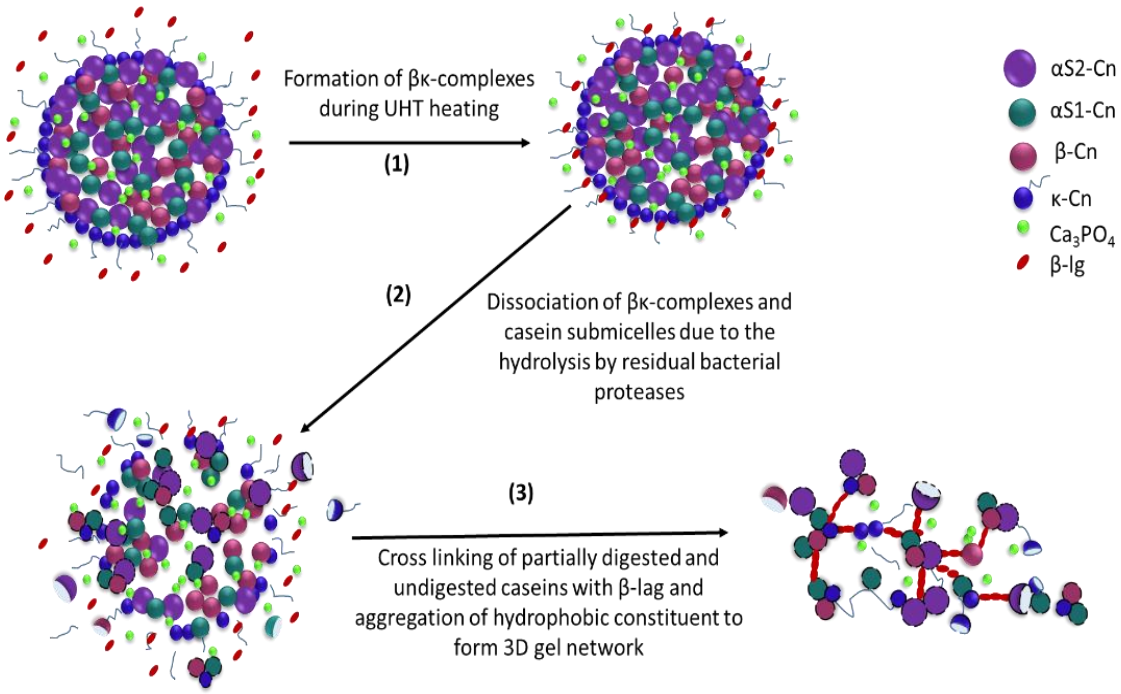


**Figure 2.6** Mechanism of age gelation of UHT milk due to bacterial proteases (based on the theory of McMahon (1996).

Milk films harbour a variety of biofilm-forming bacteria that can cause fouling (Oliveira et al. 2015). Biofilms are surface-adherent bacterial communities, which are aggregated in a matrix of self-secreted extracellular polymeric substance (EPS) that promote successful attachment and subsequent growth of microorganisms on a surface (Teh et al. 2014, Marchand et al. 2012, Toyofuku et al. 2012). Formation of biofilms comprises different stages, such as formation of a conditioning film, bacterial adhesion, bacterial proliferation, and biofilm expansion (Li & Tian 2012). In case of dairy processing, the conditioning film mainly consists of organic milk components that are attached to the equipment. This leads to alterations in the physicochemical properties of the surface, including surface free energy, hydrophobicity, and electrostatic charges, which may assist the subsequent attachment of

bacteria. The attachment of bacteria into the conditioning film is mediated by fimbriae, pili, flagella, and bacterial EPS. Biofilms can be composed of a single microbial species, but it is often a symbiotic relationship between multiple bacterial species (Oliveira et al. 2015). Gram-negative biofilm-forming bacteria that are isolated in dairy farming and processing environments include *Pseudomonas, Proteus, Enterobacter, Citrobacter, Shigella, Escherichia, Edwardsiella, Aeromonas, Plesiomonas, Moraxella, Alcaligene*s, and *Legionella* (Vlková et al. 2008, Marchand et al. 2012).

Biofilms can be formed in the cracks, corners, dead ends, gasket, valves and joints of the milking and processing equipment (Marchand et al. 2012). However, surfaces subjected to fouling are more prone to biofilm formation, in comparison to cleaned surfaces. Some bacteria such as *Pseudomonas, Aeromonas,* and *Legionella* spp. can be introduced to the dairy farm and processing environment through the rinse water used in the milking machines (Marchand et al. 2012). Similarly, bacteria present in refrigerated raw milk can enter into processing systems (Teh et al. 2014). Once established, biofilms are extremely difficult to eradicate using conventional cleaning and disinfection regimens. This is due to their various resistant phenotypes, while disinfectants are ineffective because they cannot fully penetrate the biofilm matrix (Vlková et al. 2008). For example, *Pseudomonas* spp. are able to alter their phenotypes via phase variation, thus ensuring their survival in the presence of stress such as temperature fluctuation and exposure to sanitizers during the cleaning of dairy processing and storage equipment (Marchand et al. 2012). Approximately $1 \times 10^8$ cfu/mL of bacteria can enter into milk via biofilms (Marques et al. 2007). Factors affecting the functionality of biofilms include the types of bacteria, their symbiotic interactions, pH, nutrient availability, quorum sensing molecules (e.g. N-Acyl homoserine lactones, AHL), the presence of organic and inorganic compounds and temperature (Oliveira et al. 2015). Interestingly, it was also observed that proteolytic activities of bacteria producing biofilms are higher than that of their planktonic counterparts (Teh et al. 2014). Particularly, the AHL quorum-sensing signals may regulate the production of proteolytic enzyme production and iron chelation during the spoilage of milk (Oliveira et al. 2015). Similarly, the production of

heat-stable proteolytic enzymes appears to be higher when biofilms contain multiple species compared to single species highlighting the direct relationship between biofilms and milk spoilage (Teh et al. 2012).

### 2.11.4 Post-processing contamination

The incidence of spore-forming bacilli in UHT milk has been infrequently reported in many countries around the world over past 50 years. For example, Gram-positive spore-formers including *B. cereus*, *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. coagulans*, *B. polymyxa*, *B. megaterium, B. sphaericus, Aneurinibacillus* spp*., Brevibacillus sp., Geobacillus* spp*., Paenibacillus* spp*., Ureibacillus sp.* and *Virgibacillus* spp*.* were isolated from UHT milk produced in Egypt, Belgium, Brazil, Sardinia, Africa, Algeria, China and Czech Republic (Bahout 2000, Cosentino et al. 1997, Ghellai & Moussaboudjemaa 2013, Lee 1984, Neumann et al. 2010, Pacheco-Sanchez & Massaguer 2007, Scheldeman et al. 2006, Tabit & Buys 2011, Vidal et al. 2016, Vyletelova et al. 2002, Zacarchenco et al. 2000). However, the level of these isolates in UHT milk was in the range of $10^2$-$10^3$ cfu/mL. Approximately, 37% of contaminated UHT milk samples from Italy had higher bacterial loads that were found to exceed $10^5$ cfu/mL (Montanari et al. 2004).

Despite these bacteria causing no significant spoilage, *B. sporothermodurans* is known to be problematic and is difficult to eradicate once established in the milk processing environment, thus causing sterility issues in UHT milk (Scheldeman et al. 2006). Other bacteria isolated from UHT milk include *Pseudomonas* spp., *H. alvei*, *R. aquatilis*, *S. aureus*, *Streptococcus* spp., *Lactobacillus* spp., *Shigella* spp., *Aeromonas* spp. and *Micrococcus* spp. (Chen et al. 2 011, Jensen et al. 2001, Lee 1984, Weiler et al. 2011). Food recalls notified to Food Safety Australia New Zealand (FSANZ) between 1990 and 2005 indicated contamination of flavoured UHT milk with coliforms, *Bacillus* spp. and *B. cereus* (FSANZ 2006). Even with lower initial counts in UHT milk products these psychrotrophic bacteria can exceed the regulatory limits during storage. Presence of these microorganisms in UHT milk is mainly associated with post-process contamination, especially during filling operations in the aseptic zone, as well as several other factors (Anderson et al. 2011, Boor & Murphy 2005, Chavan

et al. 2011, David et al. 1996, Hassan et al. 2009, Sarkar 2015, Samarzija et al. 2012). They include,

(i) Unsterile product due to the introduction of raw materials, adulteration, unsanitary handling, storage, or batching issues.

(ii) Higher microbial load in the raw material, where the heating regime is not adequate for complete elimination.

(iii) Improper cleaning and sanitation of the processing equipment, line turbulence, filling machines and inadequate preventive maintenance that together enable bacteriological seeding.

(iv) Pre-sterilisation issues that enable spore activation.

(v) Faulty heating, sterilisation and regeneration issues that allow the survival of high heat-resistant spores.

(vi) Defective cooling cycle including surge tanks that facilitate the accumulation of condensation.

(vii) Biofilm formation on the surfaces of filing machines, gaskets pipelines and other contact surfaces of the processing equipment.

(viii) Improper post-processing storage allowing for spore germination.

(ix) Loss of sterility due to the positive air pressure, system gasping and indexing operations, which leads to the formation of aerosols that can be contaminated by the environmental load.

(x) Loss of package integrity and poor sealing of containers.

Nevertheless, UHT processing and associated aseptic filling is often associated with a defect rate of 1/1000, but the industry standard is in the range of ≤ 1/1000 to ≤ 1/10000 (Chavan et al. 2011). Once UHT milk are being contaminated by above bacteria, bacteria can proliferate and produce exo enzymes and toxins that can cause spoilage (gelation and bitterness) and intoxication , making UHT milk undesirable for human consumption.

### 2.11.5 Food safety concerns

Milk-borne outbreaks are associated with consumption of milk and dairy products (Angulo et al. 2009, Champagne et al. 1994, De Buyser et al. 2001, Dhanashekar et al. 2012, Zeinhom & Abdel-Latef 2014).They can be sporadic and can occur in small clusters, or can be large-scale outbreaks that occur in families, schools, hospitals or in association with community gatherings. For example, in the United States, a study reported 46 milk-borne outbreaks between 1973 and 1992 (Headrick et al. 1998). About 68 milk-borne outbreaks were reported in the time period from 1993 to 2006 (Angulo et al. 2009). In England, about 233 milk-borne outbreaks were reported in 1951-1980 (Galbraith et al. 1982). It is an important public health concern, especially among very young, aged, infirm, or immuno-compromised individuals, since it can be the cause of morbidity and mortality (Angulo et al. 2009). Majority of milk-borne outbreaks are associated with the consumption of raw milk or pasteurised milk and dairy products (Newell et al. 2010).

There is little evidence for UHT-related milk-borne outbreaks. To our knowledge, there is no evidence for outbreaks of *Bacillus* spp. associated with UHT milk consumption. However, with the ability of these microbes to survive UHT heating via spore production and their high incidence in UHT milk, these bacteria may be the most important potential food safety concern in UHT processing. Additionally, many enzymes such as phoshpolipase C, beta-lactamases, proteases and collagenases are potential virulence factors of *B. cereus* (Kotiranta et al. 2000). These toxins are often produced at temperatures ranging from 6 to 25 °C, with bacterial counts in the range of $10^5$-$10^7$ cfu/mL (Griffiths 1990). Although, *B. cereus* is responsible for a large number of food-borne diseases around the world, only a few are known to be associated with milk and dairy products as the medium for transmission. *B. amyloliquefaciens* and *B. subtilis* are also known to produce a heat-stable cytotoxic component other than cereulide (de Jonghe et al. 2010). Although, food-borne intoxications are self-limiting diseases, in some cases the emetic toxin of *B. cereus s. l.* can cause severe and fatal consequences such as fulminant liver failure, septicaemia and rhabdomyolysis (Tomiyama et al. 1989).

Although the emetic toxins are often produced in starchy foods, the presence of these toxins in milk has also been reported (Agata et al. 2002). There are several case studies that have demonstrated intoxications with the diarrheic toxins due to the consumption of contaminated milk (Granum & Lund 1997). A lower incidence of outbreaks related to *Bacillus* spp. may be attributed to underreporting as a result of mild symptoms and misdiagnosis of the disease, thus confusing it with other food poisoning syndromes (intoxication of *S. aureus* and *C. perfringens*) (Anzueto 2014).

In addition to Gram-positive bacteria, the presence of some GNPs has been reported in UHT milk due to the post-processing contamination. They include several enteric isolates such as *H. alvei,* coliforms*, Shigella* and *Aeromonas* that are associated with gastroenteritis, septicaemia and urinary infections in humans (Chen et al. 2011).

## 2.12 Rapid Identification of Psychrotrophic Bacteria in Milk and Dairy Products

Accurate identification of bacterial isolates causing spoilage and food borne illnesses is one of the key functions of a dairy microbiology laboratory. Identification is conducted based on phenotypic (phenomics), metabolite (metabolomics), protein (proteomics), and genetic (genomics) characteristics of microorganisms.

### 2.12.1 Biochemical profiling of bacteria by API and Microbact

Microorganisms were originally identified on the basis of the phenotypic characteristics such as cell morphology, colony morphology, and biochemical characteristics (e.g. enzymatic characteristics, fermentation of carbohydrates and catabolism of proteins or amino acids) (Bochner 2009, Savage et al. 2017). The advantage of using these methods is that the characteristics can be easily observed and scored without expensive technology. However, they can be laborious and time consuming, and cannot normally be used for identification of some atypical stains of the same species. With the aid of computer databases that can rapidly and accurately identify the behaviours of a particular microorganism, system biologists have developed a number of standardized and miniaturised identification systems such as API and Microbact, which are based on the biochemical characteristics of

microorganisms (Savage et al. 2017, Vithanage et al. 2014). These commercial test strips contain dehydrated substrates that can be rehydrated using a solution which includes cells of a single microorganism, with colour change used as an indicator of a positive result (sometimes revealed by adding a reagent) after incubation. The positive colours are compiled to obtain a profile number that can be compared with the database either manually or with the help of semi-automated systems. This method has been used for identification of bacteria in milk (Munsch-Alatossava & Alatossava 2006). However, these systems, apart from being time consuming, also are technically demanding and require the knowledge of Gram status and other phenotypic characteristics prior to the selection of the appropriate test strip.

### 2.12.2 Metabolic profiling of bacteria by Biolog

In contrast, the Biolog system determines the metabolic activities of microorganism by their ability to utilise or oxidize 95 substrates in a 96-well microtitre plate. Each of these wells contains a redox dye and tetrazolium violet that changes to a purple colour due to respiration that occurs, when the cells begin oxidizing the specific carbon source (Holmes et al. 1994). Reactions are read after 4 hours and/or after an overnight incubation. The test results may be read either by eye and recorded manually or read with an automated plate reader. Thus, the metabolic profile of a bacterial sample can be compared with a database containing the metabolic information of a large number (> 2500) of taxa. However, this method was found to be associated with limited reproducibility for slow-growing microorganisms, when it was performed within 4.5 hours (Vithanage et al. 2014).

### 2.12.3 Proteomic profiling of bacteria using matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS categorises the bacteria based on the unique protein profile of the ribosomal proteins in the range of 2-20 kDa. Typically, a small amount of the cultured bacteria in solid or liquid media is transferred to a target plate and is air-dried for 5 min. The sample is then overlaid by an excessive amount of matrix solution (1,000-10,000 fold excess of organic acid, usually alpha-cyano-4-hydroxycinnamic acid; CHCA), forming small laser-

absorbing crystals after air drying (Seng et al. 2009). The matrix solution also contains a solvent (e.g. acetonitrile), which allows the co-crystallization of matrix and protein molecules upon evaporation (Seng et al. 2009). The target plates are transferred into a high vacuum system of the instrument and laser pulses are focussed on the sample for few nanoseconds (Reich et al. 2013). The laser pulses are absorbed by crystallised CHCA, leading to the ionization of bacterial proteins by addition or loss of protons (Singhal et al. 2015). The uptake of laser energy by those crystals causes excitation of matrix molecules (pressure pulse) leading to micro-explosions that result in desorption (release) of matrix and ionised bacterial molecules from the target plate (Reich et al. 2013). An electric field then accelerates the movement of the ionised bacterial proteins towards the detector (Reich et al. 2013). The speed of these ions in the electric field remains constant until they reach the detector, while the vacuum condition in the spectrometer prevents their collisions with the air molecules (Reich et al. 2013). The velocity of the ions depends only on the mass to charge ratio (*m/z*), where all the ions are singly charged by the MALDI process, hence the time-of-flight of the protein ions is solely dependent on their masses (Seng et al. 2009). This leads to the development of a spectrum, representing *m/z* ratio on the x-axis and intensity signifying the amount of ions on the y-axis, which can be compared with known spectra (Super spectra or reference spectra > 18,000) in the database to obtain species-level discrimination for unknown bacteria (Jadhav et al. 2015). This method is a rapid, reliable and cost-effective identification system with simple sample preparation protocols. However, ribosomal modifications that occur due to altered growth conditions can affect the species' identification (Anderson et al. 2012). Therefore, it is important to develop the reference spectra using highly conserved ribosomal proteins, which can be continuously expressed under any given condition (Unpublished data). MALDI-TOF MS has been used for identification of milk spoilage bacteria, although database limitations resulted in slightly lower identification accuracy than molecular methods (Savage et al. 2017, Vithanage et al. 2014).

### 2.12.4 Genomic profiling of bacteria based on 16S rRNA gene sequencing

Genomic-based identification systems utilise the unique nucleotide sequences in the rDNA genes, such as 16S rRNA, 23S rRNA and 16-23S internal transcribed spacer (ITS), present in the small and large ribosomal subunits of microorganisms (Janda & Abbott 2007). The rDNA transcripts have a low rate of polymorphism within species, which is why they are highly conserved in the bacteria of same genus or species, but usually differ among the microorganisms of other genera and species (Woo et al. 2008). Amplifying the gene by polymerase chain reaction (PCR) using a reliable primer, followed by purification and sequencing of the DNA products can reveal the sequence of the genes of bacteria of interest (Vithanage et al. 2014). Comparison of this sequence with quality controlled sequences available in the public (GenBank®, RDP-II and RIDOM) and commercial (MicroSeq and SmartGene) databases generally results in reliable identification of unknown bacteria (Savage et al. 2017, Vithanage et al. 2014). Thus, this method provides inter-specific comparison to elucidate phylogenetic relationships (Vithanage et al. 2014). To date, a large number of bacterial genera and species have been reclassified and renamed using 16S rRNA gene sequences. Similarly, this method also facilitates the classification of uncultivable bacteria and the discovery and classification of novel bacterial species (Woo et al. 2008). As a result of the increasing availability of PCR and DNA sequencing facilities and automated systems, the use of 16S rRNA gene sequencing has not remained limited to research applications but is also widely used for clinical diagnosis. However, there are no standardised guidelines for using the technique or interpret the results using sequence data (Woo et al. 2008). Misannotated data or limited availability of sequences for certain bacteria in the databases can affect the reliability of identification by this method (Vithanage et al. 2014).

### 2.13 Determination of the Proteolytic Activities in Milk and Dairy Products

Determination of the proteolytic activities in milk and dairy products would enable a greater understanding of the proteolytic activity and also help to better define the shelf-life of

products by early detection of proteolytic changes. This can help to minimise large-scale recalls, wastage and the resultant financial loss to commercial milk processors.

### 2.13.1 Agar diffusion assay

The proteolytic activity of bacteria is often determined on plate count agar supplemented with 1% skim milk (skim milk agar assay), optimised for the detection of protease activity by disc diffusion or well diffusion assays. The production of proteases can thus be readily determined by zones of clearance around the discs or wells (Hantsis-Zacharov & Halpern 2007, Vijayaraghavan & Vincent 2013).

### 2.13.2 Spectrophotometric assay

Protease activity was originally determined using a spectrophotometric assay that measured increases in the levels of tyrosine or tryptophan-containing peptides with the help of the Folin-Ciocalteau reagent. The rates of proteolysis were determined by calculating the regression of proteolysis over time (Hull 1947). The *o*-phthaldialdehyde (OPA) assay determines the release of α-amino groups due to the hydrolysis of casein, which forms a complex with *o*-phthaldialdehyde (OPA) in the presence of β-mercaptoethanol that strongly absorbs at 340 nm (Church et al. 1983). The OPA assay further optimises for the rapid determination of large scale samples using a microtitre plate assay coupled with a spectrofluorometer (Barba et al. 2013). Later, more sensitive spectrophotometric assays for protease activity were developed on the basis of the reaction of primary amino groups of trichloroacetic acid (TCA)-soluble peptides and amino acids, along with regents such as fluorescamine (4-phenylspiro [furan-2(3H), 1-phthalan]-3, 3-dione), trinitrobenzene sulfonic acid (TNBS) and OPA (Chism et al. 1979, Chove et al. 2013, Church et al. 1983, Humbert et al. 1990, McKellar 1981). Those assays were found to be linear in the range of 2 to 50 nmoL of amino groups per sample aliquot. The spectrophotometric assays are simple, rapid and thus suitable for routine analysis in a dairy processing environment; however, they are not able to detect relatively low levels of proteases that may occur in milk and dairy products. Additionally, since they lack specificity, proteases from bacterial or indigenous origin cannot be differentiated. Moreover, the sensitivity of the assay can be affected by the interference of

milk caseins, although addition of milk clarifying agents may actually increase the sensitivity of those assays (Saint-Denis et al. 2001).

### 2.13.3 Radiometric assay

Radiometric assays use radio-labelled substrates such as [$^{14}$C] N, N-dimethyl casein, $^{14}$C-methylated β-casein and determine the levels of TCA-soluble radioactive peptides (Drucker 1972, Donnelly et al. 1980). The method can detect the release of as little as 0.05 nmoL tyrosine/min/mL by BPs. Another assay based on radio-labelled casein (methyl-$^{14}$C) or azo-casein (Bastian & Brown 1996) measures the radioactive counts per minute using a charm analyser (Christen 1987). This method displayed $10^4$ times more sensitivity than that of the Hull method (Christen 1987). However, performing radiometric assays requires careful handling of the protocols and specialist instrumentation.

### 2.13.4 Bioluminescence assay

Bioluminescence-based protease assay methods are based on the luciferase-luciferin reaction. Protease in the sample digests the luciferase enzyme upon addition, after which, the amount of undigested luciferase is detected by adding of luciferin substrate in the presence of ATP. The amount of protease in the sample is inversely proportional to the amount of light emitted (Sutherland 1993). The time-dependant nature of the technique means that each sample has to be analysed individually. This makes the method is tedious and time-consuming. It was also observed that the assay was 100-fold less sensitive than the FITC-based flurometric assay (Sutherland, 1993).

### 2.13.5 Fluorescence assay

Twining (1984) developed an enzyme linked-fluorescent assay using a fluorescent casein substrate to determine the release of fluorescently labelled tyrosine in milk. The method directly determines the protease activity of the sample. This was further optimised by Sutherland et al. (1993), enabling the determination of the protease activity as low as 2-10 ng/mL. Both Sigma and Merck have developed different protease assay kits based on this principle and have claimed a minimum detectable limit of < 1 ng/mL. Similarly, The Merck-BIOQUANT® proteinase assay kit indirectly quantifies the BPs activity in UHT milk with a

minimum detection level of *2* mg/mL (standard alcalase R equivalent) equivalent to 0.05 u/mL, where one unit is the release of 1 mmoL fluorescein isothiocyanate per min at 40 ºC and pH 7.2 (Chen 2000). Therefore, the fluorimetric assay, using the FITC-casein substrates, currently remains the most sensitive assay method for the detection of proteinase activity causing gelation in UHT milk and dairy products.

### 2.13.6 Enzyme-linked immunosorbent (ELISA) assay

In the last decade, more sensitive ELISA assays have been developed for the detection of bacterial proteolytic activities in milk and dairy products (Table 2.7) (Birkeland et al. 1985, Chen et al. 2003, Clements et al. 1990, Gonzalez et al. 1993, Matta & Punji 2000). Direct determination of BPs by ELISA utilises monoclonal antibodies raised against purified proteases, thus the signal developed is proportional to the amount of protease in the test solution (Birkeland et al. 1985, Clements et al. 1990, Matta & Punji 2000). In contrast, inhibition ELISAs have been developed using antibodies raised against the hydrolytic product (e.g. caseinomacropeptide), specific peptide bonds ($Phe_{105}$-$Met_{106}$) or substrate (immunoglobulins), in which, the signal developed is inversely proportional to the amount of BPs (Abuknesha et al. 2010, Dupont et al. 2007, Picard et al. 1994). The major advantages of performing an ELISA are its rapidity, increased specificity and sensitivity, ability to concurrently test a large number of samples, and ability to remove the interference of milk proteins during the analysis. However, these methods cannot differentiate the active and inactive proteases, and are likely mislead one towards overestimation. Furthermore, the method can only detect structurally related proteases, thus detection of a broad range of potential proteolytic enzymes that occur in milk and dairy products may require the formation of a cocktail of antibodies that are developed against different proteolytic enzymes.

### 2.13.7 Reversed-phase high performance liquid chromatography (RP-HPLC)

The reversed-phase high performance liquid chromatography (RP-HPLC) method developed by Datta and Deeth (2003) was able to differentiate the peptides produced by BPs and plasmin, where 12% TCA-soluble peptides were those released by BPs, while peptides in the pH 4.6 filtrate were derived from either or both, BPs and plasmin. The results correlated

with the fluorescamine test. However, the method is difficult to establish for routine analysis due to its complexity and the challenge of quantifying protease activity, which in turn, can be attributed to the difficulties in finding suitable standards.

### 2.13.8 MALDI-TOF MS

MALDI-TOF MS was developed recently for the detection of protease activity by using a peptide-encoded microplate with different substrates (multiplex) and the measurement of the cleavage products by using mass spectrometry. The assay was sensitive for trypsin and chymotrypsin in the ranges of 5.0 to 500 and 10 to 500 nM, respectively (Hu et al. 2015). This assay is a rapid, reliable and cost-effective identification method; however detection of proteolytic activity of different proteases may require knowledge of substrate specificity of the protease of interest.

### 2.13.9 Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy has been used for the detection of proteolytic activities in the presence of different strains of *Yarrowia lipolytica* during the process of cheese ripening (Lucia et al. 2001). A study found that there was significant heterogeneity in Amide I and Amide II bands due to the proteolytic activity (Lucia et al. 2001). Ozen et al. (2003) used FTIR to determine the concentration and differentiation of plasmin and plasminogen in water and solutions containing sodium caseinate. The assay determined the plasmin activity in the range of 0.38 –1.8 mg/mL and claimed it to be rapid and simple, but quantitation of proteolytic activity may still be required for the preparation of an additional standard curve of known concentration of the protease of interest with substrate.

### 2.13.10 Zymography

Casein or gelatine zymography is an electrophoretic technique based on the SDS-PAGE containing protein substrate (casein or gelatine) copolymerized within the polyacrylamide gel matrix (Nicodeme et al. 2005, Stuknyte et al. 2016). Isoforms of proteases can be determined using a two-dimensional zymographic approach (2D-zymography) (Saitoh et al. 2007). The advantages of zymography include its simplicity and low-cost, as it does not require expensive materials (e.g. antibodies). Similarly, proteases with different molecular

weights showing activity towards the same substrate can be detected and quantified on a single gel (Vandooren et al. 2013). The molecular weights of proteolytic bands can be determined on the basis of molecular weight markers and the activity can be quantified using a standard curve of the protease of interest using a densitometric approach (Vandooren et al. 2013). However, zymographic techniques are time consuming, and may therefore be unsuitable for routine analysis (Vandooren et al. 2013).

**2.13.11 Molecular assay**

Most recently, molecular methods have been developed for the detection of proteolytic bacteria in milk based on the *aprx* gene (Dufour et al. 2008, Marchand et al. 2009). Machado et al. (2013) has developed a multiplex PCR assay to determine the different protease genes such as *aprx*, *apr*, *ser* genes, with a limit of detection of $10^2$ cfu/mL of *P. fluorescens,* when it is inoculated into sterilised whole milk (Machado et al. 2013). Similarly, Bach et al. (2001) developed a PCR assay to quantify different proteolytic bacteria in soil using a real time multiplex PCR assay, which may likely be more sensitive in the detection of proteolytic bacteria in milk. Genomic-based methods are specific and sensitive and exhibit a strong correlation with conventional methods. However, practical applications of those methods remain difficult since they are not rapid, require technical knowledge and are expensive. Methods for assaying the activities of bacterial proteinases in milk and dairy products are summarised in Table 2.7.

**2.13.12 Determination of Physico-chemical properties**

Alternatively, the extent of proteolysis can be measured by changing the physicochemical and functional properties in milk such as particle size, zeta-potential, casein hydration, viscosity, heat-coagulation time, phosphate stability, and sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) (Baglinière et al. 2012, Gaucher et al. 2011).

Overall, it is difficult to put forth comparative data on the concentration/activity of BPs in milk and dairy products due to the distinct differences in the assays and calculations. However, procedures based on these analyses can be proposed and optimised as diagnostic tests for

determining bacterial proteinase that are responsible for proteolysis in milk and dairy products.

## 2.14 Process Innovations for Extended Shelf-Life of UHT Milk and Dairy Products

In order to achieve a superior quality and an extended shelf-life, commercial milk processing primarily focuses on reducing or controlling the growth of psychrotrophs. Subjective attributes including taste, colour, odour, gelation, sedimentation, separation, viscosity and microbial growth can be affected by raw milk quality, pre-treatment processes, heating regimes, homogenization pressure, deaeration, post-process contamination, aseptic packaging, and package barriers.

### 2.14.1 Low temperature storage of raw milk

The utilisation of high-quality raw milk is of utmost importance in order to achieve an extended shelf-life of UHT milk and dairy products (Law et al. 1977, Murphy et al. 2016, Vithanage et al. 2016, Vithanage et al. 2017). Speck and Adams (1976) indicated that preventing the growth of psychrotrophic bacteria in raw milk would be difficult as well as expensive.

**Table 2.7** Methods of detecting protease activity in milk and dairy products.

| Method type | Principle | Reaction condition | Sensitivity | Reference |
|---|---|---|---|---|
| Agar diffusion assay using skim milk agar | Determination of the zones of clearance. | 37 °C for 24 h, and incubation at RT with Bromocresol green dye for 20-30 min | NS | (Hantsis-Zacharov & Halpern 2007, Vijayaraghavan & Vincent 2013) |
| Spectrophotometric | Increases the levels of tyrosine or tryptophan-containing peptides using Folin-Ciocalteau reagent. | 30 °C, 60 min, pH 7.5 | 8 nmoL/mL tyrosine in buffer | (Hull 1947) |
| | Determination of level of α-amino groups due to hydrolysis of casein in milk using OPA reagent. | 37 °C for 10-15 min, 340 nm | 10-11 mM α-amino acids | (Church et al. 1983) |
| | Determination of residual protease activity using OPA and casein by measuring the level of lysine. | 25 °C for 5 min, excitation 340 nm and emission 455 nm | 1 mg/mL lysine | (Barba et al. 2013) |
| | Reaction of bovine serum albumin (BSA) with N-α-benzoyl-DL-arginine-4-nitroanilide (BAPA). | 37 °C , pH 8.0, 1 h | 0.6 µmoL of TCA-soluble free amino group (7.9 x $10^7$ cfu/mL) | (Shamsuzzaman & McKellar 1987) |
| | Determination of level of α-amino groups due to hydrolysis of casein using OPA reagent. | 37 °C and 30 °C for 20 h. | 10-11 mM α-amino acids | (Church et al. 1983) |
| | Determination of primary amino groups with the reaction of trinitrobenzene sulphonic acid (TNBS). | 25 °C for 30 min, 420 nm | 20-200 µg/mL | (Chove et al. 2013) |
| | Release of primary amino group due to the reaction of protease with azo-casein. | 37 °C, 10 min, pH 8.3, 440 nm | 0.072-0.494 mg/mL | (Button et al. 2011) |
| Radiometric | Determination of the level of TCA soluble radioactive peptides using [$^{14}$C] N, N-dimethyl casein as a substrate. | 37 °C, pH 8,overnight | 0.400 pu/mL or 0-1 µg | (Drucker 1972) |
| | Reaction with radio-labelled casein (methyl-$^{14}$C) or azo-casein. | 30 °C, 20 min, pH 7.5 | 0.025 nmoL/mL tyrosine in buffer | (Bastian & Brown 1996, Christen 1987) |
| Fluorometric | Reaction with fluorescein-thiocarbamoyl-β-casein (FTC-β-CN). | 30 °C, 10 min, pH 7.5 | 0.005 nmoL/mL tyrosine in buffer | (Chen et al. 2004) |
| | Reaction with fluorescein isothiocyanate (FITC) to produce fluorescent primary amino acids. | 37 °C, pH 7.7-8.0, 1.5 h | 0.001-0.125 u/mL in buffer, raw milk | (Sutherland 1993) |
| Luminometry | Bioluminescent method with luciferase-luciferin activity. | 25 °C, 5 min | 0.125-0.25 u/mL in buffer, raw milk | (Němečková et al. 2009, Sutherland 1993) |
| Immunological (ELISA dot-blot) | Detects *Pseudomonas* AFT-36 protease antibody. | RT, 2.5 h | 1.0 ng/mL in buffer or milk | (Matta et al. 1997) |
| Immunological (ELISA) | Detects *P. fluorescens* (P1) protease using | 1 hr at 37 °C | $10^6$-$10^7$ cfu/mL or 0.25 | (Birkeland et al. 1985) |

| | | | | |
|---|---|---|---|---|
| | monoclonal antibody. | | ng/mL | |
| | Detects thermostable proteases of *Bacillus* species (B17) using polyclonal antibodies. | 37 °C for 6.5 h | 1.2 ng/mL of protease in milk or buffer | (Matta & Punji 2000) |
| Immunological (Inhibition ELISA) | Detects κ-casein degradation (CMP). | RT, 4 h | 0.1 µg/mL or < $10^7$ cfu/mL | (Picard et al. 1994) |
| | Detect $Phe_{105}$-$Met_{106}$ peptide bond of the κ-casein. | RT, 3 h | 15.3 ng/mL | (Dupont et al. 2007) |
| | Detects proteases of *P. fluorescens* (OM82, N73A, M143A, and OM186). | RT, 6 h | 0.24-7.8 ng/mL | (Clements et al. 1990) |
| | Detects protease activity of *B. licheniformis* using humans (IgA) and sheep (IgG) as universal substrates based on substrate specificity. | RT, 5-18 h | IgA: 10 µunits/test and IgG; $10^4$ µunits/test | (Abuknesha et al. 2010) |
| Casein zymography | Detects protease activity in an SDS-PAGE gel. | 7 °C, pH 8·8 for 30 min, | 3 µg of proteins | (Nicodeme et al. 2005) |
| MALDI-TOF MS | Detection of protease activity by using a peptide-encoded microplate. | RT, 15-20 min | 5.0-500 nM | (Hu et al. 2015). |
| FTIR | Detection of Amide I and Amide II bands in milk and dairy products. | RT, 15-20 min | 0.38 –1.8 mg/mL | (Lucia et al. 2001, Ozen et al. 2003) |
| RP-HPLC | RP-HPLC with UV detection for 6% TCA soluble peptide fraction. | 40 °C, 0.75 mL/min, 210 nm | NS | (Le et al. 2006, Lewis et al. 2014) |
| Molecular methods | Determination of the protease gene in milk and dairy products using specific primers by multiplex PCR assay. | 94 °C for 2 min, 35 cycles at 94 °C for 1 min, 55 °C for 40 s and 72 °C for 1 min, 72 °C for 7 min | $10^2$-$10^8$ cfu/mL | (Machado et al. 2013) |

NS: Not Specified. ELISA: enzyme linked immunosorbent assay; MALDI-TOF MS: matrix assisted laser desorption time of flight mass spectrometry; FTIR: fourier transform infrared spectroscopy; RP-HPLC: reversed phase high performance liquid chromatography; PCR: polymerase chain reaction.

However, it was found that growth of psychrotrophs in raw milk can be controlled by deep cooling of raw milk at 2 ºC or 4 ºC for a period ≤ 48 hours. However, it should be noted that the generation times of *P. fluorescens* and *E. aerogenes* at 0-2 ºC are 30.2 hours and 37.7 hours, respectively, and 16.8-25.9 hours at 4 ºC for *Y. enterocolitica*. Controlling temperature in the liquid phase is easier than in foam on the surface of milk, which tends to increase, and can accelerate the growth of psychrotrophs. Similarly, refrigeration of raw milk < 4 ºC is energy intensive and requires a large capital outlay at each stage of milk production including collection, transportation, processing and post processing.

### 2.14.2 Thermisation

Processing raw milk immediately after its reception is difficult in larger dairies, which is why it is stored in silos under refrigerated conditions for several hours or days (Giaccone et al. 2016, Sheehan 2007). This consequently facilitates the proliferation of psychrotrophs that can cause significant quality and sensory deterioration in the finished products due to the production of heat-stable enzymes (Sheehan 2007). Thus, raw milk is subjected to thermisation by heating with mild conditions at 63-65 °C for 15 s, followed by rapid cooling to ≤ 4 ºC (Giaccone et al. 2016). The heat treatment temporarily inhibits the growth of psychrotrophs, while the latter step prevents the growth of mesophilic spore-forming bacteria (Sheehan 2007). It was observed that the combination of heating to 65 °C for 15 s and storing at 2 °C was very effective in maintaining the quality of stored milk for several days (Griffiths et al. 1987, Stadhouders 1982). The most recent study conducted in our laboratory indicated significant reduction in the GNP counts and TDP counts by heating at 75 ºC for 15 s, subsequent cooling at 2 °C and storage at 4 °C, which resulted in significant increase in storage life (> 10 days) of raw milk (Vithanage et al. 2017).

### 2.14.3 Low-temperature inactivation (LTI) of BPs

Barach and others (1976) demonstrated that the heat-resistant enzymes present in milk could be inactivated by heat treatment at low temperature (55 ºC) for 30-60 min without affecting the flavour or protein content of the milk. This inactivation was shown to be effective irrespective of the protease concentration (Barach et al. 1976). The effectiveness of

this technique for enzyme inactivation is greater in milk, which has been already subjected to UHT treatment (after 1 day). However, the inactivation is significant for 30 min, where the extended time at this temperature resulted in a higher protease activity. However, some BPs were shown to be resistant to heat-treatment at 55 ºC for 60 min. Thus, it can be safely said that the feasibility of applying LTI for commercial scale is yet to be established.

### 2.14.4 Innovative steam injection

Innovative steam injection (ISI) is a novel steam injection technology that has been developed on the basis of the UHT heating principle, but with rapid heating (shorter than 0.2 s holding time) at high temperatures (150 to 180 ºC), compared to UHT technology (van Asselt et al. 2008). ISI involves pumping of the product through a narrow ended pipe (nozzle, 1-3 mm), which contains several cavities from which, high pressure steam is injected into a vacuum vessel in order to achieve rapid heating of the product (van Asselt et al. 2008). The heating is directly followed by flash cooling in a vacuum vessel. This method showed plasmin inactivation (≥ 99%) and six-decimal reduction of *B. sporothermodurans* with less product degradation (< 50% of β-lactoglobulin denaturation), extended shelf-life and consistency in the taste and freshness of milk, as compared to the typical UHT processing (van Asselt et al. 2008).

### 2.14.5 Membrane processing

Microfiltration (MF) serves as a viable alternative technology to traditional heat treatment. The technology was proven for its ability to reduce bacterial counts, while maintaining the organoleptic and sensory attributes in milk with negligible reduction in the total protein content (0.02 to 0.03%) (Kumar et al. 2013). It is primarily based on the fractionation of bacteria and spores (0.1-10.0 μm) using a semi-permeable membrane (0.2-2 μm, > 200 kDa), which depends on the particle size or molecular weight (Kumar et al. 2013). The liquid that passes through the membrane is referred to as ‘permeate’, while the liquid retained is known as “retentate” or “concentrate”. However, the efficiency of the membrane is largely associated with the pressure gradient (transmembrane pressure) across the membrane and the concentration gradient of the liquid (Kumar et al.2013). Te Giffel & van der Horst (2003)

reported that MF was able to reduce 99.1-99.9% of bacterial spores from milk. Schmidt et al. (2012) observed that MF induced decline in microbial loads (5-6 log10 units to < 1 cfu/mL). Thus, the MF can result in the extension of the shelf-life of milk by 12 to 45 days at 4 °C. The major drawback of membrane technology is its inability to remove all bacteria, which can cause membrane fouling and spore germination. These problems can be minimised by using cold assisted MF and microsieves (membrane with narrow pore sizes), however that may cause massive fouling (Kumar et al. 2013).

### 2.14.6 Pulsed electric fields

Pulsed electric field (PEF) processing is a promising alternative to conventional pasteurisation that can inactivate microbial cells and their enzymes using a combination of heat (< 50 ºC) and an electrical field in the form of very short (1 to 10 µs), high-voltage (20 to 50 kV/cm) pulses with specific energy inputs in the range 50 to 1000 kJ/kg (Sharma et al. 2014). Raw milk subjected to pre-heat treatment at 55 ºC and subsequent PEF (15.9-26.2 kV/cm for 17-101 µs) can result in the reduction of bacteria from 8.3 log cfu/mL to 2 log cfu/mL (Sharma et al. 2014). The inactivation of microorganisms with PEF treatments depends on bacterial cell structure and size, as well as the food related factors including product pH, water activity ($a_w$), soluble solids, and electrical conductivity. PEF treatment, in combination with heat, may enhance the inactivation of bacterial enzymes and plasmin in milk; however such a treatment requires more severe PEF treatment compared to bacterial inactivation. PEF-induced enzymatic inactivation was shown to be less effective in whole milk, as compared to skim milk and buffered solutions.

### 2.14.7 Ultrasound

Ultrasound technology involves transmission of a sound wave with higher frequencies (20 kHz to 10 MHz) than audible frequency (e.g. 20 kHz). The applications of ultrasound in the food industry are divided into two distinct categories according to the energy generated by the sound field, namely low energy (intensity: < 1 W/cm$^2$; frequency: > 100 kHz) and high energy (intensity: >1: 10-1000 W/cm$^2$; frequency: 20-100 kHz). Low energy ultrasound uses a low power level, which is why it is non-destructive and does not cause any changes in the

physical or chemical properties of food (Abdullah & Chin 2014, Ercan & Soysal 2013). In contrast, high energy ultrasound involves physical or mechanical disruption that accelerates certain chemical reactions. Thus, it is used to generate emulsions, homogenise milk, disrupt cells, disperse aggregations, modify and control of crystallization processes, degas liquid foods, inactivate enzymes and microbial, enhance drying and filtration and induce oxidation reactions (Abdullah & Chin 2014, Ercan & Soysal 2013).

The phenomenon involves formation, growth and explosion of acoustic cavities (micro gas bubbles) in liquids to produce localized hot spots with temperature exceeding 5500 °C and pressures of up to 50 MPa that can cause cellular disruption and free radical formation (Knorr et al. 2004).

### 2.14.8 High-pressure treatments

High-pressure processing involves minimal processing, which results in the production of safe and nutritious foods (Ghalavand et al. 2015, Hayes et al. 2005). Microfluidisation is based on high pressure processing, where fluid is divided into two channels at the inlet, and subsequent collisions of liquid in the reaction chamber using high-speed liquid jets (Dumay et al. 2013, Pereda et al. 2007). In contrast, the high pressure homogenisation (HPH) operates on a principle similar to that of the conventional homogenisation (20-60 MPa) with higher pressures (150-200 MPa) (Dumay et al. 2013). Ultra-high pressure homogenization (UHPH) is based on the same principle with pressures between 350-400 MPa (Dumay et al. 2013). High pressure processing applied to the dairy industry results in greater reduction of the microbial counts, inactivation of microbial and indigenous enzymes (lactoperoxidase, plasmin, and alkaline phosphatase) and reduction of fat globule size that reduces the creaming rate, consequently improving the shelf-life of milk and dairy products (Datta et al. 2005, Hayes & Kelly 2003, Pereda et al. 2007). UHPH resulted in 14-21 days of shelf-life in milk, as a result of microbial inactivation (Chavan et al. 2011). Greater microbial inactivation is achieved by increasing the pressure, inlet temperature, and number of passes through the machine (Pereda et al. 2007). High pressure processing can also result in changes in the in the physicochemical properties of milk including colour, viscosity, pH, and acidity, texture,

and mouth-feel that are considered as important factors affecting the consumer's perception about the freshness of milk (Pereda et al. 2007).

### 2.14.9 UV treatment

UV light is an electromagnetic radiation with wavelength in the range of 100 to 400 nm (Koutchma et al. 2016). UV treatment involves the use of radiation with the help of a monochromatic light from low-pressure mercury (LPM) lamps and polychromatic light from medium-pressure mercury (MPM) lamps. There are three different wavelengths in the UV range that are commonly used: UV-A at 320-400 nm, UV-B at 280-320 nm, and UV-C of 200-280 nm (Koutchma et al. 2016). Particularly, UV-C light is known to contain germicidal activity against a wide range of microorganisms (Koutchma et al. 2016). Thus, UV-C (at approximately 253.7 nm) is being used as a non-thermal technology for the disinfection of surfaces, water, and various liquid food products (Koutchma et al. 2016). Advantages of using UV radiation for milk processing include: no changes in the milk proteins, no significant amount of toxic by-products, removal of certain organic contaminants, maintenance of the aroma, taste, and colour, no off-flavour or off-odour production, less energy consumption in comparison to thermal processing (Cappozzo et al. 2015, Koutchma et al. 2016). However, the antibacterial effectiveness of UV radiation is dependent on direct microbial exposure, thus microbial inactivation may be reduced in milk with opaque background and a higher absorption coefficient, as well as, when treating food with irregular surfaces (Koutchma et al. 2016, Pereira et al. 2014).

### 2.14.10 Active packaging

Active antimicrobial packaging modifies the internal environment of the package by constant interaction with the food over the specified shelf-life, which alters the state of the packaged food system and its headspace (Malhotra et al. 2015). Apart from better quality and an extended shelf-life, this also leads to an enhancement of sensory qualities and maintenance of microbial safety (Malhotra et al. 2015). In the technique, packaging materials with different functionalities actively interact with the food product. For example, some of the materials are involved in absorption of moisture and ethylene, scavenging of $O_2$ or $CO_2$, $CO_2$ or ethanol

emitting systems, and the release of antimicrobials or antioxidants (Brockgreitens & Abbas 2016, Malhotra et al. 2015). Packing materials such as ascorbic acid, photo-sensitive dyes and iron powder are able to scavenge oxygen from food, thus preventing the growth of aerobic microorganisms. Active packaging of UHT skim milk results in the reduction of microbial growth and enzymatic (protease and lipase) activities within 14 days of storage, as compared to conventional packaging (Wong & Goddard 2014). Nano composites containing various antimicrobial agents in food packaging films have shown enhanced thermal, physicochemical, mechanical, and optical properties of food products (Malhotra et al. 2015). In addition, some materials can change the organoleptic properties in food (colour, texture and appearance of food) as a result of the release of antimicrobials like essential oils (Malhotra et al. 2015). Thus, interactions between the packing material and the food need to be studied further to improve this technology.

**2.14.11 Additives**

Incorporation of additives into raw milk to complement refrigeration should not affect the organoleptic properties or the cost of the milk products. The addition of $CO_2$ (20-30 mM) result in production of $HCO_3^-$ ions, which possesses antimicrobial properties (Sarkar 2015). Once it's being produced $HCO_3^-$ can be easily pumped off under vacuum after storage (Sarkar 2015). Therefore it is a promising strategy for inactivation bacteria in raw milk without developing off-flavour (Sarkar 2015). This method produced a significant reduction in the microbial count ($10^3$ cfu/mL) at 7 ºC for 144 h, although the sensory characteristics of the milk were affected due to acidification (with a reduction of pH from 6.59-6.7 to 6.2). However, addition of $N_2$ (40-120 mL/min) resulted in halting the growth of psychrotrophic proteolytic bacteria at 6 °C for 11 days with no significant alterations in the sensory properties (pH 6.6 to 7.0) (Sarkar 2015). Flushing of $N_2$ through the headspace of the milk-containing vessel is an economical strategy that can lead to reduction in the microbial count (Munsch-Alatossava et al. 2010). Furthermore, addition of $H_2O_2$ (8 ppm) with $SCN^-$ (12 ppm) can result in activation of the lactoperoxidase system in milk that can cause leakage of amino acids and $K^+$, leading to cell lysis (Champagne et al. 1994, Kriščiunaite et al. 2011). Similarly,

introduction of citrate-utilizing lactic acid bacteria (LAB) like *Lactobacillus lactis* ($10^8$ cells/mL) showed a bactericidal effect in milk, possibly due to the production of $H_2O_2$, bacteriocin or modulation of lactoferrin activity (viz. due to the citrate assimilation) in milk (Champagne et al. 1994). However, seeding with LAB cultures is unlikely to be effective in raw milk with poor bacteriological quality; on the other hand, seeding with bacterial counts as high as $10^8$ cells/mL of LAB can also result in reduction of pH from 6.2-5.3 in 7 days. Addition of a sorbate/propionate mixture (1000 ppm each) with diacetyl (50 ppm) showed reduction in the microbial counts. Incorporation of iron binding protein, viz. lactoferrin, into milk at 2.67 mg/mL was effective at inhibiting certain GNPs tested as individual cultures (Kutila et al. 2003); however a preliminary study conducted in our laboratory indicated the requirement of a slightly higher amount of lactoferrin (10.5 mg/mL) to inhibit psychrotrophs in a complex microbial mixture (Unpublished data).

Alternatively, the stability of the UHT milk can be significantly increased by using various additives such as sulfhydryl (SH) group-blocking agents (N-ethylmaleimide: NEM), protease inhibitors, sodium hexametaphosphate (SHMP) and calcium chloride (Chavan et al. 2011). Particularly, NEM inhibits denaturation of whey protein, while protease inhibitors can inactivate the residual proteases in UHT milk. Interestingly, the addition of calcium chloride (0.05%) or SHMP (0.1%) to milk, prior to the UHT processing, significantly increases the stability and shows no gelation even after 500 days at 25 ºC (Chavan et al. 2011). These agents are known to facilitate the bridging between ionized groups of casein, holding the *κ*-casein more tightly to the micelle, which may delay release of the *βκ*-complex due to proteolytic activity, thus hindering the gelation of UHT milk during storage (Chavan et al. 2011).

