Microbiological quality of raw milk attributable to prolonged refrigeration conditions

This is the Accepted version of the following publication


The publisher’s official version can be found at
Note that access to this version may require subscription.

Downloaded from VU Research Repository  https://vuir.vu.edu.au/33830/
Microbiological Quality of Raw Milk Attributable to Prolonged Refrigeration Conditions

Nuwan R. Vithanage¹,⁵, Muditha Dissanayake¹,⁵, Greg Bolge⁶, Enzo A. Palombo³, Thomas R. Yeager²,⁴,⁵* Nivedita Datta¹,⁴

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

*Corresponding author Tel.: +61 3 9919 8103, Mob: +61 468899823; E-mail address:
thomas.yeager@vu.edu.au

Keywords: Raw milk, Psychrotrophic proteolytic bacteria, Thermoduric psychrotrophs, Diversity, Protease activity, Proteolysis.
Abstract

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 °C, 4 °C, 6 °C, 8 °C, 10 °C 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) and 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 × 10^4 cfu/mL and 1 × 10^4 cfu/mL, and were defined as storage life for quality (SLQ), and storage life for safety (SLs) aspects, respectively. The storage conditions also significantly affect 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 SLQ and SLs of raw milk.
Introduction
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 ºC 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 ºC 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 (Vithanage et al., 2016; Griffiths et al., 1987). 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, 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 ºC and 7 ºC; optimum temperature is in the range of 25-
35 ºC; and maximum temperature can be as high as 45 ºC. In addition, some thermuduric
psychrotrophs are able to withstand temperatures as high as 72-74 ºC (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 pasteurized 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 (Oliveira et al.,
2015; Marchand et al., 2009a). 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 (Haryani et al., 2003; O’Connell et al., 2016; Griffiths
et al., 1987). Changes in storage conditions are also associated with the microbial composition in the
corresponding samples (Hantsis-Zacharov & Halpern, 2007; Lafarge et al., 2004; 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: 2.3 × 10^4 cells/mL) medium quality (B: 5.3 × 10^5
cells/mL) and poor quality (C: 6.7 × 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 ºC) within 2-3 h of the milking procedure. A volume (500
mL) of the samples were 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 days. Those conditions included 2 °C (deep cooling), 4 °C (standard refrigeration) and 6 °C, 8 °C, 10 °C 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, Rita, & Alatossava, 2007) and incubated for 10 days, 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 days (Buehner, Anand, & Garcia, 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 mins (Eppendorf 5415C microfuge, Hamburg, Germany) to remove the milk fat. A volume of (1 mL) 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 in 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, 300A, 250 mm x 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 spectrofluorophotometer (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).

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

(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 ºC 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 ºC, 4 ºC, 6 ºC, 8 ºC, 10 ºC and 12 ºC for 10 days. The enumeration of bacteria and analysis of protease activity and proteolysis was conducted as described before (n = 9).

**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 with different growth rates when stored under different refrigerated conditions (Fig. S1; Fig. 1). 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 ºC storage showed significant inhibition of the PPrBC and TDPC. Storage temperatures of ≥ 4 ºC resulted in significant increases in PPrBC, whereas TDPC showed significant increases in growth rate at ≥ 8 ºC (P < 0.05) (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 ºC (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 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 ºC (P < 0.05) (Fig. 2). Even the standard refrigeration condition (4 ºC) showed significant increase in PA and PL during the extended storage of raw milk (10 days) and this was observed after 6, 8 and 5 days in A, B and C samples, respectively (P < 0.05) (Fig. 2; Fig. 3). In contrast, 2 ºC 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 × 10⁴ cfu/mL at all temperature conditions, except for 2 ºC (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 × 10^4 cfu/mL in A, B and C samples at 4 ºC resulted in
protease activity of 2.8 × 10^3 RFU/mL, 1.0 × 10^2 RFU/mL and 4.0 × 10^4 RFU/mL and those values
were equivalent to 9.3 ng/mL, 3.5 ng/mL 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 %, were 12.1%, 8.4% and 15.1%. In contrast, at 6 ºC with similar PPrBC (ranging
from 5.2-5.4 × 10^4 cfu/mL), the protease activities in the samples were 3.9 × 10^4 RFU mL⁻¹, 2.9 × 10^3
RFU/mL and 5.3 × 10^4 RFU/mL (equivalent to 12.1 ng/mL, 5.4 ng/mL 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 ≥
0.90, P < 0.0001; at ≥ 4 ºC), when PPrBC reached 5.0 × 10^4 cfu/mL (Table S1). This correlation was
in the range of 0.81-0.95 (P < 0.001), when TDPC reached 5.0 × 10^4 cfu/mL at ≥ 8 ºC (Table S2). The
correlation coefficients between PBC and PA and/or PL was significant (r ≥ 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_{LO}) was defined depending on the PPrBC counts, hence time to reach PPrBC of 5.0 × 10^4
cfu/mL was defined as S_{LO} (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 × 10^4 cfu/mL was defined as S_{LS} (Table S3). Both S_{LO} and S_{LS} showed
significant correlation with initial counts ≥ 4 ºC and ≥ 8 ºC storage, respectively (Table S1; S2).