## 2.15 Conclusion

Overall, the quality and shelf-life of UHT milk and dairy products is often compromised by sensory and functional defects that emerge due to the heat-stable proteolytic activity derived from contaminating Gram-negative and Gram-positive psychrotrophic bacteria disseminated

from various sources in the farming and processing environments. The extent of diversity of these bacteria is associated with storage temperature, time and seasonality. Detection and controlling the proteolytic activity in raw milk can substantially improve the quality of UHT milk with concomitant increase in processing efficiency. Overall, this would help increase the shelf-life of the UHT-treated products, which may, in turn, facilitate the transportation of these products to far geographical regions, thus benefitting the commercial UHT milk processing.

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# CHAPTER 3: ISOLATION AND CHARACTERISATION OF PROTEOLYTIC BACTERIA FROM RAW MILK

## 3.1 Overview of Chapter

Chapter 3 presents the isolation and characterisation of psychrotrophic proteolytic bacteria in raw milk. The psychrotrophic bacteria were isolated from raw milk samples, followed by the preliminary characterisation of their extracellular proteolytic activity at refrigerated storage and heat-stability of the enzymes after 150 ºC for 20 s heating. Finally, the isolates were identified using a polyphasic approach comprising API, Microbact, Biolog, MALDI-TOF MS and 16S rRNA gene sequencing. The article entitled “Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk” by Nuwan R. Vithanage, Thomas R. Yeager, Snehal R. Jadhav, Enzo A. Palombo, and Nivedita Datta has been published in the peer-reviewed journal “International Journal of Food Microbiology” (2014), 189: 26–38. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.07.023.

## Note from the examination process of this PhD thesis:

*Bacillus cereus* considered in the present study belonged to the *B. cereus sensu lato* species complex including *B. cereus, B. thuringiensis, B. mycoides, B. weihenstephanensis,* and *B. pseudomycoides*, which are ecologically diverse bacterial group of medical and agricultural significance (Bartoszewicz & Marjańska, 2017, Okinaka & Keim, 2016).

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## GRADUATE RESEARCH CENTRE

### DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

*This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.*

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**College of Health and Biomedicine, Victoria University, Werribee Campus, Melbourne VIC, Australia**

| Name(s) of Co-Author(s) | Contribution (%) | Nature of Contribution | Signature | Date |
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| Nuwan R. Vithanage | 85% | Concept Development, research question, hypothesis, data analysis manuscript writing and submission to journals. | [redacted] | 15-Feb-2017 |
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| Snehal R. Jadhav | 2% | Concept Development, research question, hypothesis and manuscript preparation. | | 28-Feb-2017 |
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# Comparison of identification systems for psychrotrophic bacteria isolated from raw bovine milk

Nuwan R. Vithanage [a], Thomas R. Yeager [b,d], Snehal R. Jadhav [c], Enzo A. Palombo [c], Nivedita Datta [a,d,*]

[a] College of Health and Biomedicine, Victoria University, Australia
[b] College of Engineering & Science, Victoria University, Australia
[c] Faculty of Science, Engineering and Technology, Swinburne University of Technology, Australia
[d] Institute for Sustainability and Innovation, Victoria University, Australia

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ABSTRACT

Psychrotrophic bacteria in raw milk produce heat-resistant extracellular proteases, resulting in spoilage and shelf-life reduction of ultrahigh temperature treated milk and milk products. Controlling of these spoilage microbes requires rapid and reliable identification systems for screening of raw milk. This study aimed to compare commercial bacterial identification systems with a genetic method (considered as the 'gold standard' method) for the identification of heat-resistant protease producing bacteria in raw milk. Five bacterial identification systems were compared based on typability, discrimination power (i.e. Simpson's Index of Diversity), reproducibility and speed of analysis. The accuracy of 16S rRNA gene sequencing, Biolog, MALDI-TOF MS, API, and Microbact for the identification of Gram negative bacilli at the species level was 100.0%, 86.8%, 63.2%, 60.5% and 57.9%, respectively. The Gram positive bacilli were identified by 16S rRNA gene sequencing, Biolog, MALDI-TOF MS, and API with accuracies at the species level of 100.0%, 85.0%, 95.0% and 90.0%, respectively. The 16S rRNA gene sequencing and phylogenetic analysis discriminated *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Hafnia alvei*, *Bacillus cereus*, *Bacillus pumilus* and *Bacillus licheniformis* to the subspecies level. The Simpson's Index of Diversity scores were 0.966, 0.711, 0.496, 0.472, and 0.140, for 16S rRNA gene sequencing, Biolog, MALDI-TOF MS, API and Microbact, respectively. Limited reference profiles in the databases of Biolog, MALDI-TOF MS, API and Microbact systems reduced their accuracy in bacterial identification, compared to 16S rRNA gene sequencing. The rapidity of each assay is in the following order; MALDI-TOF MS > 16S rRNA gene sequencing > Biolog > Microbact > API. The reproducibility of the assays is in the order of 16S rRNA gene sequencing > API > Microbact > MALDI-TOF MS > Biolog. Thus, 16S rRNA gene sequencing appears to be the most reliable and robust system for the identification of dairy spoilage bacteria. The Biolog system is suitable for the identification of Gram negative spoilage bacteria, while MALDI-TOF MS and API systems are suitable for the identification of Gram positive spoilage bacteria isolated from raw milk. The commercial systems used in this study have been developed and extensively used for the identification of clinical microbes but only a limited number of studies used those systems to identify the environmental microorganisms that often contaminate raw milk. Therefore, comparison of those systems for the identification of spoilage microbes in raw milk would provide better understanding of their suitability for routine dairy microbiology and more extensive dairy research.



## 1. Introduction

Identification of pathogenic and spoilage microorganisms by rapid and reliable methods is a fundamental aspect of dairy microbiology. Psychrotrophic bacteria are considered as the major concern in ultrahigh temperature (UHT) processing, as they can grow under refrigeration conditions, regardless of their optimal growth temperature (Champagne et al., 1994). Psychrotrophs comprise a large heterogeneous group of Gram negative and Gram positive genera (Champagne et al., 1994). Among those problematic microbiota, *Pseudomonas* (particularly *Pseudomonas fluorescens*) and *Bacillus* are the leading causes of spoilage of milk and dairy products, and the most frequent isolates

*Abbreviations:* ATCC, American Type Culture Collection; BPs, bacterial proteases; CHCA, α-cyano-4 hydroxycinnamic acid; GNB, Gram negative bacilli; GPB, Gram-positive bacilli; MALDI-TOF MS, Matrix assisted laser desorption ionisation time of flight mass spectrometry; MCL, Maximum Composite Likelihood; MB 24E, Microbact; NF-GNB, Non-fermenter Gram-negative bacilli; qPCR, quantitative real time PCR; SIM, similarity index; SID, Simpson's Index of Diversity; SNP, Single Nucleotide Polymorphism; UHT, ultrahigh temperature.

* Corresponding author at: College of Health and Biomedicine, Victoria University, P.O. Box 14428, Melbourne, Victoria 8001, Australia. Tel.:+61 3 9919 8124.
*E-mail address:* Nivedita.Datta@vu.edu.au (N. Datta).

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from milk and dairy products at the time of spoilage despite comprising less than 10.0% of the original raw milk microbiota (Champagne et al., 1994). They are ubiquitous in nature and can be disseminated into raw milk via soil, water and vegetation at dairy farms (Vissers and Driehuis, 2009).

Psychrotrophs produce extracellular proteases and lipases that are resistant to UHT heating regimes (Sørhaug and Stepaniak, 1997) and may lead to a range of quality defects such as bitterness, increased viscosity, gel formation and rancidity in UHT milk during ambient storage (Datta and Deeth, 2001). In particular, the bacterial proteases (BPs) are the most frequent causes for UHT milk spoilage (Button et al., 2011) that can cause gelation even with $6.0 \times 10^{-2}$ U/mL, within 3 months of storage at 23 °C (Mitchell and Ewings, 1985). The spoilage of UHT-treated products can reduce the shelf-life, resulting in negative brand image and significant financial loss for commercial milk processors.

The quality of the raw milk is often predicted based on higher counts of bacteria, including psychrotrophs and thermophilic microbes (Cempíriková, 2002). Furthermore, Dogan and Boor (2003) observed variations in proteolytic and lipolytic activities even within the *P. fluorescens* population. Thus, the number and diversity of heat-stable protease producing bacteria in raw milk would be an important limiting factor of shelf-life of UHT milk.

Many species of psychrotrophs are difficult to identify rapidly by conventional biochemical based approaches which involve time-consuming and labour-intensive cultivation procedures. The most widely used commercial phenotypic methods for the identification of bacteria are the API (BioMérieux, Marcy l'Etoile, France), Microbact (MB 24E) (OXOID GmbH; Wesel, Germany) and Biolog systems (Biolog, Inc., Hayward, CA, USA). Both API and Microbact systems identify bacteria based on fermentation of sugars (carbohydrates), assimilation of certain other carbon sources, and production of certain unique metabolites and enzymes (Bosshard et al., 2006; Ling et al., 1988). The Biolog identification system is based on the exchange of electrons in microbial respiration (the ability of bacteria to oxidise the 95 substrates). The rate of respiration is determined by a colour reaction using a redox dye (tetrazolium violet-based colour reaction) which is subsequently compared with metabolic fingerprints of known bacteria in the Biolog database (Bochner, 2009).

These systems have contributed to the rapid identification and characterisation of bacteria for 30 years; however, all phenotypic systems have potential limitations, such as difficulty in determining phenotypic variation among strains, lower reproducibility, and the limited entries in the corresponding databases (Tshikhudo et al., 2013). Particularly, non-fermentative microorganisms can pose problems due to their phenotypic variations and slower growth rates (Bosshard et al., 2006).

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) identifies bacteria using a specific mass spectrum, which is generated by bacterial proteins, and subsequent comparison of the mass spectrum [mass-to-charge (m/z) ratio vs. intensity] of the unknown strain with a spectral library of reference strains, where species are assigned based on the highest percentage similarity to the reference spectral profiles (Seng et al., 2009). Two MALDI-TOF MS systems are commercially available; VITEC MS (BioMérieux, Marcy l'Etoile, France) and MALDI biotyper CA system (Bruker Daltonics, Billerica, MA). Those systems have been successfully used for the identification of bacteria in clinical microbiology and have a number of advantages in comparison to conventional and molecular methods, such as speed, cost-effectiveness and efficiency; however with limitations related to limited database facilities and determining cell viability (Seng et al., 2009).

Molecular methods have been applied for routine identification of microbes and measuring their relative abundance, diversity and phylogeny (Janda and Abbott, 2007). 16S rRNA gene sequencing is the most widely accepted identification system, which determines species or subspecies level identification due to the presence of divergent regions in the 16S rRNA gene among various bacteria, while conserved regions allow bacteria to be categorised within the same species (Janda and Abbott, 2007).

The molecular methods show higher sensitivity than phenotypic systems; however, they have certain limitations such as determining cell viability, misannotated data in the public databases, complex protocols with numerous sample preparation steps, the need for trained personnel, cost and the requirement for specialised equipment. Therefore, several studies have used the automated RiboPrinter microbial characterisation system (Qualicon Inc., Wilmington, Del) which has been developed based on the differences in 16S rRNA genes in various bacteria, but automates all the steps from cell lysis to image processing and provides identification within 8 h (Wiedmann et al., 2000).

The present study focused on comparison of five identification systems, API, Microbact, Biolog, MALDI-TOF MS and 16S rRNA gene sequencing, for the identification of spoilage bacteria isolated from raw milk. The main objective was to highlight the advantages and limitations of each system for bacterial species identification.

## 2. Materials and methods

### 2.1. Isolation of bacteria

Seven raw milk samples with high Bactoscan counts (495,000–> 600,000) were supplied by a commercial UHT milk processor in Victoria, Australia. Each of the raw milk samples was serially diluted using 10-fold volumes of 0.1% (w/v) sterile peptone water (Sigma-Aldrich, Castle Hill, Australia) and cultured (0.1 mL aliquot) on *Pseudomonas* agar base with CFC supplement (CFC; Cetrimide: 0.01 mg/mL, Fucidin: 0.01 mg/mL, Cephalosporin: 0.05 mg/mL; Oxoid, UK) (Ercolini et al., 2009), and Brilliance™ *Bacillus cereus* agar with polymyxin B supplement (BBC agar; Oxoid, UK) (Nemeckova et al., 2011), using the spread plate technique, in triplicate. The plates were incubated at 4 °C for 10 days to establish the pre-processing storage condition of raw milk used by commercial milk processors in Australia.

Bacteria with distinct colony morphologies were selected. The cell morphology was determined microscopically after Gram-stain preparation. The phenotypic characteristics were determined using tests including catalase (3.0% (w/v) $H_2O_2$), oxidase (1.0% tetramethyl *p*-phenylenediamine dihydrochloride), spore stain and growth on MacConkey agar (Smibert and Krieg, 1984).

The isolates were sub-cultured on the same medium until pure cultures were achieved. The isolated bacteria were grown in nutrient broth (Sigma-Aldrich, Castle Hill, Australia) and were then stored in 30.0% (v/v) glycerol at −80 °C and used as stock cultures in subsequent analysis.

American Type Culture Collection (ATCC) cultures including *P. fluorescens* (ATCC 13525, 17386), *B. cereus* (ATCC 10876, 10987, 14579), *Bacillus licheniformis* (ATCC 14580) and *Bacillus subtilis* (ATCC 6633) were used as positive controls.

### 2.2. Screening of thermo-resistant proteases

The extracellular protease activity of all Gram negative bacilli (GNB) and Gram positive bacilli (GPB) isolated from raw milk and ATCC cultures ($n = 167$) was determined using 1.0% (w/v) skim milk agar (Oxoid, Basingstoke, England); the appearance of clearing zones around the colonies after 10 days at 4 °C was indicative of proteolysis (Hantsis-Zacharov and Halpern, 2007). Bacteria with significant extracellular proteases activity ($n = 75$) were further screened for thermo-resistant protease activity.

Overnight cultures (0.5 mL) of the protease producing GNB and GPB were grown in 50.0 mL commercial UHT skim milk (in 250.0 mL Erlenmeyer flasks), on an orbital shaker at 120 rpm, for 72 h at 26 °C and 48 h at 30 °C, respectively. The bacterial cultures were centrifuged (Model Avanti J-26S XPI, Beckman Coulter) at 15,000 ×*g* for 15 min at 4 °C, and the cell-free supernatant containing crude BPs were used to screen

**Table 1**
An overview of identified bacterial strains using API, Microbact, Biolog, MALDI-TOF MS and 16S rRNA gene sequencing.

| Strain[a] | 16S rRNA gene sequencing | C (%)[b] | API | C (%)[b] | Microbact 24E | C (%)[b] | Biolog | SIM[c] | MALDI-TOF MS | C (%)[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| *P. fluorescens isolates* | | | | | | | | | | |
| P1 | *P. fluorescens* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 98.0 | *P. fluorescens* | 0.624 | *P. fluorescens* | 89.6 |
| P2 | *P. lurida* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 98.0 | *P. marginalis* | 0.734 | *P. fluorescens* | 87.5 |
| P3 | *P. fluorescens* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 87.9 | *P. fluorescens* | 0.660 | *Pseudomonas* sp. | 99.9 |
| P6 | *P. gessardii* | 99.0 | *P. fluorescens* | 96.9 | *P. aeruginosa* | 98.2 | *P. fluorescens* biotype C | 0.599 | *Pseudomonas* sp. | 77.1 |
| P14 | *P. proteolytica* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 89.1 | *P. fluorescens* | 0.578 | *P. fluorescens* | 80.7 |
| P20 | *P. gingeri* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 88.1 | *P. fluorescens* | 0.607 | *Pseudomonas* sp. | 99.9 |
| P23 | *P. gessardii* | 99.0 | *P. fluorescens* | 96.9 | *P. aeruginosa* | 58.3 | *P. fluorescens* biotype C | 0.646 | *P. fluorescens* | 70.8 |
| P24 | *P. poae* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 99.7 | *P. tolaasii* | 0.570 | *P. fluorescens* | 76.5 |
| P25 | *P. veronii* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 92.3 | *P. fluorescens* | 0.703 | *P. fluorescens* (*P. veronii*)[d] | 96.0<br>45.0 |
| P27 | *P. poae* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 99.7 | *P. marginalis* | 0.741 | *P. fluorescens* | 76.5 |
| P29 | *P. poae* | 100.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 99.7 | *P. marginalis* | 0.726 | *P. fluorescens* | 75.5 |
| P32 | *P. gessardii* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 98.5 | *P. fluorescens* biotype C | 0.617 | No ID[e] (*P. grimontii*)[d] | 0.0 (45.6) |
| P33 | *P. salomonii* | 99.0 | *P. fluorescens* | 99.9 | *B. pseudomellei* | 99.9 | *P. tolaasii* | 0.651 | *P. fluorescens* | 81.2 |
| ATCC 13525 | *P. fluorescens* | 99.0 | *P. fluorescens* | 99.9 | *P. fluorescens* | 99.6 | *P. fluorescens* | 0.674 | *P. fluorescens* | 95.9 |
| ATCC 17386 | *P. fluorescens* | 98.0 | *P. fluorescens* | 99.8 | *P. fluorescens* | 96.1 | *P. fluorescens biotype G* | 0.682 | *P. fluorescens* | 90.9 |
| *Non-fluorescent pseudomonas isolates* | | | | | | | | | | |
| P9 | *P. syringae* pv *syringae* | 99.0 | *P. fluorescens* | 92.9 | *B. cepacia* | 93.6 | *P. fluorescens* | 0.734 | *P. fluorescens* | 70.6 |
| P10 | *P. psychrophila* | 98.0 | *P. fluorescens* | 92.5 | *P. fluorescens* | 67.1 | *P. fluorescens* | 0.538 | *P. fluorescens* | 74.6 |
| P17 | *P. psychrophila* | 98.0 | *P. fluorescens* | 92.6 | *P. fluorescens* | 89.0 | *P. fluorescens* | 0.719 | *Pseudomonas* sp. (*P. grimontii*)[d] | 99.2 (48.6) |
| P40 | *P. fragi* | 95.0 | *P. fluorescens* | 95.8 | *P. fluorescens* | 96.0 | *P. fragi* | 0.514 | No ID[e] | 0.0 |
| P43 | *P. syringae* pv *syringae* | 96.0 | *P. fluorescens* | 95.2 | *P. fluorescens* | 95.5 | *P. fragi* | 0.734 | No ID[e] (*P. fluorescens*)[d] | 0.0 (41.6) |
| P45 | *P. fragi* | 98.0 | *P. fluorescens* | 71.0 | *P. fluorescens* | 98.1 | *P. lundensis* | 0.719 | No ID[e] | 0 |
| P46 | *P. lundensis* | 98.0 | *P. putida* | 95.4 | *P. fluorescens* | 96.7 | *P. lundensis* | 0.560 | No ID[e] | 0 |
| P47 | *P. syringae* pv *phaseiolocola* | 98.0 | *P. putida* | 99.1 | *P. fluorescens* | 71.8 | *P. fragi* | 0.591 | No ID[e] (*P. fluorescens*)[d] | 0.0 (43.6) |
| P48 | *P. lundensis* | 98.0 | *P. putida* | 94.3 | *P. fluorescens* | 99.7 | *P. lundensis* | 0.660 | No ID[e] | 0 |
| P49 | *P. fragi* | 98.0 | *P. fluorescens* | 61.9 | *P. fluorescens* | 84.7 | *P. fragi* | 0.734 | No ID[e] (*P. fluorescens*)[d] | 0.0 (44.0) |
| P61 | *P. fragi* | 98.0 | *P. fluorescens* | 71.0 | *B. pseudomallei* | 99.9 | *P. fragi* | 0.757 | No ID[e] (*P. grimontii*)[d] | 0 (40.6) |
| P62 | *P. lundensis* | 98.0 | *P. putida* | 94.5 | *P. aeruginosa* | 87.5 | *P. lundensis* | 0.697 | No ID[e] (*P. chlororaphis*)[d] | 0 (50.3) |
| *Enteric bacteria* | | | | | | | | | | |
| P4 | *H. alvei* | 99.0 | *H. alvei* | 90.4 | *H. alvei* | 99.9 | *H. alvei* | 0.603 | *H. alvei* | 99.9 |
| P36 | *H. paralvei* | 99.0 | *H. alvei* | 98.8 | *H. alvei* | 99.3 | *H. alvei* | 0.747 | *H. alvei* | 99.9 |
| P39 | *H. paralvei* | 99.0 | *H. alvei* | 98.8 | *H. alvei* | 96.0 | *H. alvei* | 0.739 | *H. alvei* | 99.9 |
| P50 | *H. alvei* | 98.0 | *H. alvei* | 92.4 | *H. alvei* | 84.8 | *H. alvei* | 0.459 | *H. alvei* | 99.9 |
| P51 | *R. aquatilis* | 98.0 | *Kluyvera* sp. | 86.2 | *R. aquatilis* | 99.1 | *R. aquatilis* | 0.542 | *R. aquatilis* | 78.0 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| P52 | *H. alvei* | 99.0 | *H. alvei* | 99.9 | *H. alvei* | 79.0 | *H. alvei* | 0.665 | *H. alvei* | 99.9 |
| P53 | *H. alvei* | 98.0 | *H. alvei* | 99.8 | *H. alvei* | 64.0 | *H. alvei* | 0.559 | *H. alvei* | 99.9 |
| P55 | *H. alvei* | 99.0 | *H. alvei* | 91.5 | *H. alvei* | 92.6 | *H. alvei* | 0.622 | *H. alvei* | 99.9 |
| P59 | *S. liquefaciens* | 99.0 | *S. proteamaculans* | 89.7 | *S. liquefaciens* | 97.7 | *S. liquefaciens* | 0.610 | *S. liquefaciens* | 85.0 |
| *Miscellaneous NF-GNB* | | | | | | | | | | |
| P11 | *A. guillouiae* | 98.0 | *A. baumannii/calcoaceticus* | 99.2 | *A. baumannii* | 92.7 | *A. guillouiae* | 0.627 | *A. guillouiae* | 85.2 |
| P60 | *S. maltophilia* | 100.0 | *S. maltophilia* | 98.4 | *S. maltophilia* | 94.9 | *S. rhizophila* | 0.330 | *S. maltophilia* | 99.9 |
| *B. cereus group* | | | | | | | | | | |
| B5 | *B. weihenstephanensis* | 99.0 | *B. cereus* 1 | 91.0 | NP[f] | NP[f] | *B. weihenstephanensis* | 0.524 | *B. weihenstephanensis* | 91.2 |
| B18 | *B. thuringiensis* serovar *finitimus* | 99.0 | *B. firmus* | 96.2 | NP[f] | NP[f] | *B. acidicola* | 0.596 | *B. cereus* | 77.0 |
| B36 | *B. weihenstephanensis* | 100.0 | *B. cereus* 1 | 92.4 | NP[f] | NP[f] | *B. weihenstephanensis* | 0.667 | *B. weihenstephanensis* | 82.5 |
| B51 | *B. thuringiensis* serovar *thuringiensis* | 99.0 | *B. cereus* 1 | 99.7 | NP[f] | NP[f] | *B. pseudomycoides* | 0.593 | *B. cereus* | 87.0 |
| B77 | *B. thuringiensis* serovar *thuringiensis* | 99.0 | *B. cereus* 1 | 99.9 | NP[f] | NP[f] | *B. thuringiensis* | 0.562 | *B. cereus* | 96.8 |
| B100 | *B. weihenstephanensis* | 100.0 | *B. cereus* 1 | 99.8 | NP[f] | NP[f] | *B. weihenstephanensis* | 0.634 | *B. weihenstephanensis* | 90.2 |
| ATCC 10876 | *B. thuringiensis* serovar *kurstaki* | 99.0 | *B. cereus* 1 | 95.5 | NP[f] | NP[f] | *B. thuringiensis* | 0.589 | *B. cereus* | 93.5 |
| ATCC 10987 | *B. thuringiensis* serovar *kurstaki* | 98.0 | *B. cereus* 2 | 61.1 | NP[f] | NP[f] | *B. acidicola* | 0.653 | *B. cereus* | 99.9 |
| ATCC 14579 | *B. thuringiensis* serovar *kurstaki* | 99.0 | *B. cereus* 1 | 99.9 | NP[f] | NP[f] | *B. thuringiensis* | 0.541 | *B. cereus* | 99.9 |
| *B. pumilus group* | | | | | | | | | | |
| B50 | *B. safensis* | 99.0 | *B. pumilus* | 99.9 | NP[f] | NP[f] | *B. pumilus* | 0.513 | *B. pumilus* | 99.9 |
| B58 | *B. pumilus* | 99.0 | *B. pumilus* | 99.9 | NP[f] | NP[f] | *B. pumilus* | 0.682 | *B. pumilus* | 99.9 |
| B88 | *B. pumilus* | 99.0 | *B. pumilus* | 99.9 | NP[f] | NP[f] | *B. pumilus* | 0.751 | *B. pumilus* | 96.6 |
| B91 | *B. altitudinis* | 99.0 | *B. pumilus* | | NP[f] | NP[f] | *B. pumilus* | 0.638 | *B. pumilus* | 85.4 |
| *B. licheniformis* group | | | | | | | | | | |
| B94 | *B. licheniformis* subsp B | 100.0 | *B. licheniformis* | 99.9 | NP[f] | NP[f] | *B. licheniformis* | 0.601 | *B. licheniformis* | 89.0 |
| B95 | *B. licheniformis* subsp B | 99.0 | *B. licheniformis* | 99.9 | NP[f] | NP[f] | *B. licheniformis* | 0.623 | *B. licheniformis* | 85.6 |
| B96 | *B. licheniformis* subsp A | 99.0 | *B. licheniformis* | 99.6 | NP[f] | NP[f] | *B. licheniformis* | 0.516 | *B. licheniformis* | 88.0 |
| B98 | *B. licheniformis* subsp A | 99.0 | *B. licheniformis* | 99.9 | NP[f] | NP[f] | *B. licheniformis* | 0.566 | *B. licheniformis* | 80.1 |
| ATCC 14580 | *B. licheniformis* subsp B | 99.0 | *B. licheniformis* | 99.9 | NP[f] | NP[f] | *B. licheniformis* | 0.575 | *B. licheniformis* | 99.9 |
| *B. subtilis group* | | | | | | | | | | |
| ATCC 6633 | *B. subtilis* | 99.0 | *B. amyloliquefaciens* | 99.9 | NP[f] | NP[f] | *B. spizizenii* | 0.573 | *B. subtilis* | 89.8 |
| *Miscellaneous Gram positive bacilli* | | | | | | | | | | |
| B99 | *B. horneckiae* | 98.0 | *B. megaterium* | 57.9 | NP[f] | NP[f] | *S. pasteurii* | 0.503 | No ID[e] | 0.0 |

[a] Total number of bacteria (n) = 58.
[b] Confidence score (C %) was obtained from the corresponding identification software, except for Biolog which gave similarity index (SIM)[c].
[c] Similarity index (SIM).
[d] Lower confidence scores.
[e] No ID: no valid identification.
[f] NP: not performed.

for thermo-resistance (Teh et al., 2011). The heat stability of crude BPs was determined by heating 5.0 mL of BPs in 9.0 mL Pyrex tubes (Sigma-Aldrich, Castle Hill, Australia) at 150 °C for 20 s in a shaking oil bath (Ratek, Boronia, Australia), followed by immediate cooling on an ice bath, and subsequent addition of 0.2% sodium azide (Sigma-Aldrich, Castle Hill, Australia) to inhibit bacterial growth (NÖrnberg et al., 2010).

### 2.3. Determination of residual protease activity

The residual BP activities of heat-treated BP samples were determined by observing the coagulation of UHT whole milk. A 1.0 mL aliquot of heated crude BPs was mixed with 2.0 mL of commercial UHT whole milk in sterile tubes, followed by incubation for 5 days at 25 °C and the coagulation pattern of UHT milk was determined visually (Teh et al., 2011). Unheated crude enzyme (1.0 mL) and uninoculated UHT (3.0 mL) whole milk were used as positive and negative controls, respectively. Fifty one raw milk isolates and ATCC cultures (7) that were positive for production of heat-resistant proteases were analysed further, as described below.

### 2.4. Identification of bacteria

The stock cultures were grown on nutrient agar (Oxoid, Basingstoke, England), and sub-cultured on tryptone soy agar containing 5.0% (v/v) sheep blood. The plates containing GNB and GPB were incubated for 24 h at 26 °C and 30 °C, respectively, prior to identification.

#### 2.4.1. 16S rRNA gene sequencing

Overnight NB cultures (0.1 mL) were transferred into sterile microcentrifuge tubes containing sterile MilliQ water and centrifuged at 14,000 ×*g* for 10 min. Cell pellets were re-suspended in water to wash the cells. This was repeated twice more prior to DNA extraction using the Instagene matrix (Biorad, Gladesville, New South Wales, Australia) according to the manufacturer's instructions.

Five different primer pairs were used for amplification of the 16S rRNA gene. The first pair comprised fD1 (AGA GTT TGA TCC TGG CTC AG) and rD1 (AAG GAG GTG ATC AGC C) universal primers (Weisburg et al., 1991) and the other primers were *Pseudomonas* genus specific primers 16S-P5SH (TGA AGA GTT TGA TCA TGG CTC) and 16S-DG74 (AGA GGA GGT GAT CCA ACC GCA) (Wiedmann et al., 2000); *Bacillus* genus specific primers PEU7 (GCA AAC AGG ATT AGA TAC CC) and DG74 (AGG AGG TGA TCC AAC CGC A) (Durak et al., 2006); *P. fluorescens* specific primers 16SPSEfluF (TGC ATT CAA AAC TGA CTG AAT) and 16SPSER (CAC ACC GTG GTA ACC G) (Scarpellini et al., 2004); and *Hafnia alvei* specific primers PA (AGA GTT TGA TCC TGG CTC AG) and PC (CCC ACT GCT GCC TCC CGT AG) (Ridell et al., 1995). The expected PCR products for each primer set were ~1500 bp, ~1500 bp, ~850 bp and 353 bp, respectively.

PCR was conducted in a 50.0 μL final volume, containing 20.0 ng template DNA, 0.5 μM primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5.0 μL of 10× PCR buffer and 1.0 U Platinum Taq DNA polymerase (Invitrogen Life Technologies, Mulgrave, Australia) (Ercolini et al., 2009). A volume of 2.0 μL sterile MilliQ water was used instead of DNA in the negative control.

PCR amplification was carried out using a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA, USA) under the following cycling conditions: initial denaturation step at 94 °C for 3 min; and 40 cycles of denaturation steps at 94 °C for 1 min, 45 s of annealing step at various temperatures for each primer set [46 °C (fD1/rD1), 52 °C (P5SH/16s-DG74), 50 °C (PEU7/DG74), 43 °C (16SPSEfluF/16SPSER) and 53 °C (PA/PC)] and elongation step at 72 °C for 2 min, followed by a final chain extension step at 72 °C for 10 min.

The PCR products were resolved in a 1.0% agarose-TBE gel electrophoresed at 90 V for 1.5 h. GeneRuler 100 bp DNA Ladder Plus (Fermentas) was used as the molecular weight marker. The resulting PCR products were purified using the Wizard SV and PCR clean-up system (Promega Corporation, Auburn, Victoria, Australia), according to the manufacturer's instructions. The purified PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the forward primers used for PCR amplification at the Micromon sequencing facility (Monash University, Victoria, Australia).

The resulting sequences were aligned using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) in the GenBank nonredundant/nucleotide collection (nr/nt) (Benson et al., 2005), Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) (Cole et al., 2009), and GreenGenes databases (http://greengenes.lbl.gov/cgi-bin/JD_Tutorial/nph-16S.cgi) (DeSantis et al., 2006). The results of 16S rRNA gene sequencing were evaluated as follows: species identification: ≥99.0%, secure species identification: ≥95.0%, and genus level identification: <95.0% (Bosshard et al., 2006), according to the criteria given in the CLSI MM18-A guideline (CLSI, 2010). Phylogenetic analysis was conducted using MEGA 6.0 software (Tamura et al., 2013). The sequence similarity was estimated using the maximum composite likely method (Tamura et al., 2004) and the phylogenetic tree was constructed using the UPGMA method (Sneath and Sokal, 1973) with bootstrap test of 1000 replicates (>50.0%) shown next to the branches (Felsenstein, 1985).

#### 2.4.2. Identification using API 20NE, API 20E and API50CH systems

Raw milk isolates were identified using a combination of API 20E/20NE (GNB) and API 50CH/20E (GPB) systems, according to the manufacturer's instructions (BioMérieux, Marcy l'Etoile, France). The API strips were examined after 24 and 48 h at 30 °C and identifications were carried out manually by submitting the seven digit code to the online apiweb™ database. The species identification was conducted using the following evaluation criteria: excellent species identification: ≥99.9%; very good species identification: ≥99.0%; good species identification: ≥90.0%; and acceptable species identification: ≥80.0% (Truu et al., 1999).

#### 2.4.3. Identification by Microbact 24E system

One-to-two colonies of the GNB were dispersed in 3.0 mL sterile saline, distributed in multiwell MB24E plates (24 wells) and incubated for 24 h at 25 °C (for *P. fluorescens*) and 35 °C (for other bacteria), respectively, according to the instructions given by the manufacturer. The wells were examined for colour changes after 24 h by adding the appropriate reagents. The results were converted to numerical codes and identified using the Computer Aided Microbact™ Identification software (MB1244) (Ling et al., 1988). Evaluation criteria for MB24E system were the same as API (Section 2.4.2).

#### 2.4.4. Identification by Biolog GEN III system

The 96 wells of Biolog GENIII plates were inoculated with 150.0 μL bacterial suspensions prepared using a fraction of a colony in IFA or IFB media adjusted to the appropriate cell density, according to the manufacturer's protocol. The inoculated plates were incubated at 30 °C or 25 °C (for slow growing microorganisms) for 18–24 h. Development of colour reactions was observed at 4 h, 18 h, 22 h and 24 h intervals, using an automated microplate reader at 590 nm until a similarity index (SIM) of ≥0.500 was obtained. Species identification was made using the reference metabolic profiles available in the MicroLog GEN III database (release 3.01A) (Holmes et al., 1994).

#### 2.4.5. Identification by MALDI-TOF MS system

*2.4.5.1. Direct smear method.* Bacterial colonies (a small fraction) and *Escherichia coli* ATCC 8739 (calibrant strain) were spotted onto disposable MALDI-TOF MS target slides (BioMérieux, Marcy l'Etoile, France) in triplicate. The bacterial spots were air dried for 5 min, subsequently overlaid with 1.0 μL of matrix solution [10.0 mg/mL α-cyano-4

hydroxycinnamic acid (CHCA)] (BioMérieux, Marcy l'Etoile, France) and allowed to air dry again prior to MALDI-TOF MS analysis (Anderson et al., 2012).

*2.4.5.2. Extraction method.* The extraction method was only conducted for the GPB which gave neither identification nor spectral profiles by the direct method. Approximately five bacterial colonies were picked from the plates and the whole proteins were extracted using the method described by Anderson et al. (2012).

*2.4.5.3. MALDI-TOF MS analysis.* The target plates were analysed using an Axima Performance MS mass spectrometer (Shimadzu Scientific Instruments, USA) equipped with a 337 nm nitrogen laser, fired at a frequency of 50 Hz in positive linear mode. Each spectrum was obtained after accumulating 100 shots per spot. Data were automatically or manually acquired using the manufacturer's settings. The spectra obtained for each isolate were imported into the SARAMIS™ (version 4.10) database (BioMérieux, Marcy l'Etoile, France) and were analysed by standard pattern matching (with default parameter settings). The spectra were analysed in the 2–20 kDa mass range for bacterial identification (Jadhav et al., 2014). The evaluation criteria recommended by the manufacturer for the species identification: scores ≥99.9–97.0% indicating highly probable species identification; scores 90.0–98.0% indicating secure genus identification, probable species identification; scores 85.0–89.0% indicating probable genus identification and scores <70.0% indicating no reliable identification; however, identifications with >75.0% confidence were considered as reliable identifications in the current study.

### 2.5. Data analysis

Identifications were considered as reliable when two or more systems were in an agreement. All identification systems were compared based on typability (% identification accuracy at genus/species level and subspecies level), reproducibility (% probability) and rapidity (time required for the identification; h). The discriminatory power of each was determined using the Simpson's Index of Diversity (SID) score. The numerical value indicates the suitability of a given method to distinguish species/subspecies by calculating the probability of discriminating two unrelated strains by a given typing method (Hunter and Gaston, 1988).

## 3. Results

### 3.1. Diversity of spoilage bacteria in raw milk

Among the psychrotrophic bacteria isolated from raw milk (n = 160), 46.9% (n = 75) showed potential extracellular protease activity and 31.3% of these (n = 51) were resistant to heating at 150 °C for 20 s. According to the 16S rRNA gene sequencing, a diverse range of heat-resistant protease producing bacteria were isolated from raw milk including 36 (70.6%) GNB from the *P. fluorescens* group (26.0%), *H. alvei* (14.0%), *Pseudomonas fragi* (10.0%), *Pseudomonas lundensis* (6.0%) and a few *Pseudomonas psychrophila*, *Pseudomonas syringae*, *Rahnella aquatilis*, *Serratia liquefaciens*, *Stenotrophomonas maltophilia* and *Acinetobactor guillouiae*. GPB comprising *B. cereus* group (14.0%; *Bacillus weihenstephanensis* and *Bacillus thuringiensis*), *B. licheniformis* (8.0%), *Bacillus pumilus* group (8.0%; *Bacillus safensis*, *B. pumilus* and *Bacillus altitudinis*) and one strain of *Bacillus horneckiae* were isolated. The identifications obtained from all identification systems are listed in Table 1.

### 3.2. Identification of bacteria using 16S rRNA gene sequencing

The 16S rRNA gene sequencing gave species and genus level identification for 100.0% of bacteria (Fig. 1), with >98.0% confidence (Table 1).

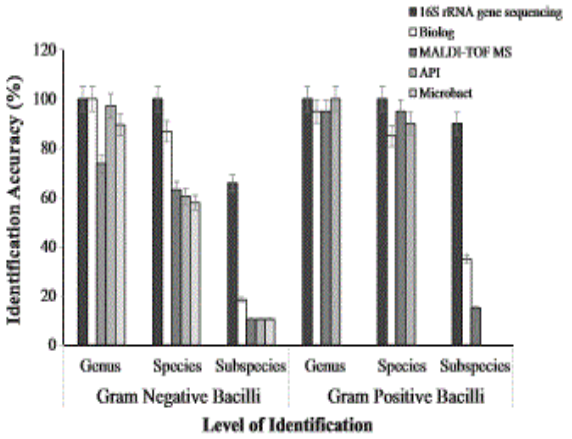


**Fig. 1.** The accuracy of five systems in the identification of spoilage bacteria (means of three replicates ± standard error of the mean). API gave no subspecies level identification for GPB. Note: Microbact was not performed for the Gram positive bacteria.

Subspecies level identifications were obtained for 59.5% of GNB, including the *P. fluorescens*, *P. syringae* and *H. alvei* groups and 85.0% of GPB (in the *B. cereus*, *B. pumilus* and *B. licheniformis* groups). The discrimination power of 16S rRNA gene sequencing system (SID score) was 0.966. The phylogenetic tree constructed based on 16S rRNA sequences indicated that the bacteria can be categorised into 10 clusters (Fig. 2), in which bacteria share >98.0% sequence homology (data not shown). The phylogenetic tree contained 10 different clusters corresponding to the genera *Pseudomonas*, *Acinetobactor*, *Stenotrophomonas*, *Burkholderia*, *Kluyvera*, *Serratia*, *Hafnia*, *Rahnella*, *Sporosarcina* and *Bacillus*, which comprised subclusters related to the species level, in which bacteria share >98.0% sequence homology (Fig. 2). Particularly, the *Pseudomonas* cluster contained two subclusters, fluorescent and non-fluorescent *Pseudomonas* species. Furthermore, subspecies of *P. fluorescens*, *P. syringae*, *H. alvei*, *B. cereus*, *B. pumilus* and *B. licheniformis* were clustered separately. The system was found to have 100.0% reproducibility and intermediate rapidity, requiring 5–8 h to obtain identification.

### 3.3. Identification of bacteria by API system

A combination of API 20E (enteric) and 20NE (non-enteric) identified 97.3%, 60.5%, 10.5% of GNB, at the genus, species and subspecies levels, respectively (Fig. 1). Accurate species level identifications were obtained for *P. fluorescens*, *H. alvei* and *S. maltophilia* strains, with >99.9% confidence (Table 1). In particular, the subspecies of the *P. fluorescens* group isolates contained clear differences in biochemical profiles (data not shown). The identification for non-fluorescent pseudomonads (i.e. *P. fragi*, *P. psychrophila*, *P. syringae* and *P. lundensis*) and *A. guillouiae* was accurate only up to the genus level, with >75.0% confidence. The majority of non-fluorescent pseudomonads were misidentified as *P. fluorescens* by the API system. *R. aquatilis* was misidentified as *Kluyvera* sp. by the API 20E system.

Combined API50CH/20E system identified GPB with 100.0% and 90.0% identification accuracies at the genus and species levels, respectively (Fig. 1). The system identified *B. cereus*, *B. licheniformis*, *B. pumilus* and *B. subtilis* to the species level, with 61.0–99.9% confidences. However, only one *B. cereus* strain was misidentified as *Bacillus firmus* (96.2% confidence), while *B. horneckiae* was misidentified as *Bacillus megaterium*, with 57.9% confidence. However, the subspecies of *P. fluorescens*, *P. syringae*, *H. alvei*, *B. cereus*, *B. pumilus* and *B. licheniformis* were found with apparent discrepancies in the biochemical characteristics (data not shown). The system was found to have lower discrimination power (0.472 SID score) and 99.0% overall reproducibility. The majority of the tests required 24 h incubation in appropriate conditions;

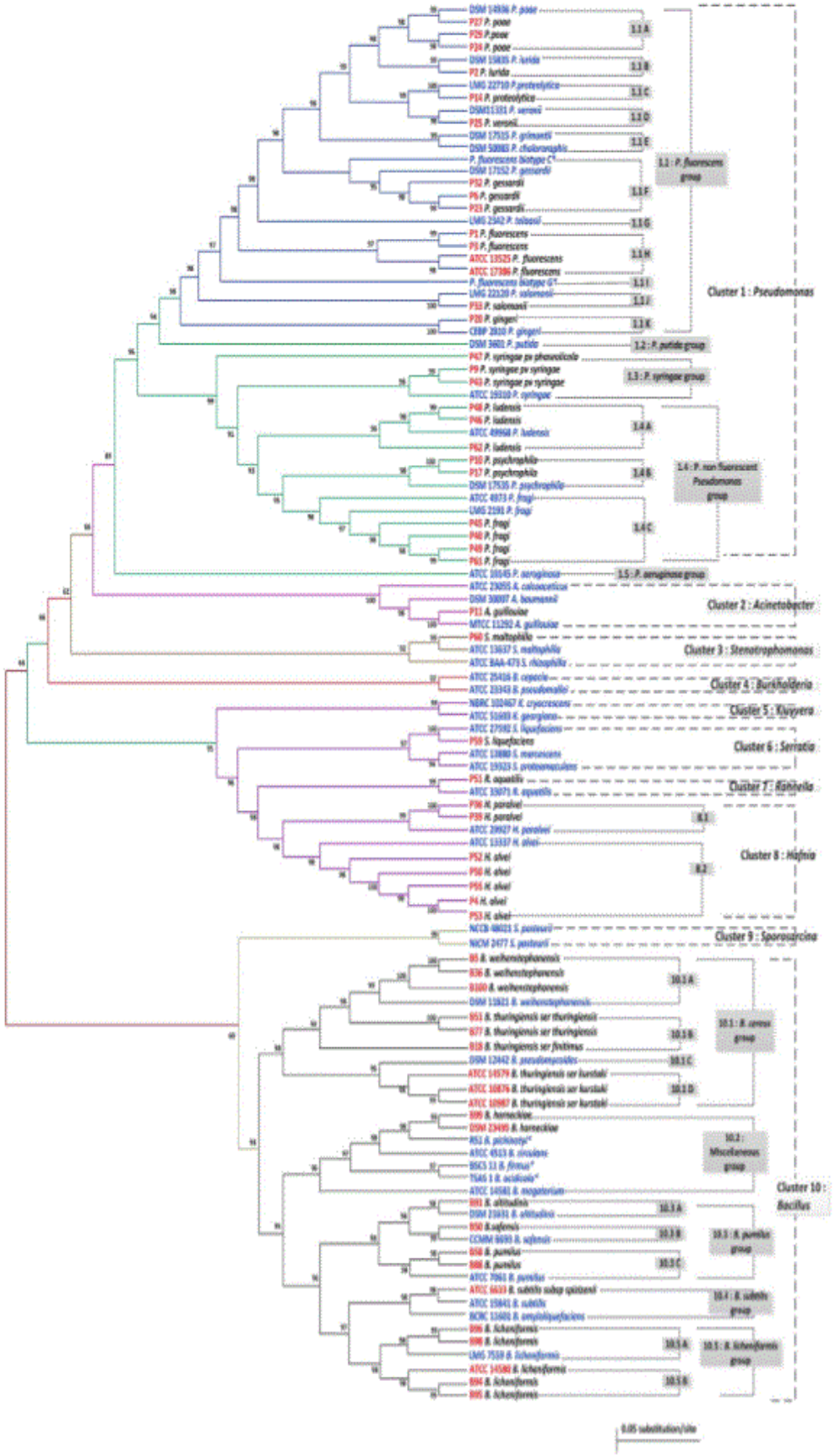


**Fig. 2.** Phylogenetic tree of milk spoilage bacteria derived from similarities of 16S rRNA gene sequences. Bootstrap percentages of 50% or more are indicated at the branch point. Red colour indicates 16S rRNA gene sequences of spoilage bacteria obtained from the current study. Blue colour indicates sequences of the type strains and blue colour with * indicates sequences of other strains, obtained from NCBI.

however, the denitrification test required 48 h for completion, while assimilation tests also required 48 h to obtained visible turbidity.

### 3.4. Identification of bacteria by Microbact 24E system

The MB24E system accurately assigned GNB with 89.5%, 57.9% and 10.5% identification accuracies to genus, species and subspecies levels, respectively (Fig. 1). The system correctly identified the majority of *P. fluorescens* strains (except for *Pseudomonas salomonii*), *H. alvei*, *R. aquatilis*, *S. liquefaciens* and *S. maltophilia* to the species level (Table 1) with >95.0% confidence. The identifications of non-fluorescent pseudomonads (8.1%) were inaccurate at the species level. Some of the *Pseudomonas* species were assigned under genus *Burkholderia* with >93.0% confidences, however with variations in the biochemical (data not shown) and genetic profiles (Fig. 2). However, subspecies of *P. fluorescens* and *H. alvei* group showed higher discrimination than that in API systems (data not shown). The system has not been developed for *Bacillus* species. Thus, discrimination power of the MB24E system was found with a low SID score of 0.140. The individual tests in the system were highly reproducible and were found with 99.0% reproducibility. Time required to obtain identity was 48 h, due to the presence of the denitrification test.

### 3.5. Identification of bacteria by Biolog system

Biolog system demonstrated 100.0%, 86.8% and 18.4% accuracies at the genus, species and subspecies levels for GNB, respectively (Fig. 1), with >0.500 SIM score. They included *P. fluorescens*, *P. lundensis* and *P. fragi* (except for strain P45), *H. alvei*, *S. liquefaciens*, *R. aquatilis* and *A. guillouiae* (Table 1). Some *P. fluorescens* strains were given reliable biovar level identification, for example *Pseudomonas gessardii* strains (i.e. P6, P23, and P32) were identified as *P. fluorescens* biotype C (Table 1). However, the system provided unreliable identifications for majority of *P. fluorescens* strains at the subspecies and biovar levels (Table 1). The system misidentified *P. psychrophila* and *P. syringae* as *P. fluorescens*, similar to other phenotypic methods. Only one *P. fragi* strain (i.e. P45) and *S. maltophilia* were misidentified as *P. lundensis* (SIM; 0.719) and *Stenotrophomonas rhizophila* (SIM; 0.330), respectively.

The Biolog system gave reliable genus level (95.0%), species level (85.0%) and subspecies level (35.0%) identifications for majority of GPB, with >0.500 SIM score, except for *B. horneckiae* which was misidentified as *Sporosarcina pasteurii* (SIM; 0.503). The majority of *B. cereus* group bacteria were identified at the subspecies level as *B. weihenstephanensis* and *B. thuringiensis* and *B. subtilis* (ATCC 6633) as *B. subtilis* subsp *spizizenii* (Table 2). However, some *B. cereus* strains were given unreliable identifications as *Bacillus acidicola* and *Bacillus pseudomycoides* (SIM; >0.593). The Biolog system had the second highest discrimination power with SID score of 0.711. However, the individual tests in the GENIII panel showed lower reproducibility, which resulted in an overall reproducibility of 92.0%. Identifications of GPB and enteric bacteria required only 4 h of incubation, while other NF-GNB required 22–24 h.

### 3.6. Identification of bacteria by MALDI-TOF MS system

The MALDI-TOF MS system gave concordant identifications of 73.7%, 63.2% and 10.8% for GNB, at genus, species and subspecies levels, respectively (Fig. 1). Reliable species level identifications were given for some *P. fluorescens* strains, *H. alvei*, *S. maltophilia*, *R. aquatilis*, *S. liquefaciens* and *A. guillouiae* (Table 1), with >75.0% confidence. However, some *P. fluorescens* strains (i.e. P3, P6, and P20) and non-fluorescent *Pseudomonas* species were identified to the reliable genus level (data not shown). Subspecies level identifications were obtained for a few *P. fluorescens* isolates, however identification of *Pseudomonas veronii* was considered as unreliable due to lower confidence score (45.0%). Only one *P. gessardii* strain (i.e. P32) was misidentified as *Pseudomonas grimontii*, while a few non-fluorescent pseudomonads were misidentified as *P. grimontii* and *Pseudomonas chlororaphis*, with lower (<51.0%) confidence and some bacteria (27.0%) were not identified by the system (Table 1).

MALDI-TOF MS system gave 95.0% accurate identification at both genus and species levels for GPB, including *B. cereus*, *B. licheniformis*, *B. pumilus* and *B. subtilis* (Fig. 1), with >77.0% confidence. Subspecies level identifications were given only for *B. weihenstephanensis* by the system (Table 1). Only the *B. horneckiae* strain gave no identification. The identifications of *Bacillus* species were more reliable with the extraction method. MALDI-TOF MS was found with lower discrimination power (SID score: 0.496) and 98.0% reproducibility. It required 0.25 h for the direct smear method and about 0.50 h for the extraction method to obtain identity.

## 4. Discussion

The present study was designed to evaluate and compare five systems identifying spoilage microbiota in raw milk, based on their typability, discrimination power, reproducibility and rapidity. All the systems demonstrated reliable identifications at the genus level. Accuracy of identification at the species level was in the following order: GNB; 16S rRNA gene sequencing > Biolog > MALDI-TOF MS > API > Microbact; and GPB: 16S rRNA gene sequencing > MALDI-TOF MS > API > Biolog. Discrimination power (SID) of the systems is in the order of 16S rRNA gene sequencing > Biolog > MALDI-TOF MS > API > Microbact. Reproducibility was higher in 16S rRNA gene sequencing followed by API and Microbact, MALDI-TOF MS, and Biolog. The rapidity

**Table 2**
Comparison of 16S rRNA gene sequencing, API, Microbact, Biolog and MALDI-TOF MS for bacterial identification.

| Methods | Quantity of sample (colony) | Number of sample/run | Typability (%) | | | | | Discrimination power (SID) | Reproducibility (%) | Rapidity (h) | No of profiles in the database |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Genus | Species | Subspecies | Mis ID[a] | No ID[b] | | | | |
| 16S rRNA gene sequencing | 1 | 96 | 100.0[c] | 100.0[c] | 65.8[c] | 0.0[c] | 0.0[c] | 0.966 | 100.0 | 5–8 | >50,000 |
| | | | 100.0[d] | 100.0[d] | 90.0[d] | 0.0[d] | 0.0[d] | | | | |
| API | 1–4 | 1 | 97.3[c] | 60.5[c] | 10.5[c] | 2.6[c] | 0.0[c] | 0.472 | 99.0 | 24–48 | ~600 |
| | | | 100.0[d] | 90.0[d] | 0.0[d] | 0.0[d] | 0.0[d] | | | | |
| Microbact 24 | 1–4 | 4 | 89.5[c] | 57.9[c] | 10.5[c] | 7.9[c] | 0.0[c] | 0.140 | 99.0 | 24–48 | ~150 |
| | | | 0.0[d] | 0.0[d] | 0.0[d] | 0.0[d] | 0.0[d] | | | | |
| Biolog | 0.2 | 1 | 100.0[c] | 86.8[c] | 18.4[c] | 0.0[c] | 0.0[c] | 0.711 | 92.0 | 18–24 | >2500 |
| | | | 95.0[d] | 85.0[d] | 35.0[d] | 5.0[d] | 0.0[d] | | | | |
| MALDI-TOF MS | 0.4 | 192 (48 × 4) | 73.7[c] | 63.2[c] | 10.5[c] | 0.0[c] | 23.7[c] | 0.496 | 98.0 | 0.25–0.50 | ~18,831 |
| | | | 95.0[d] | 95.0[d] | 15.0[d] | 0.0[d] | 5.0[d] | | | | |

[a] Mis ID: complete misidentification.
[b] No ID: no valid identification.
[c] Identification of GNB.
[d] Identification of GPB.

of the assay was higher with MAL-TOF MS and the other systems are in the following order: 16S rRNA gene sequencing >Biolog > API > Microbact.

The genus *Pseudomonas* (48.0%), particularly *P. fluorescens* (26.0%), dominated the spoilage microbiota in raw milk at 4 °C for 10 days storage, in agreement with previous findings (Dogan and Boor, 2003). Other bacteria such as *P. fragi*, *P. lundensis*, *H. alvei*, *R. aquatilis*, *S. liquefaciens*, *S. maltophilia*, *A. guillouiae*, *B. cereus* group, *B. licheniformis*, *B. pumilus* group and *B. horneckiae* were isolated at lower frequencies. The majority of these spoilage bacteria were previously described with heat-resistant extracellular protease activity (Champagne et al., 1994; Griffiths et al., 1981; NÖrnberg et al., 2010), however, this is the first description of heat-resistant protease activity in *A. guillouiae*, *B. horneckiae*, *S. maltophilia* and *R. aquatilis*. The diverse range of microbes identified in raw milk reflects the variety of potential on-farm and transport-related sources (Vissers and Driehuis, 2009).

The 16S rRNA gene sequencing demonstrated highest identification 100.0% accuracy at the species level (Fig. 1), with ≥98.0% confidence, which is consistent with previous studies that obtained 92.0% accuracy at the species level for the identification of GNB (Bosshard et al., 2006). In contrast to previous studies, subspecies and pathovar level discriminations were observed in some bacteria (65.8%), including *P. fluorescens* (Brosch et al., 1996), *P. syringae* (Chapman et al., 2012), *H. alvei* (Huys et al., 2010), *B. cereus* (Ash et al., 1991), *B. pumilus* (Liu et al., 2013) and *B. licheniformis* (Madslien et al., 2012) groups (Table 1). Spoilage microbiota may contain hypervariable signature sequences in the 16S rRNA gene (Chakravorty et al., 2007) specific for spoilage potential (Dogan and Boor, 2003) and their psychrotolerance (Pruss et al., 1999). These findings suggest that those sequences can be used as biomarkers for identifying spoilage microorganisms in milk and dairy products and tracking their sources. However, this system was not able to identify *B. subtilis* subsp *spizizenii* (ATCC 6633) at subspecies level, but shared 99.8% pairwise sequence similarity with published sequence of this bacterium (gi|418969582|gb|JX861887.1|).

The PCR amplification and sequencing were successful with both genus and species specific primers. However, amplification of Gram-positive bacilli was unsuccessful with universal primers and sequencing of some *Pseudomonas* species was unsuccessful with universal primers as well. The identification of *H. alvei* was initially obtained using universal primers which was further confirmed by sequencing of the specific 353 bp PCR product.

The heterogeneity of the 16S rRNA gene among the spoilage bacteria was further confirmed by the phylogenetic tree, which contained ten distinct clusters related to the genus level and subclusters at the species and subspecies levels (Fig. 2), as described before (Anzai et al., 2000; Cilia et al., 1996). Each subspecies level discriminated by phylogenetic analysis shares ≥98.0% sequence similarity (Fig. 2). However, the 16S rRNA gene sequences of ATCC strains (both *Pseudomonas* and *Bacillus*) varied from bacteria isolated from Australian raw milk, which may reflect geographical variation of dairy bacteria. Álvarez-Pérez et al. (2013) observed genetic heterogeneity among *P. fluorescens* isolated from various geographical regions, whereas Garcia-Martinez et al. (2002) described genetic variation in *Alteromonas macleodii* strains recovered from northern and southern hemisphere samples. The *B. horneckiae* strain had >98.0% similarity with published sequences of *Bacillus circulans*, and *B. firmus* (Fig. 2), in agreement with previous findings (Vaishampayan et al., 2010) in which *B. circulans* predominated in aseptically packaged pasteurised milk and showed growth below 2 °C (Ledenbach and Marshall, 2009). The 16S rRNA gene sequencing was found to have the highest discrimination power (SID: 0.966). Wiedmann et al. (2000) obtained an SD score of 0.955 for ribotyping. This system previously detected single nucleotide polymorphisms (SNPs) in various bacteria (Mauchline et al., 2011), further confirming its remarkable discrimination power.

The 16S rRNA gene sequencing was also found to have 100.0% reproducibility. This system was previously observed to have good intra (Cloud et al., 2010) and inter (Storms et al., 2002) laboratory reproducibility in identifying various bacteria, suggesting that identifications resulting from 16S rRNA gene sequencing are unbiased from the perspective of the protocol used. However, reliability of identification by 16S rRNA sequencing can be affected by the presence of misannotated data in the public databases such as GenBank®, RDP-II, and RIDOM (Janda et al., 2005). The commercial databases (MicroSeq and SmartGene) contain quality controlled sequences, which may be expensive for users and not as extensive as the public databases. Moreover, 16S rRNA gene sequencing requires at least 2 days to obtain identification (Morgan et al., 2009), however, 16S rRNA gene based molecular methods have been optimised for rapid detection of bacteria that may require only 5–8 h to perform the analysis (Chauhan et al., 2013; Wiedmann et al., 2000).

A combination of API 20E/NE systems provided 97.3%, 60.5% and 10.5% of identification accuracy to the genus, species and subspecies levels of GNB, respectively. Bosshard et al. (2006) observed lower identification accuracy by the API20NE system (54.0% correct) compared to 16S rRNA gene sequencing (92.0% correct) for the identification of clinical NF-GNB. On the contrary, Awong-Taylor et al. (2008) obtained higher accuracy (74.0%), than ribotyping (66.0%) for the identification GNB, using combined API20E/NE systems. Even though the API system was not able to identify to the subspecies level, intraspecies biochemical (phenotypic) variation among *P. fluorescens*, *H. alvei*, *B. cereus*, *B. pumilus* and *B. licheniformis* was apparent (data not shown). The current study is in agreement with previous studies which observed variation in phenotypic characteristics, such as gelatin hydrolysis, nitrate reduction, urea hydrolysis and citrate utilisation among *Pseudomonas proteolytica* (Reddy et al., 2004), *P. veronii* (Elomari et al., 1996) and *P. gessardii* (Verhille et al., 1999); however *Pseudomonas poae* was negative for gelatinase activity (Behrendt et al., 2003). Another study observed biovar level discrimination in the *P. fluorescens* group, similar to the present study, using both phenotypic and genotypic approaches (Johnsen et al., 1996).

However, non-fluorescent pseudomonads were misidentified as *P. fluorescens* in an agreement with previous studies (Dogan and Boor, 2003). Furthermore, *R. aquatilis* was misidentified as *Kluyvera* species but these two genera comprised only 95.0% sequence similarity, further supporting its assignment as *R. aquatilis* by other methods (Fig. 2). However, Stepien-Pysniak (2010) identified those two species using API 20E, suggesting that similar biochemical profiles may be associated with misidentification.

The API 50CH/20E system identified GPB with 100.0% and 90.0% accuracies to the genus and species levels, respectively, but no subspecies level identifications were obtained. A few strains such as B18, B99 and ATCC 6633 were misidentified as *B. firmus*, *B. megaterium*, and *B. amyloliquefaciens*; however they share <97.0% genetic similarity (Fig. 2). The API system was found with an SID score of 0.472 and 99.0% reproducibility, as observed previously with both API20NE and API50CH/20E systems (Johnsen et al., 1996; Logan and Berkeley, 1984); however the latter study observed limited discrimination power in certain tests of the API50CH panel, which is in an agreement with the present study.

The Microbact 24E system identified GNB with 89.5%, 57.9% and 10.5% accuracies at genus, species and subspecies levels, respectively. The system was found to have enhanced discrimination power to distinguish subspecies of *P. fluorescens* and *H. alvei* groups compared to the API system (data not shown), possibly due to the presence of tests with higher discrimination ability in the MB24E panel (Mugg and HillI., 1981). Both MB24E and API demonstrated higher accuracy in identification of enteric bacteria (97.0%) and NF-GNB (91.0%) isolated from clinical specimens, but with lower accuracy for environmental isolates (Ling et al., 1988). The major limitation found with this system is the lack of its ability to identify GPB. Thus, it was found to have the least discrimination power among all systems (SID score: 0.140). However, it had 99.0% reproducibility, but was less rapid (48 h), similar to

the API system, due to the presence of the nitrate reduction test. The limitations of both API and MB24E are limited reference biochemical profiles of spoilage bacteria in the database and limited number of tests (excluding API 50CH) available in the test panel that may result in lower discrimination. Prior knowledge about the bacteria such as Gram reaction, cell morphology and ability to ferment lactose, is a prerequisite for selecting the appropriate identification panel. Furthermore, both databases contain limited entries (Table 2), reducing the identification capabilities of both methods.

The Biolog system demonstrated second highest identification accuracy for GNB, with 100.0% and 86.58% to the genus and species, respectively. A previous study obtained only 68.3% accuracy to the species level, in comparison to 16S rRNA sequencing (90.6%), when identifying various clinical isolates (Morgan et al., 2009). Inconsistent with 16S rRNA sequencing, in the present study, the Biolog system identified few of the *P. fluorescens* isolates (18.4%) at either the species or biovar level (Table 1). For example, Biolog identified *P. gessardii* (by 16S rRNA sequencing) as representative of *P. fluorescens* biotype C, which share >98.0% sequence similarity (Fig. 2) and higher phenotypic similarity (data not shown) (Verhille et al., 1999). In another study, Johnsen et al. (1996) demonstrated that the phenograms provided by the Biolog system were in a good agreement with biovar classifications. Furthermore, Brosch et al. (1996) complied ribotyping data with biovar levels derived from the Biolog system and found that biovars of *P. fluorescens* formed a distinct ribogroup. Dawson et al. (2002) demonstrated that combined rep-PCR and Biolog metabolic fingerprinting resulted in a more taxonomically realistic relationship than ribotyping for characterisation of fluorescent pseudomonads. However, some *P. fluorescens* strains (i.e. ATCC 17386, *P. poae*, *P. salomonii*, and *P. lurida*) were assigned incorrectly as *Pseudomonas tolaasii* and *Pseudomonas marginalis* which is consistent with previous studies which observed incorrect or partial assignment of *P. fluorescens* strains by the Biolog system (Martins et al., 2011; Munsch-Alatossava and Alatossava, 2006); however the bacteria share distinct genetic heterogeneity with those incorrectly assigned bacteria (Fig. 2). Additionally, *P. fluorescens* biotype G and ATCC 17386 produced distinct biochemical properties (data not shown) (Brosch et al., 1996). Interestingly, the system correctly identified the majority of non-fluorescent pseudomonad isolates (i.e. *P. fragi* and *P. lundensis*), excluding *P. syringae* and *P. psychrophila*, which may be due to the limited database facilities and slow growth rate, as observed before (Shenge et al., 2008). Enteric bacteria (i.e. *H. alvei*, *R. aquatilis* and *S. liquefaciens*) provided reliable species identification within 4 h of incubation, consistent with a previous study (Klingler et al., 1992). Biolog misidentified *S. maltophilia* as *S. rhizophila* (Table 2), the former sharing only 92.2% sequence similarity with *S. rhizophila* (>gi|8980461|emb| AJ293461.1) (Fig. 2) but containing similar biochemical properties (Wolf et al., 2002), suggesting that discrimination of these two species may require additional biochemical tests. Alternatively, Pinot et al. (2011) used 16S rRNA gene sequencing in parallel to the Biolog assay to discriminate these two bacteria.

The Biolog system identified GPB to the genus level with 95.0% accuracy and 85.0% to the species level. The system was also able to discriminate *B. cereus* group microbes (35.0%), namely *B. weihenstephanensis* and *B. thuringiensis* (Fig. 2). The system identified the ATCC *B. subtilis* strain to the subspecies level (Table 1). In addition, some *B. cereus* group isolates were misidentified as *B. pseudomycoides* (i.e. B51), *B. acidicola* (i.e. B18 and ATCC 10987), in which B51 shared only 95.0% sequence identity with *B. pseudomycoides* (>gi|2331222|gb| AF013121.1|) (Fig. 2), while B18 and ATCC 10987 contained distinct biochemical profiles (data not shown) (Albert et al., 2005) and genetic profiles compared to *B. acidicola* (Fig. 2). Likewise, *B. horneckiae* was not identified by the system, possibly due to lack of an appropriate metabolic profile in the Biolog database.

The Biolog system performed with intermediate discrimination power (SID score: 0.711), and lower (92.0%) reproducibility. Wiedmann et al. (2000) observed a significantly higher number of tests (72.6%) with limited reproducibility. However, another study observed that reproducibility of individual tests in the Biolog panel can be increased by extended incubation time (24 h) (Klingler et al., 1992), indicating that rapidity of the assay is species specific. It was also observed that reproducibility of the tests can result from a lower inoculum (<$10^8$ CFU/mL) (Konopka et al., 1998) and lower absorbance ($OD_{560}$ < 40.0%) by the automated system (Johnsen et al., 1996), indicating the necessity of analysing the metabolic profiles manually to minimise the errors associated with borderline results. Furthermore, there was difficulty in identification of non-reactive NF-GNB and encapsulated bacteria (Holmes et al., 1994), suggesting the requirement of optimisation of the conditions for the identification of fastidious microorganisms, as described before (Mauchline and Keevil, 1991). A major limitation of the system is the limited number of metabolic profiles in the database (Awong-Taylor et al., 2008). However, the presence of ~2500 reference profiles warrants its ongoing optimisation using authentic isolates as it is a user-friendly platform which requires no Gram-stain and other preliminary tests for the identification. Both Biolog and API systems have previously given 86.0% accuracy for the identification of GNB at the genus level and less at the species level (Truu et al., 1999). The identifications obtained by Biolog system were occasionally more significant than ribotyping (Dawson et al., 2002).

The MALDI-TOF MS system identified GNB with 73.7% accuracy to the genus level and 63.2% to the species level, while GPB were identified with 95.0% accuracy to the genus and species levels. Consistent with the findings of Bohme et al. (2013), both fluorescent and non-fluorescent *Pseudomonas* species contained genus and species specific peaks in the spectral profile (data not shown). The system identified the majority of *P. fluorescens* isolates, except for strain P3 that was identified as *Pseudomonas* sp. with 99.0% confidence, possibly due to missing peaks or peaks with lower intensity. Subspecies level identifications were limited for a few *P. fluorescens* isolates (10.5%); however the system identified *P. veronii* with significantly lower confidence (45.0%), and thus this identification was interpreted as unreliable. Of the two commercial MALDI-TOF MS databases, SARAMIS and Bruker Biotyper, the latter system was found to have higher efficiency by Alby et al. (2013) and was able to identify *P. gessardii* (Fernández-Olmos et al., 2012). Non-fluorescent *Pseudomonas* species (27.02%) were not able to be identified by the system, probably due to the absence of superspectra in the database. However, identification of enteric bacteria was often reliable, with 99.9% confidence, except for *R. aquatilis* and *S. liquefaciens*, which had only 78.0% and 85.0% confidences, respectively. In particular, the *H. alvei* group yielded subspecies specific discrimination. Likewise, the system identified some *B. weihenstephanensis*, but did not identify the *B. thuringiensis* isolates. The SARAMIS database contains superspectra of these two subspecies, thus inability of discrimination may be associated with missing peaks. Identifications of *Bacillus* species were more efficient with the extraction method (95.0% to 99.9% confidence) compared to the direct smear method (75.0% to 99.9% confidence), which is consistent with results of Anderson et al. (2012). The relatively thicker cell wall of Gram positive bacilli (Beeby et al., 2013) may reduce the penetration of the matrix into the cell during direct analysis of samples. A recent study compared SARAMIS and Bruker Biotyper software with 16S rRNA gene sequencing for the identification of NF-GNB and found >90.0% agreement across the systems (Alby et al., 2013). MALDI-TOF MS also provided concordant identification (100.0%) to the conventional biochemical based approach (99.6%) (Baillie et al., 2013).

The discrimination power of MALDI-TOF MS was 0.496 (SID score); however the system was previously able to detect even SNP in *Erwinia* sp. (Sauer et al., 2008), indicating its potentiality for discrimination of various bacteria upon system and database optimisation. The MALDI-TOF MS system was found to have 98.0% reproducibility. Mellmann et al. (2009) also obtained 98.75% interlaboratory reproducibility for the identification of NF-GNB by MALDI-TOF MS. The system's major advantage was its remarkable rapidity that requires only 0.25 h to achieve

identification by direct smear method, and 0.5 h for extraction method (Sauer et al., 2008), which is much more rapid than other methods (Table 2). However, the MALDI-TOF MS system has been optimised for the identification of clinical bacteria (Anderson et al., 2012). Therefore, the major limitation associated with this system is the limited number of reference spectral profiles of spoilage bacteria in the current database (Seng et al., 2009). However, developing an in-house database for spoilage bacteria can possibly overcome this limitation (Bohme et al., 2013). Thus, the spectral profiles obtained in the current study may be useful for further development of MALDI-TOF MS databases. However, detailed knowledge of the potential pitfalls of the system and sound knowledge of bacterial taxonomy are prerequisites for determining subspecies, serovar and strain level identifications and successful implementation of this system in dairy microbiology (De Bel et al., 2010).

Besides comparison purposes, some studies have used a polyphasic approach with molecular and phenotypic systems to minimise the taxonomic discordances obtained by an individual system (Dogan and Boor, 2003), which can be used to develop an algorithm for the effective identification of spoilage bacteria in the routine dairy laboratory (Bosshard et al., 2006).

Unlike genotypic methods, the identification accuracy derived from phenotypic methods can be hindered by many factors. They include, type of bacteria, growth rate, growth medium, age of culture, suspension medium, inoculum density, incubation temperature and time (Praphailong et al., 1997). Particularly, as MALDI-TOF MS identification is generally performed from the primary isolation plate, identification can be affected by various medium components and salts in culture media due to variation in the protein expression and ion suppression (Anderson et al., 2012). However, API, Microbact and Biolog systems use secondary inoculation media, prior to the inoculation into the corresponding strips or plates; therefore, components in the primary media should not affect the identification. For example, Thaochan et al. (2010) obtained concordant species identification for enteric bacteria grown on tryptone soya agar (TSA) and peptone yeast extract agar (PYEA) by the API 20E system, except for higher recovery of enteric bacteria on TSA (62.3%) than PYEA (37.6%). Praphailong et al. (1997) observed no variation in identification of yeast species by the Biolog system when they were grown in various media. However, psychrotrophic bacterial species are slow-growing microbes, which may require an extended incubation at a given temperature for higher accuracy, which is not feasible from the spoilage point of view. Use of higher inoculum size showed enhanced identification accuracy and rapidity of the assay (Konopka et al., 1998; Praphailong et al., 1997). It was also found that the age of the cultures and composition of the suspension media such as saline or distilled water can affect the identification of microorganisms (Praphailong et al., 1997; Konopka et al., 1998). This suggests that identification of the diverse range of spoilage bacteria by phenotypic methods such as API, Microbact, Biolog and MALDI-TOF MS using the standard protocols recommended by the manufacturers can reduce the identification accuracy of individual isolates. Therefore, optimum growth conditions for specific bacteria need to be established prior to the identification to achieve highest accuracy by commercial systems (Klingler et al., 1992; Praphailong et al., 1997).

The major limitation of the present study is the use of a culture-based approach for all systems prior to the identification, affecting the rapidity of each method. Particularly, API, Microbact and Biolog systems require an additional subculturing step in suspension medium (i.e. saline, Aux, CHB or IFA) prior to the inoculation into the appropriate test strips or mirotitre plates and incubation steps, leading to reduced rapidity of those methods. Performing MALDI-TOF MS and molecular methods may require only a single isolated colony from solid media, eliminating this unnecessary subculturing step (Anderson et al., 2012; Chauhan et al., 2013). MALDI-TOF MS can be used to directly identify bacteria in positive blood cultures, independent from a pre-isolation step, however it has difficultly in identifying bacteria from a mixed population (La Scola and Raoult, 2009). Jacob et al. (2012) developed a PCR assay coupled with electrospray ionisation mass spectrometry (PCR/ESI-MS) to quantify and simultaneously detect multiple pathogens in a single reaction without a pre-isolation step.

However, culture-independent molecular methods have the potential for the identification of bacteria including uncultivable bacteria in any given sample, completely eliminating the isolation of bacteria (Cocolin et al., 2013). Therefore, culture-independent molecular techniques, including analysis of ribosomal RNA genes (Albertson et al., 1990; Cocolin et al., 2013), metagenomic studies (Loman et al., 2013), and quantitative real time PCR (qPCR) (Smith et al., 2004) remain the preferred methods for rapid identification of microorganisms with the highest reliability.

In conclusion, 16S rRNA gene sequencing has provided genetically unique and phenotypically coherent clusters of Gram negative and Gram positive milk spoilage bacteria. Therefore, 16S rRNA gene sequencing has the greatest potential for the identification and characterisation of spoilage bacteria and application in microbial surveillance and source tracking of contamination to ensure the quality of UHT milk. The Biolog system showed intermediate accuracy in identification of Gram negative spoilage bacteria, but was limited in terms of rapidity, and reproducibility, thus may be more suited to research laboratories for characterisation purposes. The MALDI-TOF MS system gave identification with intermediate accuracy for Gram positive spoilage bacteria, with remarkable rapidity. The identification accuracy of API system was higher for Gram positive bacteria but the identification accuracies of both API and Microbact systems were hindered by limited database facilities that may require extensive optimisation to be suitable for dairy microbiology.

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# CHAPTER 4: OPTIMISATION OF MATRIX-ASSISTED LASER DESORPTION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS) FOR IDENTIFICATION OF GRAM-NEGATIVE PSYCHROTROPHIC PROTEOLYTIC BACTERIA FROM RAW MILK

## 4.1 Overview of Chapter

Chapter 4 represents the optimisation of psychrotrophic proteolytic bacteria in raw milk by MALDI-TOF MS. The Gram-negative psychrotrophic bacteria characterised by 16S rRNA gene sequencing were used to optimise MALDI-TOF MS using a combined MALDI-TOF MS-bioinformatics approach for species level discrimination. This method can be used to improve the assignment of Gram-positive bacteria in raw milk, and will be focussed on in the future. The article entitled “Species Level Discrimination of Psychrotrophic Pathogenic and Spoilage Gram-Negative Raw Milk Isolates using a Combined MALDI-TOF MS Proteomics-Bioinformatics Based Approach” by Nuwan R. Vithanage, Jeevana Bhongir, Snehal R. Jadhav, Chaminda S. Ranadheera, Enzo A. Palombo, Thomas R. Yeager and Nivedita Datta has been submitted in the peer-reviewed journal “Journal of Proteome research” (Under review).

**Note from the examination process of this PhD thesis:**

*Bacillus cereus* considered in the present study belonged to the *B. cereus sensu lato* species complex including B*. cereus, B. thuringiensis, B. mycoides, B. weihenstephanensis,* and *B. pseudomycoides*, which are ecologically diverse bacterial group of medical and agricultural significance (Bartoszewicz & Marjańska, 2017, Okinaka & Keim, 2016).

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## GRADUATE RESEARCH CENTRE

### DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

*This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.*

### 1. PUBLICATION DETAILS (to be completed by the candidate)

| | |
|---|---|
| Title of Paper/Journal/Book: | Species Level Discrimination of Psychrotrophic Pathogenic and Spoilage Gram-Negative Raw Milk Isolates using a Combined MALDI-TOF MS Proteomics-Bioinformatics Based Approach |
| Surname: | Vithanage |
| First name: | Nuwan |
| College: | College of Health and Biomedicine |
| Candidate's Contribution (%): | 85% |

Status: **Submitted (under review)**

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### 2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

| [redacted] | 27-Jul-2017 |
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| **Signature** | **Date** |

### 3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
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| **College of Health and Biomedicine, Victoria University, Werribee Campus, Melbourne VIC, Australia** |

| Name(s) of Co-Author(s) | Contribution (%) | Nature of Contribution | Signature | Date |
|---|---|---|---|---|
| Nuwan R. Vithanage | 85% | Concept Development, research question, hypothesis, data analysis manuscript writing and submission to journals. | [redacted] | 15-Feb-2017 |
| Jeevana Bhongir | 3% | Laboratory experiment and manuscript preparation. | | 22-Feb-2017 |
| Chaminda S. Ranadheeraa | 1% | Data analysis. | | 15-Feb-2017 |
| Snehal R. Jadhav | 1% | Manuscript preparation. | | 28-Feb-2017 |
| Enzo A. Palombo | 4% | Concept Development, research question, hypothesis and manuscript preparation. | | 15-Feb-2017 |
| Thomas R. Yeager | 4% | Concept Development, research question, hypothesis, manuscript preparation and submission to journals. | | 15-Feb-2017 |
| Nivedita Datta | 2% | Concept Development, research question, hypothesis. | | 20-Feb-2017 |

*Species-Level Discrimination of Psychrotrophic Pathogenic and Spoilage Gram-Negative Raw Milk Isolates Using a Combined MALDI-TOF MS Proteomics–Bioinformatics-based* by *N.R. Vithanage, J. Bhongir, S.R. Jadhav, C.S. Ranadheera, E.A. Palombo, T.R. Yeager, and N. Datta* was published in the peer review journal, *Journal of Proteome Research, 16/6, 2188-2203, 2017*.



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# CHAPTER 5: FACTORS AFFECTING MICROBIOLOGICAL QUALITY OF RAW MILK

## 5.1 Overview of Chapter

Chapter 5 demonstrates the intrinsic and extrinsic factors such as initial microbial counts, temperature, time, season on microbial counts, diversity and their spoilage potential on microbiological quality and biodiversity of raw milk. The article entitled "Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential" by Nuwan R. Vithanage, Muditha Dissanayake, Greg Bolge, Enzo A. Palombo, Thomas R. Yeager and Nivedita Datta has been published in the peer-reviewed journal "International Dairy Journal" (2016), 57: 80–90. http://dx.doi.org/10.1016/j.idairyj.2016.02.042.

## Note from the examination process of this PhD thesis:

*Bacillus cereus* considered in the present study belonged to the *B. cereus sensu lato* species complex including B*. cereus, B. thuringiensis, B. mycoides, B. weihenstephanensis,* and *B. pseudomycoides*, which are ecologically diverse bacterial group of medical and agricultural significance (Bartoszewicz & Marjańska, 2017, Okinaka & Keim, 2016).

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

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| **College of Health and Biomedicine, Victoria University, Werribee Campus, Melbourne VIC, Australia** |
|---|

| **Name(s) of Co-Author(s)** | **Contribution (%)** | **Nature of Contribution** | **Signature** | **Date** |
|---|---|---|---|---|
| Nuwan R. Vithanage | 85% | Concept Development, research question, hypothesis, data analysis manuscript writing and submission to journals. | [redacted] | 15-Feb-2017 |
| Muditha Dissanayake | 2% | Laboratory experiment and manuscript preparation. | | 15-Feb-2017 |
| Greg Bolge | 1% | Sample provision. | | 15/02/17 |
| Enzo A. Palombo | 5% | Concept Development, research question, hypothesis and manuscript preparation. | | 15-Feb-2017 |
| Thomas R. Yeager | 5% | Concept Development, research question, hypothesis, manuscript preparation and submission to journals. | | 15-Feb-2017 |
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# Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential

Nuwan R. Vithanage [a, f], Muditha Dissanayake [a, f], Greg Bolge [e], Enzo A. Palombo [c], Thomas R. Yeager [b, d, f, *], Nivedita Datta [a, d]

[a] College of Health and Biomedicine, Victoria University, Werribee, Victoria 3030, Australia
[b] College of Engineering and Science, Victoria University, Werribee, Victoria 3030, Australia
[c] Faculty of Science, Engineering and Technology, Swinburne University of Technology, Hawthorn, Victoria 3122, Australia
[d] Institute for Sustainability and Innovation, Victoria University, Werribee, Victoria 3030, Australia
[f] Advanced Food Systems Research Unit, Victoria University, Werribee, Victoria 3030, Australia
[e] Murray Goulburn Co-operative Co Ltd, Leongatha, Victoria 3953, Australia

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ABSTRACT

Refrigerated storage of raw milk promotes the growth of psychrotrophic bacteria, some of which produce heat-stable exoenzymes causing dairy product spoilage. The effects of storage conditions and season on the biodiversity of psychrotrophs in raw milk were examined using matrix-assisted laser desorption time of flight mass spectrometry and 16S rRNA analysis. The ability of psychrotrophs to produce protease, lipase and phospholipase C was determined. The predominant genera found were *Pseudomonas* (19.9%), *Bacillus* (13.3%), *Microbacterium* (5.3%), *Lactococcus* (8.6%), *Acinetobacter* (4.9%) and *Hafnia* (2.8%); a considerable number of isolates were hitherto unknown species and genera. Diversity varied significantly ($P < 0.05$), depending on the storage temperature, time, initial microbiota and season. The predominant isolates showed significantly higher heat-stable exoenzyme activities after heating at 142 °C for 4 s. Improving the quality of milk products may require differential processing of raw milk depending on the type of microbiota present, storage temperature and seasonality.



## 1. Introduction

Raw milk provides an ideal medium for the growth of contaminating microorganisms due to its pH close to neutral value, high water and rich nutrient and mineral contents (Champagne et al., 1994). Microorganisms in bulk tank milk at the farming environment originate from the interior of the teat due to mastitis, via dirt attached to the teat surfaces, for example, cowshed, feed, bedding material and soil, and the surfaces of milking equipment (Vacheyrou et al., 2011; Vissers & Driehuis, 2009). Bacteria that are able to grow below 7 °C, regardless of their higher optimal growth temperature, are defined as psychrotrophs. These bacteria often account for <10% of the initial microflora in raw milk, especially under hygienic conditions; however, this figure can be >75% in situations of poor hygiene (Cousin, 1982; Hantsis-Zacharov & Halpern, 2007). The psychrotrophic bacterial count even in hygienically-produced raw milk depends on the storage temperature and time (Griffiths, Phillips, & Muir, 1987; Griffiths, Phillips, West, & Muir, 1988). A wide variety of genera including Gram-negative genera (*Pseudomonas, Aeromonas, Alcaligenes, Acromobactor Acinetobacter, Flavobacterium, Chryseobacterium, Enterobacteriaceae* such as *Serratia, Hafnia, Klebsiella, Enterobacter* and *Rahnella*) and Gram-positive genera (*Bacillus, Clostridium, Corynebacterium, Micrococcus Streptococcus, Staphylococcus, Microbacterium, Lactococcus and Lactobacillus*) are frequently found in raw milk (Quigley et al., 2013; Samarzija, Zamberlin, & Pogacic, 2012; Vithanage, Yeager, Jadhav, Palombo, & Datta, 2014). Of these, *Pseudomonas* and *Bacillus* are the most frequently isolated from refrigerated raw milk (Sørhaug & Stepaniak, 1997).

Raw milk produced in Australian farms is mostly held under refrigerated conditions for three to five days in the farm bulk tank before delivering to dairy processing plants. The extent of time

* Corresponding author. Tel.: +61 3 9919 8103.
*E-mail address:* thomas.yeager@vu.edu.au (T.R. Yeager).

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that raw milk is stored prior to processing may vary depending on the milk collection intervals and transport distances. Raw milk can also be stored under refrigerated conditions at the plant prior to processing. This practice can lead to the selection and outgrowth of psychrotrophic bacteria in raw milk. Failure to maintain the appropriate refrigeration conditions can even lead to changes in the population dynamics of raw milk microbiota (Lafarge et al., 2004).

During refrigerated storage, many of these psychrotolerant microorganisms are known to produce protease and lipases, which are able to survive at pasteurisation or even ultra-high temperature (UHT) treatment (Champagne et al., 1994; Chen, Daniel, & Coolbear, 2003; Marchand et al., 2009b; Sørhaug & Stepaniak, 1997). Moderate levels of these microbial enzymes may be beneficial for cheese manufacture due to the development of desirable flavours and aromas, but may be detrimental to other milk and dairy products, especially those with extended shelf-life (UHT milk and milk powders; Champagne et al., 1994). For example, residual proteolytic activity can lead to an increase in viscosity, induce a bitter flavour and cause gelation (Datta & Deeth, 2003), while lipolytic enzymes can result in rancidity due to the hydrolysis of milk fat (Chen et al., 2003) before the products complete their shelf-life.

Since it is difficult to inactivate these microbial enzymes by existing technological means, it is important to reduce the risk of their contamination in raw milk. Therefore, understanding the psychrotrophic microbial consortia present in raw milk prior to the processing and their spoilage potential under different optimal and suboptimal conditions are critical. Thus, the aim of the current study was to monitor the biodiversity of raw milk microbiota under different temperature conditions as well as in different seasons using analytical and molecular techniques. To evaluate those groups with the highest relevance to spoilage in the final products, the enzymatic characteristics of the bacteria were also evaluated.

## 2. Materials and methods

### 2.1. Sampling sites

Three commercial dairy farms (designated as A, B, and C) located in south-east Victoria, Australia, were selected for raw milk sampling and these samples were kindly provided by commercial dairy processor in Australia. Samples were taken under aseptic conditions and delivered to the laboratory in ice within 2–3 h of the milking procedure. Three samples per farm were analysed each month over the period of June 2013 to June 2014.

### 2.2. Storage conditions

Samples were stored at 2, 4, 6, 8 or 10 °C for 10 days in a refrigerated shaking incubator (Innova 4230, New Brunswick Scientific, Edison, NJ, USA) set at 120 rpm. Then, an increment trial was conducted, starting at 2 °C with a temperature increment of 1 °C every 24 h, to a maximum of 12 °C, to simulate the effect of chilling milk to low temperatures followed by suboptimal storage in an insulated silo and/or temperature fluctuations that occur during transportation (Griffiths et al., 1987). Triplicate samples were analysed daily, commencing from day 0, when the raw milk was collected, until day 10.

### 2.3. Enumeration of microorganisms

The samples were subjected to decimal dilutions with 0.1% (w/v) sterile peptone water (Sigma–Aldrich, Castle Hill, Australia) and plated on sterile plate count agar supplemented with 1.0% (w/v) skim milk (PCM agar) using the drop plate method (Munsch-Alatossava, Rita, & Alatossava, 2007) in triplicate. The plates were incubated at 7 °C for 10 days and 30 °C for 48 h for the enumeration of psychrotrophs and mesophilic bacteria, respectively. The thermoduric psychrotrophic count (TDC) was determined as described before, with minor modifications; briefly, the raw milk was heated at 63 ± 0.5 °C for 30 min and cultured on the PCM agar and incubated at 7 °C for 10 days (Buehner, Anand, & Garcia, 2014).

### 2.4. Isolation and preliminary identification of psychrotrophic bacteria

The diversity of the culturable psychrotrophic bacteria in raw milk was determined each month. An average of 20 bacterial colonies with distinct morphologies that grew on PCM plates after 10 days at 7 °C per farm per month were randomly selected and subcultured on nutrient agar (NA) to obtain pure cultures. Isolated colonies were subcultured on NA at least four times prior to Gram staining and microscopic examination of cell morphology. The phenotypic characteristics of bacterial isolates were determined using the tests including catalase (3.0%, w/v, $H_2O_2$), oxidase (1.0% tetramethyl *p*-phenylenediamine dihydrochloride), spore stain and growth on MacConkey agar (Smibert & Krieg, 1984). The isolated bacteria were finally grown in nutrient broth (Sigma–Aldrich, Castle Hill, Australia) and were then stored in 30.0% (v/v) glycerol at −80 °C and used as stock cultures in subsequent analysis.

American Type Culture Collection (ATCC) cultures including *Pseudomonas fluorescens* (ATCC 13525, 17386), *Bacillus cereus* (ATCC 10876, 10987, 14579), *Bacillus licheniformis* (ATCC 14580), *Bacillus subtilis* (ATCC 6633) and *Bacillus circulans* (ATCC 66) were used as positive controls.

### 2.5. Identification of isolates using matrix-assisted laser desorption time of flight mass spectrometry

Identification of isolates using matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) was conducted using the direct method; those which were not able to be identified by direct method were subsequently identified by the extraction method described by Anderson et al. (2012). MALDI-TOF MS identifications were made using the spectra obtained from samples of each isolate to develop a consensus spectrum. All spectra were compared using cluster analysis. As MALDI-TOF MS is able to discriminate isolates to the strain level, the identifications derived from the method were used to reduce the number of isolates for further analysis by 16S rRNA sequencing. The cluster analysis was conducted based on the freely-available web-based application, SPECLUST (http://bioinfo.thep.lu.se/speclust.html) (Alm et al., 2006). A hierarchical cluster analysis (HCA) was defined as a group of consensus spectra having spectral distances of $\leq$0.3. For each cluster, one or more isolates (depending on the cluster size) were selected and used in further investigation. The average linkage method was used to merge the two clusters with the smallest average of pairwise distances and the width in peak match score was set to 10 Da.

### 2.6. Identification of isolates using 16S rRNA gene sequencing and phylogenetic analysis

All representative isolates of each cluster (depending on the cluster size) were further identified using 16S rRNA gene sequencing using universal primers, as described previously (Vithanage et al., 2014). The sequence similarity was calculated using the maximum composite likely method (MEGA 6.0) (Tamura,

Nei, & Kumar, 2004; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), based on alignments from CLUSTAL W. A phylogenetic tree was constructed using the UPGMA method (Sneath & Sokal, 1973). The bootstrap values obtained were from 1000 iterations (<50.0%) (Felsenstein, 1985).

### 2.7. Screening for the enzyme production

All isolates were tested for proteolytic or lipolytic enzyme activity by agar diffusion assays at 7 °C for 10 days. Proteolytic and lipolytic enzyme production was tested using skim milk agar (1% skim milk powder, 0.5% yeast extract, 1.5% agar) and tributyrin agar (von Neubeck et al., 2015). The appearance of clearing zones around the colonies after 10 days at 7 °C was indicative of proteolysis. Formation of dark blue zones around the colonies was signified lipolytic activity. Production of phospholipase C was determined using egg-yolk agar which gives a white opaque zone of precipitation that spreads beyond the edge of the colony (Rossignol et al., 2008).

### 2.8. Heat stability of bacterial enzymes

The production of heat-stable extracellular enzymes by the predominant isolates in UHT skim milk was determined according to the method described by Vithanage et al. (2014). The extracellular enzymes were heated 142 °C for 4 s, followed by immediate storage in ice. The residual protease activity was determined using a fluorescein isothiocyanate casein (FITC) assay in a microtitre plate procedure (protease fluorescent detection kit; Sigma–Aldrich, Castle Hill, Australia) (Cupp-Enyard, 2009). The residual lipase in the raw milk was determined using *p*-nitrophenyl butyrate in acetonitrile as the substrate (Humbert, Guingamp, & Linden, 1997). Phospholipase C activity was determined colourimetrically using *p*-nitrophenylphosphorylcholine (NPPC) as the substrate (Sacherer, Défago, & Haas, 1994).

### 2.9. Statistical analysis

The association between mesophilic and psychrotrophic bacterial populations in raw milk samples was tested by Pearson correlation. Differences between the two kinds of populations and between the species isolated on different dates were tested by one-sample t test. Repeated analysis of variance was used to determine the significance of the differences between the bacterial populations on different sampling dates. Differences between species richness and enzymatic activities of isolates belonging to different taxa were calculated by means of $\chi^2$. The entire statistical analysis was conducted with SPSS for Windows (Version 21 software; IBM Corp. in Armonk, NY). The significance (*P* values) of each criterion was calculated by MANOVA, when appropriate and $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Differences in bacterial counts and their alterations under refrigerated storage

The average mesophilic count initially present in raw milk from three farms, as determined by total plate count (TPC), varied by more than two orders of magnitude and was in the range of $1.17 \times 10^4$ cfu mL$^{-1}$ to $1.34 \times 10^5$ cfu mL$^{-1}$, while the psychrotrophic bacteria counts (PBC) ranged from $5.75 \times 10^2$ to $6.72 \times 10^3$ cfu mL$^{-1}$ (Fig. 1). Thus, the PBC represented 4.91–5.01% (±6.7%) of the TPC. Also, the differences in the initial PBC of three types of milk samples were statistically significant ($F_{3, 15} = 4.56$ P = 0.0284).

### 3.2. Microbial biodiversity and relative abundance in raw milk

The diversity of the microbial consortia in raw milk collected from three farms was investigated between June 2013 and June 2014. Microbial population dynamics significantly varied among the three farms studied. The majority of isolates (n = 927) were able to grow at both 7 and 30 °C, indicating the ability of these bacteria to grow under low temperature conditions.

These isolates were initially identified using MALDI-TOF MS, but some of species remained unidentified (data not shown). A total of 604 representative isolates identified through analysis of MALDI-TOF MS spectra, including the unidentified isolates, were further identified using gold standard 16S rRNA sequencing. Phylogenetic analysis of these represented sequences indicated that these bacteria belonged to seven clusters representing the classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacilli*, *Actinobacteria*, *Flavobacteria* and *Sphingobacteria* (Fig. 2). Bacterial

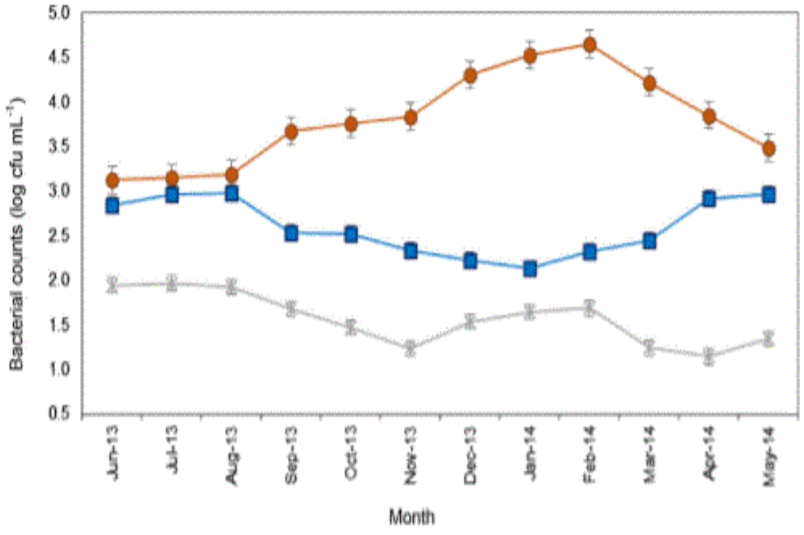


**Fig. 1.** Mesophilic (—●—) and psychrotrophic (—■—) and thermoduric (—▽—) bacterial population dynamics in raw milk sampled between June 2013 and May 2014. Each sampling month represents an average of samples from three farms (mean ± the standard error).

isolates from raw milk, sampled from three farms (A, B, and C) that were identified by 16S rRNA sequencing were listed in the Supplementary material Table S1.

Three predominant bacterial classes were detected, viz., *Gammaproteobacteria*, *Bacilli*, and *Actinobacteria*. On average, the largest group of isolates belonged to *Gammaproteobacteria* (including pseudomonads) at 42%, followed by *Bacilli* (32%) and *Actinobacteria* (15%). Only 2–3.5% of isolates belonged to other four classes. Although raw milk from all three farms comprised of those three predominant bacterial classes, each farm exhibited significantly different dominancy patterns between each class of bacteria ($\chi^2_{15} = 10.6367$, $P = 0.031$). For example, both sample A and C was dominated by *Gammaproteobacteria* and to a lesser extent *Bacilli* and *Actinobacteria*, with relative abundances of 82%, 8%, 6% and 52%, 34%, 10%, respectively. On the contrary, sample B was dominated by *Bacilli* with 44% (mainly Gram-positive cocci and lactic acid bacteria), followed by 28% *Gammaproteobacteria* (mainly non-fluorescent pseudomonades) and 16% *Actinobacteria* (Supplementary material Table S1).

The most abundant bacterial genera among the predominant bacterial classes were class *Gammaproteobacteria: Pseudomonas, Acinetobacter, Hafnia; class Bacilli*: *Bacillus, Paenibacillus, Leuconostoc, Lactococcus, Streptococcus, Staphylococcus, Enterococcus; class Actinobacteria: Microbacterium, Micrococcus* and *Rhodococcus* (Fig. 2). Of these, *Pseudomonas* exhibited the highest biodiversity, followed by the genus *Bacillus*, in which 33 and 29 species, respectively, were detected.

Among the members of the genus *Pseudomonas*, fluorescent pseudomonads (*P. fluorescens, Pseudomonas gessardii, Pseudomonas poae, Pseudomonas proteolytica, Pseudomonas Brennerii* and

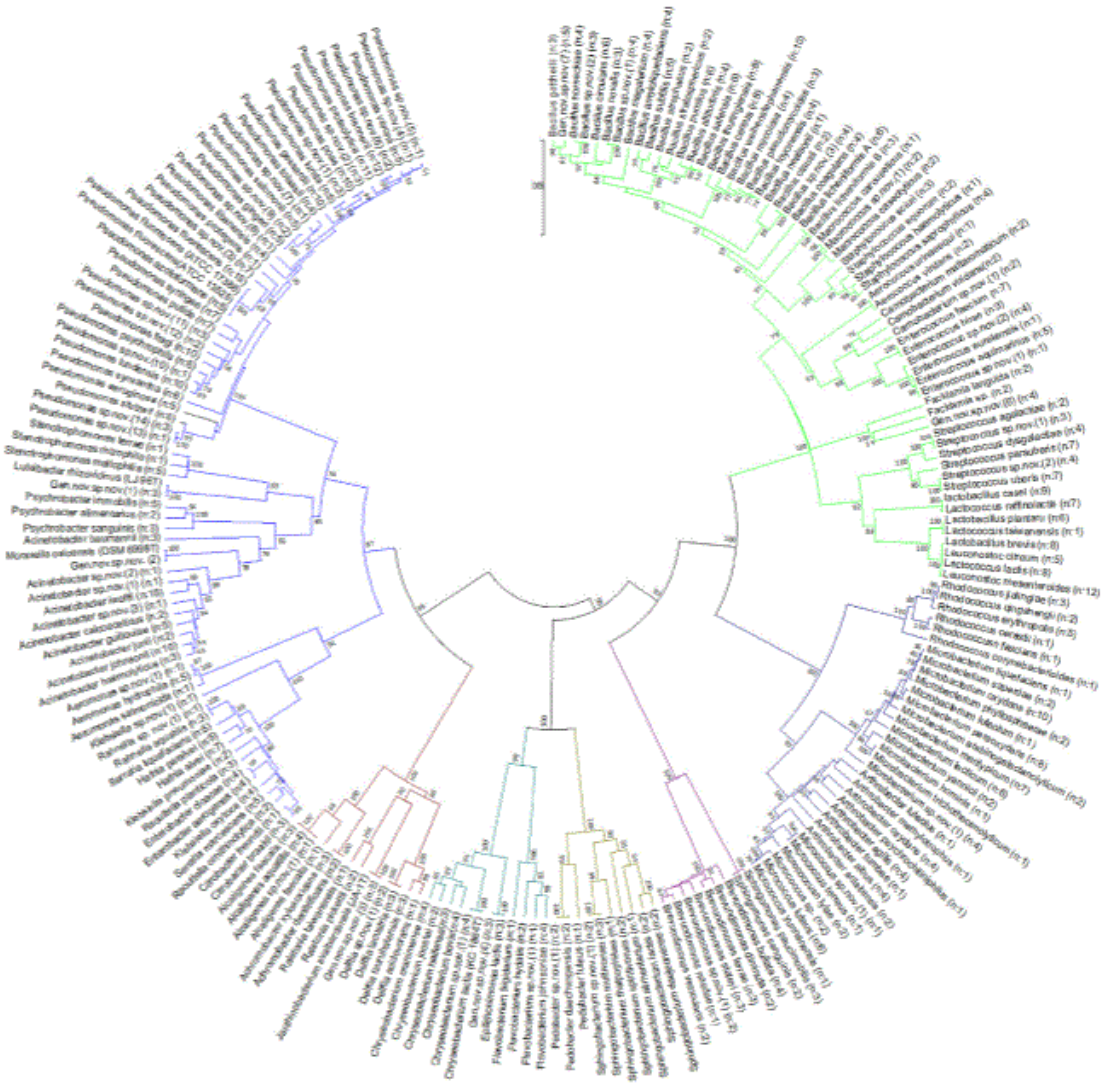

**Fig. 2.** Phylogenetic tree constructed using 16S rRNA gene sequences of predominant raw milk isolates. Bootstraps (%) are based on the 1000 replications and percentages of 50% or more are indicated at the branch point. Scale bar, 0.1 substitutions per site. Different colours indicate 16S rRNA gene sequences of different bacterial classes of spoilage bacteria obtained from the current study.