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

Heating of raw milk samples at 75 ºC for 15 s followed by storage at different refrigeration conditions
resulted in a significant reduction of PPrBC (P < 0.05) (Table S3). This consequently decreased the
PA and PL with concomitant increased in the S_{LO} (P < 0.05), especially the temperature conditions ≤ 8
ºC storage (Table S3). In contrast, the S_{LS} showed only slight increase (P > 0.05). The most
significant increase in storage life (both $S_{LO}$ 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 (Table S3).

Discussion

Raw milk collected from three farms showed significantly different initial TPC, PBC, PPrBC and TDPC, possibly related to the different the 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 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 ≥ 5 × 10^4 cfu/mL at ≥ 4 °C, and TDPC ≥ 1 × 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 × 10^4 cfu/mL and TDPC of 1 × 10^4 cfu/mL for shelf life extension and product safety. This is consistent with a PPrBC count of 4.5 × 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 food safety concern when it reaches 1.0 × 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 (O’Connell et al., 2016; Haryani et al., 2003; Griffiths et al., 1987). 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 ≥ 5 × 10^4 cfu/mL with elevated PA and PL within 4-7 days at 6 °C storage. The TDPC with similar initial counts can increased to ≥ 1 × 10^4 cfu/mL within 5-9 days at 8 °C. At 4 °C, the PPrBC counts reached the corresponding levels within 5-8 days storage and less than 2 days 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 (Ma et al., 2014; Vine et al., 2004). The fluctuation in the microbial counts also accompanied by 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 belong to *B. cereus* group was 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 and 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; Machado et al., 2013; Marchand et al., 2009b; Dufour et al., 2008). 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 (Griffiths et al., 1987; Stadhouders, 1982). 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, however resulted in significant decrease in protease activity. This in turn showed significantly higher $S_{LO}$, 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 (Vithanage et al., 2016; Anzueto, 2014). 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 good 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.

Acknowledgments

The authors wish to acknowledge financial assistance from Victoria University and Murray Goulburn Co-operative Co. Limited. We are also grateful to the staff of the Murray Goulburn Co-operative Co. Limited for their help organizing the sampling campaign and to the sampled dairy farms for their hospitality. Professor Todor Vasiljevic, Dr. Jayani Chandrapala are gratefully acknowledged for their constructive suggestions during the reviewing process. The assistance of Mr. Anuja Manchanayake and Mr. Jamith Jayasekara for manuscript preparation is gratefully acknowledged.

References


Cupp-Enyard C 2009 Use of the Protease Fluorescent Detection Kit to Determine Protease Activity. Journal of Visualized Experiments 30(1) 1514-1521.

Datta N & Deeth HC 2003 Diagnosing the cause of proteolysis in UHT milk. LWT - Food Science and Technology 36(2) 173-182.


characterization of an extracellular protease produced by one of them. International Journal of Food Microbiology 125(2) 188-196.


Silveira IA, Carvalho EP, Teixeira D & Barrios BE 1999 Verification of the proteolytic and lipolytic activities of the microbial flora isolated from raw, refrigerated, type B milk. II. Psychrotrophic microorganisms. Rev Latinoam Microbiology 41(2) 85-89.


Vine NG, Leukes WD & Kaiser H 2004 In vitro growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus. FEMS Microbiology Letters 231(1) 145-152.


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.

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.

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 ºC, 4 ºC, 6 ºC, 8 ºC, 10 ºC and 12 ºC storage. The results were presented as mean ± SE, (n = 9).

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 ºC, 4 ºC, 6 ºC, 8 ºC, 10 ºC and 12 ºC storage. The results were presented as mean ± SE, (n = 9).