*Pseudomonas veronii*) and non-fluorescent pseudomonads (*Pseudomonas fragi*, *Pseudomonas lundensis*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Pseudomonas psychrophila* and *Pseudomonas syringae*) were isolated frequently from raw milk.

In addition to pseudomonads, a relatively large proportion of *Acinetobacter* spp. (*Acinetobacter johnsonii*, *Acinetobacter iwoffii*, *Acinetobacter baumannii* and *Acinetobacter guillouiae*) were detected with varied proportions. Among the enteric isolates, *Hafnia alvei*, *H. paralvei*, *Serratia liquefaciens*, *Serratia marcescens*, *Klebsiella oxytoca*, *Klebsiella pneumonia* and *Enterobacter aerogenes* were the most detected species, especially in samples B and C.

Psychrotropic bacilli that were frequently isolated from raw milk include *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephensis*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus subtilis*. Additionally, lactic acid bacteria (*Lactococcus lactis*, *Lactococcus raffinolactis*, *Lactobacillus fermentum* and *Leuconostoc mesenteroides*) and Gram-positive cocci (*Streptococcus*, *Enterococcus* and *Staphylococcus* species) were frequently isolated, especially from samples B and C.

Four bacteria belong to the class *Actinobacteria* were often isolated from raw milk; they include *Microbacterium oxydans*, *Microbacterium lacticum*, *Microbacterium maritypicum*, *Micrococcus* spp., *Arthrobacter* spp. and *Rhodococcus* spp. Species belonging to the genera *Aeromonas*, *Stenotrophomonas*, *Psychrobacter*, *Rahnella*, and *Raoutella* were isolated rarely from raw milk.

### 3.3. Effects of extended refrigerated storage conditions on the biodiversity of raw milk

Samples were stored at 2, 4, 6, 8, or 10 °C and 2–12 °C for 10 days to simulate the optimal and suboptimal conditions that could possibly occur in the dairy farming and processing environment. This resulted in significant changes ($P < 0.01$) in the microbial population (Fig. 3). The diversity of the raw milk microbiota was substantially increased when the raw milk samples were stored at the temperatures ≥6 °C (i.e., 6 °C, 8 °C, 10 °C: $F_{3, 15} = 22.58$, 46.7 and 57.2; for all $P < 0.0001$). Bacteria belonging to genus *Pseudomonas*, *Bacillus* and *Micrococcus* accounted for the majority of microbiota after 10 days storage, depending on the temperature applied. As the storage temperature decreased to 2 °C and 4 °C, only certain *Pseudomonas* spp. (mainly *P. fluorescens* group), and certain *Bacillus* isolates (*B. cereus*, *B. weihenstephensis* and *B. circulans*) increased with a concomitant reduction in the levels of *Streptococcus*, *Enterococcus*, *Staphylococcus*, lactic acid bacteria and enteric bacteria (*Hafnia*, *Rahnella*, *Klebsiella*, *Enterobactor*, and *Serratia*). The emergence of potentially pathogenic and spoilage bacteria (*Aeromonas*, *Stenotrophomonas* and *Listeria*) was observed even at 4 °C after 72 h, but not at 2 °C. In contrast to the low temperature storage, temperatures above 6 °C facilitated the outgrowth of mesophilic microorganisms such as *Lactobacilli*, *Enterococcus*, *Streptococcus*, *Micrococcus*, *Serratia*, *Klebsiella*, *Escherichia* and *Stenotrophomonas*. Intermediate storage temperatures (2–12 °C), resembling the conditions that occurred in insulated milk tanks, resulted in increased levels of psychrotrophic bacteria, and the overall trend was appeared to be in between 8 °C and 10 °C storage.

### 3.4. Effect of seasonal variation on the raw milk microbiota

The levels of psychrotrophic and mesophilic counts of raw milk in various seasons were significantly different (*t* test: $t_A$, $t_B$, $t_C$: 3.67, 4.52, 6.87; for all $P < 0.001$). The mesophilic counts increased noticeably in summer (December–February), whereas psychrotrophic counts were at their peak level in winter (June–August) and to a lesser extent in spring (September–November). The PBC was at its lowest in summer months ($P < 0.05$) (Fig. 1). The counts of thermoduric psychrotrophic bacteria (TDC) were highest during the summer (Fig. 1).

The diversity of the microbiota present in all raw milk samples appeared to be markedly different ($P < 0.05$) depending on the season. The average of species richness in different seasons is presented in Fig. 4. Psychrotrophic bacterial genera belonging to the class *Gammaproteobacteria* (*Pseudomonas*, *Acinetobacter* and *Psychrobacter*) and certain *Bacillus* species (*B. cereus*, *B. weihenstephensis* and *B. circulans*) were more prevalent during the winter season. On the contrary, the incidence of other *Gammaproteobacteria*, mainly enteric bacteria (*Hafnia*, *Serratia*, *Stenotrophomonas*, *Klebsiella* and *Enterobactor*) and non-fermenters (*Acinetobactor*, *Pseudomonas*, *Aeromonas*, *Brevundimonas* and *Pseudoalteromonas*) were significant in spring and autumn. The majority of *Bacillus* isolates (*Bacillus thurigiensis*, *B. licheniformis*, *B. pumilus* and *B. subtilis*) and *Paenibacillus* sp. were prevalent in numbers during the summer months. *Actinobacteria* (*Microbacterium*, *Micrococcus*, *Rhodococcus* and *Arthrobacter*) were dominant in autumn (Fig. 4). The remaining four groups, namely

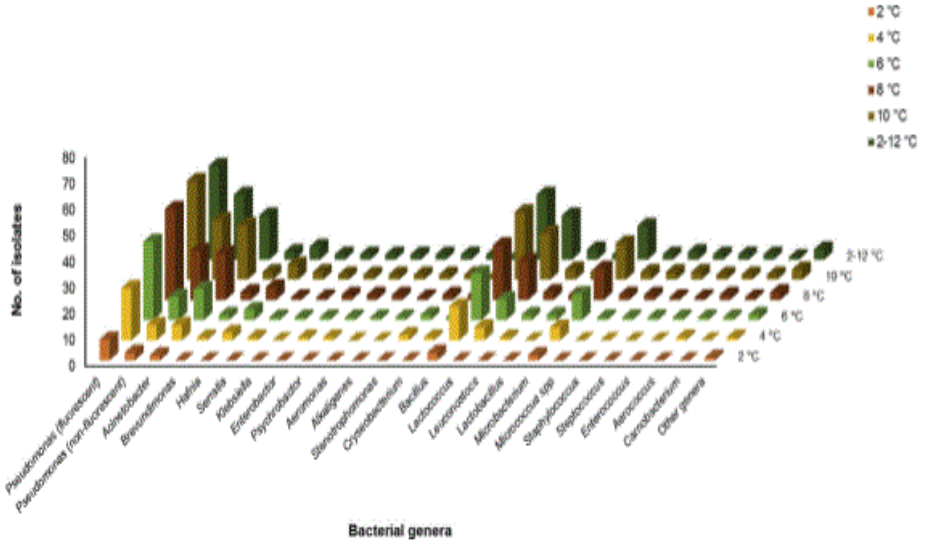


**Fig. 3.** Predominant bacterial genera isolated from raw milk from three farms under various storage conditions.

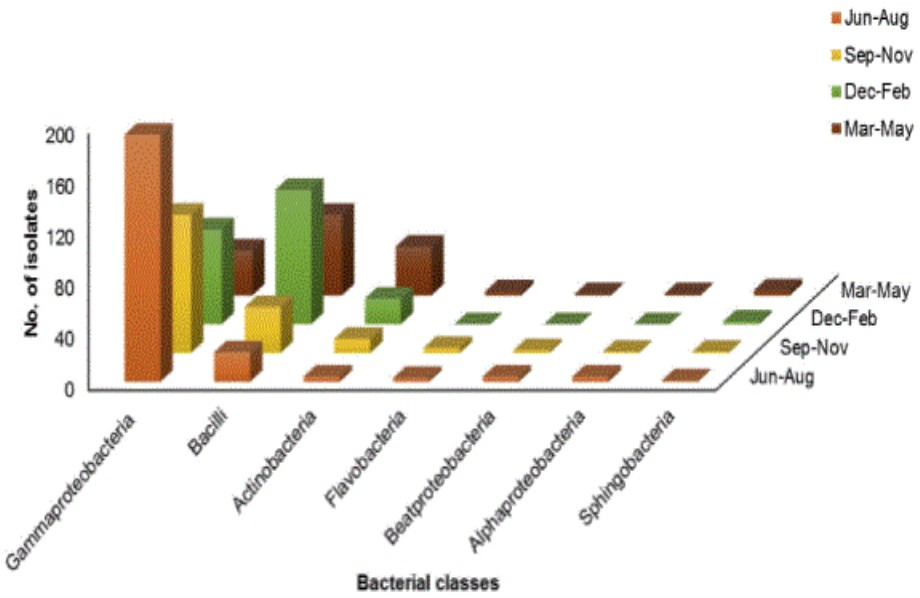


**Fig. 4.** The average number of species in every bacterial classes isolated on different months corresponding to the different seasons.

*Sphingobacteria*, *Alphaproteobacteria*, *Betaproteobacteria* and *Flavobacteria* were only detected sporadically throughout the year at low levels.

### 3.5. Potential novel species and genera in raw milk

A considerable fraction (74 out of 604) of the raw milk isolates were not able to be assigned to a species level due to their lower homology (<98%) in the 16S rRNA gene sequences of the closet relative in the GenBank/RDP databases. Of these, eleven isolates showed identity below 95% to the closet relative in the GenBank/RDP databases. The delineation of these isolates may require additional physiological and chemotaxonomic markers, as substantial differences in the 16S rRNA gene of a known species or genera are a strong indication for a hitherto unknown species (<98%) or genera (<95.0%). Interestingly, these potential novel species were detected across all bacterial classes; *Gammaproteobacteria*: 31; *Bacilli*: 20; *Flavobacteria*: 7; *Actinobacteria*: 7; *Betaproteobacteria*: 4; *Alphaproteobacteria*: 3; and *Sphingobacteria*: 2 (Supplementary material Table S2). The distinct phylogenetic positions of these isolates within the corresponding genus or related genera were confirmed using the neighbour joining method and calculated using the sequences of type strain of related species as references (Fig. 2). A total of 42 novel species and eleven novel genera were assigned using the sequences generated in the current study, which is approximately 27% of all species and 13% of all isolates. The presence of these novel species in more than one sample indicates a relatively frequent occurrence.

### 3.6. Enzymatic characteristics of raw milk microbiota

All the isolates were screened for their ability to produce exoenzymes (protease, lipase and phospholipase C) at 6 °C using agar diffusion assays. The expression of spoilage enzymes was absent in 14.2% of the isolates, while 12.1% showed no growth at 6 °C. The potential enzymatic activities were present in the raw milk isolates with varying combinations and expressional levels (Fig. 5). Representatives of the genus *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Hafnia* and *Microbacterium* were not only the most predominant isolates, but also the most enzymatically active genera. Approximately 56.3% of the fluorescent *Pseudomonas* isolates (*P. gessardii*, *P. proteolytica*, *P. poae*, *P. veronii* and some *P. fluorescens*) showed strong proteolytic activity, while 21.6% (*P. fluorescens*, *Pseudomonas salomonii*, and *P. veronii*) and 13.8% (*P. fluorescens*) exhibited lipase and phospholipase C activities, respectively. Of the proteolytic *P. fluorescens*, 64% were able to produce lipase, while 52% of the lipolytic strains were able to produce phospholipase C. In contrast, the non-fluorescent pseudomonads (*P. fragi*, *P. lundensis*, *P. syringae*, *P. stutzeri*, *P. putida* and *Pseudomonas azotoformans*) were highly lipolytic, while some species of these isolates showed both proteolytic and lipolytic activity. The *Bacillus* spp. were strongly proteolytic and lipolytic, and these species also showed comparatively high phospholipase C activity. The majority of the *Microbacterium* spp. showed strong lipolytic and proteolytic activities. Gram-negative bacteria, such as *Hafnia*, *Stenotrophomonas*, *Serratia*, *Aeromonas* spp. displayed a very strong tendency to produce proteases rather than lipases, whereas *Psychrobacter*, *Brevundimonas*, *Acinetobactor*, *Enterobactor* and *Alcaligenes* showed relatively high lipolytic, but weak proteolytic activities and phospholipase C activity with varying proportions. In general, about 87% of lactic acid bacteria (*Lactococcus*, *Leuconostoc* and *Lactobacillus*) grew at 6 °C; however only 15% of them showed no enzymatic activity and the expression of all exoenzymes in these bacteria was almost absent or minimal. The activity of all exoenzymes was minimal in Gram-positive cocci genera such as *Staphylococcus*, *Streptococcus*, *Enterococcus*, Aerococcus and Carnobacterium. However, *Flavobacteria*, including *Chryseobacterium* and *Flavobacterium* spp., showed variation in their enzymatic activities, depending on the species. Sample C contained significantly higher number of bacteria with a high spoilage potential. Although, sample A had relatively low PBC, the proportion of bacteria with spoilage potential was higher bacteria were higher than that than that in sample B.

### 3.7. Heat stability of extracellular enzymes of selected raw milk isolates

The extracellular enzymes of the predominant raw milk isolates were heated at 142 °C for 4 s to mimic conditions often used in

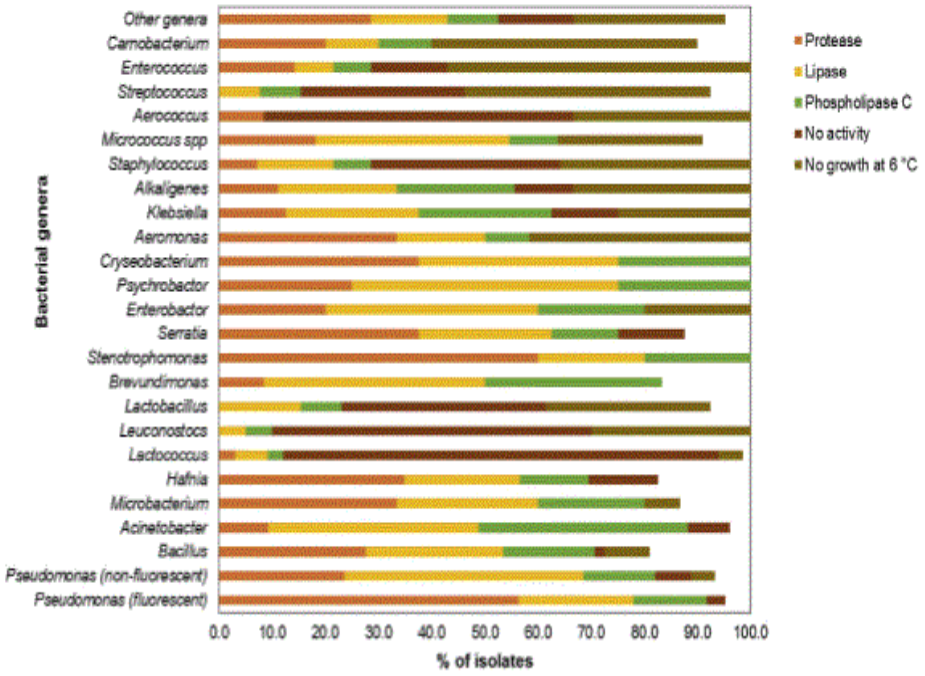


**Fig. 5.** Ability of raw milk isolates to produce protease, lipase, and phospholipase C enzymes, and inability of producing enzymes and growth at 6 °C by raw milk isolates.

commercial UHT milk processing. The residual protease, lipase and phospholipase C activities were determined using colourimetric and/or fluorescent assays. *Pseudomonas*, *Bacillus* and *Microbacterium* isolates retained 50–75% activity after the heat treatment. The enzymes from a variety of isolates showed significant variation after the heat treatment (Table 1).

## 4. Discussion

Psychrotrophic bacteria play a major role in spoilage of milk and dairy products due to the production of heat-stable enzymes (Champagne et al., 1994). The results of the current study excluded the contribution of non-cultivable and strictly anaerobic bacteria. Besides the bacteria causing enzymatic spoilage, a broader spectrum of enzymatically active and/or technologically relevant microorganisms in raw milk were evaluated.

The initial mesophilic and psychrotrophic counts in all three raw milk samples were in the range of $10^3$–$10^5$ and $10^2$–$10^3$ cfu mL$^{-1}$, respectively. The counts of thermoduric microbes with the capacity to grow under refrigeration conditions were below $10^3$ cfu mL$^{-1}$. According to the guidelines of the Pasteurised Milk Ordinance (PMO), premium quality raw milk must contain < $10^5$ cfu mL$^{-1}$ of mesophilic and psychrotrophic bacteria, as the major quality criteria. However, the European Union standards also require psychrotrophic counts of < $10^3$ cfu mL$^{-1}$ as a technological requirement (Cempirkova, 2002). The thermoduric counts, which are critical for pasteurisation and UHT milk processing, also required to be < $10^4$ cfu mL$^{-1}$ (Buehner et al., 2014). Overall, this indicates that the raw milk from three farms investigated in this study met all quality criteria and commercial standards, with the exception of TPC for farm C. Nevertheless, the differences in the average initial microbial counts were significant between the farms ($P < 0.05$). This is most probably attributed to the differences in the individual farm management systems, or suboptimal conditions that may have occurred during transport and storage (Cempirkova, 2002, 2007).

The extended storage of raw milk under various optimal and suboptimal conditions that could have possibly occurred in the farming and processing environments has markedly affected both microbial counts and diversity at the end of the cold storage. Both bacterial counts and microbial diversity were highly significantly different between the farms ($P < 0.0001$). This effect was greatest within 2–3 days of storage at ≥ 6 °C or after 4 days of storage at 4 °C, depending on the farm. These results are consistent with previous studies (Griffiths et al., 1987, 1988; Haryani, Datta, Elliott, & Deeth, 2003; Lafarge et al., 2004). Therefore, improving the

**Table 1**
Percentage of enzymes produced from predominant raw milk isolates showing enzyme activity before and after heat-treatment.[a]

| Genera | Before heat-treatment | | | After 142 °C, 4 s treatment | | |
|---|---|---|---|---|---|---|
| | Protease | Lipase | Phospholipase C | Protease | Lipase | Phospholipase C |
| *Pseudomonas* | 62.5$^a$ | 48.9$^a$ | 47.2$^a$ | 58.3$^a$ | 34.5$^a$ | 28.9$^a$ |
| *Bacillus* | 53.3$^a$ | 41.5$^a$ | 27.5$^a$ | 50.1$^a$ | 38.7$^a$ | 24.4$^a$ |
| *Microbacterium* | 70.2$^a$ | 57.4$^a$ | 57.4$^a$ | 65.2$^a$ | 53.2$^b$ | 37.4$^b$ |
| *Lactococcus* | 5.3$^c$ | 7.9$^c$ | 0.0 | 1.3$^c$ | 1.0$^c$ | 0.0 |
| *Acinetobacter* | 25.6$^b$ | 83.7$^a$ | 58.1$^a$ | 19.8$^a$ | 76.8$^b$ | 48.3$^b$ |
| *Hafnia* | 40.0$^b$ | 16.0$^c$ | 12.0$^c$ | 34.2$^b$ | 15.4$^c$ | 12.3$^c$ |

[a] Means significance levels by t-test shown by $^a$ $P < 0.001$; $^b$ $P < 0.05$; $^c$ $P > 0.05$.

quality of finished products with concomitant processing flexibility would require raw milk to be processed based on the storage temperature and time.

Three main classes of bacteria (*Gammaproteobacteria*, *Bacilli* and *Actinobacteria*) were isolated frequently from all three samples with 94.8% of the bacterial genera belonging to these classes and 30 species accounting for 85% of all isolates. Similar findings have been reported by several studies using culture-dependant and culture-independent approaches (Hantsis-Zacharov & Halpern, 2007; Lafarge et al., 2004; Quigley et al., 2013; von Neubeck et al., 2015). However, the pattern of dominant bacterial species varied significantly in the samples from different farms.

Phylogenetic analysis based on partial sequencing of 16S rRNA gene grouped the raw milk microbiota into seven different clusters. Each class of bacteria comprised different sub-clusters based on the genus and species. The class *Gammaproteobacteria*, mainly the genus *Pseudomonas*, accounted for the most predominant part of the raw milk microbiota. Interestingly, fluorescent and non-fluorescent pseudomonads were grouped in two separate sub-clusters, as observed before (Marchand et al., 2009b; Vithanage et al., 2014). Both fluorescent and non-fluorescent pseudomonads are considered as the major cause of spoilage of milk and other food products (Ercolini et al., 2007; Marchand et al., 2009b). In addition to the *Pseudomonas* spp., other *Gammaproteobacteria* such as *Acinetobacter*, *Aeromonas*, *Stenotrophomonas*, *Hafnia*, *Rahnella* and *Serratia* were often detected in raw milk, as reported previously (Quigley et al., 2013; Vithanage et al., 2014; von Neubeck et al., 2015).

The second most predominant bacterial class found in raw milk was the *Bacilli*, comprising genera of *Bacillus*, *Paenibacillus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, and *Staphylococcus*. In particular, the genus *Bacillus* contributed the second most to species richness after *Pseudomonas*. They were grouped in different clusters based on the species. The most frequent milk borne *Bacilli* were members of the *B. cereus* group, viz., *B. weihenstephensis*, *B. thuringiensis*, and to a lesser extent *B. pumilus*, *B. subtilis* and *B. licheniformis*. Among the *Paenibacillus* spp., *P. polymyxa*, *P. odorifer* were isolated frequently from raw milk. These bacteria have been previously described as mesophilic or thermophilic microorganisms; however we found that they were able to survive refrigerated conditions below 8 °C. This suggests that psychrotolerance is likely to be a common characteristic in *Bacilli*, which has not yet been fully explored.

The third predominant bacteria isolated from raw milk was the class *Actinobacteria*, mainly *Microbacterium* spp. Raw milk also comprised considerable amounts of *Micrococcus*, *Rhodococcus*, *Aerococcus* and *Rothia* spp., that were classified in the class *Actinobacteria*. In addition to the most predominant bacterial classes, members of the class *Flavobacteria*, including *Chryseobacterium* and *Flavobacterium*, were isolated frequently from raw milk.

Growth of these raw milk microbiota differed under various refrigeration conditions. This is possibly related to their growth rate under the corresponding conditions, as well as their initial counts prior to the refrigerated storage (Griffiths et al., 1987; McKellar, 1989; Muir, 1996a, 1996b). The genus *Pseudomonas* comprises species with the shortest generation time at 0–7 °C (Chandler & McMeekin, 1985; Sørhaug, 1992). In contrast, psychrotrophic bacilli possess longer generation times and lag phase duration at 2–7 °C compared with *Pseudomonas* spp. (Chandler & McMeekin, 1985). Thus, Gram-negative psychrotrophs, mainly *Pseudomonas* spp., can become most competitive psychrotolerant microorganisms under regular refrigerated conditions, and can invariably outgrow other bacteria (Hibbing, Fuqua, Parsek, & Peterson, 2010). In the present study, non-fermentative microorganisms like *Aeromonas*, *Psychrobacter*, *Stenotrophomonas* and enteric bacteria were observed after 4–5 days of storage at 4 °C, and even after 2–3 days, as the temperature increased to ≥6 °C, depending on the sample used. Furthermore, growth of other Gram-positive bacteria like *Lactobacilli*, *Enterococcus*, *Streptococcus*, *Micrococcus* and certain Gram-negative genera belonging to family *Enterobacteriaceae* was enhanced at temperatures above 6 °C. Another study observed that the contaminant microbiota in raw milk reached > $10^8$ cfu $mL^{-1}$ after 4 days of incubation at 7 °C, in which the presence of *Acinetobacter* spp., *P. fluorescens and S. marcescens* was detected on the fourth day of incubation (Machado, Bazzolli, & Vanetti, 2013). In general, the data of the current study was in an agreement with previous descriptions, which observed changes in microbial population under various refrigerated conditions by both culture-dependant (Griffiths et al., 1987) and culture-independent approaches (Lafarge et al., 2004). Notwithstanding, these studies have been used only limited temperature profiles, compared with the current study. Furthermore, the microbial composition appeared to vary depending on the season. This is consistent with previous studies (Buehner et al., 2014; Hantsis-Zacharov & Halpern, 2007; Lafarge et al., 2004; Marchand et al., 2009a), which may be related to the optimum growth temperature of these bacteria (Griffiths et al., 1987). For example, *Bacilli* possess relatively high temperature optima, which may contribute to their higher counts in summer, while *Gammaproteobacteria*, i.e., *Pseudomonas*, *Acinetobacter* and *Psychrobacter* have relatively lower optimal temperatures and were seen to predominate in winter months. Alternatively, the feeding patterns of cows during the summer (grazing) and winter seasons (silage) may have contributed to the diversity of the microbial populations in raw milk. Thus, spoilage of milk by *Gammaproteobacteria* could be common in winter months, while *Bacilli*-associated spoilage could be more common in the summer months. Since seasonal variation cannot be avoided, improved cleaning procedures need to be implemented at the farm level to minimise bacterial contamination depending on the season.

A relatively large proportion of contaminating bacteria can be transferred from stable to milking parlour, then to milk, depending on their potential sources at the farm (Vacheyrou et al., 2011) and different farm management systems (Mallet et al., 2012). For example, the presence of pseudomonads and enteric bacteria in raw milk, implies intensive washing cycles of milking equipment (Richard, 1981), whereas the presence of higher *Bacillus* counts in raw milk is often related to temperature abuse conditions in the farm bulk tank (Meer, Baker, Bodyfelt, & Griffiths, 1991; Stepaniak, 1991). The majority of these bacteria are involved in biofilm production, thus can be disseminated via milking equipment, poor hygienic conditions used in the milking process as well as mastitis and can lead to increase the numbers of *Micrococcus*, *Arthrobacter*, *Microbacterium*, *Brevibacterium*, *Staphylococcus*, *Lactobacillus* and *Lactococcus* (Desmasures, Opportune, & Guéguen, 1997). Most importantly, time and temperature combinations of raw milk storage profoundly effects the numbers and the diversity of microbial populations (De Jonghe et al., 2011; Griffiths et al., 1987, 1988; Haryani et al., 2003; Lafarge et al., 2004). To understand the population dynamics of microbial consortia in raw milk, their potential sources at the farm and their effects during storage in commercial silos it may require extensive sample analysis with an active surveillance program using a combination of the molecular and proteomic approaches used in this study.

Despite the fact that microbial diversity has been extensively explored over recent decades, potential novel species based on <98.0% identity to the 16 S rRNA gene (Kim, Oh, Park, & Chun, 2014) have been reported in the current study as well as in several studies described before (De Jonghe et al., 2011; Fricker, Skanseng, Rudi, Stessl, & Ehling-Schulz, 2011; Hantsis-Zacharov & Halpern, 2007; Schmidt, Kaufmann, Kulozik, Scherer, & Wenning, 2012; Vaz-

Moreira et al., 2011; von Neubeck et al., 2015). This highlights that raw milk microbiota is rather underexplored. The lower relative abundance of certain species in raw milk can make it challenging to completely characterise the raw milk microbiota profile. It was nevertheless surprising that some of the novel species may be underdetermined even with novel molecular approaches such as next-generation-sequencing or cloning of 16S rRNA gene sequences (Janda & Abbott, 2007).

Bacteria isolated from raw milk were able to produce cold-active exoenzymes such as protease, lipase, and phospholipase C. Spoilage by psychrotrophic bacteria is often proteolytic and lipolytic in nature, which is possibly the most frequent cause of milk spoilage (von Neubeck et al., 2015). In the present study, species of *Pseudomonas* (mainly *P. fluorescens* group), *Bacillus*, *Stenotrophomonas*, *Serratia*, *Aeromonas*, *Chryseobacterium*, *Hafnia* and *Microbacterium* exhibited strong proteolytic potential, as described before in some studies (Hantsis-Zacharov & Halpern, 2007). Other species of *Pseudomonas* (mainly non-fluorescent pseudomonads), *Bacillus*, *Microbacterium*, *Chryseobacterium*, *Enterobacter*, *Psychrobacter*, *Brevundimonas* and *Acinetobacter* were strongly lipolytic, as has also been observed before (Hantsis-Zacharov & Halpern, 2007). According to Hanamant & Bansilal, 2013 only a limited number of Gram-positive cocci isolates, namely *M. luteus*, *Serratia equorum*, *K. rosea* and *Kocuria* spp., showed lipolytic activity.

The proteolytic and lipolytic potential of the genus *Pseudomonas* has been extensively studied. The gene encoded for the extracellular metalloprotease in pseudomonads is *aprX* (also known as *aprA*) (Loper et al., 2012; Marchand et al., 2009b). This gene is located at the beginning of a polycistronic operon with the *lipA* gene that encodes an extracellular lipase (Burger, Woods, McCarthy, & Beacham, 2000; Hassan et al., 2010; McCarthy, Woods, & Beacham, 2004; Woods, Burger, Beven, & Beacham, 2001). Therefore, it is obvious that *Pseudomonas* spp. have both proteolytic and lipolytic activity, probably due to simultaneous expression. However, expression of these enzymes can be tightly regulated (Blumer, Heeb, Pessi, & Haas, 1999; Heeb & Haas, 2001; Reimmann et al., 1997), which may be attributed to the absence of one or both the enzymes in some isolates. Therefore, intra and inter species variation in protease and lipase activity is possible, as observed in the present study, which is, possibly related to genetic and/or expression variability. Consistent with previous studies, *Acinetobactor* species were found with strongly lipolytic, and but with minor to moderate levels of proteolytic activity.

In the present study, it was observed the production phospholipase C enzyme by certain species of *Bacillus Pseudomonas*, *Serratia*, *Acinetobacter*, *Aeromonas*, *Flavobacteria* and *Microbacterium* spp. which is in an agreement with previous studies (McKellar, 1989; Titball, 1993).

Representatives of the predominant raw milk isolates showed >50% residual enzymatic activity after heating at 142 °C for 4 s, with varying intensities. Similarly, the heating of bacterial enzymes by high-temperature short time pasteurisation (HTST: 72 ± 0.5 °C for 15 s), low-temperature long time pasteurisation (LTLT: 63 ± 0.5 °C for 30 min) resulted in >85% residual activity (data not shown), highlighting the highest possibility of these enzymes to cause spoilage in pasteurised dairy products. This is consistent with previous studies that observed the ability of these enzymes to withstand either pasteurisation or UHT heat treatment, and underpinning their potential for spoilage of pasteurised and UHT milk products (Champagne et al., 1994; Koka & Weimer, 2001).

Extended studies conducted by the authors revealed that these heated enzymes can markedly affect UHT milk stability, functional properties and hence the UHT milk quality (Unpublished results). It was also previously observed that these enzymes have been involved in the production of various off-tastes, putrefaction and gelation (Champagne et al., 1994; Koka & Weimer, 2001). The bacterial proteolytic enzymes can hydrolyse β and κ casein fractions leading to complete hydrolysis of milk proteins at the end of the storage period (McKellar, 1989; Varnam & Sutherland, 1984). The bacterial lipolytic enzymes are associated with hydrolysis of free fat; however, these enzymes have the potential to hydrolyse milk fat even of intact membranes (Fitz-Gerald & Deeth, 1983). Bacterial phospholipase C can hydrolyse phosphatidylcholine and other phospholipids in the milk fat globule membrane (McKellar, 1989). A classic example of bacterial contamination that results in 'bitty' spoilage of the cream layer is the production of extracellular phospholipase C (lecithinase) by *B. cereus*. However, in practice, soft clotting may be a more common form of spoilage (Meer et al., 1991).

In a parallel study, raw milk samples were subjected to HTST (72 ± 0.5 °C for 15 s), LTLT (63 ± 0.5 °C for 30 min) pasteurisation conditions and UHT treatment (142 °C for 4 s) to determine the effect of these conditions on microbial diversity (data not shown). It has been described that these heating processes can activate endospores causing germination during the storage of milk and dairy products (Anzueto, 2014; Champagne et al., 1994). In the present study, the pasteurisation conditions resulted in survival of heat-stable endospores (*Bacillus* and *Paenibacillus*), and heat-resistant vegetative cells (*Microbacterium*, *Enterococcus* and *Micrococcus*), while UHT processing resulted in survival of certain spore-forming *Bacillus* and *Paenibacillus* spp. (Unpublished results). Thermoduric bacteria have long been recognised as the major cause of flat-sour spoilage role in low-acid foods (Champagne et al., 1994). These two groups of bacteria are recognised as the most important technologically relevant microorganisms in milk processing (Chauhan et al., 2013; Scheldeman, Herman, Foster, & Heyndrickx, 2006).

More importantly, sample C contained higher level of both Gram-negative psychrotrophs and thermoduric psychrotrophs with higher heat-stable enzymatic potential. Sample A contained higher amounts of Gram-negative psychrotrophic bacteria with higher heat-stable enzymatic activities. In contrast, sample B contained Gram-negative psychrotrophs as well as thermoduric bacteria such as *Enterococcus* and *Micrococcus* with limited enzymatic potential. Therefore, sample C may not be suitable for production of both pasteurised and UHT milk due to the presence of bacteria with higher heat-stable enzyme production and/or spore production. The UHT milk produced using sample A can be affected by heat-stable bacterial enzymes during the extended storage under ambient temperature however it can be suitable for the production of shorter shelf-life dairy products (Barbano, Ma, & Santos, 2006; Samarzija et al., 2012). On the contrary, sample B may not be suitable for the production of pasteurised milk due to the presence of thermoduric vegetative bacteria, but that can be used for the production of UHT milk, as these bacteria had limited enzymatic activities. These observations further highlight that diversity of bacteria plays a significant role in maintaining the quality of these milk products, thus, raw milk can be recommended for processing based on the individual microbial populations. Therefore, it is important to determine the diversity of raw milk microbiota using a rapid and reliable method, prior to mixing in bulk tanks in dairies. Similarly, it is important to establish hygienic precautions based on the sources at the farm level by identifying the critical control points (CCPs) and implementing CCPs at various points of the production process according to standards and regulations of the food industry.

Moreover, the presence of pathogenic spore formers, like *B. cereus* and *B. licheniformis* can withstand UHT treatment and cause foodborne illnesses upon germination after the processing (Champagne et al., 1994). Especially, presence of *B. cereus* with the counts above $1.0 \times 10^4$ cfu $mL^{-1}$ are known to be unsafe for human consumption due to the production of toxins, and some of these

toxins are also known to be heat-stable (Zwietering, de Wit, & Notermans, 1996). It has also been hypothesised that the emergence of psychrotrophic bacteria during cold storage can cause chronic diseases like Crohn's disease (Hugot, Alberti, Berrebi, Bingen, & Cezard, 2003).

## 5. Conclusions

The cold chain of storage and transportation together with seasonal and temperature fluctuations can result in significant changes in the microbial composition of raw milk. The presence of novel species and genera in the milk microbiome highlights that there is still a great deal to be studied and many potential novel bacteria to be characterised. Although sophisticated farm management systems reduce the initial raw milk microbial loads, the presence of thermoresistant enzymes, like protease and lipase, originating from psychrotrophs will be the limiting factor in maintaining the quality and the shelf life of milk and dairy products. The predominant bacteria, such as *Pseudomonas*, *Bacillus* and *Microbacterium* spp., account for >85% of the total microbial population and these were found to produce heat-stable enzymes. Proteases, lipases and, to a lesser extent, phospholipase C need to be given particular attention with respect to the relative abundance of the originating microbes, intensity of enzyme expression and extent of heat-stability. To improve the quality of milk products it may require differential processing of raw milk depending on the microbiota present. In this respect, it is also important to develop more sensitive and efficient tools to rapidly monitor the microorganisms with higher spoilage potential. Thus, the heat-stability and molecular characteristics of secreted enzymes and the development of novel screening methods need to be addressed in future studies. Overall, producing superior quality fluid milk and dairy products with longer shelf life may facilitate the distribution of those products over wider geographic regions and through new commercial channels, resulting in overall benefits to the dairy industry.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.idairyj.2016.02.042.

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# CHAPTER 6: IMPORTANCE OF PSYCHROTROPHIC PROTEOLYTIC BACTERIA AND THERMODURIC PSYCHROTROPHS IN RAW MILK

## 6.1 Overview of Chapter

Chapter 6 describes the importance of psychrotrophic proteolytic bacterial counts (PPrBC) and thermoduric psychrotrophic counts (TDPC) as quality and safety criteria in raw milk and the effect of different refrigeration conditions including 2 ºC, 4 ºC, 6 ºC, 8 ºC, 10 ºC and 12 ºC on these counts, related protease activity, proteolysis and their correlation in raw milk. The raw milk samples were collected from three different farms with a distinct variation in the quality, representing high quality, medium quality and poor quality milk. The article entitled “Microbiological Quality of Raw Milk Attributable to Prolonged Refrigeration Conditions” by Nuwan R. Vithanage, Muditha Dissanayake, Greg Bolge, Enzo A. Palombo, Thomas R. Yeager and Nivedita Datta has been published in peer-reviewed journal “Journal of Dairy Research” (2017) 84: 92-101. https://doi.org/10.1017/S0022029916000728.

## Note from the examination process of this PhD thesis:

*Bacillus cereus* considered in the present study belonged to the *B. cereus sensu lato* species complex including B*. cereus, B. thuringiensis, B. mycoides, B. weihenstephanensis,* and *B. pseudomycoides*, which are ecologically diverse bacterial group of medical and agricultural significance (Bartoszewicz & Marjańska, 2017, Okinaka & Keim, 2016).

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

**GRADUATE RESEARCH CENTRE**

**DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION**

*This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.*

## 1. PUBLICATION DETAILS (to be completed by the candidate)

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## 2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

| [redacted] | 24-Mar-2017 |
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| **Signature** | **Date** |

## 3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

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| **College of Health and Biomedicine, Victoria University, Werribee Campus, Melbourne VIC, Australia** |

| Name(s) of Co-Author(s) | Contribution (%) | Nature of Contribution | Signature | Date |
|---|---|---|---|---|
| Nuwan R. Vithanage | 85% | Concept Development, research question, hypothesis, data analysis manuscript writing and submission to journals. | [redacted] | 15-Feb-2017 |
| Muditha Dissanayake | 2% | Laboratory experiment and manuscript preparation. | | 15-Feb-2017 |
| Greg Bolge | 1% | Sample provision. | | 15/02/17 |
| Enzo A. Palombo | 5% | Concept Development, research question, hypothesis and manuscript preparation. | | 15-Feb-2017 |
| Thomas R. Yeager | 5% | Concept Development, research question, hypothesis, manuscript preparation and submission to journals. | | 15-Feb-2017 |
| Nivedita Datta | 2% | Concept Development, research question, hypothesis. | | 20-Feb-2017 |

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# Microbiological quality of raw milk attributable to prolonged refrigeration conditions

Nuwan R Vithanage[1,2], Muditha Dissanayake[1,2], Greg Bolge[3], Enzo A Palombo[4], Thomas R Yeager[2,5,6]* and Nivedita Datta[1,6]

[1] College of Health and Biomedicine, Victoria University, Werribee, Victoria 3030, Australia
[2] Advanced Food Systems Research Unit, Victoria University, Werribee, Victoria 3030, Australia
[3] Murray Goulburn Co-operative Co Ltd, Leongatha, Victoria 3953, Australia
[4] Faculty of Science, Engineering and Technology, Swinburne University of Technology, Hawthorn, Victoria 3122, Australia
[5] College of Engineering and Science, Victoria University, Werribee, Victoria 3030, Australia
[6] Institute for Sustainability and Innovation, Victoria University, Werribee, Victoria 3030, Australia

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Refrigerated storage of raw milk is a prerequisite in dairy industry. However, temperature abused conditions in the farming and processing environments can significantly affect the microbiological quality of raw milk. Thus, the present study investigated the effect of different refrigeration conditions such as 2, 4, 6, 8, 10 and 12 °C on microbiological quality of raw milk from three different dairy farms with significantly different initial microbial counts. The bacterial counts (BC), protease activity (PA), proteolysis (PL) and microbial diversity in raw milk were determined during storage. The effect of combined heating (75 ± 0·5 °C for 15 s) and refrigeration on controlling those contaminating microorganisms was also investigated. Results of the present study indicated that all of the samples showed increasing BC, PA and PL as a function of temperature, time and initial BC with a significant increase in those criteria ≥6 °C. Similar trends in BC, PA and PL were observed during the extended storage of raw milk at 4 °C. Both PA and PL showed strong correlation with the psychrotrophic proteolytic count (PPrBC: at ≥4 °C) and thermoduric psychrotrophic count (TDPC: at ≥8 °C) compared to total plate count (TPC) and psychrotrophic bacterial count (PBC), that are often used as the industry standard. Significant increases in PA and PL were observed when PPrBC and TDPC reached $5 \times 10^4$ cfu/ml and $1 \times 10^4$ cfu/ml, and were defined as storage life for quality ($S_{LQ}$), and storage life for safety ($S_{LS}$) aspects, respectively. The storage conditions also significantly affected the microbial diversity, where *Pseudomonas fluorescens* and *Bacillus cereus* were found to be the most predominant isolates. However, deep cooling (2 °C) and combination of heating and refrigeration (≤4 °C) significantly extended the $S_{LQ}$ and $S_{LS}$ of raw milk.

**Keywords:** Raw milk, psychrotrophic proteolytic bacteria, thermoduric psychrotrophs, diversity, protease activity, proteolysis.

Since the introduction of storage and transportation of raw milk under refrigerated conditions in the 1950s, the spoilage of raw milk by mesophilic microbiota has been substantially reduced. According to the guidelines of Food Standards Australia and New Zealand (FSANZ), raw milk is required to be stored at 5 °C within 3·5 h from the start of the milking process, whereas the European Union (EU) standards state that raw milk is required to be stored at 6–8 °C within 2 h from the end of milking (FSANZ, 2012). While this practice hinders the growth of mesophiles, cold storage of raw milk provides favourable conditions for the growth of psychrotrophic microorganisms (Quigley et al. 2013). Thus, the level of psychrotrophs in raw milk after the milking process is dependent on both the storage temperature and time (Griffiths et al. 1987; Vithanage et al. 2016). The initial psychrotrophic bacterial load typically accounts for <10% of the total microbiota when milking is conducted under hygienic conditions, however, these bacteria can become >75% of the total population when milking is conducted using unhygienic protocols (Cousin,

*For correspondence; e-mail: thomas.yeager@vu.edu.au

1982). The dairy farm environment comprises a variety of potential sources of psychrotrophs that can contaminate raw milk, mainly during the milking process (Vissers & Driehuis, 2009).

Psychrotrophic bacteria isolated from raw milk predominantly include the Gram negative genera of *Pseudomonas, Acinetobacter, Hafnia, Rahnella, Alcaligenes, Achromobacter, Aeromonas, Serratia, Enterobacter, Chryseobacterium, Chromobacterium*, and *Flavobacterium*, and the Gram positive genera of *Bacillus, Clostridium, Corynebacterium, Streptococcus, Micrococcus, Staphylococcus, Enterococcus, Lactobacillus*, and *Microbacterium*. Of these, *Pseudomonas* and *Bacillus* are the most frequently reported raw milk isolates (Vithanage et al. 2016). Psychrotrophic bacteria are able to grow at minimum temperatures between −10 and 7 °C; optimum temperature is in the range of 25–35 °C; and maximum temperature can be as high as 45 °C. In addition, some thermoduric psychrotrophs are able to withstand temperatures as high as 72–74 °C (McKellar, 1989).

During cold storage, these bacteria can produce extracellular proteases (mainly) and lipases that are resistant to pasteurisation and even ultra-high temperature (UHT) processing, contributing to the spoilage in milk and dairy products (Oliveira et al. 2015). Proteolytic enzymes induce the hydrolysis of casein, which may be evident as a greyish colour, bitter taste and gelation of spoiled milk (Vyletělová & Hanuš, 2000a). UHT milk is more susceptible to proteolysis than pasteurised milk due to longer storage times under ambient temperature condition (McKellar, 1981). Psychrotrophs with higher protease expression can produce this level of protease activity within a few hours under suboptimal storage conditions (Renner, 1988).

The relationship between psychrotrophs and milk quality has been widely investigated (Marchand et al. 2009a; Oliveira et al. 2015). To date, limited evidence has been found associating the effect of storage conditions with the growth of psychrotrophic bacteria, their proteolytic potential and deterioration of milk proteins due to proteolysis (Griffiths et al. 1987; Haryani et al. 2003; O'Connell et al. 2016). Changes in storage conditions are also associated with the microbial composition in the corresponding samples (Lafarge et al. 2004; Hantsis-Zacharov & Halpern, 2007; von Neubeck et al. 2015). However, the experimental data demonstrating the relationship between microbial counts and proteolysis in raw milk is not well established, due to the distinct variation in the proteolytic potential and heat-resistance of those proteolytic enzymes produced by raw milk microbiota (Dogan & Boor, 2003; Marchand et al. 2009b). Hence, the current study investigated the effects of microbiological quality and associated proteolysis on storage life of raw milk under different refrigeration conditions for a prolonged period with a focus on psychrotrophic proteolytic counts (PPrBC). The effect of high-temperature short-time pasteurisation (HTST) of raw milk prior to the UHT processing on microbiological and proteolytic parameters was also evaluated.

## Materials and methods

### *Raw milk samples*

Raw milk samples from three commercial farms (designated as A, B and C) were provided by a commercial UHT milk processor in Victoria, Australia. These samples were selected from seven potential samples to represent high quality (A: $2{\cdot}3 \times 10^4$ cells/ml) medium quality (B: $5{\cdot}3 \times 10^5$ cells/ml) and poor quality (C: $6{\cdot}7 \times 10^6$ cells/ml) raw milk based on Bactoscan counts as well as statistics of the respective commercial processor (Vithanage et al. 2014). Three representative samples were collected directly from the bulk milk tank at each of the farms under aseptic conditions and delivered to the laboratory on ice (at 4–5 °C) within 2–3 h of the milking procedure. A volume (500 ml) of the samples was transferred into a sterile Erlenmeyer flask (1 l) under aseptic conditions and stored under various experimental conditions (as described below). Samples were analysed daily, commencing from day 0, representing three biological (three separate samples of milk from each bulk tank) and three technical (three sub samples from each 500 ml) replicates ($n = 9$).

### *Storage conditions*

Raw milk samples were incubated under various temperature conditions in a refrigerated shaking incubator (Innova 4230, New Brunswick Scientific, Edison, NJ, USA) and subjected to constant agitation at 120 rpm for 10 d. Those conditions included 2 °C (deep cooling), 4 °C (standard refrigeration) and 6, 8, 10 or 12 °C (elevated temperatures in the farm bulk tank and commercial silo).

### *Enumeration of bacteria in raw milk*

The total plate count (TPC) was determined according to the method described in the International Dairy Federation (IDF) standard: 101A: 1991 with slight modification. Raw milk samples were serially diluted (10-fold) and cultured on plate count agar (Sigma-Aldrich, Castle Hill, Australia) supplemented with 1·0% (w/v) skim milk (PCM agar) using the drop plate method (Munsch-Alatossava et al. 2007) and incubated for 10 d, at 7 °C (for psychrotrophic bacterial counts: PBC) and 48 h at 30 °C (for total plate count: TPC) in duplicate. Clearing zones around colonies of psychrotrophic bacteria were indicative of proteolysis and these colonies were used to calculate PPrBC counts (Cempírkova, 2007).

The thermoduric psychrotrophic count (TDPC) was determined by heating the raw milk at 63 ± 0·5 °C for 30 min, in a shaking oil bath (Ratek, Boronia, Victoria, Australia), excluding the come up time (i.e., time required to reach the corresponding temperature). Samples were cultured on PCM and incubated at 7 °C for 10 d (Buehner et al. 2014).

### *Identification of predominant raw milk microbiota*

Identification of predominant isolates was conducted using matrix-assisted laser desorption time of flight mass

spectrometry (MALDI-TOF MS) as well as 16S rRNA sequencing according to the method described by Vithanage et al. (2014) in duplicate.

### *Sample preparation for protease activity and peptide analysis*

Raw milk samples were prepared by centrifugation of raw milk at 16 000 ***g*** for 5 min (Eppendorf 5415C microfuge, Hamburg, Germany) to remove the milk fat. A volume (1 ml) of raw milk was mixed with 12% trichloroacetic acid (TCA) and incubated at 37 °C for 30 min. The mixture was filtered through 0·45 µm syringe filter (Minisart® Regenerated Cellulose; Sartorius, Victoria, Australia) and the filtrate was used for protease assays. The same procedure was used for obtaining the TCA-soluble peptides for the peptide analysis.

### *Determination of protease activity*

Protease activity in the raw milk samples stored under different storage conditions was determined using the Protease Fluorescent Detection Kit (Sigma-Aldrich, Castle Hill, Australia) according to the manufacturer's instructions. The fluorescence intensity due to release of trichloroacetic acid (TCA)-soluble fluorescent peptides was determined using a spectrofluorophotometer (POLARstar Omega; BMG LABTECH, Mornington, Victoria, Australia) with excitation at a wavelength of 485 nm and the emission at a wavelength of 535 nm in duplicate. The increase in fluorescence intensity obtained due to hydrolysis of the protein was expressed as relative fluorescence units (RFU/ml). Thermolysin (Sigma-Aldrich, Castle Hill, Australia) was used as the positive control, and it was also used to generate a standard curve (0–25 ng) when determining the detection limit (ng/ml) (Cupp-Enyard, 2009).

### *Determination of proteolysis by reversed-phase high performance liquid chromatography (RP-HPLC)*

Separation of TCA-soluble peptides was performed on a reversed-phase HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with C-18 monomeric column (5 µm, 300 A, 250 mm × 4·6 mm; Grace Vydac, Hesperia CA, USA) at 35 °C and a UV/Vis detector at 214 nm according to the method described by Datta & Deeth (2003), with some modifications. A volume (50 µl) of TCA-soluble peptides was injected and the peptides were eluted by a linear gradient from 100 to 0% of solvent A (0·1% trifluoroacetic acid (TFA) in Milli-Q water) in solvent B (0·1% TFA in 90%, v/v HPLC-grade acetonitrile in Milli-Q water) over 40 min at a flow rate of 0·75 ml/min in duplicate.

### *Determination of proteolysis by degree of hydrolysis by O-phathaldialdehyde (OPA) method*

The extent of proteolysis was also determined using the modified OPA method (Zarei et al. 2012) in duplicate. A volume (5 µl) of TCA-soluble peptides was mixed with 245 µl of OPA reagent (Thermo Fisher Scientific, Victoria, Australia) in microtiter plates and the absorbance was determined using a spectrofuorophotometer (POLARstar Omega; BMG LABTECH, Mornington, Victoria, Australia) with a wavelength of 340 nm in duplicate. The degree of hydrolysis (DH %) was calculated based on the following formula (i.e., equation (1)) (Slattery & Fitzgerald, 1998).

$$DH\% = \left(\frac{100}{N}\right)(\Delta A \times M \times d/\varepsilon \times c) \quad (1)$$

where $\Delta A$ is the difference between the absorbance of test sample and un-hydrolysed sample at 340 nm, $M$ is the molecular mass of the test protein (Da), $d$ is the dilution factor, $\varepsilon$ is the molar extinction coefficient at 340 nm (6000 l/mol/cm), $c$ is the protein concentration (g/l) and $N$ is the total number of peptide bonds per protein molecule.

### *Determination of the effect of combined pasteurisation and low temperature storage*

Raw milk samples from all three farms were heated at 75 ± 0·5 °C for 15 s in a shaking oil bath (Ratek, Boronia, Victoria, Australia), excluding the come up time (Griffiths et al. 1987). Following heat treatment, the samples were aseptically transferred into 1 l sterile Erlenmeyer flasks and stored under different temperature at 2, 4, 6, 8, 10 and 12 °C for 10 d. The enumeration of bacteria and analysis of protease activity and proteolysis was conducted as described before.

### *Data processing and statistical analysis*

The analysis was conducted in triplicate. Correlation coefficients and significance levels (MANOVA) of the tested sets (TPC; PBC; PPrBC; TDPC) were calculated using the SPSS software for Windows (Version 21 software; IBM Corp. in Armonk, NY). $P < 0·05$ was considered statistically significant.

## Results

### *The initial microbiological counts of raw milk of different farms*

The total plate count in A, B and C raw milk samples were 2·84 (±1·21), 3·79 (±1·54) and 5·86 (±2·32) log cfu/ml, respectively. Similarly, the initial PBC in the corresponding samples were in the following order; A: 2·66 (±1·11); B: 2·87 (±1·01); C: 4·85 (±1·21) log cfu/ml. Interestingly, the PPrBC counts showed a different ascending order, of B: 1·38 (±1·05) log cfu/ml; A: 2·37 (±1·04) log cfu/ml; C: 3·79 (±1·10) log cfu/ml. The TDPC in the A, B and C samples were 1·03 (±0·14) log cfu/ml, 2·70 (±0·20) log cfu/ml and 3·61(±0·11) log cfu/ml, respectively.

### *Effects of different storage conditions on the microbial growth in raw milk*

Bacterial growth curves comprising TPC, PBC, PPrBC and TDPC showed the characteristic sigmoidal growth pattern

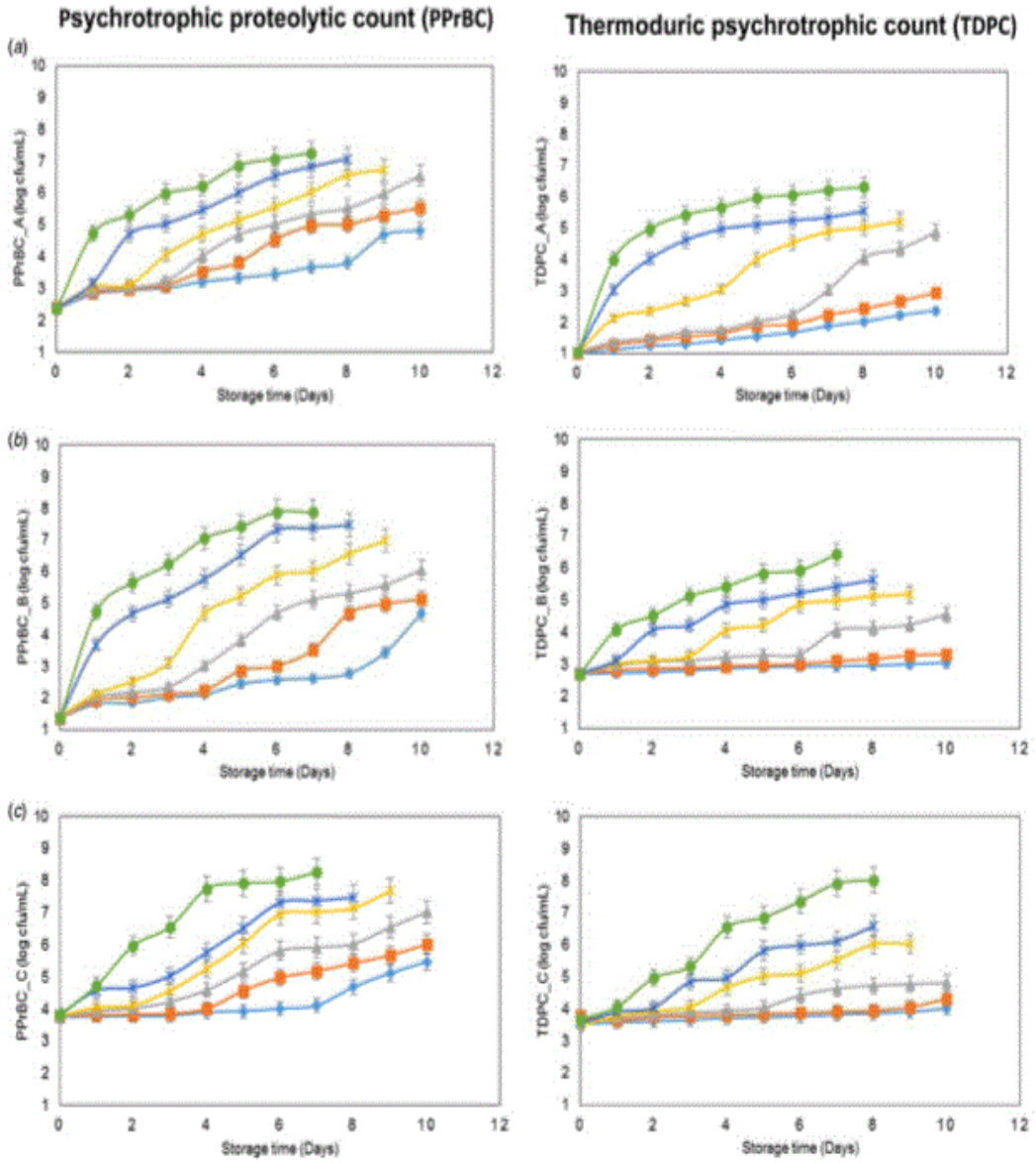


**Fig. 1.** Effect of different storage conditions on the proteolytic psychrotrophic counts (PPrBC) and thermoduric psychrotrophic counts (TDPC) of a, b and c raw milk samples; at 2 °C, 4 °C, 6 °C, 8 °C, 10 °C and 12 °C storage. The results were presented as mean±SE, ($n = 9$).

with different growth rates when stored under different refrigerated conditions (Fig. 1 and Supplementary Fig. S1). The growth curves of PPrBC, TDPC of sample A, B and C showed a double-sigmoidal shape (Fig. 1). However, storage of raw milk at 2 °C storage showed significant inhibition of the PPrBC and TDPC. Storage temperatures of ≥4 °C resulted in significant increases in PPrBC, whereas TDPC showed significant increases in growth rate at ≥8 °C ($P < 0{\cdot}05$; Fig. 1).

### *Diversity of raw milk microbiota under refrigerated conditions*

The predominant microorganisms isolated were *Pseudomonas, Bacillus*, and *Microbacterium* and, to a lesser extent, members of the family *Enterobacteriaceae* (Table 1). The most predominant genera found in refrigerated raw milk were *Pseudomonas* (mainly *Pseudomonas fluorescens)* and *Bacillus* (*Bacillus cereus, Bacillus weihenstephensis* and *Bacillus circulans*). This diversity varied depending on the sample and temperature tested. For example, the level of enteric, non-fermenter Gram negative bacilli (NF-GNB), Gram positive cocci and Gram positive bacillus were higher at temperatures ≥8 °C (Table 1).

### *Effects of different storage conditions on the protease activity and proteolysis in raw milk*

The initial protease activities (PA) of A, B and C raw milk samples were 404·5 (±4·76), 257 (±2·82) and 604·3 (±5·13) RFU/ml. Consequently, the initial proteolysis (PL) that has

**Table 1.** Percentages of predominant bacteria belong to each taxon isolated from three samples throughout the simulations of the cold dairy chain using different storage conditions

| | % of isolates | | | | | |
|---|---|---|---|---|---|---|
| Microorganisms ($n = 927$) | 2 °C | 4 °C | 6 °C | 8 °C | 10 °C | 12 °C |
| *Pseudomonadaceae*† | 87·3 | 80·9 | 76·6 | 69·5 | 52·2 | 39·2 |
| GPB | 8·7 | 9·4 | 9·6 | 13·5 | 25·2 | 30·3 |
| *Enterobacteriaceae*‡ | 3·1 | 5·8 | 6·1 | 7·3 | 9·8 | 12·3 |
| Miscellaneous NF-GNB | 0·9 | 1 | 3·4 | 4·2 | 6·4 | 8·6 |
| GPC | 0 | 0·8 | 2·3 | 3·2 | 5·2 | 7·3 |
| Un-identified | 0 | 2·1 | 2 | 2·3 | 1·2 | 2·3 |

GPB, Gram positive Bacilli; 80% of the GPB was belong to *B. cereus* and *M. lacticum.*; GPC, Gram Positive Cocci mainly *Streptococci* and *Staphylococci* spp.; NF-GNB, Non-Fermenting Gram Negative Bacilli with 75% of *Acinetobacter* and *Stenotrophomonas* spp.
†85% of this genera was belong to *P. fluorescens*
‡Approximately 76% of the isolates from family *Enterobacteriaceae* were belong to *Hafnia* and *Serratia*

been denoted by degree of hydrolysis (%DH) of each samples was in the following ascending order; B: 0·88 (±0·51)%, A: 1·32 (±1·02)% and C: 2·42 (±1·13)%. A significant increase in PA and PL (denoted by %DH) was apparent at storage conditions ≥6 °C ($P < 0·05$; Fig. 2). Even the standard refrigeration condition (4 °C) showed significant increase in PA and PL during the extended storage of raw milk (10 d) and this was observed after 6, 8 and 5 d in A, B and C samples, respectively ($P < 0·05$; Figs. 2 & 3). In contrast, 2 °C storage resulted in significant reduction in the PA and DH in all three raw milk sample ($P < 0·0001$; Fig. 2).

### *Correlation of protease activity and proteolysis with bacterial counts in raw milk*

An increase in protease activity and proteolysis were observed when the PPrBC counts reached $5·0 \times 10^4$ cfu/ml at all temperature conditions, except for 2 °C (Table 2; Fig. 2). However, the corresponding protease activity and proteolysis varied as function of temperature (Table 2; Fig. 2). For example, the presence of PPrBC in the range of $5·1$ to $5·4 \times 10^4$ cfu/ml in A, B and C samples at 4 °C resulted in protease activity of $2·8 \times 10^3$ RFU/ml, $1·0 \times 10^2$ RFU/ml and $4·0 \times 10^4$ RFU/ml and those values were equivalent to 9·3, 3·5 and 11·9 ng/ml as calculated using thermolysin as the positive control by the FITC method, respectively (Table 2; Fig. 2). The proteolysis of the samples, denoted by DH %, was 12·1, 8·4 and 15·1%. In contrast, at 6 °C with similar PPrBC (ranging from $5·2–5·4 \times 10^4$ cfu/ml), the protease activities in the samples were $3·9 \times 10^4$ RFU/ml, $2·9 \times 10^3$ RFU/ml and $5·3 \times 10^4$ RFU/ml (equivalent to 12·1, 5·4 and 13·4 ng/ml) with DH % of 18·2, 10·4 and 21·3%, representing farms A, B and C, respectively (Table 2; Fig. 2).

Interestingly, the correlation coefficients ($r$) between PPrBC and PA/PL were highly significant ($r \geq 0·90$, $P < 0·0001$; at ≥4 °C), when PPrBC reached $5·0 \times 10^4$ cfu/ml (Supplementary Table S1). This correlation was in the range of 0·81–0·95 ($P < 0·001$), when TDPC reached $5·0 \times 10^4$ cfu/ml at ≥8 °C (Supplementary Table S2). The correlation coefficients between PBC and PA and/or PL was significant ($r \geq 0·82–0·95$, $P < 0·05$), however, the TPC showed poor correlation with PA/PL ($r = 0·55–0·62$, $P > 0·05$; data not shown).

### *Storage life of raw milk attributable to different temperature conditions*

Besides the significant correlation in increase in PA and PL with PPrBC, both parameters appear to vary depending on the temperature condition. Therefore, the storage life in the aspect of raw milk quality ($S_{LQ}$) was defined depending on the PPrBC counts, hence time to reach PPrBC of $5·0 \times 10^4$ cfu/ml was defined as $S_{LQ}$ (Supplementary Table S3). However, the storage life in the aspect of raw milk safety ($S_{LS}$) was dependent on the counts of pathogenic thermoduric psychrotrophs such as *B. cereus* and the time to reach TDPC of $1·0 \times 10^4$ cfu/ml was defined as $S_{LS}$ (Supplementary Table S3). Both $S_{LQ}$ and $S_{LS}$ showed significant correlation with initial counts ≥4 and ≥8 °C storage, respectively (Supplementary Tables S1 and S2).

### *Extension of storage life of raw milk by a combination of pasteurisation and low-temperature storage*

Heating of raw milk samples at 75 °C for 15 s followed by storage at different refrigeration conditions resulted in a significant reduction of PPrBC ($P < 0·05$; Supplementary Table S3). This consequently decreased the PA and PL with concomitant increased in the $S_{LQ}$ ($P < 0·05$), especially the temperature conditions ≤8 °C storage (Supplementary Table S3). In contrast, the $S_{LS}$ showed only slight increase ($P > 0·05$). The most significant increase in storage life (both $S_{LQ}$ and $S_{LS}$) was observed when raw milk was stored at 2 °C, while storage life was significantly reduced when it was stored at ≥8 °C (Supplementary Table S3).

## Discussion

Raw milk collected from three farms showed significantly different initial TPC, PBC, PPrBC and TDPC, possibly

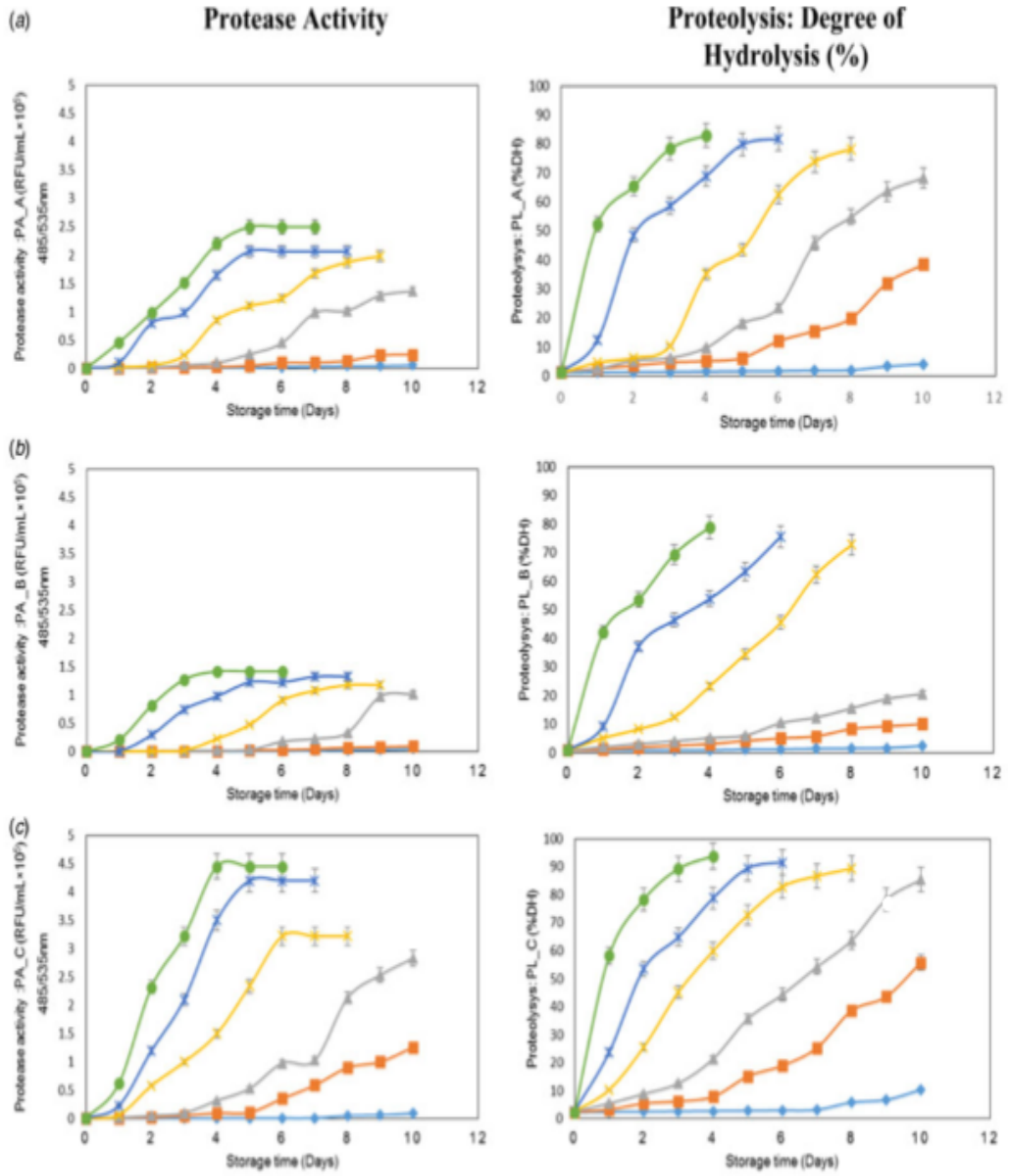


**Fig. 2.** Effect of different storage conditions on the protease activity (PA) and proteolysis (PL: %DH: degree of hydrolysis) of a, b and c raw milk samples; at 2 °C, 4 °C, 6 °C, 8 °C, 10 °C and 12 °C storage. The results were presented as mean ±SE, ($n = 9$).

related to the different farm management systems and hygienic protocols used during the milking process of these farms (Cempírkova, 2007; Srairi et al. 2009). Interestingly, the PPrBC was higher in sample A compared to sample B. This may result in significantly greater protease activity and proteolysis in the corresponding sample, regardless of its lower TPC, compared to sample B. Furthermore, proteolysis and protease activity showed a more significant correlation with PPrBC (≥4 °C) and TDPC (≥8 °C) than that with TPC and PBC in raw milk. This indicates that PPrBC and TDPC are the most important quality criteria that can be incorporated into the guidelines for the production of high quality milk and dairy products. Moreover, the maximum production of proteolytic enzymes and subsequent proteolysis was observed when PPrBC counts were above $\geq 5 \times 10^4$ cfu/ml at ≥4 °C, and TDPC $\geq 1 \times 10^4$ cfu/ml at ≥8 °C and those limits were used for predicting storage life of raw milk with respect to both quality and safety. Thus, according to the results of the present study, it can be speculated that production of UHT milk requires PPrBC counts below $5 \times 10^4$ cfu/ml and TDPC of $1 \times 10^4$ cfu/ml for shelf life extension and product safety. This is consistent with a PPrBC count of $4{\cdot}5 \times 10^4$ cfu/ml representing the threshold with respect to milk quality (Silveira et al. 1999; Vyletelova et al. 2000b). Similarly, the TDPC comprising significantly higher numbers of *B. cereus* can be a

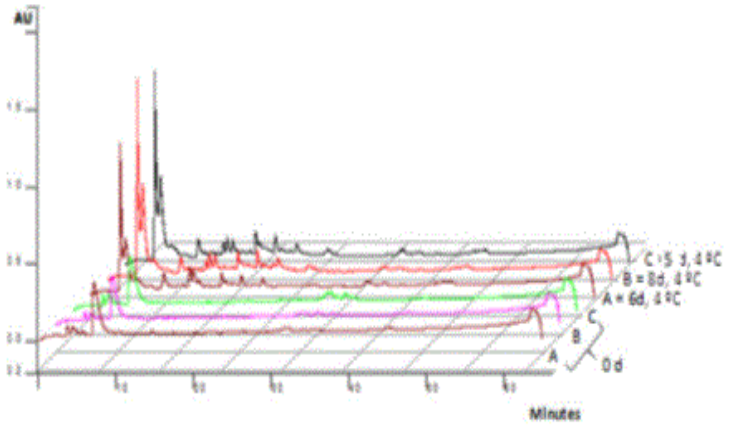


**Fig. 3.** The reversed-phase high-performance liquid chromatography (RP-HPLC) chromatograms of trichloroacetic acid (TCA) soluble peptide fractions of A, B and C raw milk samples stored at 4 °C, in 0 d and after 6, 8 and 5 d (when significant increase in proteolysis occurred), respectively.

**Table 2.** Relationship between psychrotrophic proteolytic count (PPrBC) and thermoduric psychrotrophic count (TDPC) with protease activity and degree of hydrolysis (proteolysis) in raw milk, when PPrBC reach $5 \times 10^4$ cfu/ml and TDPC reach $1 \times 10^4$ cfu/ml under different storage conditions

| Sample | Storage temperature (°C) | Time†‡ (d) | PPrBC (log cfu/ml) | TDPC (log cfu/ml) | Protease activity (RFU/ml§) | Protease concentration (ng/ml¶) | DH (proteolysis) (%) |
|---|---|---|---|---|---|---|---|
| A | 2 | 9†, >10‡ | 4·68 | 2·87 | $1{\cdot}2 \times 10^{2***}$ | 5·0*** | 3·4*** |
| | 4 | 6†, >10‡ | 4·67 | 2·97 | $2{\cdot}8 \times 10^{3**}$ | 9·3** | 12·1*** |
| | 6 | 5†, 8‡ | 4·69 | 4·06 | $3{\cdot}9 \times 10^{4**}$ | 12·1** | 18·2** |
| | 8 | 4†, 5‡ | 4·70 | 4·01 | $4{\cdot}4 \times 10^{4*}$ | 13·3* | 35·2* |
| | 10 | 2†, ‡ | 4·71 | 4·02 | $5{\cdot}0 \times 10^{4*}$ | 15·1* | 48·5* |
| | 12 | 1†, ‡ | 4·73 | 4·01 | $4{\cdot}3 \times 10^{5*}$ | 15·9* | 52·3* |
| B | 2 | 10†, >10‡ | 4·69 | 3·05 | $9{\cdot}8 \times 10^{1***}$ | 2·4*** | 2·5*** |
| | 4 | 8†, >10‡ | 4·69 | 3·32 | $1{\cdot}0 \times 10^{2***}$ | 3·5*** | 8·4*** |
| | 6 | 6†, 7‡ | 4·69 | 4·06 | $2{\cdot}9 \times 10^{3**}$ | 5·4*** | 10·4*** |
| | 8 | 4†‡ | 4·68 | 4·06 | $3{\cdot}4 \times 10^{4**}$ | 10·6** | 23·3** |
| | 10 | 2†‡ | 4·67 | 4·07 | $3{\cdot}4 \times 10^{4*}$ | 11·7* | 37·1* |
| | 12 | 1† | 4·73 | 4·08 | $3{\cdot}8 \times 10^{4*}$ | 12·9* | 42·2* |
| C | 2 | 8†, 10‡ | 4·69 | 4·02 | $2{\cdot}8 \times 10^{3**}$ | 9·3** | 5·8*** |
| | 4 | 5†, 9‡ | 4·68 | 4·06 | $4{\cdot}0 \times 10^{4**}$ | 11·9** | 15·1** |
| | 6 | 4†, 5‡ | 4·69 | 4·05 | $5{\cdot}3 \times 10^{4*}$ | 13·2* | 21·3* |
| | 8 | 3†‡ | 4·70 | 4·07 | $5{\cdot}5 \times 10^{4*}$ | 15·6* | 45·2* |
| | 10 | 2†‡ | 4·71 | 4·05 | $5{\cdot}5 \times 10^{5*}$ | 17·1* | 53·5* |
| | 12 | 1†‡ | 4·67 | 4·06 | $6{\cdot}2 \times 10^{5*}$ | 18·7* | 58·2* |

DH, Degree of hydrolysis, which denotes the extent of proteolysis that was determined using OPA-method; PPrBC, Psychrotrophic proteolytic count; TDPC, Thermoduric psychrotrophic count

Multiple samples were analysed with SD ± 1·5 ($n = 9$)

*, **, ***Means significance levels by MANOVA (SPSS Windows Ver 21) *$P < 0{\cdot}001$; **$P < 0{\cdot}05$; ***$P > 0{\cdot}05$

†Time to PPrBC of $5 \times 10^4$ cfu/ml

‡Time to reach TDPC of $1 \times 10^4$ cfu/ml

§Protease activity determined by relative fluorescence units

¶Protease concentration determined by standard curve of Thermolysin (EC 3·4·24·27)

food safety concern when it reaches $1{\cdot}0 \times 10^4$ cfu/ml (Valik et al. 2003). In contrast, several other studies determined the relationship between proteolysis with slightly higher bacterial counts in the range of $10^6$–$10^7$ cfu/ml (Griffiths et al. 1987; Haryani et al. 2003; O'Connell et al. 2016). However, Gillis et al. (1985) also demonstrated significant decrease in proteolysis and bitter peptide production with raw milk microbiota less than $10^4$ cfu/ml.

Even an initial PPrBC and TDPC as low as $10^1$–$10^2$ cfu/ml can give rise to $\geq 5 \times 10^4$ cfu/ml with elevated PA and PL

within 4–7 d at 6 °C storage. The TDPC with similar initial counts increased to $\geq 1 \times 10^4$ cfu/ml within 5–9 d at 8 °C. At 4 °C, the PPrBC counts reached the corresponding levels within 5–8 d storage and less than 2 d of storage at ≥8 °C. Thus, 2 °C is highly recommended as a storage temperature, while temperatures below 6 °C can be recommended for the purpose of pre-processing storage of raw milk, depending on the initial bacterial counts and the duration of storage.

Interestingly, some of the growth curves of bacteria exhibited a double-sigmoidal shape at ≥8 °C. It can be speculated that an increasing growth rate and production of antimicrobial metabolites under elevated temperature conditions may result in antagonistic effects within the mixed microbial population (Vine et al. 2004; Ma et al. 2014). The fluctuation in the microbial counts was also accompanied by a slight fluctuation in the protease activity and proteolysis. This is possibly related to the balance between production and utilisation of small peptides by indigenous microbiota or due to the presence of artefacts especially in FITC method (Haryani et al. 2003).

The extended storage of raw milk under various refrigeration conditions resulted in significant diversity in the raw milk microbiota. For example, storage temperatures below 4 °C resulted in an increase in the level of *Pseudomonas* spp. and some *Bacillus* spp. with simultaneous reduction in the enteric and miscellaneous NF-GNB isolates. However, the counts of isolates that belong to family *Bacillaceae* and *Enterobacteriaceae* were significantly increased above 8 °C storage. Among the thermoduric psychrotrophic isolates, species belonging to *B. cereus* group were predominantly isolated especially ≥8 °C. *B. cereus* is known to produce emetic type toxin under refrigeration conditions that can cause public health concerns when the isolates reach $1 \times 10^3$ cfu/ml (Christiansson et al. 1989). Most importantly, the spores produced by these isolates are able to withstand pasteurisation and UHT processing (Champagne et al. 1994). According to FSANZ guidelines, the counts of *P. fluorescens* and *B. cereus* in premium quality raw milk are required to be maintained below $10^7$ cfu/ml and $10^5$ cfu/ml, respectively (FSANZ, 2014). These two genera are considered as the major cause of concern in commercial milk processing. Additionally, the diversity of raw milk microbiota can be affected by seasonal differences, for example, psychrotolerant PPrBC, PBC and TDPC appear to increase during the winter months, while thermoduric counts representing mesophilic bacteria were at their highest during the summer months (Marchand et al. 2009a; Vithanage et al. 2016).

In the present study, sample B showed significantly lower protease activity and proteolysis. This can be related to the diversity of psychrotolerant bacteria in the respective sample. Previously, we observed that sample B comprised psychrotrophic isolates with limited proteolytic potential (Vithanage et al. 2016). Dogan & Boor (2003) also observed variation in the proteolytic potential even within the *P. fluorescens* population isolated from milk. *Pseudomonas* produce a heat-stable serralysin family extracellular protease, referred to as AprX (EC 3·4·24·40), while *Bacillus* spp. produce serine family proteases known as thermolysin (EC 3·4·24·27), substilisin (EC 3·4·21·62) (Bach et al. 2001; Dufour et al. 2008; Marchand et al. 2009b; Machado et al. 2013). Expression of the genes encoding these proteases was shown to be regulated by incubation temperature (Morita et al. 1997; Burger et al. 2000). Alternatively, differences in proteolysis can be related to the characteristics of proteolytic enzymes such as their cold-active nature, specificity and temperature-dependence (McKellar, 1989).

The growth of spoilage bacteria in raw milk can be controlled by thermisation (at 65 °C for 15 s), followed by storing of the heated milk under refrigeration conditions (Stadhouders, 1982; Griffiths et al. 1987). In contrast to these earlier studies, the current study used heating of raw milk at 75 °C for 15 s, which is typically used in HTST pasteurisation. This practice is often used upon receiving raw milk at dairy processing plants prior to UHT treatment. This resulted in significant reduction (1-log) in PPrBC counts, but not TDPC, and also resulted in significant decrease in protease activity. This in turn showed significantly higher $S_{LQ}$, but no significant difference in $S_{LS}$. Thus, the knowledge of number and diversity of psychrotrophic proteolytic bacteria in raw milk can be used for appropriate production of milk and dairy products (Anzueto, 2014; Vithanage et al. 2016). Similarly, reliable control of raw milk isolates with higher proteolytic potential would be important for the extension of raw material storage with concomitant increase in flexibility of the manufacturing process (Griffiths et al. 1987).

Although the current study used raw milk representing various quality levels, a large-scale analysis would provide a more comprehensive understanding of the effect of storage conditions on raw milk quality. However, these results are in general agreement with the results of large scale studies (O'Connell et al. 2016).

In conclusion, storage temperature, time and initial counts can affect microbiological quality of raw milk, in which PPrBC and TDPC are better indicators than other microbiological criteria for predicting the quality and safety of raw milk. It is important to determine a particular predictive model to estimate the PPrBC and TDPC in samples for improving the quality and reducing large-scale wastage of raw milk. Thus, PPrBC and TDPC data can be used to evaluate specific on-farm technological requirements when deciding on quality-dependent incentive schemes for raw milk suppliers. Additionally, deep cooling of raw milk at 2 °C may be a reliable alternative for dairy farms when raw milk collection does not occur on a regular basis. Alternatively, extension in the storage-life of raw milk can be achieved by thermisation at 75 °C for 15 s (instead of 65 °C) followed by 2 °C storage. However, profiling of individual species with higher spoilage potential using rapid and reliable screening would be more informative and will be the focus of future studies. This would allow for the production of superior quality dairy products with extended

shelf life that can be distributed to wider geographical regions, benefitting commercial milk processing.

## Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029916000728.

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# CHAPTER 7: PREDICTION OF STORAGE LIFE OF RAW MILK WITH RESPECT TO *PSEUDOMONAS* SPP. AND *BACILLUS CEREUS* GROWTH UNDER ISOTHERMAL TEMPERATURE CONDITIONS

## 7.1 Overview of Chapter

Chapter 7 presents a study of the prediction of storage life of raw milk, while focussing on its quality and safety, based on the growth of *Pseudomonas* spp. and *Bacillus cereus* as a function of temperature using primary, secondary and tertiary models. The aim is to minimise the risks of spoilage and food-borne diseases associated with UHT milk and dairy products with simultaneous production flexibility.

## Prediction of storage life of raw milk with respect to *Pseudomonas* spp. and *Bacillus cereus* growth under isothermal temperature conditions

### 7.2 Introduction

Refrigerated storage of raw milk facilitates the growth of some psychrotrophic bacteria that are able to produce heat-stable extracellular proteases (Quigley et al. 2013a). The proteolytic enzymes are remarkably heat-stable, and some of them are also able to withstand UHT heating to 150 ºC for 20 s (Vithanage et al. 2014). The residual proteases in the UHT milk hydrolyse the milk proteins leading to the formation of bitter peptides as well as age gelation during storage under ambient conditions (Stoeckel et al. 2016). It was observed that a trace amount of bacterial protease activity, i.e. $6.0 \times 10^{-2}$ u/mL (~0.3 ng/mL), resulted in gelation within 3 months of ambient storage of UHT milk (Mitchell and Ewings, 1985). Bacteria with higher proteolytic activity can produce the same amount of proteases witihin a few hours of their growth (Datta & Deeth 2001). In addition to the implications for spoilage, thermoduric spore-forming psychrotrophs, including *Bacillus cereus s.l.*, can result in food safety issues due to the production of heat-stable and heat-labile toxins as well as heat-stable spores that can withstand UHT heating (Burgess et al. 2010, Gopal et al. 2015).

Raw milk is known to be contaminated with a wide range of proteolytic bacteria including *Pseudomonas, Bacillus, Acinetobacter, Hafnia, Rahnella, Alcaligenes, Achromobacter, Aeromonas, Serratia, Enterobacter, Chryseobacterium, Chromobacterium*, *Flavobacterium*, *Clostridium, Corynebacterium, Streptococcus, Micrococcus, Staphylococcus*, *Enterococcus, Lactobacillus*, and *Microbacterium,* with the first two genera dominating the total population (Machado et al. 2017, Quigley et al. 2013b, Vithanage et al. 2016).

Although the incidence and diversity of the microbial population in raw milk has been widely investigated, little information is available with respect to the relationship of psychrotrophic counts with protease production or proteolysis (Griffiths et al. 1987, Haryani et al. 2003, O'Connell et al. 2016). However, these studies demonstrated conflicting information about bacterial counts in terms of protease activity and proteolysis. For example, Griffiths et al. (1987) reported that the quality of raw milk is acceptable until the psychrotrophic bacterial

count (PBC) reaches $10^6$ cfu/mL. However, Haryani et al. (2003) defined the minimum PBC required for significant proteolysis as $1.0 \times 10^7$ cfu/mL. On the contrary, Silveira et al. (1999) reported that PBC of $2.7 \times 10^4$ cfu/mL would be sufficient to increase the protease activity in raw milk under refrigerated conditions. Another study showed that on exceeding $4.5 \times 10^4$ cfu/mL, the psychrotrophic proteolytic count (PPrBC) becomes the major cause of milk spoilage (Vyletelova et al. 2000). Most recently, we observed that there was a significant increase in protease production and proteolysis with PPrBC and thermoduric psychrotrophic count (TDPC) of $4.5 \times 10^4$ cfu/mL and $1 \times 10^4$ cfu/mL, respectively (Vithanage et al. 2017). These findings concur with the study of Gillis et al. (1985) in which significantly less proteolysis of UHT milk was observed with raw milk microbiota at less than $10^4$ cfu/mL.

The time required for psychrotrophic proteolytic bacteria and thermoduric psychrotrophs to reach significant levels can vary depending on the refrigeration temperature maintained in the farm bulk tank as well as during the transportation and storage in commercial silos prior to the processing and the involvement of the bacterial species (Griffiths et al. 1987, Vithanage et al. 2016).

Thus, a comprehensive understanding of the temperature behaviour of these bacteria would enable commercial milk processing and regulatory agencies to develop effective production and risk management strategies. In this regard, predictive microbiology needs to be used in order to obtain a systematic approach with a statistically reliable and mathematically meaningful model that describes the necessary microbial responses to distinct temperature conditions (Fu et al. 1991, Pla et al. 2015, Valik et al. 2003). This would minimise the risk of spoilage and food borne illnesses, leading to a better raw milk quality and thereby better quality of UHT milk and dairy products. This would also assist in managing the supply chain with concomitant production flexibility.

Several mathematical models have been developed to predict the growth of various bacteria as a function of temperature in different food matrixes (Fu et al. 1991, Pla et al. 2015). To our knowledge, no predictive models have been developed for PPrBC and TDPC in raw milk. Thus, the objective of the present study was to develop mathematical models that

could predict the storage life in relation to the growth of psychrotrophic proteolytic bacteria and thermoduric psychrotrophs as functions of temperature.

## 7.3 Materials and Methods

### 7.3.1 Raw milk samples

Raw milk samples from three commercial farms (designated as A, B and C), possessing high quality (A: 2.3 × $10^4$ cells/mL), medium quality (B: 5.3 × $10^5$ cells/mL) and poor quality (C: 6.7 × $10^6$ cells/mL), were provided by a commercial UHT milk processor in Victoria, Australia over one year period (n=72) (Vithanage et al. 2016, Vithanage et al. 2017). The total bacterial counts in raw milk were based on the data obtained from the corresponding dairy processor by BactoScan™ FC+ (Foss analytical, Hillerød, Denmark).

### 7.3.2 Storage conditions

Samples were collected directly from the bulk milk tank at each of the farms under aseptic conditions, and delivered to the laboratory on ice (at 4-5 ºC) within 2-3 hours of the milking procedure. 500 mL of each sample was stored at 2 °C, 4 °C, 6 °C, 8 °C, 10 °C, 12 °C, 14 °C, 16 °C, 18 °C, 20 °C, 26 °C, 30 °C and 35 °C in a shaking incubator (Innova 4230, New Brunswick Scientific, Edison, NJ, USA) with constant agitation at 120 rpm (Table 7.1) (Vithanage et al. 2017). Samples were analysed daily, commencing from day 0, signifying three biological (three separate samples of milk from each bulk tank) and three technical (three sub-samples from each 500 mL) replicates (n = 9).

### 7.3.3 Enumeration of psychrotrophic proteolytic *Pseudomonas* spp. in raw milk

The psychrotrophic proteolytic *Pseudomonas* spp. were enumerated by subjecting raw milk samples to a serial 10-fold dilution using 0.1% peptone water. Diluted samples were cultured onto *Pseudomonas* agar base with CFC supplement (CFC; Cetrimide: 0.01 mg/mL, Fucidin: 0.01 mg/mL, Cephalosporin: 0.05 mg/mL; Oxoid, UK) using the drop plate method (Munsch-Alatossava et al. 2007) and were incubated at 7 ºC for 10 days (Ercolini et al. 2009, Vithanage et al. 2014). These samples comprised psychrotrophic bacteria *Pseudomonas* (n=72) *B. cereus S.I* (n=48) and population.

**Table 7.1** The storage conditions considered in the current study are as follows:

| Storage Temperature (ºC) | Storage time (days) |
|---|---|
| 2 | 25 |
| 4 | 22 |
| 6 | 18 |
| 8 | 12 |
| 10 | 10 |
| 12 | 8 |
| 14 | 6 |
| 16 | 6 |
| 18 | 6 |
| 20 | 3 |
| 26 | 2 |
| 30 | 2 |
| 35 | 2 |

The extracellular proteolytic activity of the representative isolates was determined on the basis of Plate Count Agar, supplemented with 1.0% (w/v) skim milk (PCM agar: Sigma-Aldrich, Castle Hill, Australia) and a subsequent incubation at 7 ºC for 10 days. Clearing zones around bacterial colonies were indicative of proteolysis (Cempírkova 2007).

### 7.3.4 Enumeration of psychrotrophic and pathogenic *Bacillus cereus* in raw milk

The level of *B. cereus* in raw milk was determined using the method described by Buehner et al. (2014), with slight modifications. To explain it briefly, the raw milk was heated at 63 ± 0.5 °C for 30 min, and subsequently cultured on Brilliance™ *Bacillus cereus* agar supplemented with polymyxin B (BBC agar; Oxoid, UK) and incubated at 7 ºC for 10 days. Representative isolates with characteristic colony morphology from *B. cereus* agar were further subcultured on the Tryptone Soya Agar supplemented with 5% sheep blood for determination of haemolytic activity. Production of emetic toxin (cereulide) by *B. cereus s.l.* was detected by both liquid chromatography–mass spectrometry (Jääskeläinen et al. 2003), and by detection of cereulide gene synthase (ces) using PCR assay (Ehling-Schulz et al. 2005).

### 7.3.5 Development of primary models

The primary growth models for proteolytic psychrotrophic bacteria and thermoduric psychrotrophs were developed as functions of time at constant temperature conditions, using modified Gompertz and Baranyi models. The Gompertz model describes the growth of a microorganism using the following mathematical formula (Zwietering et al. 1996);

$$N = N_0 + (N_{max} - N_0) \times \exp\left\{-\exp\left[\frac{24k_{max}e}{(N_{max}-N_0)(\boldsymbol{\lambda}-\mathbf{t})} + 1\right]\right\}, \textbf{(1)}$$

where N is the logarithm of microbial counts ($log_{10}$ cfu /mL); $N_0$ is the logarithm of initial cell count at time t = 0 ($log_{10}$ cfu/mL); $N_{max}$ is the maximum cell number increase at the stationary phase in logarithm ($log_{10}$ cfu/mL); $k_{max}$ is the maximum specific growth rate at time t [per day] and λ or $t_{Lag}$ is the lag-phase duration (days).

Baranyi and Roberts (1994) originally introduced the Baranyi model. It is given by following equations (2-4) (Heo et al. 2014):

$$y(t) = y0 + [\left(\frac{y1}{\ln(10)}\right) + (\frac{y2}{\ln(10)})] \textbf{(2)}$$

$$y1 = \mu.t + \ln[e^{-\mu.t} - e^{-\mu(t+\mathrm{tLag})} + e^{-\mu.\mathrm{tLag}}] \textbf{(3)}$$

$$y2 = \ln[1 + 10^{(y0-ymax)}.(e^{\mu\,(t-\mathrm{tLag})} - e^{(-\mu t.\mathrm{tLag})}] \textbf{(4)}$$

where y(t) is the bacterial count in $log_{10}$ cfu/mL at time t; $y_0$ is the initial bacteria count in $log_{10}$ cfu/mL at time 0; $y_{max}$ is the maximum bacterial count in $log_{10}$ cfu/mL; $t_{lag}$ is the lag time and $\mu_{max}$ is the maximum specific growth rate, $log_{10}$ cfu/mL/h.

The average plate count data were transformed to $log_{10}$ values prior to the analysis. The average parameters (ymax, lag time and μmax) were determined using DMFit curve-fitting software v3.5 (courtesy of the Institute of Food Research, Norwich, United Kingdom) to estimate the growth rate ($log_{10}$ cfu/mL), according to the Baranyi and Gompertz models.

**7.3.6 Development of secondary models**

The effects of temperature on microbial growth parameters like maximum specific growth rate and lag phase durations, calculated using the primary models (Equations 5-6), were further evaluated using polynomial model of Belehradek-type equations as described by Koutsoumanis (2001).

$$\sqrt{\mu max} = b\mu.(T - Tmin) \textbf{(5)}$$

$$\frac{\sqrt{1}}{tlag} = bL.(T - Tmin) \textbf{(6)}$$

Where *T* is the temperature (°C), *b* is a constant, and $T_{min}$ is the nominal minimum temperature for growth, estimated by extrapolation of the regression line to $\sqrt{\mu max} = 0$.

To estimate values of regression coefficients, the SPSS software for Windows (Version 21 software; IBM Corp. in Armonk, NY) was used to make the data suitable for the polynomial model.

### 7.3.7 Prediction of raw milk storage life using tertiary model

The storage life for quality aspect ($S_{LQ}$) was defined as the time required for attaining the *Pseudomonas* counts of 5 × $10^4$ cfu/mL, while the storage life for safety aspect ($S_{LS}$) was defined as the time required for attaining *B. cereus* counts of 1 × $10^4$ cfu/mL. The prediction of storage life of raw milk was conducted using the growth kinetics data derived (by primary models) and validated (by secondary models) using an exponential model as described by Fu & Labuza (1993) using the following equation;

$ts = \ln(Nt/No)/\mu + \mathrm{tLag}$ **(7)**

where ts is the $S_{LQ}$ or $S_{LS}$, $N_0$ is the initial microbial count ($\log_{10}$ cfu/mL), $N_t$ is the microbial level at time t ($\log_{10}$ cfu/mL), Ns is the microbial level ($\log_{10}$ cfu/mL) corresponding to the end of storage life; for $S_{LQ,}$ 5 × $10^4$ cfu/mL and for $S_{LS,}$ 1 × $10^4$ cfu/mL, μ is specific growth rate and $t_{Lag}$ is the lag time.

### 7.3.8 Data processing, validation and statistical analysis

The experimental data and predicted data were compared using the correlation coefficient ($R^2$), and root mean squares error (RMSE) (McKellar & Lu 2004). The performance of the models was further validated using bias ($B_f$) and accuracy ($A_f$) factors, in which, $B_f$ measures the relative deviation of predicted and observed data, while $A_f$ is the measurement of absolute deviation of predictions, obtained from observations (Baranyi et al. 1999).

$RMSE = \sqrt{\sum(obs - pred)^2/n}$ **(8)**

$Bf = 10^{\sum \log(\frac{pred}{obs})/n}$ **(9)**

$Af = 10^{\sum \left|\log(\frac{pred}{obs})\right|/n}$ **(10)**

In the above equations, obs is the observed value of counts, pred is the predicted value and n is the number of observations (n = 9). A perfect agreement between predictions and

observations leads to bias and accuracy values equal to 1.0. $A_f$ values greater than 1 indicate that the predicted values are larger than the observed values.

Additionally, the percentage discrepancy (%D) between the predicted value and observed storage life value determined by means of exponential model was further evaluated using following equation (Koutsoumanis, 2001);

$$\%D = \left[\frac{SLobs - Slpred}{SLobs}\right] * 100 \quad \textbf{(11)}$$

where $SL_{obs}$ is the observed storage life; $SL_{pred}$ is the predicted storage life.

Regression analysis and significance levels of the data were calculated using two-way ANOVA and MANOVA with the help of the SPSS software for Windows (Version 21 software; IBM Corp. in Armonk, NY). $P < 0.05$ was considered to be statistically significant.

## 7.4 Results

### 7.4.1 Development of primary growth model for isothermal temperature conditions

Primary growth models for psychrotrophic *Pseudomonas* and *B. cereus S. I.* in raw milk were developed at three inoculation levels (1, 2 and 4 cfu/mL) at six different temperatures (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 26, 30 and 35 ºC) (Table 7.1). The storage temperature significantly affected the growth kinetics of psychrotrophic *Pseudomonas* sp. (n = 72) and *B. cereus S.I.* (n = 48) population in raw milk ($P < 0.05$). For example, in the range of 2-12 °C, the temperature-dependent growth rates and lag phase durations were graphically fitted to both Baranyi and Gompertz models with the characteristic sigmoidal growth pattern (Figure 7.1). Similarly, temperatures higher than 12 °C showed significant increase in the growth rates and a concomitant reduction in the lag phase duration, but could still fit with both the primary growth models, graphically (data not shown).

According to the Baranyi model, there was a 2-log increase in *Pseudomonas'* counts observed after about 240 h, 95 h, 8.75 h, 57 h, 42.75 h, 28.5, 19 h, 14.25 h, 4.75 h, 3.82 h, as the temperature increased from deep cooling at 2 °C, standard refrigeration (4 °C) and elevated refrigeration conditions (6-20 °C). However, *Pseudomonas* spp. had a 2-log increase between 0-10 h at higher temperatures (26 °C: optimum, 30 °C and 35 °C). The

specific growth rate (μ) and lag phase duration (λ) of *Pseudomonas* spp. varies in the range of 0.05-269.3/h and 0.03-60.5/ h, depending on the temperature condition used (Table 7.2). In case of *B. cereus*, a 2-log increase in the counts was observed after 85.5 h, 61.75 h, 42.75 h, 28.5 h, 23.75 h, 14.25 h, 9.5 h, 4.75 h, 3.21 h, as the temperature increases from 2 to 20 °C. However, these values were increased up to 0-5 h under optimum conditions (26 °C, 30 °C and 35 °C: optimum). The specific growth rate was in the range of 0.029-3.42/h, while lag phase duration was in the range of 104.09-0.93 h (Table 7.2). The maximum counts ($N_{max}$) exceeded 8 log cfu/mL at temperature > 4 °C (data is not shown).

The maximum specific growth rates of both *Pseudomonas* spp. and *B. cereus* estimated by Gompertz and Baranyi models were correlated with increased temperature during storage. The growth rates calculated by the Gompertz model were slightly higher than that of the Baranyi model (Table 7.3). For instance, at 4 °C, the growth rates predicted by the Gompertz model were 0.068/h, 0.067/h and 0.069/h for *Pseudomonas* spp. As compared to 0.0453/h, 0.0455/h and 0.0458/h for *B. cereus* in A, B and C samples, respectively. In contrast, the Baranyi model resulted in specific growth rates of *Pseudomonas* with 0.066/h, 0.064/h and 0.068/h with 0.0447/h, 0.0445/h and 0.0453/h in A, B and C samples, respectively. However, on a general scale, both graphs resembled each other (Figure 7.1).