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 ºC, in 0 day and after 6, 8 and 5 days (when significant increase in proteolysis occurred), respectively.
### Table 1

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>% of isolates</th>
<th>2 °C</th>
<th>4 °C</th>
<th>6 °C</th>
<th>8 °C</th>
<th>10 °C</th>
<th>12 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pseudomonadaceae</strong>§</td>
<td>87.3</td>
<td>80.9</td>
<td>76.6</td>
<td>69.5</td>
<td>52.2</td>
<td>39.2</td>
<td></td>
</tr>
<tr>
<td>GPB¥</td>
<td>8.7</td>
<td>9.4</td>
<td>9.6</td>
<td>13.5</td>
<td>25.2</td>
<td>30.3</td>
<td></td>
</tr>
<tr>
<td><strong>Enterobacteriaceae</strong>£</td>
<td>3.1</td>
<td>5.8</td>
<td>6.1</td>
<td>7.3</td>
<td>9.8</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous NF-GNB*</td>
<td>0.9</td>
<td>1</td>
<td>3.4</td>
<td>4.2</td>
<td>6.4</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>GPC‡</td>
<td>0</td>
<td>0.8</td>
<td>2.3</td>
<td>3.2</td>
<td>5.2</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Un-identified</td>
<td>0</td>
<td>2.1</td>
<td>2</td>
<td>2.3</td>
<td>1.2</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

*NF-GNB: Non-Fermenting Gram Negative Bacilli with 75% of Acinetobacter and Stenotrophomonas spp.*

£Approximately 76% of the isolates from family Enterobacteriaceae were belong to *Hafnia* and *Serratia*.

§Approximately 85% of this genera was belong to *P. fluorescens*.

*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.
Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Storage Temperature (°C)</th>
<th>Time (days)</th>
<th>PPrBC (log cfu/mL)</th>
<th>TDPC (log cfu/mL)</th>
<th>Protease activity (RFU/mL)</th>
<th>Protease concentration (ng/mL)</th>
<th>DH (proteolysis) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>9, &gt;10†</td>
<td>4.68</td>
<td>2.87</td>
<td>1.2×10^2‡</td>
<td>5.0‡</td>
<td>3.4‡</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6, &gt;10†</td>
<td>4.67</td>
<td>2.97</td>
<td>2.8×10^3§§</td>
<td>9.3§</td>
<td>12.1‡</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5, 8†</td>
<td>4.69</td>
<td>4.06</td>
<td>3.9×10^4§§</td>
<td>12.1§</td>
<td>18.2‡</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4, 5†</td>
<td>4.70</td>
<td>4.01</td>
<td>4.4×10^4§</td>
<td>13.3‘</td>
<td>35.2‘</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2, †</td>
<td>4.71</td>
<td>4.02</td>
<td>5.0×10^4*</td>
<td>15.1’</td>
<td>48.5‘</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1, †</td>
<td>4.73</td>
<td>4.01</td>
<td>4.3×10^5*</td>
<td>15.9’</td>
<td>52.3’</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>10, &gt;10†</td>
<td>4.69</td>
<td>3.05</td>
<td>9.8×10^1‡</td>
<td>2.4‡</td>
<td>2.5‡</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8, &gt;10†</td>
<td>4.69</td>
<td>3.32</td>
<td>1.0×10^2‡</td>
<td>3.5‡</td>
<td>8.4‡</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6, 7†</td>
<td>4.69</td>
<td>4.06</td>
<td>2.9×10^3§</td>
<td>5.4‡</td>
<td>10.4‡</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4, †</td>
<td>4.68</td>
<td>4.06</td>
<td>3.4×10^4§</td>
<td>10.6§</td>
<td>23.3§</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2, †</td>
<td>4.67</td>
<td>4.07</td>
<td>3.4×10^4*</td>
<td>11.7’</td>
<td>37.1’</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1, †</td>
<td>4.73</td>
<td>4.08</td>
<td>3.8×10^4*</td>
<td>12.9’</td>
<td>42.2’</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>8, 10†</td>
<td>4.69</td>
<td>4.02</td>
<td>2.8×10^3§</td>
<td>9.3§</td>
<td>5.8‡</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5, 9†</td>
<td>4.68</td>
<td>4.06</td>
<td>4.0×10^4§</td>
<td>11.9§</td>
<td>15.1§</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4, 5†</td>
<td>4.69</td>
<td>4.05</td>
<td>5.3×10^4*</td>
<td>13.2’</td>
<td>21.3’</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3, †</td>
<td>4.70</td>
<td>4.07</td>
<td>5.5×10^4*</td>
<td>15.6’</td>
<td>45.2’</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2, †</td>
<td>4.71</td>
<td>4.05</td>
<td>5.5×10^4*</td>
<td>17.1’</td>
<td>53.5’</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1, †</td>
<td>4.67</td>
<td>4.06</td>
<td>6.2×10^5*</td>
<td>18.7’</td>
<td>58.2’</td>
</tr>
</tbody>
</table>