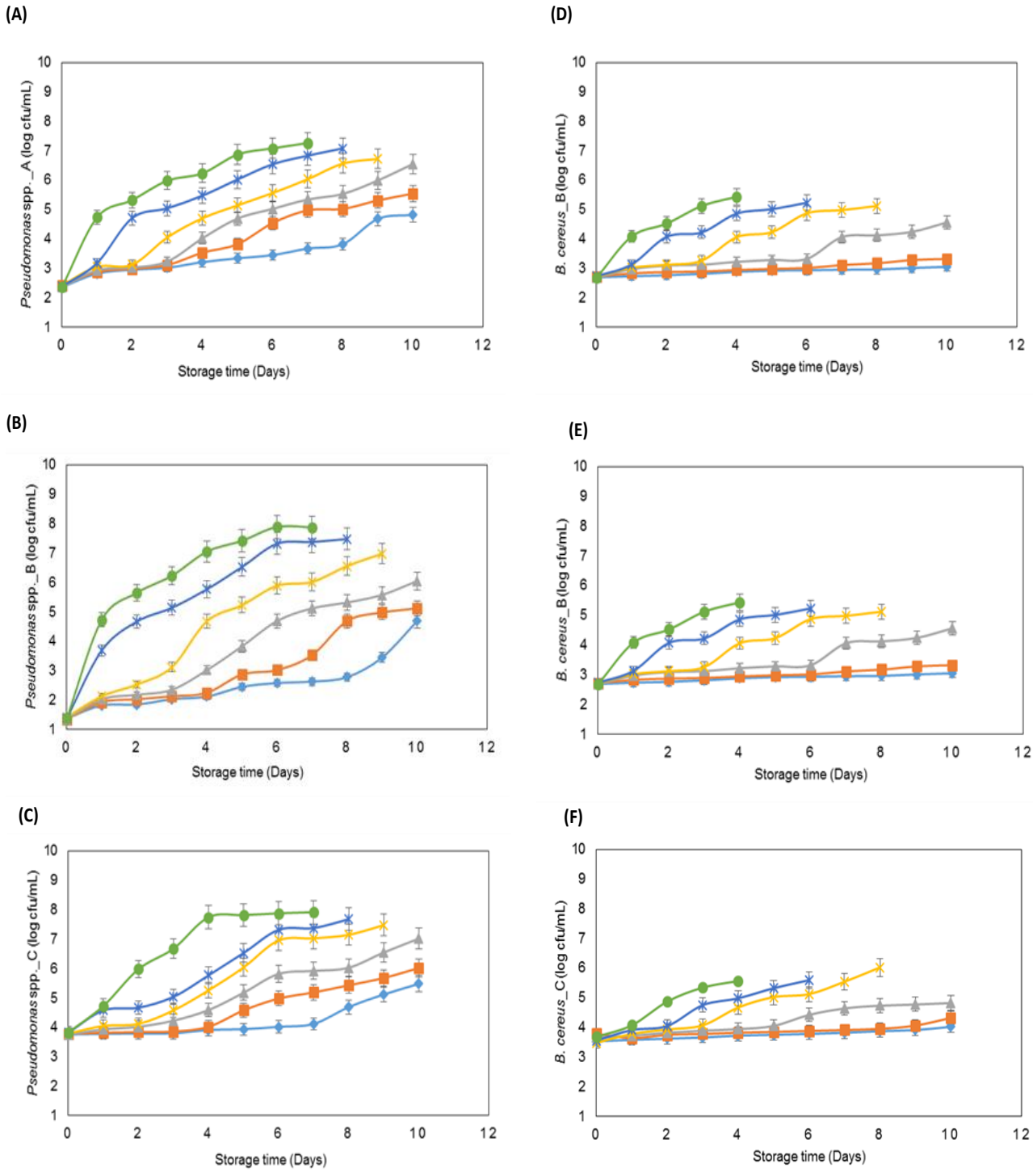


**Figure 7.1** The effect of different temperature conditions ( 2 °C , 4 °C , 6 °C, 8 °C , 10 °C , 12 °C ) on the bacterial counts in A, B and C raw milk samples with different initial counts; (A-C) *Pseudomonas* spp.; (D-F) *B. cereus* that were fitted with primary (Baranyi and Gompertz) models. The results were presented as mean ± SE, (n = 9).

**Table 7.2** Growth kinetic parameters of *Pseudomonas* spp. and *B. cereus* in raw milk under different storage temperature according to the Baranyi model.

| Storage Temp (°C) | Source | *Pseudomonas* spp. | | | | *B. cereus* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $\mu_{max}$ [†](/h) | λ [Ø] (h) | $R^2$, [§] | RMSE* | $\mu_{max}$ [†](/h) | λ (h) | $R^2$, [§] | RMSE* |
| 2 | A | 0.052 | 60.76 | 0.97 | 0.0854 | 0.029 | 104.12 | 0.99 | 0.0932 |
| | B | 0.051 | 60.74 | 0.99 | 0.0832 | 0.025 | 104.09 | 0.98 | 0.0926 |
| | C | 0.050 | 60.67 | 0.98 | 0.0856 | 0.032 | 103.98 | 0.99 | 0.0924 |
| 4 | A | 0.066 | 46.99 | 0.98 | 0.0959 | 0.0447 | 69.31 | 0.99 | 0.0932 |
| | B | 0.064 | 47.02 | 0.98 | 0.0943 | 0.0445 | 69.38 | 0.98 | 0.0928 |
| | C | 0.068 | 46.92 | 0.98 | 0.0928 | 0.0453 | 69.28 | 0.98 | 0.0936 |
| 6 | A | 0.088 | 35.13 | 0.99 | 0.0854 | 0.066 | 46.84 | 0.99 | 0.0863 |
| | B | 0.088 | 35.16 | 0.99 | 0.0854 | 0.064 | 46.88 | 0.98 | 0.0876 |
| | C | 0.089 | 35.12 | 0.98 | 0.0863 | 0.068 | 46.82 | 0.99 | 0.0857 |
| 8 | A | 0.122 | 25.39 | 0.97 | 0.1154 | 0.096 | 32.15 | 0.98 | 0.1231 |
| | B | 0.121 | 25.41 | 0.99 | 0.1147 | 0.094 | 32.18 | 0.99 | 0.1243 |
| | C | 0.123 | 25.35 | 0.98 | 0.1153 | 0.098 | 32.21 | 0.98 | 0.1254 |
| 10 | A | 0.175 | 17.74 | 0.97 | 0.1077 | 0.138 | 22.41 | 0.98 | 0.1113 |
| | B | 0.172 | 17.83 | 0.98 | 0.1068 | 0.143 | 22.45 | 0.99 | 0.1124 |
| | C | 0.178 | 17.69 | 0.99 | 0.1073 | 0.145 | 22.53 | 0.98 | 0.1132 |
| 12 | A | 0.259 | 11.98 | 0.98 | 0.1067 | 0.195 | 15.87 | 0.97 | 0.1113 |
| | B | 0.248 | 12.01 | 0.98 | 0.1064 | 0.197 | 15.89 | 0.98 | 0.1142 |
| | C | 0.268 | 11.93 | 0.98 | 0.1058 | 0.199 | 15.91 | 0.98 | 0.1156 |
| 14 | A | 0.396 | 7.82 | 0.98 | 0.1284 | 0.272 | 11.41 | 0.97 | 0.1254 |
| | B | 0.398 | 7.85 | 0.99 | 0.1278 | 0.274 | 11.43 | 0.98 | 0.1232 |
| | C | 0.394 | 7.79 | 0.98 | 0.1269 | 0.278 | 11.47 | 0.99 | 0.1228 |
| 16 | A | 0.628 | 4.94 | 0.99 | 0.2612 | 0.372 | 8.33 | 0.98 | 0.2732 |
| | B | 0.626 | 4.96 | 0.99 | 0.2609 | 0.374 | 8.36 | 0.98 | 0.2728 |
| | C | 0.632 | 4.92 | 0.99 | 0.2616 | 0.376 | 8.39 | 0.98 | 0.2763 |
| 18 | A | 1.029 | 3.01 | 0.98 | 0.2611 | 0.502 | 6.18 | 0.97 | 0.2711 |
| | B | 1.025 | 3.12 | 0.98 | 0.2608 | 0.508 | 6.23 | 0.98 | 0.2746 |
| | C | 1.032 | 2.98 | 0.98 | 0.2622 | 0.512 | 6.26 | 0.99 | 0.2755 |
| 20 | A | 1.744 | 1.78 | 0.99 | 0.2631 | 0.667 | 4.65 | 0.98 | 0.2742 |
| | B | 1.732 | 1.83 | 0.98 | 0.2628 | 0.669 | 4.67 | 0.99 | 0.2741 |
| | C | 1.748 | 1.75 | 0.99 | 0.2626 | 0.672 | 4.69 | 0.98 | 0.2732 |
| 26 | A | 10.42 | 0.297 | 0.99 | 0.2612 | 1.423 | 2.18 | 0.99 | 0.2456 |
| | B | 10.23 | 0.293 | 0.99 | 0.2614 | 1.425 | 2.22 | 0.99 | 0.2461 |
| | C | 10.53 | 0.276 | 0.99 | 0.2623 | 1.428 | 2.27 | 0.98 | 0.2443 |
| 30 | A | 40.632 | 0.08 | 0.99 | 0.2624 | 2.18 | 1.42 | 0.99 | 0.2602 |
| | B | 40.628 | 0.09 | 0.99 | 0.2609 | 2.22 | 1.44 | 0.99 | 0.2654 |
| | C | 40.635 | 0.07 | 0.98 | 0.2616 | 2.24 | 1.47 | 0.98 | 0.2648 |
| 35 | A | 269.353 | 0.01 | 0.99 | 0.2621 | 3.422 | 0.91 | 0.98 | 0.2632 |
| | B | 269.312 | 0.02 | 0.99 | 0.2636 | 3.424 | 0.92 | 0.99 | 0.2664 |
| | C | 269.362 | 0.03 | 0.98 | 0.2643 | 3.428 | 0.93 | 0.99 | 0.2658 |

All values are means ± standard deviations in the range of 1.01-2.43 (n = 9). Means sharing different letters in the same column are significantly different ($P < 0.05$). [†]Maximum specific growth rate (log cfu/mL/h); [Ø]λ: lag time duration (h); [§]$R^2$: Correlation coefficient.*RMSE: Root mean square error.

**Table 7.3** Growth kinetic parameters of *Pseudomonas* spp. and *B. cereus* in raw milk under different storage temperature according to the Gompertz models.

| Storage Temp (°C) | Source | *Pseudomonas* spp. | | | | *B. cereus* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $\mu_{max}$ [†](/h) | λ[Ø] (h) | $R^{2,\S}$ | RMSE* | $\mu_{max}$ [†](/h) | λ[Ø] (h) | $R^{2,\S}$ | RMSE* |
| 2 | A | 0.058 | 58.23 | 0.98 | 0.0823 | 0.032 | 104.09 | 0.98 | 0.0912 |
| | B | 0.056 | 60.76 | 0.99 | 0.0828 | 0.028 | 104.11 | 0.99 | 0.0925 |
| | C | 0.052 | 60.70 | 0.99 | 0.0833 | 0.038 | 104.01 | 0.98 | 0.0936 |
| 4 | A | 0.068 | 46.99 | 0.98 | 0.0959 | 0.0453 | 69.31 | 0.99 | 0.0932 |
| | B | 0.067 | 47.02 | 0.98 | 0.0943 | 0.0455 | 69.38 | 0.98 | 0.0928 |
| | C | 0.069 | 46.92 | 0.98 | 0.0928 | 0.0458 | 69.28 | 0.98 | 0.0936 |
| 6 | A | 0.092 | 35.27 | 0.98 | 0.0867 | 0.071 | 46.92 | 0.98 | 0.0871 |
| | B | 0.093 | 35.28 | 0.99 | 0.0864 | 0.069 | 46.98 | 0.99 | 0.0879 |
| | C | 0.092 | 35.23 | 0.98 | 0.0868 | 0.072 | 46.93 | 0.98 | 0.0862 |
| 8 | A | 0.126 | 25.44 | 0.98 | 0.1159 | 0.098 | 32.23 | 0.99 | 0.1238 |
| | B | 0.128 | 25.49 | 0.99 | 0.1153 | 0.096 | 32.26 | 0.98 | 0.1254 |
| | C | 0.125 | 25.39 | 0.99 | 0.1158 | 0.099 | 32.26 | 0.99 | 0.1258 |
| 10 | A | 0.178 | 17.81 | 0.98 | 0.1082 | 0.143 | 22.45 | 0.98 | 0.1123 |
| | B | 0.176 | 17.87 | 0.99 | 0.1072 | 0.146 | 22.49 | 0.99 | 0.1128 |
| | C | 0.179 | 17.72 | 0.98 | 0.1074 | 0.147 | 22.56 | 0.99 | 0.1136 |
| 12 | A | 0.262 | 11.99 | 0.99 | 0.1072 | 0.198 | 15.89 | 0.99 | 0.1123 |
| | B | 0.253 | 12.06 | 0.99 | 0.1068 | 0.199 | 15.92 | 0.99 | 0.1146 |
| | C | 0.272 | 11.96 | 0.99 | 0.1063 | 0.201 | 15.93 | 0.99 | 0.1161 |
| 14 | A | 0.399 | 7.86 | 0.98 | 0.1286 | 0.275 | 11.44 | 0.98 | 0.1256 |
| | B | 0.399 | 7.89 | 0.99 | 0.1279 | 0.277 | 11.45 | 0.98 | 0.1236 |
| | C | 0.396 | 7.82 | 0.98 | 0.1272 | 0.282 | 11.52 | 0.99 | 0.1232 |
| 16 | A | 0.632 | 4.96 | 0.99 | 0.2616 | 0.376 | 8.36 | 0.99 | 0.2736 |
| | B | 0.629 | 4.98 | 0.98 | 0.2612 | 0.376 | 8.38 | 0.99 | 0.2732 |
| | C | 0.639 | 4.96 | 0.98 | 0.2622 | 0.379 | 8.43 | 0.98 | 0.2768 |
| 18 | A | 1.032 | 3.13 | 0.98 | 0.2616 | 0.508 | 6.23 | 0.98 | 0.2721 |
| | B | 1.028 | 3.16 | 0.98 | 0.2611 | 0.510 | 6.26 | 0.98 | 0.2753 |
| | C | 1.036 | 2.99 | 0.99 | 0.2626 | 0.516 | 6.28 | 0.99 | 0.2758 |
| 20 | A | 1.746 | 1.82 | 0.99 | 0.2636 | 0.669 | 4.68 | 0.99 | 0.2743 |
| | B | 1.736 | 1.86 | 0.99 | 0.2632 | 0.672 | 4.69 | 0.98 | 0.2745 |
| | C | 1.753 | 1.78 | 0.99 | 0.2634 | 0.674 | 4.71 | 0.98 | 0.2733 |
| 26 | A | 10.43 | 0.299 | 0.99 | 0.2613 | 1.426 | 2.22 | 0.99 | 0.2458 |
| | B | 10.25 | 0.296 | 0.98 | 0.2612 | 1.423 | 2.23 | 0.98 | 0.2462 |
| | C | 10.56 | 0.279 | 0.98 | 0.2624 | 1.429 | 2.28 | 0.99 | 0.2445 |
| 30 | A | 40.66 | 0.081 | 0.99 | 0.2626 | 2.19 | 1.44 | 0.99 | 0.2608 |
| | B | 40.64 | 0.092 | 0.99 | 0.2611 | 2.25 | 1.46 | 0.99 | 0.2656 |
| | C | 40.65 | 0.071 | 0.98 | 0.2622 | 2.26 | 1.48 | 0.98 | 0.2651 |
| 35 | A | 269.36 | 0.01 | 0.99 | 0.2622 | 3.423 | 0.92 | 0.98 | 0.2633 |
| | B | 269.37 | 0.02 | 0.99 | 0.2632 | 3.426 | 0.93 | 0.98 | 0.2662 |
| | C | 269.39 | 0.03 | 0.98 | 0.2641 | 3.429 | 0.95 | 0.99 | 0.2648 |

All values are means ± standard deviations in the range of 1.11-2.87 (n = 9). Means sharing different letters in the same column are significantly different ($P < 0.05$). [†]Maximum specific growth rate (log cfu/mL/h); [Ø]λ: lag time duration (h); [§]$R^2$: Correlation coefficient.*RMSE: Root mean square error.

### 7.4.2 Development of secondary models

Analysis of the combined effect of initial counts and temperatures on the bacterial growth parameters by two-way ANOVA indicated that this effect was not significant in all three samples ($P > 0.05$: Table 7.1 and 7.2). In contrast, the effects of the temperature on the growth parameters were significantly different ($P < 0.05$). Thus, fitting of the data derived from primary models as a function of temperature was evaluated using Belehradek-type equations. This model showed that the maximum specific growth rate (μ) and lag phase duration ($t_{Lag}$) of both *Pseudomonas* spp. and *B. cereus* were significantly affected by

storage temperature, which graphically fitted very well with the so-called secondary models (Figure 7.2).

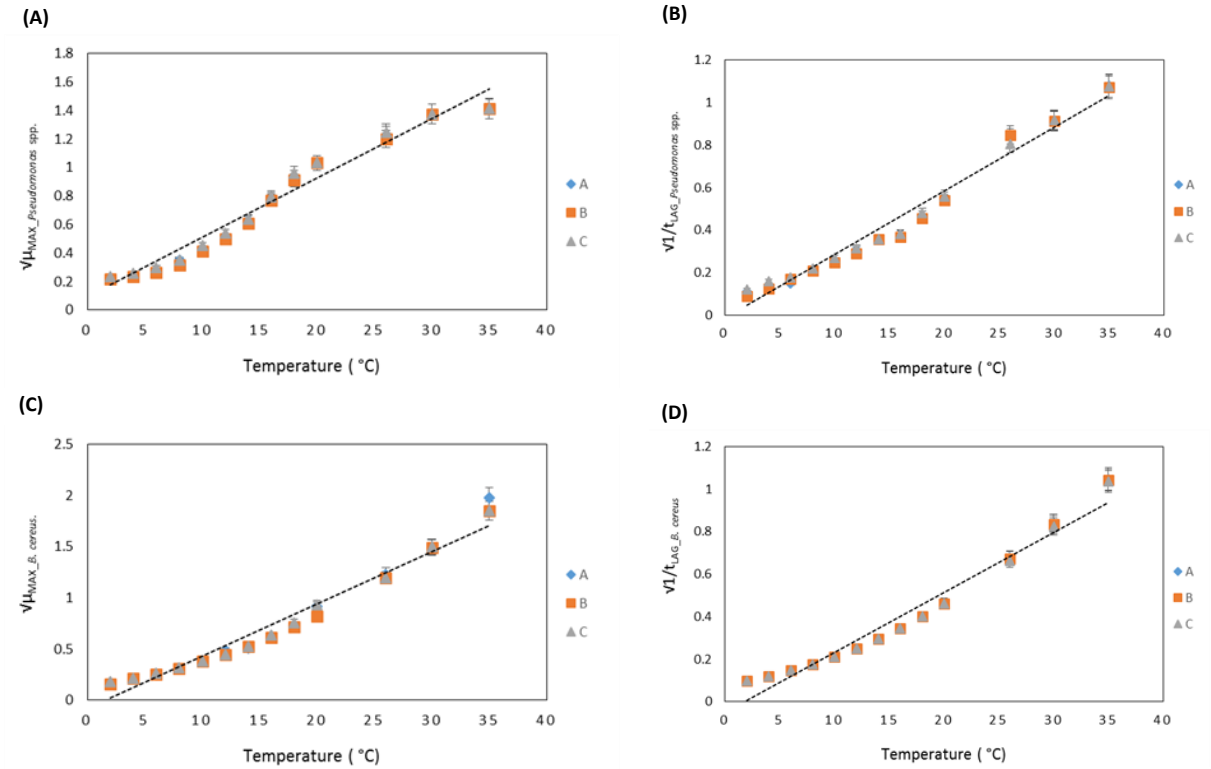


**Figure 7.2** The effect of temperature on maximum specific growth rates of (A) *Pseudomonas* spp. (B) *B. cereus* and lag phase duration of (C) *Pseudomonas* spp., (D) *B. cereus* estimated using Belehradek-type equations. Results are presented as mean ± SE (n = 9).

### 7.4.3 Validation of predictive models

All primary models for growth of *Pseudomonas* and *B. cereus* were characterized by high $R^2$, and the values derived for both Gompertz and Baranyi models were similar to each other (Table 7.2, 7.3). Although, both equations resulted in small RMSE values, the values resulting from the Baranyi model was slightly lower than those of the Gompertz model. Additionally, the bias ($B_f$) and accuracy ($A_f$) factors were used to determine the virtuousness of fit of the models, in which, Af was in the range of 1.034–1.113 and 1.010–1.046 in the two models. The values of $R^2$, RMSE, $B_f$ and $A_f$ for polynomial secondary models were statistically significant (Table 7.4).

**Table 7.4** Validation of polynomial secondary model of Belehradek-type equations.

| Model | Sample | Polynomial secondary models | $R^{2,\S}$ | RMSE* | $B_f^{€}$ | $A_f^{¤}$ |
|---|---|---|---|---|---|---|
| Maximum specific growth rates[œ] | P | P: y = 0.256x - 4.1492 | 0.9751 | 0.153 | 0.997 | 1.321 |
| | | B: y = 0.1458x - 3.5121 | 0.9833 | 0.172 | 0.998 | 1.278 |
| | B | P: y = 0.256x - 4.1573 | 0.9746 | 0.152 | 0.997 | 1.336 |
| | | B: y = 0.1479x - 3.5531 | 0.9789 | 0.179 | 0.998 | 1.313 |
| Lag phase duration[œ] | P | P: y = 0.266x - 4.1192 | 0.9751 | 0.198 | 0.996 | 1.270 |
| | | B: y = 0.1452x - 3.4121 | 0.9833 | 0.175 | 0.998 | 1.276 |
| | B | P: y = 0.252x - 4.1568 | 0.9746 | 0.172 | 0.997 | 1.268 |
| | | B: y = 0.1482x - 3.5538 | 0.9789 | 0.177 | 0.993 | 1.132 |

[œ] Estimated by Baranyi model and Gompertz models; P: *Pseudomonas* spp.; B: *B. cereus*; [§] Correlation coefficient. *****Root mean square error. [€]Bias factors. [¤]Accuracy factors.

### 7.4.4 Prediction of storage life of raw milk

The storage life for quality aspect ($S_{LQ}$) and storage life for safety aspect ($S_{LS}$) of raw milk were defined as the time to reach $5 \times 10^4$ log cfu/mL of *Pseudomonas*, or $1 \times 10^4$ log cfu/mL of *B. cereus*, respectively (Figure 7.3). The 2 °C storage resulted in a significant increase in $S_{LQ}$ in the range of 65.1-84.8 h, and $S_{LS}$ in the range of 107.9-151.6 h, and for 4 °C these values varied in the range of 50.2-65.9 h and 70.5-99.9 h, respectively (Table 7.5). However, a sharp decrease was observed in the storage life of raw milk, when the temperature increased to ≥ 14 °C. The shelf-life prediction based on the Gompertz model was observed to possess values that were similar to the ones in the Baranyi model (Table 7.6).

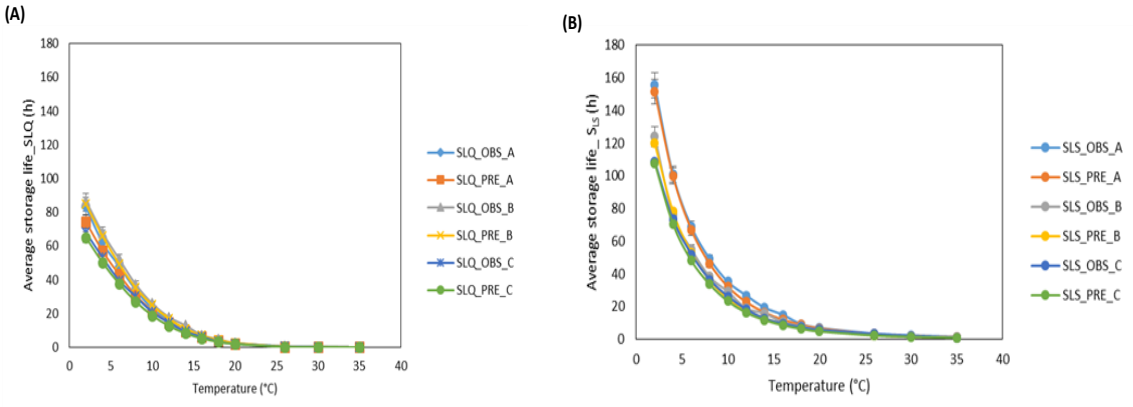


**Figure 7.3** Relationship between storage life of quality and safety aspects of raw milk from three initial counts with storage temperature; (A) storage life of quality aspect was defined as time to reach *Pseudomonas* of $5 \times 10^4$ cfu/mL, (B) the storage life of safety aspect was defined as time to reach *B. cereus* of $1 \times 10^4$ cfu/mL. Means of nine replicates ± SE (± 2.43).

**Table 7.5** Storage life of raw milk under different temperature conditions according to the Baranyi model.

| Sample | Storage Temp (ºC) | SLQ Pre | SLQ Obs | $R^{2,\S}$ | RMSE* | %D† | *Bf€* | *Af¤* | SLS Pre | SLS Obs | $R^{2,\S}$ | RMSE* | %D† | *Bf€* | *Af¤* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 2 | 83 | 74.3 | 0.98 | 2.87 | 10.53 | 0.988 | 1.105 | 155.6 | 151.6 | 0.98 | 0.89 | 2.57 | 0.997 | 1.026 |
| | 4 | 62 | 57.1 | 0.98 | 1.20 | 7.89 | 0.991 | 1.079 | 100.9 | 99.9 | 0.99 | 0.11 | 0.99 | 0.999 | 1.010 |
| | 6 | 48 | 43.0 | 0.99 | 1.24 | 10.41 | 0.988 | 1.104 | 69.1 | 67.1 | 0.99 | 0.31 | 2.89 | 0.997 | 1.029 |
| | 8 | 34 | 30.8 | 0.98 | 0.63 | 9.33 | 0.989 | 1.093 | 49.3 | 46.3 | 0.99 | 0.58 | 6.09 | 0.993 | 1.061 |
| | 10 | 23 | 21.6 | 0.97 | 0.18 | 5.97 | 0.993 | 1.060 | 35.2 | 32.2 | 0.98 | 0.58 | 8.52 | 0.990 | 1.085 |
| | 12 | 16 | 14.6 | 0.98 | 0.18 | 8.51 | 0.990 | 1.085 | 26.8 | 22.8 | 0.97 | 0.89 | 14.93 | 0.982 | 1.149 |
| | 14 | 9.5 | 9.3 | 0.98 | 0.01 | 2.46 | 0.997 | 1.025 | 19.4 | 16.4 | 0.98 | 0.58 | 15.46 | 0.982 | 1.155 |
| | 16 | 6.5 | 6.1 | 0.98 | 0.03 | 6.95 | 0.992 | 1.069 | 15.0 | 12.0 | 0.99 | 0.58 | 20.00 | 0.976 | 1.200 |
| | 18 | 3.9 | 3.7 | 0.99 | 0.01 | 4.85 | 0.994 | 1.048 | 9.3 | 8.9 | 0.99 | 0.03 | 4.30 | 0.995 | 1.043 |
| | 20 | 2.4 | 2.2 | 0.99 | 0.01 | 8.40 | 0.990 | 1.084 | 7.0 | 6.7 | 0.98 | 0.02 | 4.29 | 0.995 | 1.043 |
| | 26 | 0.6 | 0.4 | 0.99 | 0.01 | 35.49 | 0.952 | 1.355 | 3.5 | 3.1 | 0.99 | 0.03 | 11.43 | 0.987 | 1.114 |
| | 30 | 0.23 | 0.12 | 0.99 | 0.00 | 20.75 | 0.924 | 1.507 | 2.4 | 2.0 | 0.98 | 0.03 | 16.67 | 0.980 | 1.167 |
| | 35 | 0.01 | 0.01 | 0.99 | 0.00 | 10.00 | 0.988 | 1.000 | 1.5 | 1.3 | 0.99 | 0.01 | 13.33 | 0.984 | 1.133 |
| B | 2 | 86.8 | 84.8 | 0.99 | 0.31 | 2.31 | 0.997 | 1.023 | 124.0 | 120.0 | 0.98 | 0.89 | 3.23 | 0.996 | 1.032 |
| | 4 | 67.9 | 65.9 | 0.98 | 0.31 | 2.94 | 0.997 | 1.029 | 76.1 | 78.1 | 0.97 | 0.43 | 2.63 | 1.003 | 0.974 |
| | 6 | 52.3 | 49.3 | 0.98 | 0.58 | 5.73 | 0.993 | 1.057 | 55.1 | 53.1 | 0.99 | 0.31 | 3.63 | 0.996 | 1.036 |
| | 8 | 37.3 | 35.3 | 0.98 | 0.31 | 5.36 | 0.994 | 1.054 | 38.3 | 36.3 | 0.99 | 0.31 | 5.22 | 0.994 | 1.052 |
| | 10 | 26.0 | 25.0 | 0.98 | 0.11 | 3.85 | 0.996 | 1.039 | 29.1 | 25.1 | 0.98 | 0.89 | 13.75 | 0.984 | 1.137 |
| | 12 | 18.0 | 17.0 | 0.98 | 0.11 | 5.57 | 0.994 | 1.056 | 18.9 | 17.9 | 0.98 | 0.11 | 5.29 | 0.994 | 1.053 |
| | 14 | 12.9 | 10.9 | 0.98 | 0.31 | 15.47 | 0.981 | 1.155 | 15.9 | 12.9 | 0.98 | 0.58 | 18.87 | 0.977 | 1.189 |
| | 16 | 7.2 | 6.9 | 0.98 | 0.01 | 3.56 | 0.996 | 1.036 | 10.4 | 9.4 | 0.97 | 0.11 | 9.62 | 0.989 | 1.096 |
| | 18 | 4.8 | 4.3 | 0.98 | 0.04 | 10.38 | 0.988 | 1.104 | 8.0 | 7.0 | 0.99 | 0.11 | 12.50 | 0.985 | 1.125 |
| | 20 | 2.6 | 2.5 | 0.98 | 0.00 | 3.79 | 0.996 | 1.038 | 5.9 | 5.3 | 0.99 | 0.05 | 10.17 | 0.988 | 1.102 |
| | 26 | 0.5 | 0.4 | 0.98 | 0.00 | 19.50 | 0.976 | 1.195 | 2.9 | 2.5 | 0.97 | 0.03 | 13.79 | 0.984 | 1.138 |
| | 30 | 0.25 | 0.14 | 0.99 | 0.00 | 36.81 | 0.950 | 1.368 | 1.9 | 1.6 | 0.99 | 0.02 | 15.79 | 0.981 | 1.158 |
| | 35 | 0.01 | 0.01 | 0.97 | 0.00 | 10.00 | 1.000 | 1.023 | 1.2 | 1.0 | 0.97 | 0.01 | 16.67 | 0.980 | 1.167 |
| C | 2 | 68.1 | 65.1 | 0.97 | 0.58 | 4.40 | 0.995 | 1.044 | 108.9 | 107.9 | 0.97 | 0.11 | 0.92 | 0.999 | 1.009 |
| | 4 | 53.2 | 50.2 | 0.98 | 0.58 | 5.64 | 0.994 | 1.056 | 73.5 | 70.5 | 0.98 | 0.58 | 4.08 | 0.995 | 1.041 |
| | 6 | 39.7 | 37.7 | 0.99 | 0.31 | 5.04 | 0.994 | 1.050 | 52.2 | 48.2 | 0.98 | 0.89 | 7.66 | 0.991 | 1.077 |
| | 8 | 30.1 | 27.1 | 0.99 | 0.58 | 9.97 | 0.988 | 1.100 | 36.6 | 33.6 | 0.98 | 0.58 | 8.20 | 0.991 | 1.082 |
| | 10 | 20.9 | 18.9 | 0.98 | 0.31 | 9.59 | 0.989 | 1.096 | 26.3 | 23.3 | 0.99 | 0.58 | 11.41 | 0.987 | 1.114 |
| | 12 | 14.7 | 12.7 | 0.97 | 0.31 | 13.61 | 0.984 | 1.136 | 18.3 | 16.3 | 0.97 | 0.31 | 10.93 | 0.987 | 1.109 |
| | 14 | 9.3 | 8.3 | 0.98 | 0.11 | 10.74 | 0.987 | 1.107 | 12.8 | 11.8 | 0.99 | 0.11 | 7.81 | 0.991 | 1.078 |
| | 16 | 6.2 | 5.2 | 0.99 | 0.11 | 16.02 | 0.981 | 1.160 | 9.6 | 8.6 | 0.99 | 0.11 | 10.42 | 0.988 | 1.104 |
| | 18 | 3.4 | 3.2 | 0.99 | 0.01 | 5.92 | 0.993 | 1.059 | 7.4 | 6.4 | 0.99 | 0.11 | 13.51 | 0.984 | 1.135 |
| | 20 | 2.0 | 1.9 | 0.98 | 0.00 | 5.08 | 0.994 | 1.051 | 5.8 | 4.8 | 0.99 | 0.11 | 17.24 | 0.979 | 1.172 |
| | 26 | 0.4 | 0.3 | 0.98 | 0.00 | 25.29 | 0.968 | 1.253 | 3.3 | 2.3 | 0.99 | 0.11 | 30.30 | 0.961 | 1.103 |
| | 30 | 0.26 | 0.17 | 0.98 | 0.00 | 27.13 | 0.910 | 1.571 | 2.1 | 1.5 | 0.98 | 0.05 | 28.57 | 0.963 | 1.186 |
| | 35 | 0.02 | 0.02 | 0.99 | 0.00 | 10.00 | 0.995 | 1.000 | 1.0 | 1.0 | 0.99 | 0.00 | 10.00 | 1.000 | 1.000 |

§Correlation coefficient. *****Root mean square error. €Bias factors. ¤Accuracy factors; † percentage discrepancy (%D). $S_{LQ}$_Pre: predicted storage life of raw milk in quality aspect; $S_{LQ}$_Obs: observed storage life of raw milk in quality aspect; $S_{LQ}$_ Pre: predicted storage life of raw milk in safety aspect; $S_{Ls}$_Obs: observed storage life of raw milk in quality aspect.

**Table 7.6** Storage life of raw milk under different temperature conditions according to the Gompertz models.

| Sample | Storage Temp (ºC) | $S_{LQ}$ Pre | $S_{LQ}$ Obs | $R^{2,\S}$ | RMSE* | %D† | $B_f$€ | $A_f$¤ | $S_{LS}$ Pre | $S_{LS}$ Obs | $R^{2,\S}$ | RMSE* | %D† | $B_f$€ | $A_f$¤ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 2 | 84.2 | 75.4 | 0.98 | 2.90 | 10.45 | 0.988 | 0.988 | 154.4 | 152.1 | 0.98 | 0.39 | 1.49 | 0.998 | 0.998 |
| | 4 | 63.1 | 56.9 | 0.98 | 1.72 | 9.83 | 0.989 | 0.989 | 101.2 | 98.6 | 0.99 | 0.47 | 2.57 | 0.997 | 0.997 |
| | 6 | 47.9 | 43.6 | 0.99 | 0.99 | 8.98 | 0.990 | 0.990 | 67.9 | 65.4 | 0.99 | 0.44 | 3.68 | 0.996 | 0.996 |
| | 8 | 36.3 | 31.6 | 0.98 | 1.13 | 12.95 | 0.985 | 0.985 | 51.3 | 47.6 | 0.99 | 0.79 | 7.21 | 0.992 | 0.992 |
| | 10 | 23.6 | 22.3 | 0.97 | 0.16 | 5.51 | 0.994 | 0.994 | 34.9 | 33.5 | 0.98 | 0.18 | 4.01 | 0.995 | 0.995 |
| | 12 | 15.9 | 14.3 | 0.98 | 0.22 | 10.06 | 0.988 | 0.988 | 27.9 | 23.6 | 0.97 | 0.99 | 15.41 | 0.982 | 0.982 |
| | 14 | 9.7 | 9.2 | 0.98 | 0.04 | 5.15 | 0.994 | 0.994 | 19.9 | 16.2 | 0.98 | 0.79 | 18.59 | 0.977 | 0.977 |
| | 16 | 6.6 | 6.0 | 0.98 | 0.05 | 9.09 | 0.989 | 0.989 | 17.1 | 15.9 | 0.99 | 0.15 | 7.02 | 0.992 | 0.992 |
| | 18 | 4.3 | 3.9 | 0.99 | 0.03 | 9.30 | 0.989 | 0.989 | 9.6 | 9.1 | 0.99 | 0.04 | 5.21 | 0.994 | 0.994 |
| | 20 | 2.6 | 2.1 | 0.99 | 0.04 | 19.23 | 0.977 | 0.977 | 7.5 | 6.9 | 0.98 | 0.05 | 8.00 | 0.991 | 0.991 |
| | 26 | 0.7 | 0.2 | 0.99 | 0.04 | 71.43 | 0.870 | 0.870 | 3.8 | 3.2 | 0.99 | 0.05 | 15.79 | 0.981 | 0.981 |
| | 30 | 0.2 | 0.1 | 0.99 | 0.00 | 20.00 | 0.926 | 0.926 | 2.3 | 2.1 | 0.98 | 0.01 | 8.70 | 0.990 | 0.990 |
| | 35 | 0.2 | 0.1 | 0.99 | 0.00 | 20.00 | 0.926 | 0.926 | 1.7 | 1.1 | 0.99 | 0.05 | 35.29 | 0.953 | 0.953 |
| B | 2 | 86.9 | 83.7 | 0.99 | 0.64 | 3.68 | 0.996 | 0.996 | 122.3 | 119.4 | 0.98 | 0.55 | 2.37 | 0.997 | 0.997 |
| | 4 | 69.9 | 66.8 | 0.98 | 0.61 | 4.43 | 0.995 | 0.995 | 79.1 | 76.9 | 0.97 | 0.36 | 2.78 | 0.997 | 0.997 |
| | 6 | 54.3 | 46.3 | 0.98 | 2.51 | 14.73 | 0.982 | 0.982 | 56.1 | 52.1 | 0.99 | 0.89 | 7.13 | 0.992 | 0.992 |
| | 8 | 39.4 | 33.2 | 0.98 | 1.72 | 15.74 | 0.981 | 0.981 | 39.3 | 34.3 | 0.99 | 1.24 | 12.72 | 0.985 | 0.985 |
| | 10 | 27.5 | 24.4 | 0.98 | 0.61 | 11.27 | 0.987 | 0.987 | 29.8 | 23.1 | 0.98 | 1.93 | 22.48 | 0.972 | 0.972 |
| | 12 | 19.3 | 16.9 | 0.98 | 0.41 | 12.44 | 0.985 | 0.985 | 19.9 | 16.2 | 0.98 | 0.79 | 18.59 | 0.977 | 0.977 |
| | 14 | 13.9 | 11.8 | 0.98 | 0.34 | 15.11 | 0.982 | 0.982 | 16.4 | 13.9 | 0.98 | 0.44 | 15.24 | 0.982 | 0.982 |
| | 16 | 7.5 | 7.1 | 0.98 | 0.03 | 5.33 | 0.994 | 0.994 | 10.9 | 8.4 | 0.97 | 0.44 | 22.94 | 0.971 | 0.971 |
| | 18 | 5.3 | 4.1 | 0.98 | 0.15 | 22.64 | 0.972 | 0.972 | 8.6 | 7.4 | 0.99 | 0.15 | 13.95 | 0.983 | 0.983 |
| | 20 | 2.8 | 2.2 | 0.98 | 0.05 | 21.43 | 0.974 | 0.974 | 6.3 | 5.1 | 0.99 | 0.15 | 19.05 | 0.977 | 0.977 |
| | 26 | 0.6 | 0.4 | 0.98 | 0.01 | 23.33 | 0.956 | 0.956 | 3.2 | 2.9 | 0.97 | 0.02 | 9.38 | 0.989 | 0.989 |
| | 30 | 0.3 | 0.2 | 0.99 | 0.00 | 21.33 | 0.956 | 0.956 | 2.1 | 1.8 | 0.99 | 0.02 | 14.29 | 0.983 | 0.983 |
| | 35 | 0.2 | 0.1 | 0.97 | 0.00 | 10.00 | 0.926 | 0.926 | 1.6 | 1.2 | 0.97 | 0.03 | 25.00 | 0.969 | 0.969 |
| C | 2 | 69.5 | 64.9 | 0.97 | 1.10 | 6.62 | 0.992 | 0.992 | 110.2 | 103.6 | 0.97 | 1.88 | 5.99 | 0.993 | 0.993 |
| | 4 | 56.4 | 52.6 | 0.98 | 0.82 | 6.74 | 0.992 | 0.992 | 76.4 | 71.3 | 0.98 | 1.28 | 6.68 | 0.992 | 0.992 |
| | 6 | 42.5 | 39.3 | 0.99 | 0.64 | 7.53 | 0.991 | 0.991 | 53.6 | 49.7 | 0.98 | 0.86 | 7.28 | 0.992 | 0.992 |
| | 8 | 36.6 | 29.4 | 0.99 | 2.15 | 19.67 | 0.976 | 0.976 | 37.4 | 31.2 | 0.98 | 1.72 | 16.58 | 0.980 | 0.980 |
| | 10 | 22.8 | 19.9 | 0.98 | 0.55 | 12.72 | 0.985 | 0.985 | 27.6 | 22.6 | 0.99 | 1.24 | 18.12 | 0.978 | 0.978 |
| | 12 | 15.8 | 11.7 | 0.97 | 0.92 | 25.95 | 0.967 | 0.967 | 18.7 | 15.9 | 0.97 | 0.52 | 14.97 | 0.982 | 0.982 |
| | 14 | 9.9 | 8.1 | 0.98 | 0.27 | 18.18 | 0.978 | 0.978 | 13.2 | 11.9 | 0.99 | 0.16 | 9.85 | 0.989 | 0.989 |
| | 16 | 6.6 | 5.1 | 0.99 | 0.20 | 22.73 | 0.972 | 0.972 | 9.8 | 8.5 | 0.99 | 0.16 | 13.27 | 0.984 | 0.984 |
| | 18 | 4.3 | 2.9 | 0.99 | 0.18 | 32.56 | 0.957 | 0.957 | 7.7 | 6.3 | 0.99 | 0.18 | 18.18 | 0.978 | 0.978 |
| | 20 | 2.3 | 1.9 | 0.98 | 0.03 | 17.39 | 0.979 | 0.979 | 5.9 | 4.3 | 0.99 | 0.22 | 27.12 | 0.965 | 0.965 |
| | 26 | 0.6 | 0.4 | 0.98 | 0.01 | 23.33 | 0.956 | 0.956 | 3.6 | 2.8 | 0.99 | 0.08 | 22.22 | 0.972 | 0.972 |
| | 30 | 0.3 | 0.1 | 0.98 | 0.01 | 26.67 | 0.885 | 0.885 | 2.6 | 1.8 | 0.98 | 0.08 | 30.77 | 0.960 | 0.960 |
| | 35 | 0.2 | 0.1 | 0.99 | 0.00 | 10.00 | 0.926 | 0.926 | 1.3 | 1.1 | 0.99 | 0.01 | 15.38 | 0.982 | 0.982 |

§Correlation coefficient. *Root mean square error. €Bias factors. ¤Accuracy factors; † percentage discrepancy (%D).

$S_{LQ}$_Pre: predicted storage life of raw milk in quality aspect; $S_{LQ}$_Obs: observed storage life of raw milk in quality aspect; $S_{LQ}$_Pre: predicted storage life of raw milk in safety aspect; $S_{Ls}$_Obs: observed storage life of raw milk in quality aspect.

## 7.5 Discussion

The ability of psychrotrophic proteolytic bacteria to produce heat-stable extracellular enzymes during refrigerated pre-processing storage causes a significant financial burden to the commercial milk processing units (Quigley et al. 2013). The present study evaluated the applicability of different predictive microbiological models to estimate the storage life of raw milk based on two predominant raw milk species, namely *Pseudomonas* spp. and *B. cereus*. In this regard, the study evaluated the raw milk from three farms with distinct microbial counts representing high quality, medium quality and poor quality raw milk.

According to the guidelines of the Pasteurized Milk Ordinance (PMO, US Food and Drug Administration: FDA), the quality of raw milk is determined based on total plate counts ($1 \times 10^5$ cfu/mL), psychrotrophic bacterial counts ($1 \times 10^3$ cfu/mL) and themoduric counts ($1 \times 10^3$ cfu/mL) (Cempírkova 2007). However, gudielines for raw milk by Food Standards Australia and New Zealand (FSANZ) specify that the counts of *Pseudomonas* spp. and *Bacillus cereus s.l.* need to be maintained below $10^7$ cfu/mL and $10^5$ cfu/mL, respectively (FSANZ 2012). Previously, we observed the onset of protease production and associated proteolysis in raw milk, when the psychrotrophic proteolytic counts reached $5 \times 10^5$ cfu/mL with the predominant bacteria being *Pseudomonas* spp. (Vithanage et al. 2017). However, the study also demonstrated heterogeneity in the proteolytic potential of the *Pseudomonas* population isolated from raw milk and dairy products (Dogan & Boor 2003, Marchand et al. 2009). Despite the description of slightly higher psychrotrophic counts ($10^6$-$10^7$ cfu/mL) in relation to protease activity (Griffiths et al. 1987, Haryani et al. 2003, O'Connell et al. 2016), only a few studies described values related to the ones recorded by our group (Gillis et al. 1985, Silveira et al. 1999, Vyletelova et al. 2000). Additionally, the presence of *B. cereus* at ≥ $1 \times 10^4$ cfu/mL is believed to have food safety implications in milk and dairy products (Valik et al. 2003).

The results of the present study indicated that the storage temperature significantly affected the growth parameters (maximum specific growth rate and lag time duration) of *Pseudomonas* spp. and *B. cereus,* when calculated using primary (Baranyi and Gompertz)

models. Furthermore, the data derived from the study was found to befit these two models graphically; however, with slightly lower maximum specific growth rates in the Baranyi model, as compared to the Gompertz model. This has been previously observed (Heo et al. 2014, Liu et al. 2006, Pla et al. 2015) and is attributed to the differences associated in calculating the growth kinetic parameters of bacteria by each method (Baranyi et al. 1993). Thus, it can be speculated that there is no single solution for non-linear regression analysis, and that the results obtained should not be considered as absolute values but rather as comparative values (McKellar & Lu 2003).

Furthermore, it is important to determine the combined effect of initial bacterial load and temperature on the microbiological growth parameters prior to the prediction of storage life of raw milk. In the present study, the combined effect was not significantly correlated ($P > 0.05$), thus highlighting the fact that the initial bacterial load of raw milk from 1-4 log cfu/mL did not affect the growth rate of these two bacteria, as compared to the temperature.

Therefore, the present study further determines the effect of temperature in relation to the specific growth rate and lag phase duration, using the secondary model Belehradek-type equations (Koutsoumanis 2001). This involves square root transformation of the specific growth rates and square root of the reciprocal of lag phase duration of these two bacteria obtained by Baranyi and Gompertz models, which were graphed as a function of temperature. All of these polynomial secondary models fitted well with the predicted growth kinetic parameters of *Pseudomonas* spp. and *B. cereus,* which were obtained from Baranyi and Gompertz models. There was only a slight variation observed for different initial counts in raw milk, which were not significant compared to the temperature effect.

Data obtained from the two primary models were evaluated using the experimentally derived values using several statistical parameters such as $R^2$ and RMSE values. Although both primary models showed high $R^2$, it was previously described that higher $R^2$ does not necessarily indicate a good fit (Johnson 1992). The RMSE, in contrast, measures how well can the experimental data fit with the predicted data (Gonçalves et al. 2017, Heo et al. 2014, Koutsoumanis 2001). A lower RMSE value indicates a good fit of experimental data with

predictive data. In the present study, the RMSE values for the Baranyi model were lower compared to the Gompertz model, as observed previously (Heo et al. 2014). In addition, accuracy and bias factors were also used to determine the reliability of these models to predict the growth kinetic parameters. Here, the $B_f$ measures the overall agreement between predicted and observed $S_L$, which is why the perfect agreement would result in $B_f = 1$, while $B_f > 1$ indicates that the predictions were larger than the observed values (Baranyi et al. 1999). However, in $B_f$, the positive and negative predictions can cancel each other, which can be corrected by calculating $A_f$, which measures the absolute error. In the present study, we observed $B_f$ in the range of 0.97-1.003 and $A_f$ in the range of 0.97-1.186 for the predictions given by the primary models. Models describing the growth rate of pathogenic bacteria should comprise $B_f$ in the range of 0.9-1.05 for a good fit, although the ranges of 0.7-0.9 or 1.06-1.15 are acceptable, $B_f$ is unacceptable in the range of < 0.7 or > 1.5 (Ross 1996, Ross et al. 2000). Thus, the $A_f$ and $B_f$ values obtained for the current study indicated that the observations were close to the predictions of primary models. Besides the primary models, the behaviour of secondary models was also evaluated based on the aforementioned criteria, which indicates that those models are reliable in predicting the isothermal behaviour of *Pseudomonas* spp. and *B. cereus* in raw milk under different temperature conditions.

Finally, the storage life of raw milk was predicted on the basis of the growth kinetic parameter derived from Baranyi and Gompertz models using the exponential model as described by Fu and Labuza (1993). In the present study, *Pseudomonas* spp. were considered as the major influencing factor affecting raw milk quality, owing to their higher relative occurence and significantly higher spoilage potential (Vithanage et al. 2016). Thus, the time required for *Pseudomonas* spp. to reach $5 \times 10^4$ cfu/mL was defined as the storage life of raw milk with repesct to quality (Vithanage et al. 2017). Similarly, the presence of higher numbers of *B. cereus* in raw milk indicated possible food safety concerns, due to their ability to prodcue heat-stable spores that can withstand pasteurisation and UHT heating and subsequent production of toxins after germination (Heo et al. 2014, Valik et al. 2003). Thus,

the counts of *B. cereus* that are ≥ $1 \times 10^4$ cfu/mL, are considered to be the threshold limits for this bacteria in milk. These threshold limits are consistent with the previous description of higher spoilage implications and milkborne diseases that can be used as quality and safety criteria for raw milk screening, when producing milk and dairy products (Gillis et al. 1985, Silveira et al. 1999, Valik et al. 2003, Vithanage et al. 2017, Vyletelova et al. 2000).

In the present study, we observed that *Pseudomonas* spp. and *B. cerus* can reach their threshold limits within 48 hours of storage at 6 °C. This indicates that temperature abuse conditions can occur frequently during the storage of raw milk in refrigerated bulk tanks or insulated storage tanks on the farm. Unreliable refrigeration conditions in milk tanks during transportation or storage conditions where the raw milk is subjected to elevated temperature conditions in commercial silos would enable bacterial growth to a critical level. As it is challenging to maintain the required refrigeration and cold-chain conditions in extreme weather conditions, maintaining lower refrigerator temperatures and hygienic working conditions are alternative strategies to minimise spoilage and food safety issues.

The storage life of raw milk is significantly affected by lag phase duration, and this was in the range of 0.01-60.7 hours for *Pseudomonas* spp. depending on the storage temperature used. The lag phase for *B. cereus,* under corresponding conditions, was in the range of 0.91-104.1 hours. These times are 20-50% of the total storage life of raw milk. Unlike the specific growth rate, the lag phase duration represents a transition period of bacteria to adjust to a new environment (Koutsoumanis 2001). Thus, lag time not only depends on the current growth conditions, but also on previous conditions (Koutsoumanis 2001). It is therefore important to accurately predict lag phase duration to estimate the storage life of raw milk.

In this regard, temperature abuse is often random; therefore, extensive analysis is required to determine the effect of different temperature scenarios that could occur in the actual cold chain, and while calculating the lag phase under these conditions (Koutsoumanis 2001). It is also important to understand the biodiversity of bacteria with higher spoilage potential, their

relative occurrence in raw milk and factors affecting their presence in raw milk (Hantsis-Zacharov & Halpern 2007, Vithanage et al. 2017, von Neubeck et al. 2015).

Moreover, it is important to develop a user-friendly but complex mathematical modelling software to predict the storage life of raw milk depending on variables in this complex microbial niche, allowing for application of these models by people without detailed mathematical knowledge.

A large number of indicator microorganisms, including coliforms, *Escherichia coli*, *B. cereus* and *Staphylococcus aureus*, are currently used in modelling software for prediction of food safety aspects (Fang et al. 2003, Fernandez-Piquer et al. 2011, Heo et al. 2014, Little & Knøchel 1994, Pla et al. 2015, Ross et al. 2000). However, only a few of these models have incorporated spoilage microorganisms (Gonçalves et al. 2017, Koutsoumanis 2001, Lin 2015). Particularly, psychrotrophic proteolytic bacteria other than *Pseudomonas* spp. may have higher spoilage potential, while thermoduric psychrotrophs other than *B. cereus* may have higher toxigenic potential. Thus, to improve the reliability of these predictions and implement the modelling software for routine quality assurance purposes in raw milk, it is important to incorporate more spoilage causing microorganisms and toxigenic spore-formers. This will assist in defining incentive schemes for dairy farmers on the basis of the raw milk quality as well as facilitate the distribution and marketing in the milk industry.

A major limitation of the current study was the use of a few samples to represent all parameters that were utilized to evaluate the quality in the processing environment. Thus, a more extensive study incorporating large numbers of raw milk samples would provide a more comprehensive understanding of the effects of storage conditions on the raw milk quality. Nonetheless, the results of the current study are in general agreement with those of large scale studies (O'Connell et al. 2016).

## 7.6 Conclusion

Overall, the primary models exhibited a good fit to the experimental data, thus they can be used to predict the growth dynamic parameters of *Pseudomonas* spp. and *B. cereus* under

different isothermal conditions. The secondary modelling indicated that the data of growth kinetic parameters such as the maximum specific growth rate and lag phase duration derived from Baranyi and Gompertz models were well-fitted to the Belehradek-type equations, which further exemplified the temperature dependence of those growth kinetic parameters. The data derived and evaluated from primary (i.e. Baranyi, Gompertz) and polynomial secondary models (Belehradek-type equations) can be used to predict the storage life of raw milk using an exponential model. Thus, the use of these predictive microbiological models would be an efficient and accurate way of monitoring or controlling the quality of raw milk by avoiding the risk of spoilage and food safety implications in milk and dairy products at variable storage temperatures. This would allow for the production of superior quality dairy products with extended shelf-life that can be distributed to wider geographical regions, thus amassing profits to the commercial milk processing industry.

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# CHAPTER 8: DETERMINATION OF INACTIVATION KINETICS AND THERMODYNAMIC PARAMETERS OF PSYCHROTROPHIC BACTERIAL PROTEASES OF DAIRY ORIGIN

## 8.1 Overview of Chapter

Chapter 8 presents the thermal inactivation kinetics and thermodynamics properties of selected proteolytic enzymes of psychrotrophic bacteria. The study comprised screening for heat-stability of bacterial proteases (BPs) from different bacteria at 65 °C, 95 °C and 150 °C, followed by the heating of selected BPs at a wide range of temperature conditions ranging from 55 to 160 °C. This may allow determination of inactivation kinetics of a variety of BPs that are likely to be present during Ultra High Temperature (UHT) milk processing, thus defining new time-temperature parameters for UHT processing to control BPs in UHT milk and, thereby, increasing the shelf-life of UHT milk, especially of the products that are destined for the export market.

## Determination of Inactivation Kinetics and Thermodynamic Parameters of Psychrotrophic Bacterial Proteases of Dairy Origin

### 8.2 Introduction

The refrigerated storage of raw milk in the farming and processing environments favours the growth of psychrotrophic bacteria (Adams et al. 1975, Fairbairn & Law 1986, Oliveira et al. 2015, Vithanage et al. 2016). These bacteria synthesise the extracellular metalloproteases during the late exponential or stationary growth phases (Griffiths 1989). Once entering milk, these bacterial proteases (BPs) are extremely difficult to inactivate using existing UHT heating regimes (Chavan et al. 2011, Stoeckel et al. 2016b). Even low concentrations of BPs can lead to significant reduction in the shelf-life of the UHT milk during ambient storage (Mitchell & Ewings 1985). Although psychrotrophic bacteria produce a wide range of heat-stable proteases, *Pseudomonas* and *Bacillus* are of major concern because of their higher relative occurrence, cold-adapted propagation and spoilage potential (Vithanage et al. 2016).

The BPs can hydrolyse the milk protein (mainly caseins) during the pre-processing refrigerated storage of raw milk, as well as post-processing storage in the finished products (Baglinière et al. 2012, Gaucher et al. 2011). Typical defects of the UHT milk products caused by BPs include bitterness, particle formation, and increase in viscosity and age gelation (Champagne et al. 1994, Samarzija et al. 2012, Stoeckel et al. 2016a). Alternatively, BPs are also involved in the activation of the indigenous plasmin system that is known to be heat-stable. Thus, these two types of proteases can synergistically act on milk proteins (Marchand et al. 2008).

Therefore, controlling the presence of BPs in UHT-treated milk and dairy products is of utmost importance, especially in the ones that are produced for the export markets and require an extended shelf-life. A few studies have demonstrated that this can be achieved by controlling the growth of psychrotrophs in raw milk, by introducing deep cooling (0-2 °C), or maintaining the cold chain at appropriate temperatures during storage and transportation (Griffiths et al. 1987, Haryani et al. 2003, Vithanage et al. 2016). Apparently, maintaining these conditions in the dairy farms is extremely difficult, especially during the hot summer season, thus the possible benefits do not justify the cost of deep cooling.

In addition, the reduction of proteolytic activities in the UHT milk can be minimised by inactivation through thermal processing (Stoeckel et al. 2016b). In this regard, understanding the inactivation kinetics of these enzymes is required. Several studies describe the kinetics of thermal inactivation of BPs in milk or in model systems (Adams et al. 1975, Baur et al. 2015, Glück et al. 2016, Mu et al. 2009); however, those data are not consistent and this is likely to be associated with the diversity of BPs and their complicated behaviour in milk during heat-inactivation (Marchand et al. 2008). For example, Patel et al. (1986) observed different calcium ion requirements and amino acid compositions, which resulted in different heat-stabilities in four different BPs of *Pseudomonas* spp. Furthermore, those studies are limited to the inactivation kinetics of *Pseudomonas* proteases, which is why only limited information is available for the inactivation kinetics of proteolytic enzymes of other bacteria (Adams et al. 1975, Adams 1991, Baur et al. 2015, Glück et al. 2016, Mu et al. 2009, Patel & Bartlett 1988). Thus, the aim of the present study was to screen for heat-stability of wide range of bacterial proteases (n = 119) at 65 °C, 95 °C and 150 °C, followed by the determination of inactivation kinetics and associated thermodynamic parameters of selected BPs (n = 6) using thermal treatments in the range of 55-160 °C in UHT whole milk, skimmed milk and simulated milk ultra-filtrate.

## 8.3 Materials and Method

### 8.3.1 Chemicals

All chemicals used for the analysis, including Protease Fluorescent Detection Kit, were of analytical grade and purchased from Sigma Aldrich (Castle Hill, New south Wales, Australia). UHT full cream milk and skimmed milk (Devondale, full cream: 3% fat and skimmed milk: 0.1% fat) from the same batch were kindly provided by a commercial milk processor in Australia (Murray Goulburn Co-operative Co. Limited, Southbank, Victoria, Australia).

### 8.3.2 Isolation and identification of microorganisms

Representative psychrotrophic isolates belonging to different bacterial families including (i) *Moraxellaceae* (n = 4), (ii) *Aeromonadaceae* (n = 3), (iii) *Xanthomonadaceae* (n = 3) (iv) *Alcaligenaceae* (n = 3), (v) *Enterobacteriaceae* (n = 24), (vi) *Pseudomonadaceae* (n = 47), (vii) *Flavobacteriaceae* (n = 4), (viii) *Microbacteriaceae* (n = 6) and (ix) *Bacillaceae* (n = 36), that exhibited apparent extracellular proteolytic activities were considered in the analysis (Vithanage et al. 2016, Vithanage et al. 2014). These isolates were previously identified using matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing (Vithanage et al. 2016, Vithanage et al. 2014).

American Type Culture Collection (ATCC) cultures including *P. fluorescens* (ATCC 13525, 17386), *B. cereus* (ATCC 10876, 10987, 14579), *Bacillus licheniformis* (ATCC 14580), *Bacillus subtilis* (ATCC 6633), *Acinetobacter baumanii* (ATCC 19606) and *Serratia marsecens* (ATCC 14756) and thermolysin from *Bacillus thermoproteolyticus* were used as positive controls.

### 8.3.3 Production of extracellular proteases

Overnight culture of psychrotrophic bacteria (with higher extracellular protease activity: (n = 118) in Nutrient broth containing $1 \times 10^8$ cfu/mL counts were inoculated into 250 mL of sterile UHT skimmed milk with 1% (v/v) ratio in 1 L Erlenmeyer flasks and subsequent incubation at 7 °C for 10 days in a refrigerated shaking incubator (Innova 4230, New Brunswick Scientific, Edison, NJ, USA) at 120 rpm. Following incubation, the bacterial cultures were centrifuged (Model Avanti J-26S XPI, Beckman Coulter) at 16,000 × g for 15 min at 4 °C, and the cell-free supernatant containing crude BPs were used for the inactivation experiments.

### 8.3.4 Screening for thermo-resistant proteases

The crude BPs were initially screened for heat stability in UHT skimmed milk (ratio: 950 µL UHT skimmed milk: 50 µL of crude BPs) by heating 1.0 mL of BPs in 9.0 mL Pyrex tubes (Sigma-Aldrich, Castle Hill, Australia) at 65 °C for 15 min, 95 °C for 15 min and 150 °C for 20 s (with additional temperature come-up time) in a shaking oil bath (Ratek, Boronia, Australia). This was followed by immediate cooling in an ice bath, and subsequent addition of 0.2% sodium azide (Sigma-Aldrich, Castle Hill, Australia) to inhibit bacterial growth (Vithanage et al. 2014).

### 8.3.5 Determination of the inactivation kinetics of selected enzymes

The inactivation kinetics of representative BPs (n = 6) with higher heat-stabilities were determined under a wide range of temperature (55-160 °C) and time conditions (Table 2.1). The inactivation of BPs was determined in three different heating media, including UHT whole milk, skimmed milk and simulated milk ultra-filtrate (SMUF) buffer. SUMF buffer was prepared daily according to the following formulation: 11.6 mM $KH_2PO_4$, 3.70 mM $C_6H_5K_3O_7.H_2O$ (tripotassium citrate), 6.09 mM $C_6H_5Na_3O_7.2H_2O$ (trisodium citrate), 1.03 mM $K_2SO_4$, 8.05 mM KCl, 8.97 mM $CaCl_2.2H_2O$ and 3.20 mM $MgCl_2.6H_2O$ (Glück et al. 2016). A volume of 950 µL of each heating medium was mixed with 50 µL of crude BPs at room temperature, and heated at different time-temperature conditions, while accommodating the come-up time (Table 8.1). The heated samples were then frozen immediately in liquid $N_2$ and stored at 80 °C until measurement of residual enzyme activity.

**Table 8.1** Time/temperature combinations and BPs considered inactivation experiments.

| Temperature (°C) | Time |
|---|---|
| 55 | 0, 15, 30, 60, 90 min |
| 65 | 0, 15, 30, 60, 90 min |
| 60 | 0, 15, 30, 60, 90 min |
| 75 | 0, 15, 30, 60, 90 min |
| 85 | 0, 15, 30, 60, 90 min |
| 95 | 0, 15, 30, 60, 90 min |
| 105 | 0, 15, 30, 60, 90 min |
| 110 | 0, 15, 30, 60, 90 min |
| 115 | 0, 15, 30, 60, 90 min |
| 125 | 0, 15, 30, 60, 90 min |
| 135 | 0, 20, 30, 60, 90 s |
| 140 | 0, 20, 30, 60, 90 s |
| 142 | 0, 20, 30, 60, 90 s |

| | |
|---|---|
| 145 | 0, 20, 30, 60, 90 s |
| 150 | 0, 20, 30, 60, 90 s |
| 155 | 0, 20, 30, 60, 90 s |
| 160 | 0, 20, 30, 60, 90 s |

### 8.3.6 Determination of the residual activity by milk coagulation assay

The residual activities of heat-treated BPs were determined qualitatively using milk coagulation assays by observing the coagulation pattern after mixing with UHT skimmed milk (Teh et al. 2011). Briefly, 0.5 mL of heated BPs were mixed with 1.0 mL of UHT skimmed milk under aseptic conditions, followed by incubation at 25 °C for 5 days (Vithanage et al. 2014). Following incubation, the coagulation pattern of the heated BPs was visually evaluated. Unheated crude enzymes (0.5 mL) and uninoculated UHT skimmed milk (1.5 mL) were used as positive and negative controls, respectively.

### 8.3.7 Determination of the residual activity by automated fluorescens isothiocyanate casein (FITC) assay

The residual protease activity in the heated samples was determined using the Protease Fluorescent Detection Kit (Sigma-Aldrich, Castle Hill, Australia), according to the manufacturer's instructions. The fluorescence intensity, caused by the release of trichloroacetic acid (TCA)-soluble fluorescent peptides, was determined using a spectrofluorophotometer (POLARstar Omega; BMG LABTECH, Mornington, Victoria, Australia) with excitation at a wavelength of 485 nm and emission at a wavelength of 535 nm, in duplicate. The increase in fluorescence intensity, obtained due to hydrolysis of the protein, was expressed as relative fluorescence units (RFU/mL). Thermolysin (Sigma-Aldrich, Castle Hill, Australia) was used as the positive control (Vithanage et al. 2016).

### 8.3.8 Estimation of kinetic parameters

The heat-inactivation of BPs has been described as a first-order reaction and is algebraically described in the following equation (Sant'Anna et al. 2012):

$$\frac{A}{A0} = \exp(-kt) \quad \textbf{(1)}$$

where A/Ao is the residual protease activity at treatment time t (min), and k (per min) is the inactivation rate constant at a determined temperature. The inactivation rate constants (k-values) can be estimated using the non-linear regression analysis.

Half-life ($t_{1/2}$) value of inactivation is given by the expression (Sant'Anna et al. 2012):

$t_{1/2=ln2/k}$ **(2)**

D-value representing the time required to reduce the initial activity by 90% (or 1-log) is related to the k-values and is mathematically expressed as follows (Sant'Anna et al. 2012):

$D = \ln(10)/k$ **(3)**

The Z-value refers to the temperature required to vary D-value by one log unit, and was obtained by plotting log values of the D-values on a log scale versus the corresponding temperatures (Sant'Anna et al. 2012).

**8.3.9 Estimation of the thermodynamic parameters**

The temperature dependence of k-values was described by Arrhenius' law, and is algebraically given by Sant'Anna et al. (2012):

$\ln(k) = \ln(C) - E_a/R.T$ **(4)**

where C is the Arrhenius constant, $E_a$ (kJ/mol) the activation energy, R (8.3144 J/mol K) the universal gas constant and T (K) is the absolute temperature. The $E_a$ can be estimated by the slope of linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature.

The activation enthalpy for each temperature was calculated using the obtained $E_a$ value for each enzyme with the following equation (Sant'Anna et al. 2012);

$\Delta H^{\#} = E_a - R.T$ **(5)**

The free energy of inactivation ($\Delta G^{\#}$) can be determined according to the following expression (Sant'Anna et al. 2012):

$\Delta G^{\#} = -R.T.\ln(k.h/K_B.T)$ **(6)**

where h is the Planck's constant ($6.6262 \times 10^{-34}$ J s), $K_B$ is the Boltzmann's constant

(1.3806 × $10^{-23}$ J/K). and k the inactivation rate constant for each recorded temperature (per s).

The activation entropy can be calculated using the equation 5 and 6 as follows (Sant'Anna et al. 2012);

$\Delta \boldsymbol{S}^{\#} = \Delta H^{\#} - \Delta G^{\#}/T$ **(7)**

### 8.3.10 Statistical analysis

Mean values of the residual activities of BPs were calculated from two independent experiments for each condition and duplicate assays of proteolytic activity were performed for each experiment. Statistical analysis of the data was performed using the SPSS software for Windows (Version 21 software; IBM Corp. in Armonk, NY) and plots using Microsoft Excel 2010 (version 14.0; Microsoft Corporation, North ryde, NSW, Australia). Obtained k-values were then compared using the t-test and one-way ANOVA, and a $P < 0.05$ was considered statistically significant.

## 8.4 Results

### 8.4.1 Screening for thermal inactivation of bacterial proteases using selected heating conditions

The preliminary study of heating BPs showed a wide range of residual activities after heat-treatment in UHT skimmed milk at 65 °C for 15 min, 95 °C for 15 min and 150 °C for 20 s (Table 8.2). Almost all the crude BPs samples (n = 118) and thermolysin (from *B. thermoproteolyticus*) were found to be positive for the milk coagulation assay, with varying intensities, when they were heated at 65 °C for 15 min. However, 10.08% of the BPs showed negative reactions, while 1.68% showed doubtful coagulation, when heated at 95 °C for 15 min. About 14.3% of BPs showed no reaction for milk coagulation at 150 °C after 20 s of heating. All of these negative results were found with bacteria belonging to *Alcaligenaceae, Xanthomonadaceae, Enterobacteriaceae, Pseudomonadaceae* and *Flavobacteriaceae* with ≤ 15% residual activity by FITC method (Table 8.2). However, the BPs showed residual activity in the range of 5.1-104.6% after 15 min heating at 65 °C, while

this was in the range of 1.4-91.4% and 0.8-88.6% for the 95 °C/15 min and 150 °C/20 s heating regimes, respectively (Table 8.2).

### 8.4.2 Inactivation kinetics of selected bacterial proteases using different time temperature parameters

Representative BPs, possessing high stability and originating from *Pseudomonas poae* (P24), *Hafnia alvei* (P50), *Microbacterium oxydans* (A79), *Bacillus weihenstephanensis* (B5), *Bacillus licheniformis* (B96), and thermolysin (PC; positive control), were determined for their inactivation kinetics by heating at 55-160 °C for different times (Table 8.1) in UHT whole milk (WM: pH 6.9), UHT skimmed milk (SM: pH 6.6) and SUMF buffer (pH 6.7). This was followed by measuring the residual activity with time using the FITC method.

**Table 8.2** Screening for thermal inactivation of BPs with selected time-temperature conditions.

| **Bacterial strains** | **Milk coagulation assay** | | | **Residual activity (%)** | | |
|---|---|---|---|---|---|---|
| | **65 °C*** | **95 °C*** | **150 °C§** | **65 °C*** | **95 °C*** | **150 °C§** |
| ***Family: Moraxellaceae*** | | | | | | |
| *Acinetobacter baumannii* (B71) | + | + | + | 77.5 | 65.3 | 41.2 |
| *Acinetobacter guillouiae* (P11) | + | + | + | 89.1 | 66.1 | 31.5 |
| *Acinetobacter johnsonii* (C95) | + | + | + | 73.1 | 63.2 | 39.8 |
| ***Family: Aeromonadaceae*** | | | | | | |
| *Aeromonas hydrophila* (C125) | + | + | + | 94.2 | 85.3 | 59.2 |
| *Aeromonas hydrophila* (C126) | + | + | + | 89.3 | 53.2 | 59.1 |
| *Aeromonas salmonicida* (C127) | + | + | + | 68.3 | 44.5 | 39.8 |
| ***Family: Alcaligenaceae*** | | | | | | |
| *Alcaligenes feacalis* (B3) | + | + | - | 62.1 | 35.4 | 12.3 |
| *Achromobacter xylosoxidans* (B25) | + | - | - | 43.3 | 12.2 | 8.1 |
| *Achromobacter denitrificans* (B293) | + | - | - | 33.6 | 11.9 | 6.7 |
| ***Family: Xanthomonadaceae*** | | | | | | |
| *Stenotrophomonas maltophila* (P60) | + | + | + | 66.6 | 33.7 | 20.8 |
| *Stenotrophomonas rhizophila* (A47) | + | + | + | 48.1 | 35.3 | 23.4 |
| *Stenotrophomonas chelatiphaga* (A48) | + | - | - | 19.6 | 9.3 | 6.2 |
| ***Family: Enterobacteriaceae*** | | | | | | |
| *Citrobacter freundii* (C119) | + | - | - | 23.5 | 11.5 | 9.8 |
| *Enterobacter aerogenes* (B82) | + | - | - | 15.3 | 7.4 | 5.2 |
| *Escherichia coli* (B23) | + | - | - | 19.2 | 10.9 | 7.6 |
| *Hafnia alvei* (P4) | + | + | + | 54.5 | 28.4 | 26.4 |
| *Hafnia alvei* (P50) | + | + | + | 82.2 | 51.2 | 39.7 |
| *Hafnia alvei* (P52) | + | + | + | 67.2 | 52.1 | 42.5 |
| *Hafnia alvei* (P53) | + | + | + | 56.3 | 43.2 | 39.7 |
| *Hafnia alvei* (P55) | + | + | + | 68.7 | 53.2 | 44.8 |
| *Hafnia paralvei* (P36) | + | + | + | 66.3 | 46.7 | 41.7 |
| *Hafnia paralvei* (P39) | + | + | + | 74.3 | 55.7 | 37.2 |
| *Klebsiella oxytoca* (C105) | + | + | + | 85.2 | 68.9 | 49.5 |
| *Klebsiella pneumoniae* (C109) | + | + | + | 62.4 | 48.9 | 42.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Rahnella aquatilis* (P51) | + | + | + | 58.7 | 43.4 | 37.6 |
| *Raoultella planticola* (B85) | + | ?+ | - | 29.5 | 15.6 | 11.2 |
| *Serratia liquifaciens* (P59) | + | + | + | 53.2 | 45.6 | 34.2 |
| *Serratia liquifaciens* (C103) | + | + | + | 92.4 | 77.4 | 67.3 |
| *Serratia marcescens* (C287) | + | + | + | 96.4 | 90.9 | 88.1 |
| *Serratia marcescens* (C216) | + | + | + | 86.5 | 72.4 | 57.2 |
| *Serratia proteomaculans* (B112) | + | + | + | 75.4 | 65.2 | 61.1 |
| *Serratia proteomaculans* (C235) | + | + | + | 69.9 | 59.8 | 55.5 |
| *Serratia proteomaculans* (A181) | + | + | + | 65.4 | 57.8 | 54.3 |
| *Citrobacter freundii* (ATCC 43864) | + | + | + | 51.2 | 43.8 | 39.9 |
| *Escherichia coli* (ATCC 25922) | + | + | + | 39.8 | 23.4 | 19.9 |
| *Serratia marsecens* (ATCC 14756) | + | + | + | 61.6 | 47.9 | 35.6 |
| ***Family: Pseudomonadaceae*** | | | | | | |
| *Pseudomonas fluorescens* (P3) | + | + | + | 88.4 | 52.5 | 48.3 |
| *Pseudomonas fluorescens* (B23) | + | - | - | 9.6 | 2.4 | 0.8 |
| *Pseudomonas fluorescens* (B27) | + | ?+ | - | 32.5 | 14.5 | 12.3 |
| *Pseudomonas azotoformans* (P1) | + | + | + | 72.3 | 59.7 | 49.5 |
| *Pseudomonas azotoformans* (C39) | + | + | + | 77.7 | 63.1 | 56.7 |
| *Pseudomonas lurida* (P2) | + | + | + | 65.3 | 55.9 | 52.9 |
| *Pseudomonas poae* (P24) | + | + | + | 97.3 | 87.7 | 72.2 |
| *Pseudomonas poae* (P27) | + | + | + | 67.7 | 54.6 | 42.8 |
| *Pseudomonas poae* (P29) | + | + | + | 55.6 | 44.6 | 36.6 |
| *Pseudomonas gessardii* (P6) | + | + | + | 68.7 | 54.6 | 36.7 |
| *Pseudomonas gessardii* (P23) | + | + | + | 62.3 | 51.2 | 35.2 |
| *Pseudomonas gessardii* (P32) | + | + | + | 33.9 | 50.6 | 43.9 |
| *Pseudomonas proteolytica* (P14) | + | + | + | 77.2 | 47.3 | 38.5 |
| *Pseudomonas proteolytica* (A22) | + | + | + | 82.7 | 66.3 | 58.7 |
| *Pseudomonas proteolytica* (C22) | + | + | - | 56.9 | 23.1 | 12.1 |
| *Pseudomonas proteolytica* (C23) | + | + | + | 80.8 | 61.3 | 45.4 |
| *Pseudomonas protegens* (A8) | + | + | + | 104.6 | 91.4 | 78.2 |
| *Pseudomonas gingeri* (P20) | + | + | + | 49.8 | 32.4 | 29.1 |
| *Pseudomonas veronii* (P25) | + | + | + | 67.7 | 50.2 | 41.4 |
| *Pseudomonas salomonii* (P33) | + | + | + | 60.7 | 45.6 | 34.1 |
| *Pseudomonas brennerii* (B24) | + | + | + | 63.8 | 52.4 | 43.5 |
| *Pseudomonas brennerii* (B26) | + | + | + | 51.8 | 38.1 | 21.3 |
| *Pseudomonas meridiana* (B29) | + | + | - | 45.9 | 22.9 | 12.8 |
| *Pseudomonas meridiana* (B32) | + | - | - | 18.7 | 9.7 | 2.1 |
| *Pseudomonas fragi* (P40) | + | + | + | 72.6 | 49.4 | 57.2 |
| *Pseudomonas fragi* (P45) | + | + | + | 64.4 | 48.7 | 43.1 |
| *Pseudomonas fragi* (P49) | + | + | + | 55.6 | 41.2 | 82.6 |
| *Pseudomonas fragi* (P61) | + | + | + | 58.6 | 47.6 | 42.1 |
| *Pseudomonas lundensis* (P46) | + | + | + | 59.3 | 46.3 | 44.3 |
| *Pseudomonas lundensis* (P48) | + | + | + | 51.2 | 39.9 | 36.9 |
| *Pseudomonas lundensis* (P62) | + | + | + | 58.2 | 47.3 | 42.2 |
| *Pseudomonas psychrophila* (P10) | + | + | + | 59.2 | 47.4 | 44.8 |
| *Pseudomonas psychrophila* (P17) | + | + | + | 54.2 | 44.6 | 40.8 |
| *Pseudomonas putida* (C61) | + | + | + | 49.6 | 37.8 | 34.5 |
| *Pseudomonas stutzeri* (C70) | + | + | + | 46.7 | 36.2 | 32.3 |
| *Pseudomonas syringae* (P9) | + | + | + | 40.3 | 31.9 | 26.4 |
| *Pseudomonas syringae* (P43) | + | + | + | 64.7 | 56.7 | 53.1 |
| *Pseudomonas syringae* (P47) | + | + | + | 55.6 | 43.4 | 38.5 |
| *Pseudomonas sp.nov. 1* (A23) | + | + | + | 76.2 | 62.3 | 53.5 |
| *Pseudomonas sp.nov. 2* (B25) | + | + | + | 80.6 | 61.5 | 42.6 |
| *Pseudomonas sp.nov. 3* (B16) | + | + | + | 78.2 | 59.9 | 41.2 |
| *Pseudomonas sp.nov. 4* (C48) | + | + | + | 73.5 | 57.3 | 37.6 |
| *Pseudomonas sp.nov. 5* (A30) | + | + | + | 58.3 | 38.6 | 23.5 |
| *Pseudomonas aeruginosa* (B57) | + | + | + | 68.9 | 53.2 | 44.5 |
| *Pseudomonas aeruginosa* (ATCC 27853) | + | + | + | 63.2 | 43.5 | 34.4 |
| *Pseudomonas fluorescens* (ATCC 13525) | + | + | + | 58.6 | 43.2 | 35.8 |
| *Pseudomonas fluorescens* (ATCC 17386) | + | + | + | 55.2 | 39.6 | 34.4 |
| ***Family: Flavobacteriaceae*** | | | | | | |
| *Chryseobacterium piscium* (C242) | + | - | - | 5.1 | 1.4 | 0.9 |
| *Chryseobacterium oncorhynchi* (C247) | + | - | - | 11.6 | 6.4 | 3.1 |
| *Chryseobacterium jejuense* (A211) | + | - | - | 11.6 | 8.3 | 6.3 |
| *Elizabethkingia meningoseptica* (ATCC 13253) | + | - | - | 6.5 | 2.1 | 1.2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Family: Microbacteriaceae*** | | | | | | |
| *Microbacterium oxydans* (C263) | + | + | + | 68.1 | 53.6 | 42.1 |
| *Microbacterium oxydans* (C265) | + | + | + | 73.1 | 61.2 | 52.4 |
| *Microbacterium oxydans* (B174) | + | + | + | 77.6 | 62.3 | 43.1 |
| *Microbacterium oxydans* (A79) | + | + | + | 79.5 | 62.3 | 43.1 |
| *Microbacterium maritypicum* (A81) | + | + | + | 68.0 | 58.9 | 48.7 |
| *Microbacterium maritypicum* (C271) | + | + | + | 68.7 | 53.2 | 49.4 |
| ***Family: Bacillaceae*** | | | | | | |
| *Bacillus weihenstephanensis* (B5) | + | + | + | 89.3 | 78.2 | 58.6 |
| *Bacillus weihenstephanensis* (B36) | + | + | + | 62.3 | 54.3 | 44.3 |
| *Bacillus weihenstephanensis* (B100) | + | + | + | 54.3 | 42.3 | 38.7 |
| *Bacillus thuringiensis* (B18) | + | + | + | 46.4 | 36.7 | 32.8 |
| *Bacillus thuringiensis* (B51) | + | + | + | 64.2 | 56.3 | 54.2 |
| *Bacillus thuringiensis* (B77) | + | + | + | 63.2 | 46.7 | 42.7 |
| *Bacillus cereus s.l.* (ATCC 14579) | + | + | + | 64.3 | 46.6 | 40 |
| *Bacillus cereus s.l.* (ATCC 10876) | + | + | + | 56.3 | 44.3 | 42.4 |
| *Bacillus cereus s.l.* (ATCC 10987) | + | + | + | 54.6 | 46.7 | 44.5 |
| *Bacillus licheniformis* (B94) | + | + | + | 50.2 | 42.1 | 39 |
| *Bacillus licheniformis* (B95) | + | + | + | 59.7 | 49.3 | 45.4 |
| *Bacillus licheniformis* (B96) | + | + | + | 95.6 | 90.9 | 71.1 |
| *Bacillus licheniformis* (B98) | + | + | + | 57.2 | 49.7 | 47.1 |
| *Bacillus licheniformis* (ATCC 14580) | + | + | + | 59.4 | 49.1 | 46 |
| *Bacillus safensis* (B50) | + | + | + | 49.5 | 43.4 | 41.4 |
| *Bacillus pumilus* (B58) | + | + | + | 45.7 | 32.4 | 27.1 |
| *Bacillus pumilus* (B88) | + | + | + | 51.2 | 43.2 | 40.1 |
| *Bacillus pumilus* (C136) | + | + | + | 52.2 | 41.9 | 88.6 |
| *Bacillus altitudinis* (B91) | + | + | + | 87.3 | 77.8 | 73.4 |
| *Bacillus subtilis* (A55) | + | + | + | 84.3 | 74.5 | 47.8 |
| *Bacillus subtilis* (C152) | + | + | + | 81.3 | 71.2 | 67.5 |
| *Bacillus subtilis* (ATCC 6633) | + | + | + | 84.5 | 75.6 | 71.4 |
| *Bacillus sp.nov. 1* (B99) | + | + | + | 76.4 | 65.3 | 31.9 |
| *Bacillus sp.nov. 2* (B101) | + | + | + | 76.7 | 63.4 | 58.7 |
| ***Positive control*** | | | | | | |
| Thermolysin | + | + | + | 63.2 | 53.2 | 49.9 |

*for 15 min, § for 20 s. The results were presented as means of two independent experiments ± SE, ?+ = Doubtful coagulation.

The inactivation followed an exponential decay under all conditions tested, as illustrated for BPs of *P. poae* at 55-125 °C for 0-90 min holding time (Figure 8.1). Temperature-time combinations of 135-160 °C showed a similar pattern, but with significant inactivation (data not shown). The semi logarithmic plots of % residual activity against time were observed to possess linearity at all temperatures tested for the enzymes heated in milk (Figure 8.2). The BPs heated in UHT whole milk and skimmed milk showed the highest residual activity at 55-65 °C, while BPs in SUMF showed moderate residual activity under the respective conditions. *Pseudomonas* spp. and *Hafnia alvei* proteases showed lower residual activities at 55 °C, while *Bacillus* and *Microbacterium* proteases showed lower residual activities at 60 °C and 65 °C, respectively.

Regardless of the heating medium, all enzymes showed lowest residual activity, when heated at 160 °C. The residual activities of BPs were exhibited in the ascending order of SUMF, skimmed milk and whole milk, at temperature of ≥ 75 °C.

Depending on the enzyme used, inactivation rate constants (k) of BPs were at their lowest, when heated at 55-65 °C in both skimmed and full cream milk. However, there was a moderate inactivation constant observed for BPs heated in SUMF under similar conditions. Regardless of the medium used, the k-values of BPs increased with increase in temperature at ≥ 75 °C and were at their maximum, when heated at 160 °C (Table 8.3). Similarly, the half-life of BPs were shown to be at their maximum at 55-65 °C, when BPs were heated in both whole milk and skimmed milk, but medium with SUMF (Table 8.3). These values were at their lowest at 160 °C (Table 8.3). A similar trend was observed in D-values of BPs heated between 55 °C and 160 °C, with a distinct variation at 55-65 °C, when heated in SUMF (Table 8.3).

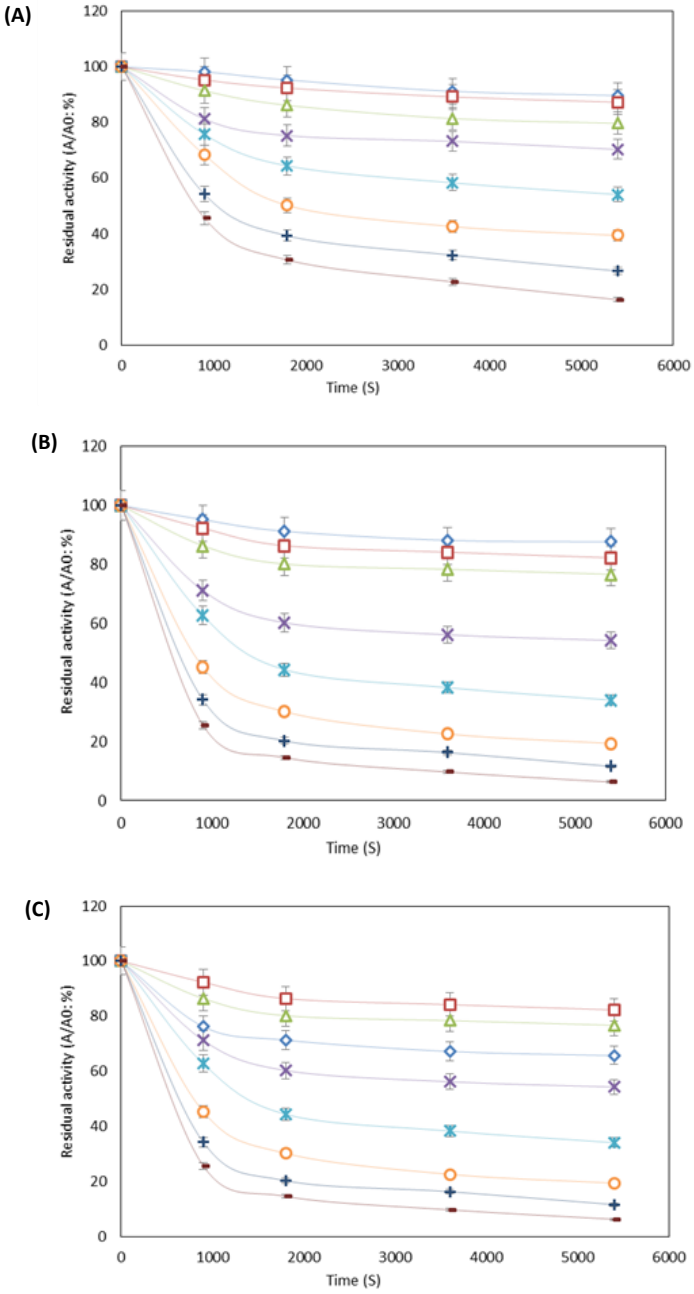


**Figure 8.1** Schematic representation of inactivation of protease of *P. poae* (P24) in (A) whole milk (B) skimmed milk and SUMF (C). Residual activity was determined after heating at 55 °C ( ), 65 °C ( ), 75 °C ( ), 85 °C ( ), 95 °C ( ), 105 °C ( ), 115 °C ( ) and 125 °C ( ) with 0-90 min holding time (i.e. the inactivation of BPs at 135-160 ˚C was conducted for 0-90 s, which was not able to accommodate in the same figure). The results were presented as means of two independent experiments ± SE (n = 2).

The semi-logarithmic plots of D-value with temperature were linear at all times (Figure 8.3) and were used to calculate the Z value for BPs. They were in the range of 48.5-72.5 °C, 38.5-57.5 °C, 32.5-40.2 °C for full cream, skimmed milk and SUMF, respectively (Table 8.3).

### 8.4.3 Thermodynamics analysis

The activation energies were estimated on the basis of Arrhenius' law (Figure 8.4) and indicated that the BPs in whole milk, skimmed milk and SUMF buffer solution were in the range of 123.2-151.8 KJ/mol, 78.4-105.7 KJ/mol and 65.3-78.9 KJ/mol, respectively (Table 8.4). A slight increasing trend of $\Delta H^{\#}$ and significant increasing trend of $\Delta G^{\#}$ with increasing temperatures from 55-160 °C was observed. However, the $\Delta S^{\#}$ values presented a heterogeneous behaviour. The thermodynamic inactivation parameters of BPs in UHT whole milk, skimmed milk and fat milk are listed in Table 8.4.

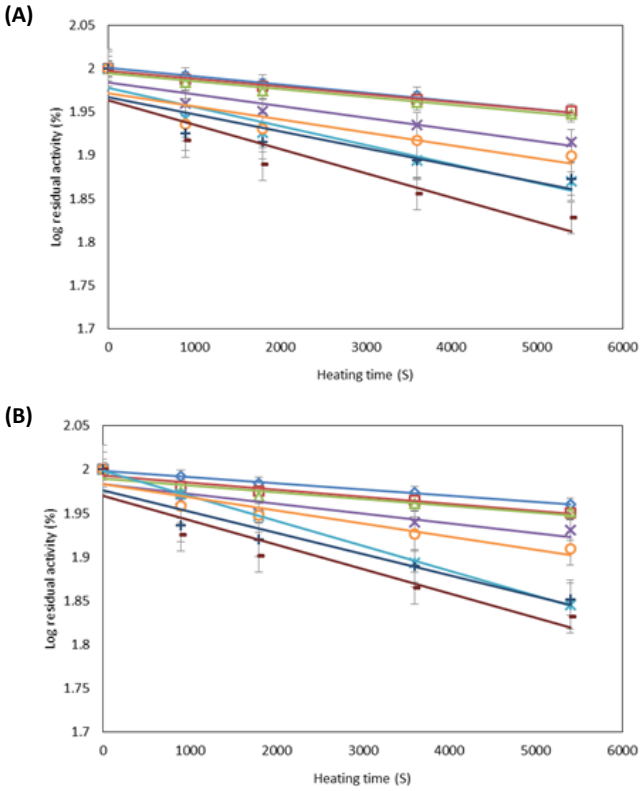


**Figure 8.2** Thermal Inactivation of the extracellular proteases from (A) *P.* poae (P24) (B) *B. licheniformis* (B96) after heating at 55 °C ( ), 65 °C ( ), 75 °C ( ), 85 °C ( ), 95 °C ( ), 105 °C ( ), 115 °C ( ) and 125 °C ( ) in UHT skimmed milk. Residual proteolytic activity is represented as log (relative fluorescens unit/mL) as a function of heating time. The results were presented as means of two independent experiments ± SE (n = 2).

**Table 8.3** Comparison of the kinetic parameters for inactivation of selected BPs in buffer, skimmed and whole milk.

| Heating Medium | Temp (°C) | Inactivation rate constant (k: /min) | | | | | | Half-life (min) | | | | | | D-value (min) | | | | | | Z-value | $R^2$* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | (°C) | |
| SUMF | 55 | 0.0098 | 0.0088 | 0.0032 | 0.0026 | 0.0014 | 0.0041 | 70.7 | 78.8 | 216.6 | 266.6 | 495.1 | 169.1 | 235.0 | 261.7 | 719.7 | 885.8 | 1645.0 | 561.7 | 1: 55.4<br>2: 69.6<br>3: 63.4<br>4: 67.6<br>5: 72.5<br>6: 48.5 | 0.921 |
| | 60 | 0.0088 | 0.0072 | 0.0093 | 0.0076 | 0.0065 | 0.0079 | 78.8 | 96.3 | 74.5 | 91.2 | 106.6 | 87.7 | 221.7 | 319.9 | 247.6 | 303.0 | 354.3 | 291.5 | | 0.987 |
| | 65 | 0.0059 | 0.0058 | 0.0085 | 0.0087 | 0.0078 | 0.0082 | 117.5 | 119.5 | 74.5 | 79.7 | 88.9 | 84.5 | 390.3 | 397.1 | 247.6 | 264.7 | 295.3 | 280.9 | | 0.967 |
| | 75 | 0.0071 | 0.003 | 0.0051 | 0.0035 | 0.0023 | 0.0055 | 97.6 | 231.0 | 135.9 | 198.0 | 301.4 | 126.0 | 324.4 | 767.7 | 451.6 | 658.0 | 1001.3 | 418.7 | | 0.946 |
| | 85 | 0.0083 | 0.0036 | 0.0062 | 0.0043 | 0.0034 | 0.0079 | 83.5 | 192.5 | 111.8 | 161.2 | 203.9 | 87.7 | 277.5 | 639.7 | 371.5 | 535.6 | 677.4 | 291.5 | | 0.889 |
| | 95 | 0.0097 | 0.0043 | 0.0067 | 0.0056 | 0.0047 | 0.0083 | 71.5 | 161.2 | 103.5 | 123.8 | 147.5 | 83.5 | 237.4 | 535.6 | 343.7 | 411.3 | 490.0 | 277.5 | | 0.899 |
| | 105 | 0.0168 | 0.0043 | 0.0069 | 0.0078 | 0.0032 | 0.0092 | 41.3 | 161.2 | 100.5 | 88.9 | 216.6 | 75.3 | 137.1 | 535.6 | 333.8 | 295.3 | 719.7 | 250.3 | | 0.945 |
| | 115 | 0.0216 | 0.0059 | 0.0088 | 0.0069 | 0.0068 | 0.0123 | 32.1 | 117.5 | 78.8 | 100.5 | 101.9 | 56.4 | 106.6 | 390.3 | 261.7 | 333.8 | 338.7 | 187.2 | | 0.967 |
| | 125 | 0.0248 | 0.0065 | 0.0148 | 0.0068 | 0.0073 | 0.0134 | 27.9 | 106.6 | 46.8 | 101.9 | 95.0 | 51.7 | 92.9 | 354.3 | 155.6 | 338.7 | 315.5 | 171.9 | | 0.956 |
| | 135 | 0.025 | 0.0069 | 0.0164 | 0.0111 | 0.0083 | 0.014 | 0.5 | 1.7 | 0.7 | 1.0 | 1.4 | 0.8 | 1.5 | 5.6 | 2.3 | 3.5 | 4.6 | 2.7 | | 0.931 |
| | 140 | 0.0288 | 0.0089 | 0.0223 | 0.0134 | 0.0107 | 0.0181 | 0.4 | 1.3 | 0.5 | 0.9 | 1.1 | 0.6 | 1.3 | 4.3 | 1.7 | 2.9 | 3.6 | 2.1 | | 0.991 |
| | 145 | 0.0297 | 0.0145 | 0.0249 | 0.0213 | 0.0118 | 0.0267 | 0.4 | 0.8 | 0.5 | 0.5 | 1.0 | 0.4 | 1.3 | 2.6 | 1.5 | 1.8 | 3.3 | 1.4 | | 0.955 |
| | 150 | 0.0338 | 0.0154 | 0.0254 | 0.0217 | 0.0177 | 0.0376 | 0.3 | 0.8 | 0.5 | 0.5 | 0.7 | 0.3 | 1.1 | 2.5 | 1.5 | 1.8 | 2.2 | 1.0 | | 0.935 |
| | 155 | 0.0354 | 0.0156 | 0.0374 | 0.0243 | 0.0183 | 0.0404 | 0.3 | 0.7 | 0.3 | 0.5 | 0.6 | 0.3 | 1.1 | 2.5 | 1.0 | 1.6 | 2.1 | 1.0 | | 0.968 |
| | 160 | 0.0535 | 0.0296 | 0.0456 | 0.0436 | 0.0328 | 0.0567 | 0.2 | 0.4 | 0.3 | 0.3 | 0.4 | 0.2 | 0.7 | 1.3 | 0.8 | 0.9 | 1.2 | 0.7 | | 0.972 |
| SM | 55 | 0.0032 | 0.0025 | 0.003 | 0.0028 | 0.0016 | 0.0041 | 216.6 | 277.3 | 231.0 | 247.6 | 433.2 | 189.6 | 719.7 | 921.2 | 767.7 | 822.5 | 1739.4 | 661.7 | 1: 45.2<br>2: 53.2<br>3: 43.5<br>4: 42.1<br>5: 57.5<br>6: 38.5 | 0.966 |
| | 60 | 0.035 | 0.0026 | 0.0035 | 0.0029 | 0.0019 | 0.0045 | 169.8 | 266.6 | 198.0 | 239.0 | 364.8 | 154.0 | 665.8 | 885.8 | 658.0 | 794.1 | 1212.1 | 531.8 | | 0.953 |
| | 65 | 0.0039 | 0.0028 | 0.0039 | 0.0031 | 0.0021 | 0.0048 | 177.7 | 254.3 | 157.4 | 231.0 | 330.1 | 169.1 | 590.5 | 593.4 | 590.5 | 787.7 | 896.7 | 511.3 | | 0.942 |
| | 75 | 0.0071 | 0.003 | 0.0051 | 0.0035 | 0.0023 | 0.0055 | 97.6 | 247.0 | 135.9 | 231.0 | 311.4 | 126.0 | 324.4 | 407.3 | 491.6 | 754.4 | 601.6 | 418.7 | | 0.979 |
| | 85 | 0.0081 | 0.0032 | 0.0057 | 0.0036 | 0.0028 | 0.0071 | 85.6 | 239.0 | 135.9 | 231.0 | 301.4 | 97.6 | 284.3 | 207.7 | 455.6 | 666.6 | 301.3 | 324.4 | | 0.929 |
| | 95 | 0.009 | 0.0035 | 0.006 | 0.0046 | 0.0034 | 0.0076 | 77.0 | 201.0 | 115.5 | 150.7 | 203.9 | 81.2 | 255.9 | 92.4 | 383.8 | 500.7 | 107.4 | 303.0 | | 0.908 |
| | 105 | 0.0164 | 0.0037 | 0.0064 | 0.0067 | 0.0039 | 0.0088 | 0.7 | 3.1 | 1.8 | 1.9 | 3.0 | 1.3 | 2.3 | 25.4 | 6.0 | 8.1 | 32.8 | 4.4 | | 0.916 |
| | 115 | 0.021 | 0.0053 | 0.0081 | 0.0066 | 0.006 | 0.0113 | 0.6 | 2.2 | 1.4 | 1.9 | 1.9 | 1.0 | 2.1 | 18.2 | 4.7 | 6.4 | 26.4 | 3.4 | | 0.967 |
| | 125 | 0.0244 | 0.0062 | 0.0145 | 0.0062 | 0.0067 | 0.0124 | 0.6 | 1.9 | 0.8 | 1.9 | 1.7 | 0.9 | 1.9 | 16.2 | 2.6 | 5.7 | 15.7 | 3.1 | | 0.955 |
| | 135 | 0.025 | 0.0069 | 0.0164 | 0.0111 | 0.0083 | 0.014 | 0.5 | 1.7 | 0.7 | 1.0 | 1.4 | 0.8 | 1.6 | 5.6 | 2.3 | 3.5 | 4.6 | 2.7 | | 0.953 |
| | 140 | 0.0281 | 0.0081 | 0.0216 | 0.0127 | 0.0097 | 0.0175 | 0.4 | 1.4 | 0.5 | 0.9 | 1.2 | 0.7 | 1.4 | 4.7 | 1.8 | 3.0 | 4.1 | 2.2 | | 0.932 |
| | 145 | 0.0284 | 0.014 | 0.0242 | 0.0205 | 0.0111 | 0.0258 | 0.4 | 1.0 | 0.5 | 0.6 | 1.0 | 0.4 | 1.3 | 2.7 | 1.6 | 1.9 | 3.5 | 1.5 | | 0.976 |
| | 150 | 0.0332 | 0.0145 | 0.0244 | 0.0207 | 0.0173 | 0.0368 | 0.3 | 0.8 | 0.5 | 0.6 | 0.7 | 0.3 | 1.2 | 2.6 | 1.4 | 1.8 | 2.2 | 1.0 | | 0.988 |
| | 155 | 0.0345 | 0.0147 | 0.0366 | 0.0239 | 0.0175 | 0.0394 | 0.2 | 0.5 | 0.3 | 0.5 | 0.5 | 0.3 | 1.1 | 2.3 | 1.0 | 1.6 | 2.1 | 1.0 | | 0.975 |
| | 160 | 0.0524 | 0.0288 | 0.0449 | 0.0431 | 0.0322 | 0.0556 | 0.2 | 0.4 | 0.3 | 0.3 | 0.4 | 0.2 | 0.7 | 1.3 | 0.9 | 0.9 | 1.2 | 0.7 | | 0.934 |
| WM | 55 | 0.0026 | 0.0009 | 0.0025 | 0.0023 | 0.0011 | 0.0033 | 266.6 | 770.2 | 277.3 | 277.3 | 630.1 | 210.0 | 885.8 | 2558.9 | 921.2 | 921.2 | 2093.6 | 697.9 | 1: 35.2<br>2: 40.2<br>3: 33.5<br>4: 32.1<br>5: 37.5<br>6: 32.5 | 0.996 |
| | 60 | 0.0310 | 0.0016 | 0.0034 | 0.0025 | 0.0017 | 0.0041 | 22.4 | 433.2 | 203.9 | 277.3 | 407.7 | 169.1 | 74.3 | 1439.4 | 677.4 | 921.2 | 1354.7 | 561.7 | | 0.923 |
| | 65 | 0.0035 | 0.0024 | 0.0039 | 0.0027 | 0.0021 | 0.0048 | 177.7 | 288.8 | 177.7 | 301.4 | 330.1 | 144.4 | 590.5 | 959.6 | 590.5 | 1001.3 | 1096.7 | 479.8 | | 0.972 |
| | 75 | 0.0065 | 0.0026 | 0.0048 | 0.0031 | 0.0016 | 0.0043 | 106.6 | 266.6 | 144.4 | 223.6 | 433.2 | 161.2 | 354.3 | 885.8 | 479.8 | 742.9 | 1439.4 | 535.6 | | 0.959 |
| | 85 | 0.0075 | 0.0028 | 0.0052 | 0.0034 | 0.0023 | 0.0065 | 92.4 | 247.6 | 133.3 | 203.9 | 301.4 | 106.6 | 307.1 | 822.5 | 442.9 | 677.4 | 1001.3 | 354.3 | | 0.899 |
| | 95 | 0.0083 | 0.0031 | 0.0056 | 0.0042 | 0.0030 | 0.0068 | 83.5 | 223.6 | 123.8 | 165.0 | 231.0 | 101.9 | 277.5 | 742.9 | 411.3 | 548.3 | 767.7 | 338.7 | | 0.898 |
| | 105 | 0.0162 | 0.0035 | 0.0062 | 0.0065 | 0.0037 | 0.0089 | 42.8 | 198.0 | 111.8 | 106.6 | 187.3 | 77.9 | 142.2 | 658.0 | 371.5 | 354.3 | 622.4 | 258.8 | | 0.958 |
| | 115 | 0.0212 | 0.0057 | 0.0080 | 0.0065 | 0.0059 | 0.0112 | 32.7 | 121.6 | 86.6 | 106.6 | 117.5 | 61.9 | 108.6 | 404.0 | 287.9 | 354.3 | 390.3 | 205.6 | | 0.978 |
| | 125 | 0.0246 | 0.0064 | 0.0146 | 0.0063 | 0.0068 | 0.0125 | 28.2 | 108.3 | 47.5 | 110.0 | 101.9 | 55.5 | 93.6 | 359.8 | 157.7 | 365.6 | 338.7 | 184.2 | | 0.965 |
| | 135 | 0.0252 | 0.0071 | 0.0165 | 0.0116 | 0.0085 | 0.0142 | 0.5 | 1.6 | 0.7 | 1.0 | 1.4 | 0.8 | 1.5 | 5.4 | 2.3 | 3.3 | 4.5 | 2.7 | | 0.943 |
| | 140 | 0.0282 | 0.0082 | 0.0224 | 0.0130 | 0.0098 | 0.0174 | 0.4 | 1.4 | 0.5 | 0.9 | 1.2 | 0.7 | 1.4 | 4.7 | 1.7 | 3.0 | 3.9 | 2.2 | | 0.919 |
| | 145 | 0.0284 | 0.0140 | 0.0242 | 0.0205 | 0.0111 | 0.0258 | 0.4 | 0.8 | 0.5 | 0.6 | 1.0 | 0.4 | 1.4 | 2.7 | 1.6 | 1.9 | 3.5 | 1.5 | | 0.955 |
| | 150 | 0.0353 | 0.0146 | 0.0243 | 0.0210 | 0.0174 | 0.0365 | 0.3 | 0.8 | 0.5 | 0.6 | 0.7 | 0.3 | 1.1 | 2.6 | 1.6 | 1.8 | 2.2 | 1.1 | | 0.993 |
| | 155 | 0.0365 | 0.0153 | 0.0368 | 0.0242 | 0.0178 | 0.0396 | 0.3 | 0.8 | 0.3 | 0.5 | 0.6 | 0.3 | 1.1 | 2.5 | 1.0 | 1.6 | 2.2 | 1.0 | | 0.988 |
| | 160 | 0.0528 | 0.0292 | 0.0450 | 0.0432 | 0.0327 | 0.0562 | 0.2 | 0.4 | 0.3 | 0.3 | 0.4 | 0.2 | 0.7 | 1.3 | 0.9 | 0.9 | 1.2 | 0.7 | | 0.992 |

Values are means ± standard deviations of duplicate assays of two independent experiments. All the values were statistically different ($P < 0.05$) in the range of 55–160 °C for each treatment.k: inactivation rate constant (per min); $t_{1/2}$: half-life (time required for 50% of inactivation); D-value: decimal reduction time (time required for 90% inactivation); Z-value: microbial thermal death time (number of degrees the temperature has to be increased to achieve a tenfold or 1 $\log_{10}$ reduction in the D-value; SMUF: simulated milk ultrafiltrate; SM: UHT skimmed milk; WM: UHT whole milk; 1: *H alvei* (P50); (C216); 2: *P. poae* (P24); 3: *M. oxydans* (A79); 4: *B. weihenstephanensis* (B5); 5: *B. licheniformis* (B96); 6: thermolysin (PC).

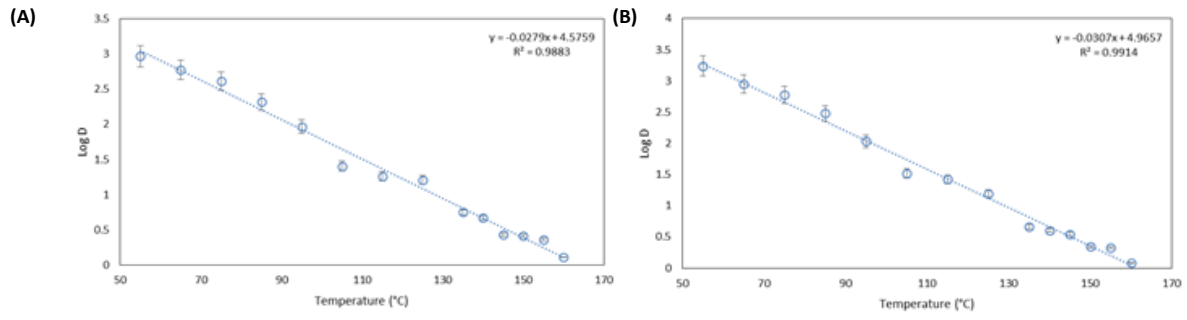


**Figure 8.3** Decimal reduction time (D-value) variation of BPs (A) *P. poae* (P24) (B) *B. licheniformis* (B96) in skimmed milk as a function of temperature. The results were presented as means of two independent experiments ± SE (n = 2).

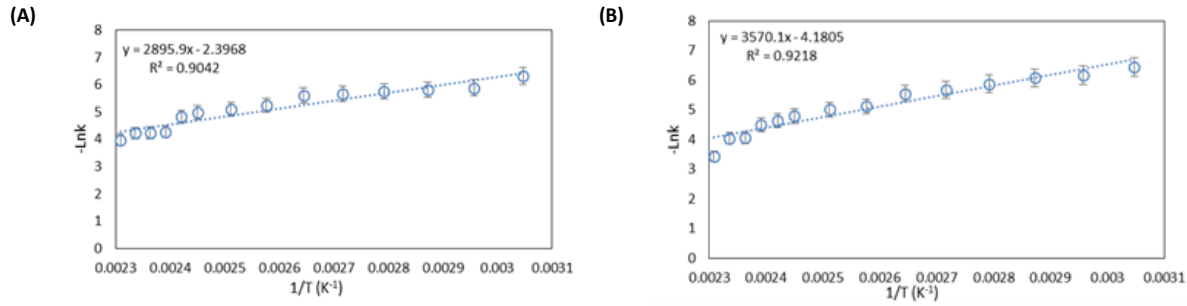


**Figure 8.4** Arrhenius' plots of thermal inactivation of BPs in skimmed milk (A) *P. poae* (P24) (B) *B. licheniformis* (B96).The results were presented as means of two independent experiments ± SE (n = 2).

## 8.5 Discussion

Controlling the BPs in UHT milk is of utmost importance due to the potential negative consequences of spoilage and reduction of shelf-life during storage under ambient conditions (Stoeckel et al. 2016a). Therefore, the present study was designed to establish the inactivation kinetics of representative heat-stable BPs in milk using a wide range of thermal treatments (55-160 °C). The experimental design enabled an assessment of the effect of milk proteins and fat on thermal inactivation of BPs. Results of the present study indicated an exponential decay in the residual activity of BPs with increased time, under all conditions tested, except for heating in SUMF buffer solution at 55-65 °C. This has been previously observed for BPs (Glück et al. 2016, Marchand et al. 2008) and is typical for thermal inactivation of proteins or peptides (Sant'Anna et al. 2012).

**Table 8.4** Comparison of the thermodynamic parameters calculated for thermal inactivation of BPs in buffer, skimmed milk and whole milk.

| Heating medium | $E_a$ (kJ/mol) | Temp (K) | $\Delta H^{\#}$ (KJ/mol) | | | | | | $\Delta G^{\#}$ (KJ/mol) | | | | | | $\Delta S^{\#}$(J/mol K) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 |
| SMUF | 1: 72* | 328 | 69.3 | 86.3 | 61.3 | 66.3 | 89.3 | 59.3 | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC |
| | 2: 89* | 333 | 69.2 | 86.2 | 61.2 | 66.2 | 89.2 | 59.2 | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC |
| | 3: 64* | 338 | 69.2 | 86.2 | 61.2 | 66.2 | 89.2 | 59.2 | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC |
| | 4: 69* | 348 | 69.1 | 86.1 | 61.1 | 66.1 | 89.1 | 59.1 | 80.5 | 78.0 | 79.5 | 78.4 | 77.2 | 79.7 | -32.7 | 23.3 | -52.9 | -35.4 | 34.2 | -59.3 |
| | 5: 92* | 358 | 69.0 | 86.0 | 61.0 | 66.0 | 89.0 | 59.0 | 83.3 | 80.9 | 82.5 | 81.4 | 80.7 | 83.2 | -40.0 | 14.4 | -59.9 | -42.9 | 23.3 | -67.5 |
| | 6: 62* | 368 | 68.9 | 85.9 | 60.9 | 65.9 | 88.9 | 58.9 | 86.2 | 83.7 | 85.1 | 84.5 | 84.0 | 85.7 | -47.0 | 6.0 | -65.6 | -50.5 | 13.4 | -72.8 |
| | | 378 | 68.9 | 85.9 | 60.9 | 65.9 | 88.9 | 58.9 | 90.4 | 86.1 | 87.6 | 88.0 | 85.2 | 88.5 | -56.9 | -0.6 | -70.7 | -58.5 | 9.8 | -78.4 |
| | | 388 | 68.8 | 85.8 | 60.8 | 65.8 | 88.8 | 58.8 | 93.7 | 89.5 | 90.8 | 90.0 | 89.9 | 91.8 | -64.1 | -9.5 | -77.3 | -62.4 | -3.0 | -85.2 |
| | | 398 | 68.7 | 85.7 | 60.7 | 65.7 | 88.7 | 58.7 | 96.6 | 92.2 | 94.9 | 92.3 | 92.6 | 94.6 | -70.1 | -16.3 | -85.9 | -66.9 | -9.7 | -90.1 |
| | | 408 | 68.6 | 85.6 | 60.6 | 65.6 | 88.6 | 58.6 | 99.2 | 94.8 | 97.7 | 96.4 | 95.4 | 97.2 | -74.8 | -22.5 | -90.9 | -75.4 | -16.7 | -94.5 |
| | | 413 | 68.6 | 85.6 | 60.6 | 65.6 | 88.6 | 58.6 | 100.9 | 96.9 | 100.0 | 98.3 | 97.5 | 99.3 | -78.3 | -27.4 | -95.5 | -79.2 | -21.6 | -98.6 |
| | | 418 | 68.5 | 85.5 | 60.5 | 65.5 | 88.5 | 58.5 | 102.3 | 99.8 | 101.7 | 101.1 | 99.1 | 101.9 | -80.7 | -34.1 | -98.4 | -85.1 | -25.2 | -103.7 |
| | | 423 | 68.5 | 85.5 | 60.5 | 65.5 | 88.5 | 58.5 | 104.0 | 101.2 | 103.0 | 102.4 | 101.7 | 104.4 | -83.9 | -37.2 | -100.4 | -87.3 | -31.3 | -108.4 |
| | | 428 | 68.4 | 85.4 | 60.4 | 65.4 | 88.4 | 58.4 | 105.4 | 102.5 | 105.6 | 104.1 | 103.1 | 105.9 | -86.4 | -39.9 | -105.5 | -90.3 | -34.2 | -110.8 |
| SM | 1: 93 | 328 | 90.3 | 109.3 | 82.3 | 90.3 | 112.3 | 105.3 | 90.3 | 73.5 | 72.8 | 73.3 | 73.2 | 71.6 | 72.4 | 51.1 | 5.6 | -60.4 | 52.2 | 11.6 |
| | 2: 112 | 333 | 90.2 | 109.2 | 82.2 | 90.2 | 112.2 | 105.2 | 90.2 | 81.3 | 74.1 | 74.9 | 74.4 | 73.2 | 73.8 | 26.8 | 14.4 | -56.3 | 47.5 | 19.9 |
| | 3: 85 | 338 | 90.2 | 109.2 | 82.2 | 90.2 | 112.2 | 105.2 | 90.2 | 76.4 | 75.5 | 76.4 | 75.8 | 74.7 | 75.1 | 40.8 | 17.7 | -50.5 | 42.7 | 23.3 |
| | 4: 95 | 348 | 90.1 | 109.1 | 82.1 | 90.1 | 112.1 | 105.1 | 90.1 | 80.5 | 78.0 | 79.5 | 78.4 | 77.2 | 78.7 | 27.7 | 21.2 | -47.9 | 33.5 | 30.3 |
| | 5: 115 | 358 | 90.0 | 109.0 | 82.0 | 90.0 | 112.0 | 105.0 | 90.0 | 83.3 | 80.5 | 82.2 | 80.9 | 80.1 | 81.2 | 18.9 | 29.1 | -44.4 | 25.6 | 34.9 |
| | 6: 84 | 368 | 89.9 | 108.9 | 81.9 | 89.9 | 111.9 | 104.9 | 89.9 | 86.0 | 83.1 | 84.8 | 83.9 | 83.0 | 83.7 | 10.7 | 33.9 | -39.5 | 16.3 | 39.7 |
| | | 378 | 89.9 | 108.9 | 81.9 | 89.9 | 111.9 | 104.9 | 89.9 | 90.3 | 85.6 | 87.3 | 87.5 | 85.8 | 86.8 | -1.2 | 41.8 | -33.0 | 6.3 | 48.7 |
| | | 388 | 89.8 | 108.8 | 81.8 | 89.8 | 111.8 | 104.8 | 89.8 | 93.6 | 89.1 | 90.5 | 89.8 | 89.5 | 89.5 | -9.8 | 50.6 | -22.5 | -0.2 | 57.3 |
| | | 398 | 89.7 | 108.7 | 81.7 | 89.7 | 111.7 | 104.7 | 89.7 | 96.6 | 92.0 | 94.8 | 92.0 | 92.3 | 92.5 | -17.3 | 61.4 | -14.5 | -5.9 | 68.9 |
| | | 408 | 89.6 | 108.6 | 81.6 | 89.6 | 111.6 | 104.6 | 89.6 | 99.2 | 94.8 | 97.7 | 96.4 | 95.4 | 96.6 | -23.4 | 70.2 | -7.6 | -16.6 | 78.6 |
| | | 413 | 89.6 | 108.6 | 81.6 | 89.6 | 111.6 | 104.6 | 89.6 | 100.8 | 96.5 | 99.9 | 98.1 | 97.2 | 97.9 | -27.2 | 79.6 | -0.5 | -20.6 | 89.1 |
| | | 418 | 89.5 | 108.5 | 81.5 | 89.5 | 111.5 | 104.5 | 89.5 | 102.1 | 99.7 | 101.6 | 101.0 | 98.8 | 102.0 | -30.1 | 89.4 | 7.4 | -27.4 | 100.2 |
| | | 423 | 89.5 | 108.5 | 81.5 | 89.5 | 111.5 | 104.5 | 89.5 | 103.9 | 101.0 | 102.8 | 102.3 | 101.6 | 103.8 | -34.1 | 99.7 | 17.1 | -30.2 | 111.0 |
| | | 428 | 89.4 | 108.4 | 81.4 | 89.4 | 111.4 | 104.4 | 89.4 | 105.3 | 102.3 | 105.5 | 104.0 | 102.9 | 106.0 | -37.1 | 105.4 | 21.9 | -34.1 | 117.0 |
| WM | 1: 137 | 328 | 134.3 | 152.3 | 126.3 | 128.3 | 155.3 | 126.3 | 73.0 | 70.1 | 72.8 | 72.6 | 70.6 | 73.6 | 58.3 | 104.8 | 41.2 | 110.8 | 39.3 | 58.3 |
| | 2: 155 | 333 | 134.2 | 152.2 | 126.2 | 128.2 | 155.2 | 126.2 | 81.0 | 72.8 | 74.8 | 74.0 | 72.9 | 75.4 | 65.2 | 114.4 | 46.4 | 120.2 | 45.8 | 65.2 |
| | 3: 129 | 338 | 134.2 | 152.2 | 126.2 | 128.2 | 155.2 | 126.2 | 76.1 | 75.0 | 76.4 | 75.4 | 74.7 | 77.0 | 69.3 | 119.2 | 53.5 | 124.8 | 50.2 | 69.3 |
| | 4: 131 | 348 | 134.1 | 152.1 | 126.1 | 128.1 | 155.1 | 126.1 | 80.2 | 77.6 | 79.3 | 78.1 | 76.2 | 79.0 | 75.1 | 124.0 | 57.3 | 133.1 | 56.8 | 75.1 |
| | 5: 158 | 358 | 134.0 | 152.0 | 126.0 | 128.0 | 155.0 | 126.0 | 83.0 | 80.1 | 81.9 | 80.7 | 79.5 | 82.6 | 79.2 | 133.1 | 61.8 | 138.9 | 63.9 | 79.2 |
| | 6: 127 | 368 | 133.9 | 151.9 | 125.9 | 127.9 | 154.9 | 125.9 | 85.7 | 82.7 | 84.5 | 83.7 | 82.6 | 85.1 | 84.3 | 139.0 | 68.3 | 144.8 | 69.5 | 84.3 |
| | | 378 | 133.9 | 151.9 | 125.9 | 127.9 | 154.9 | 125.9 | 90.3 | 85.4 | 87.2 | 87.4 | 85.6 | 88.4 | 93.2 | 149.6 | 77.4 | 156.6 | 78.7 | 93.2 |
| | | 388 | 133.8 | 151.8 | 125.8 | 127.8 | 154.8 | 125.8 | 93.6 | 89.4 | 90.5 | 89.8 | 89.5 | 91.5 | 103.5 | 160.8 | 91.0 | 168.2 | 88.2 | 103.5 |
| | | 398 | 133.7 | 151.7 | 125.7 | 127.7 | 154.7 | 125.7 | 96.6 | 92.1 | 94.9 | 92.1 | 92.3 | 94.4 | 115.3 | 175.6 | 102.1 | 183.1 | 99.1 | 115.3 |
| | | 408 | 133.6 | 151.6 | 125.6 | 127.6 | 154.6 | 125.6 | 99.2 | 94.9 | 97.7 | 96.6 | 95.5 | 97.2 | 130.9 | 188.0 | 112.4 | 196.4 | 110.8 | 130.9 |
| | | 413 | 133.6 | 151.6 | 125.6 | 127.6 | 154.6 | 125.6 | 100.8 | 96.6 | 100.0 | 98.2 | 97.2 | 99.2 | 142.4 | 200.8 | 123.1 | 210.8 | 121.2 | 142.4 |
| | | 418 | 133.5 | 151.5 | 125.5 | 127.5 | 154.5 | 125.5 | 102.1 | 99.7 | 101.6 | 101.0 | 98.8 | 101.8 | 154.8 | 214.1 | 134.3 | 226.7 | 135.2 | 154.8 |
| | | 423 | 133.5 | 151.5 | 125.5 | 127.5 | 154.5 | 125.5 | 104.1 | 101.0 | 102.8 | 102.3 | 101.7 | 104.3 | 159.9 | 228.2 | 147.2 | 238.1 | 145.5 | 159.9 |
| | | 428 | 133.4 | 151.4 | 125.4 | 127.4 | 154.4 | 125.4 | 105.5 | 102.4 | 105.6 | 104.1 | 103.0 | 105.8 | 171.8 | 238.5 | 154.2 | 247.0 | 152.7 | 171.8 |

Values are means ± standard deviations of duplicate assays of two independent experiments. All the values were statistically different ($P < 0.05$) in the range of 55–160 °C for each treatment.$E_a$: activation energy; ΔH: activation enthalpy; ΔG: Gibbs free energy for inactivation; ΔS: activation entropy; * the activation energy for SUMF was calculated, when temperature > 65 °C; NC: Not calculated due to different phenomenon for low temperature inactivation. SMUF: simulated milk ultrafiltrate; SM: UHT skimmed milk; WM: UHT whole milk; 1: *H alvei* (P50); (C216); 2: *P. poae* (P24); 3: *M. oxydans* (A79); 4: *B. weihenstephanensis* (B5); 5: *B. licheniformis* (B96); 6: thermolysin (PC).

The semi-logarithmic plots of residual activities of all BPs showed a linear relationship with time, suggesting that the inactivation of BPs followed a first order monophasic kinetics process ($R^2$ = 0.889-0.9996; $P < 0.05$). This has been previously observed in several studies (Glück et al. 2016, Marchand et al. 2008), and validates the monomeric nature of these BPs (Sekine et al. 2002). Irrespective of the medium used, significant inactivation rate constants ($P < 0.0001$) were observed for the BPs at 145-160 °C, suggesting that the BPs may be less heat-stable at these temperatures (Deylami et al. 2014, Gouda et al. 2003, Sant'Anna et al. 2012). Therefore, UHT heating below these values may not be adequate for controlling BPs in the finished products. Besides the limited number of reported cases of UHT milk spoilage, many studies observed gelation, bitterness, fat separation of combination of these quality defects in UHT milk due to the proteolysis by bacterial enzymes during the ambient storage (Glück et al. 2016, Marchand et al. 2008).

The higher inactivation rate of BPs was associated with a significantly lower half-life (i.e. 50% reduction from the initial activity), decimal reduction time (i.e. 90% reduction from the initial activity) and high Z-values (temperature dependence of the decimal reduction time of enzyme activity/the range of temperature increase needed for a log10 reduction in the D value) than those commonly used for the estimation of thermal inactivation of proteins and enzymes (Deylami et al. 2014, Gouda et al. 2003, Marchand et al. 2008, Sant'Anna et al. 2012, Stepaniak & Fox 1983, Stoeckel et al. 2016b). These values were the highest at 55-65 °C, and lowest at 145-160 °C and maintain consistency with the previous description (Stepaniak & Fox 1983). In the present study, we also observed total inactivation of some BPs at 150-160 °C after 60-90 s in milk and SUMF (data not shown). This is possibly related to the lower heat-stabilities of the respective BPs, or their relatively lower expression (Marchand et al. 2009). However, some BPs showed higher activity upon UHT heating at 135-140 °C (data not shown), which may possibly be related to the reversible structural changes in the corresponding BPs (Joshi & Satyanarayana 2013). For example, circular dichroism (CD) spectral analysis of *Pseudomonas* protease (T16) indicated that the enzyme undergoes an additional conformational change to form a more ordered and stable structure.

This structure was further stabilised by $Ca^{2+}$ ion, which protected against heat inactivation at 60-95 °C (Patel & Bartlett 1988).

The inactivation of BPs was more prominent in the SUMF buffer solution as compared to milk. Stabilisation of BPs can be attributed to interactions of milk proteins and fat content (Adams 1991). Metal ions are believed to be essential for maintenance of the structural conformation, thermostability and integrity of the catalytic site (Patel & Bartlett 1988). It was concluded that high temperature stability can be achieved due to the binding of $Ca^{2+}$ to the enzyme structure, causing correct re-folding (Adams 1991, Kroll 1989, Patel et al. 1986). This may also be associated with the amino acid composition, especially the presence of threonine in the N-terminal region of the BPs (Patel et al. 1986). Besides thermal inactivation, we also observed low temperature inactivation (LTI) at temperatures between 55-65 °C.

In addition to the inactivation kinetics, knowledge of thermodynamic parameters is essential for determining the stability of BPs and for elucidation of the associated thermal inactivation mechanisms (Adams 1991, Deylami et al. 2014, Gouzi et al. 2012). This may involve any secondary stabilization or destabilization effects that might be disregarded if only the half-lives and D-values are taken into consideration (Adams 1991). The parameters include activation energy ($E_a$), Gibbs free energy change ($\Delta G$), enthalpy change ($\Delta H$) and entropy change ($\Delta S$), (Table 8.4). The $E_a$ refers to the absorption of energy required for molecules to move faster with a proper collision orientation to initiate the inactivation reaction, which involves stretching, bending, and ultimately breaking of bonds. This can be estimated by Arrhenius' law (Deylami et al. 2014, Gouzi et al. 2012, Sant'Anna et al. 2012). In contrast, the $\Delta G$ represents the energy barrier that can inactivate the enzyme or achieve high energy transition state during the inactivation process; thus, high $\Delta G$ depicts the difficulty that may be faced in forming transition high energy complex or higher enzyme stability (Deylami et al. 2014, Gouzi et al. 2012). Both $\Delta H$ and $\Delta S$ indicate the amount of bonds broken and the amount of intra- and inter- (with solute) molecular bonds being formed during inactivation (Deylami et al. 2014).

In the present study, the activation energies of the BPs for thermal inactivation at 55-160 °C were significantly higher. This is consistent with previous descriptions of higher $E_a$ of several BPs (Diermayr et al. 1987, Stoeckel et al. 2016b). This was the highest in UHT whole milk, intermediate in skimmed milk and lowest in SUMF buffer solution. Those BPs having higher $E_a$ indicate that the energy from the medium is not enough to start the inactivation reaction. Thus, high activation energy indicates strong temperature dependence of these enzymes, and that the reaction will proceed at a slower rate at low temperature, but relatively faster at high temperatures (Van Boekel 2008).

In general, these thermodynamic parameters are at their lowest, when the enzymes are in their native globular state, as a result of the inter- (with solute) and intra-molecular interactions that produce highly compact, organised structures with more hydrophobic portions buried inside the structure, and more hydrophilic regions located on the surface (Deylami et al. 2014, Gouzi et al. 2012, Sant'Anna et al. 2012). The weak interactions in the α-helixes and β-barrels in the native structure are subjected to unfolding upon heating, and thus lead to an expanded as well as less organised structure (Deylami et al. 2014, Gouzi et al. 2012, Roman & González Flecha 2014, Sant'Anna et al. 2012). A shift from β-structures to random coils was observed, when the temperature increased from 25 °C to 95 °C, thus indicating the unfolding (Patel & Bartlett 1988). This unfolding of proteins/enzymes is also characterised by the melting temperature ($T_m$), at which, 50% of the enzyme is unfolded (Duy & Fitter 2005). This, in turn, leads to an increase the $\Delta G$ and $\Delta S$ with reduction in the $\Delta H$, due to the formation of a high energy transition state, reforming of bonds within the molecules and solute molecules with both hydrophilic and hydrophobic parts, and disruption of bonds in the native structure (Deylami et al. 2014, Gouzi et al. 2012, Sant'Anna et al. 2012).

These data further confirm the three-stage thermal inactivation theory of BPs (Stoeckel et al. 2016b). It was described that denaturation of BPs was likely to begin below the optimum temperature condition between 40 °C and 50 °C, at which, the BPs are subjected to reversible unfolding, leading to a gradual increase in activity. This stage is called active

enzyme stage. This was observed in the current project (data not shown) and has also been observed in other studies (Patel & Bartlett 1988, Stoeckel et al. 2016b).

Secondly, BPs are known to be subjected to intermolecular autoproteolysis between 55 °C and 65 °C, referred to as LTI (Barach et al. 1976, Stoeckel et al. 2016b). At this stage, the enzymes are believed to be minimally unfolded and are present as a cocktail of folded (active) and unfolded or partially unfolded (inactive) BPs. The unfolded proteins are more susceptible to autolysis, compared to folded enzymes (Stoeckel et al. 2016b). In this study, LTI was only observed in SMUF buffer and it was possibly related to the higher substrate concentration for BPs in milk, presumably due to substrate binding, compared to that in SMUF (Stepaniak & Fox 1983, Stoeckel et al. 2016b). Similarly, LTI appeared to be enzyme specific, where *Pseudomonas* and *Hafnia* proteases showed higher inactivation at 55 °C, while *Microbacterium* and *Bacillus* proteases showed LTI at 60 °C and 65 °C. Previous studies also observed differences in the LTI at different temperatures ranging from 45-55 °C in *Pseudomonas* proteases, and this is likely to be associated with differences in the enzyme structure, amino acid composition and differences in the temperature optima and specificities of different BPs (Patel & Bartlett 1988, Stepaniak & Fox 1983). It was contended that conformational transition occurred at this temperature to facilitate the inactivation by autolysis (Patel & Bartlett 1988, Stepaniak & Fox 1983). However, the protease was not able to auto-inactivate on cooling to 55 °C from high temperature such as 80 °C, 90 °C, 100 °C or 150 °C (Diermayr et al. 1987, Stepaniak & Fox 1983). This may be related to the conformational stability that BPs acquire at high temperatures (Diermayr et al. 1987, Patel & Bartlett 1988, Stepaniak & Fox 1983). Since this stage is associated with enzyme-mediated autolysis, the $\Delta H$, $\Delta G$ and $\Delta S$ appeared to be slightly lower, along with the higher inactivation rate constants. However, the rapid inactivation of BPs at 55-65 °C is possible only if the enzyme is heated from lower temperatures, and does not hold true if it is cooled from 90 °C; this phenomenon is possibly related to the conformational changes that occurred at higher temperature (Diermayr et al. 1987). Theoretically, many proteins exhibit folding intermediates, comprising non-native structures that are compact and partly folded with

reduced tertiary interactions, with a rather large content of secondary structure, and a fluctuating hydrophobic core (Hoyoux et al. 2004). These are termed as molten globule states and are formed rather early during the unfolding-refolding process (Hoyoux et al. 2004). This stage is concentration-dependant rather than time-dependent, and follows the second order inactivation kinetics (Barach et al. 1976, Stoeckel et al. 2016b).

The thermal inactivation of BPs occurs, when the temperature increases to ≥ 75 °C. This process is comprised of a long linear relationship of residual activity with time. This linearity suggests that the inactivation of BPs can occur via a unique temperature-dependent mechanism, such as protein unfolding (Deylami et al. 2014). However, at temperatures above ≥ 75 °C, the proteases undergo additional conformational changes to form a more ordered structure that results in an increase in heat-stability (Patel & Bartlett 1988). This may, presumably, be the result of a general rearrangement of ionic groups to form a more ordered protein molecule by ionic binding, which is unlikely to achieve its native conformation (Patel and Bartlett, 1988). However, it has not been elucidated whether it is catalytically active or inactive. This partially contradicts the view of Stepaniak and Fox (1983), who suggested that the enzyme molecules undergo unfolding to a greater extent at temperatures ≥ 55 °C. If the enzyme undergoes unfolding to form an aggregation, there should be an abrupt biphasic curve, but we observed a very gradual and continuous relationship even at UHT conditions (Patel & Bartlett 1988). This stage is characterised by the highest activation energy, higher $\Delta G$, $\Delta S$ and lower $\Delta H$, as observed in the typical denaturation reactions with breaking and forming non-covenant interactions within the enzyme molecule and with solutes that occurs in the surrounding environment during the formation of a transition state (Gouzi et al. 2012). However, some BPs show variability in $\Delta S$, and this possibly indicates the net enzyme and solvent disorder, which occurs due to the presence of the surrounding water molecules and structural effects, which in turn, can be attributed to the conformational changes occurring in the enzyme molecule during reaction (Deylami et al. 2014). The mechanisms involved in thermal inactivation include deamination of asparagine or glutamine residues, succinimide formation from aspartate residues,

oxidation of amino acid side chains, aggregation of proteins and reactions of the disulphide and thiol groups. The possible reason for the negative $\Delta S$ in the present study may be related to the formation of inter-and intramolecular bonds that are prevalent during the aggregation process (Anema & McKenna 1996, Deylami et al. 2014). The positive values of $\Delta H$ indicate that denaturation of BPs is an endothermic reaction (Deylami et al. 2014).

The changes in thermodynamic parameters and secondary structure can be elucidated experimentally using the fourier transform infrared spectroscopy (FTIR) and circular dichroism spectroscopy (CD), as well as intrinsic fluorescence and differential scanning calorimetry (DSC) measurements (Barth 2007, D'Amico et al. 2003, Gouda et al. 2003). The changes in the molecular dimensions of BPs, caused by the thermal unfolding, can be elucidated by size exclusion chromatography on high performance liquid chromatography (HPLC) or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under native, reducing or non-reducing conditions (Gouda et al. 2003, Roy et al. 2012).

Overall, the data suggests that higher temperatures in the range of 145-160 °C can result in total or partial inactivation of BPs. Thus, higher temperatures (i.e. 150 to 180 ºC), which have relatively shorter holding time (i.e. 0.2-0.5 s), and which, use innovative steam injections (ISI), may be more effective for inactivation of BPs (van Asselt et al. 2008). The time-temperature combinations and inactivation kinetics can be utilised for extrapolation of the time required to achieve a similar degree of inactivation using the $F_0$ value (combined effect of time and temperature on the inactivation of BPs), especially in the extreme high temperature region, as occurs in ISI heating (Marchand et al. 2008). Alternatively, raw milk can be inactivated by LTI, but will require prior removal of other milk constituents by ultra-filtration (to control the effect of milk components on the heat-stability of BPs) and re-addition of the milk components into milk after LTI processing to the correct concentrations. This approach will enable concomitant reduction in the activity of BPs, while increasing the nutritional and sensory qualities. However, this approach may require further optimisation prior to adoption by milk processors.

Nonetheless, achieving sufficient inactivation of BPs in milk is extremely difficult without affecting its nutritional quality and flavour (Stoeckel et al. 2016b). Therefore, it is important to establish a more constructive approach instead of trying to inactivate these enzymes using heat treatment. The onset of protease activity was observed in psychrotrophic proteolytic bacteria, when the counts reached ≤ 5 × $10^4$ cfu/mL during the exponential phase, thus controlling their multiplication and metabolism can be achieved by means of more stringent refrigeration conditions (Vithanage et al. 2017). Furthermore, proper hygiene management systems at the farm, storage of the raw milk for a shorter period at the farm, short distribution channels, as well as the use of thermisation prior to the processing, are some factors that can contribute to the reduction of BPs in UHT milk. One alternative can be the production of long shelf-life milk destined for export using high quality raw milk (Stoeckel et al. 2016a). However, this may require the use of rapid and reliable screening methods for BPs or protease-producing bacteria (Machado et al. 2013, Vithanage et al. 2014).

## 8.6 Conclusion

The extracellular proteases of different bacteria showed significantly higher heat-stabilities in both UHT skimmed and full cream milk as compared to SMUF buffer. This highlights the fact that BPs cannot be inactivated using current time-temperature conditions, which are used in most commercial UHT processes. The BPs followed the first order monophasic thermal inactivation with time under all tested conditions, and also followed the Arrhenius equation, except for LTI, which occurred at 55-65 °C in SMUF buffer. Thus, 90% of the respective protease activity can be reduced by heating at 145-160 °C for several min, depending on the type of BPs. However, this may be detrimental to the sensory and nutritional qualities of UHT milk. However, these temperature-time parameters can be utilised for defining a new time-temperature combinations for UHT processing on the basis of the severity of the heat-treatment calculations using $F_0$ values, especially in ISI heating. The presence of milk proteins and fats increases the thermal stability of BPs thus making it difficult to bring about their thermal inactivation in milk. The protective effect of milk proteins and fats also

contributes to this phenomenon. Therefore, this study will contribute to further elucidation of heat-stabilities and establishing novel UHT heating parameters to control BPs in UHT milk. Alternatively, use of other technologies or controlling the growth and metabolism of these problematic bacteria may allow for superior product quality with an extended shelf-life. This will allow for a worldwide distribution within the shelf-life defined by the respective commercial UHT milk processers and bring overall benefit to the UHT milk processing industry.

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# CHAPTER 9: CONCLUSION AND FUTURE DIRECTIONS

## 9.1 Overall Conclusions

Raw milk microbiota is comprised of a variety of Gram-negative and Gram-positive bacterial genera, including *Pseudomonas, Bacillus, Hafnia, Acinetobacter, Microbacterium, Stenotrophomonas*, *Klebsiella, Aeromonas* and *Serratia* that are able to secrete heat-stable extracellular proteases. In the current study, these bacteria were characterised and assigned by multiple identification techniques, including API, Microbact, Biolog, MALDI-TOF MS and 16S rRNA gene sequencing. This polyphasic approach allowed for the accurate classification of these psychrotrophs at the genus, species and even subspecies level, but benefits and limitations were noted for each method.

For example, 16S rRNA gene sequencing is based on the genetic heterogeneity in the ribosomal RNA of prokaryotic 30S subunit and provides accurate species and even subspecies level assignment to almost all the bacteria (n = 55) in the current study, including ATCC cultures (Chapter 3). This suggests that there is greater potential for classification of genetically unique and phenotypically coherent clusters of psychrotrophs, and may be able to assist in tracking their origins at the farm source. However, this method is time-consuming, technically demanding and requires costly materials in terms of instrumentation and sample preparation, prior to the identification. The lowest identification rates for psychrotrophic bacteria were provided by both API and Microbact, which are based on bacterial phenomics. A major limitation is that the Microbact system does not have any identification tests for Gram-positive bacteria. Thus, selecting the type of identification

system to be used requires prior knowledge of Gram stain status. Similarly, both API and Microbact methods are comprised of only a limited number of biochemical reactions, which are insufficient for the characterisation of phenotypically indistinguishable bacteria. For example, if the *Pseudomonas fluorescens* group and *Bacillus cereus s.l.* group comprised a heterogeneous collection of subspecies with significantly similar phenotypic characteristics; they cannot be readily discriminated by these methods. Furthermore, these two systems comprised limited entries in the database for bacteria isolated from food and environmental specimens. Although these two methods are reliable in comparison to the conventional phenotypic characterisation, performing the API and Microbact assays are still time consuming and technically challenging, and may therefore require extensive optimisation to be suitable for dairy microbiology. In contrast, Biolog assigns unknown bacteria based on bacterial metabolism of 96 substrates, which is why, this system can provide higher identification accuracy for Gram-negative isolates and intermediate accuracy for Gram-positive isolates compared to other methods. However, this method still requires knowledge of the Gram reaction for selection of the appropriate system. Additionally, the Biolog method requires costly instrumentation and sample preparation materials, but with lower running time (18-24 h) as compared to API, MB and 16S rRNA gene sequencing. It is therefore better suited for research purposes rather than large-scale sample analysis in the routine dairy microbiology laboratory.

Likewise, MALDI-TOF MS also provided intermediate and accurate identification to the genus and species levels, especially for Gram-positive psychrotrophs. Although MALDI-TOF MS requires expensive instrumentation, its running costs are comparatively low. The method also comprises simple sample preparation protocols that can be performed without specialist technical knowledge. One of the major limitations of MALDI-TOF MS is the limited number of reference spectra of milk spoilage bacteria and environmental isolates in commercial databases. Additionally, the identification of bacteria can be affected by different culture conditions and sample preparation methods used prior to the analysis. Thus, we evaluated the effect of (i) different media formulations that are routinely encountered in the

microbiology laboratory, (ii) incubation time and temperature conditions, and (iii) smear preparation on the accuracy of species-level identification of unknown bacteria (Chapter 4). The results of the study indicated that these pre-analytical factors significantly affected the identification rates of raw milk isolates by MALDI-TOF MS. Furthermore, the consensus mass peak lists of milk spoilage bacteria grown under different conditions obtained in this study and reference spectra, found from FoodBIMS (an on-line library of reference spectra of food microorganisms), were analysed with the freely-available web-based mass spectral clustering tool, SPECLUST. This approach further enhanced the assignment of bacteria to the corresponding species level. The peaks lists generated for all isolates were further analysed by MASCOT to determine the identity of each peak. The majority of peaks were found to be generated by ribosomal proteins that are constitutively expressed, regardless of the media or the culture conditions. Thus, with the help of the typed sequences available in the NCBI and RDP databases, we developed customised ribosomal protein databases containing prokaryotic 30S small and 50S large sub-unit proteins and matched the obtained consensus peptide masses with the ribosomal database. This proteomic-bioinformatics-based approach allowed for a significantly higher assignment of bacteria to the correct species level in the resultant dendogram. These results were further evaluated using 16S rRNA gene sequencing for further clarification, and a comparable clustering pattern was found in the phylogenetic tree. Thus, this approach enabled the elucidation of the subtle differences in the bacterial whole cell proteome between closely related species, which provided a ‘snapshot’ of the significant part of the entire bacterial proteome in relation to the genome. Thus, the MALDI-TOF MS proteomics-bioinformatics approach showed comparable bacterial assignments to 16S rRNA gene sequencing and would be more appropriate for discrimination of psychrotrophic spoilage and pathogenic bacteria in raw milk, especially for extensive sample analysis, due to its cost-cost-effective nature, rapidity, reproducibility and reliability.

The incidence and diversity spoilage and pathogenic microorganisms in raw milk can be affected by various factors, including different refrigeration conditions, prolonged storage and

seasonality. To evaluate these factors, the present study (Chapter 5) involved an investigation of raw milk from three different farms representing high quality, medium quality and poor quality raw milk. The milk was stored at 2 °C, 4 °C, 6 °C, 8 °C, and 10 °C for 10 days, in order to mimic the various refrigeration conditions used in farm bulk tanks, insulated tanks and commercial silos and commercial milk processing. In the present study, we observed a shift of microbial composition from primarily Gram-negative psychrotrophs to Gram-positive psychrotrophs, when the temperature increase was ≥ 6 °C. Also, the microbial composition was affected by the extended storage of raw milk, even under standard refrigeration condition (6 °C), while deep cooling at 2 °C resulted in significant reduction in the raw milk microbiota, compared to other refrigeration conditions. The seasonality was also found to affect the microbial composition, which resulted in a significant rise in the number of Gram-negative psychrotrophs and some Gram-positive populations in winter, while significantly higher mesophilic populations were detected in summer. However, we observed a large number of isolates that could be assigned to the correct taxonomic level by either 16S rRNA gene sequencing or MALDI-TOF MS. Among the isolates, only three genera, namely *Pseudomonas*, *Bacillus* and *Microbacterium* spp., showed higher heat-stable proteolytic potential, while the rest of them showed different heat-stable enzymatic potential with varying combinations and intensities. Therefore, improving the quality of UHT milk and dairy products may require differential processing of raw milk, depending on the microbiota present.

Although profiling of microorganisms is important to understand the diversity and spoilage potential, defining raw milk quality based on each and every microorganism in raw milk is extremely difficult, due to the their distinct enzymatic properties. Thus, it is important to define the raw milk quality, with respect to the food safety based on the overall bacterial counts. Currently, commercial processors use TPC, PBC and TDC as the major quality criteria. Our study (Chapter 6) observed that high quality raw milk, which had the lowest TPC, PBC, TDC and TDPC but intermediate PPrBC, showed higher proteolysis than medium quality raw milk. This highlights that PPrBC is an important quality criteria that

needs to be included in the current raw milk screening methods. Likewise, both medium quality and poor quality raw milk comprise higher TDPC counts and a majority of them are represented by *B. cereus* group microorganisms, which are known to produce heat-stable endospores and toxins that can be harmful upon consumption of UHT milk. Thus, it is important to incorporate TDPC, when screening and assessing the safety of raw milk. Alternatively, these counts can be used to evaluate the specific on-farm technological requirements and when deciding on quality-dependent incentive schemes for raw milk suppliers. An increase in the protease activity and proteolysis was apparent, when the PPrBC reached $5 \times 10^4$ cfu/mL. TDPC representing *B. cereus* can indicate increased risk, when the counts exceed $1 \times 10^4$ cfu/mL, a level, which could be used to predict the storage life of raw milk. However, PPrBC and TDPC counts were significantly lower at 2 °C storage. Likewise, HTST pasteurisation of raw milk, upon arrival at the laboratory, also resulted in the reduction of PPrBC, but not TDPC.

Thus, PPrBC (representing *Pseudomonas* spp.) and TDPC (representing *B. cereus*) were selected for the prediction of the storage life of raw milk. These experimental data, derived from the analysis, were fitted well with primary models such as the modified Gompertz and Baranyi primary models (Chapter 7). The growth kinetic parameters such as maximum specific growth rate and lag phase duration, calculated using the primary models, are fitted well with the secondary model of Belehradek-type equations. Finally, the information derived from primary and secondary models was used for the prediction of storage life of raw milk, using an exponential model. This may avoid the risk of spoilage and food safety implications that can occur in the UHT milk and dairy products, when the raw milk is stored under suboptimal refrigeration conditions.

The extracellular proteases secreted by psychrotrophic proteolytic bacteria exhibited significant higher heat stabilities (Chapter 8), which are difficult to inactivate by the current time-temperature combinations used in the commercial UHT processing. Thus, we determined the inactivation kinetics and associated thermodynamic parameters of selected BPs from different bacterial genera in the range of 55-160 °C, in order to elucidate the

possible heat stability mechanisms. This may help define the new time-temperature combinations for use in commercial UHT processing. The results showed that complete or 90% inactivation of BPs occurred at 145-160 °C after several min. This combination may; however, be detrimental to the nutritional aspects and mouth-feel of UHT milk. Therefore, we suggest that the new time-temperature combinations can be defined in the ISI heating region, where raw milk is heated at 150-180 °C for 0.2-0.5 s. However, this needs to be defined on the basis of the $F_0$ values. In addition, the milk constituents such as proteins and milk fat showed protective effects on the heat stability of BPs under high temperature conditions. However, the BPs showed LTI only in SMUF compared to milk. Thus, LTI can be used as an alternative inactivation strategy along with ultra-filtration to remove the milk constituents prior to the heat-treatment. Overall, this information may contribute to the production of superior quality UHT milk and dairy products, with extended shelf-life facilitating the distribution of those products over wider geographic regions and through new commercial channels, thus benefiting the commercial UHT milk processing industry, as well as providing the necessary nutritional requirements to the consumers using this value-added product

## 9.2 Future Directions

There is an increasing demand for UHT milk, especially in global markets, due to its remarkable characteristics, such as being free from pathogenic microorganisms, ability to be stored under ambient conditions for extended periods and nutritional quality that can be consumed “on-the-go”. Indirectly, UHT milk has other societal benefits linked to increased urbanisation, which include the reduction of greenhouse gas emissions, resulting from the lower utilisation of refrigeration. However, the challenges associated with gelation and other deterioration issues, resulting from BPs that can occur during storage and transportation, are becoming increasingly apparent, especially at relatively high ambient temperatures that can occur during shipping. The impact of BPs on milk and dairy products has been extensively studied and reviewed for more than 40 years. Many of these studies were limited to the BPs

secreted by *Pseudomonas* spp., which become dominant during the refrigerated storage. However, researchers are still struggling to elucidate the mechanism associated with enzyme-linked gelation, as a result of which, controlling strategies have not been established yet to improve the quality of UHT milk. This is likely to have some relation with the remarkable heterogeneity in the BPs in terms of proteolytic potential and heat-stabilities, even within the same enzyme super family. Thus, it is important to understand the proteolytic potential, specificities on milk proteins, the minimal amount of protease that can cause quality defects, and associated gelation properties of a variety of BPs. In addition, it is important to develop rapid and reliable methods that can detect the presence of these proteolytic enzymes in milk and finally, it is also important to establish appropriate control strategies using non-thermal techniques need.