*§‡Means significance levels by MANOVA (SPSS Windows Ver 21) * P < 0.001; § P < 0.05; ‡ P > 0.05.
PPrBC: Psychrotrophic proteolytic count; TDPC: Thermoduric psychrotrophic count
† Time to PPrBC of 5×10^4 cfu/mL; † time to reach TDPC of 1×10^4 cfu/mL
§ Protease activity determined by relative fluorescence units; ‡ Protease concentration determined by standard curve of Thermolysin (EC 3.4.24.27)
* DH Degree of hydrolysis, which denotes the extent of proteolysis that was determined using OPA-method.
Multiple samples were analysed with SD ±1.5 (n = 9)
Fig. 1

(A) Psychrotrophic proteolytic count (PPrBC)

(B) Thermoduric psychrotrophic count (TDPC)

(C) Psychrotrophic proteolytic count (PPrBC)

(D) Thermoduric psychrotrophic count (TDPC)
Fig. 2

Protease Activity

Proteolysis: Degree of Hydrolysis (%)

(A)

(B)

(C)
Fig. 3

[Graph showing data with labels A, B, and C.]

Minutes
Caption of Supplementary Tables

Table S1
Relationship between the psychrotrophic proteolytic count (PPrBC) with protease activity (PA), proteolysis (PL) and storage life in the aspect of quality (SLQ) of raw milk stored under different conditions at the end of the storage life.

Table S2
Relationship between the thermoduric psychrotrophic count (TDPC) with protease activity (PA), proteolysis (PL) and storage life in the aspect of safety (SLs) of raw milk stored under different conditions at the end of the storage life.

Table S3
The effect of refrigerated storage and combined high temperature short time (HTST) pasteurisation and refrigerated storage on storage life/shelf life of raw milk stored under different conditions.

Caption of Supplementary Figures

Fig. S1
Effect of different storage conditions on the total plate counts (TPC) and psychrotrophic bacterial counts (PBC) 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, \( n = 9 \).
Multiple samples were analysed with SD ±1.5 (n = 9).

¥Degree of hydrolysis, which denotes the extent of proteolysis that was determined using OPA-method.

¤Protease activity determined by relative fluorescence units/mL.

†
CC: Correlation coefficient; TDPC: Thermoduric psychrotrophic count; PA: protease activity; PL: proteolysis.

*,‡,§Means significance levels by MANOVA (SPSS Windows Ver 21) * P < 0.001; † P < 0.05; ‡ P > 0.05.

Table S1

<table>
<thead>
<tr>
<th>Storage Temperature (°C)</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (r) (PPrBC × PA)</td>
<td>CC (r) (PPrBC × PL)</td>
<td>CC (r) (PPrBC × PA)</td>
</tr>
<tr>
<td>2</td>
<td>0.65‡</td>
<td>0.67‡</td>
<td>0.72†</td>
</tr>
<tr>
<td>4*</td>
<td>0.98*</td>
<td>0.97*</td>
<td>0.90*</td>
</tr>
<tr>
<td>6</td>
<td>0.99‡</td>
<td>0.98‡</td>
<td>0.95‡</td>
</tr>
<tr>
<td>8</td>
<td>0.97*</td>
<td>0.98*</td>
<td>0.95*</td>
</tr>
<tr>
<td>10</td>
<td>0.95‡</td>
<td>0.93‡</td>
<td>0.90‡</td>
</tr>
<tr>
<td>12</td>
<td>0.96‡</td>
<td>0.95‡</td>
<td>0.94‡</td>
</tr>
</tbody>
</table>

After 6, 8 and 5 days of storage of A, B and C samples.

Correlation coefficient; PPrBC: Psychrotrophic proteolytic count; PA: protease activity; PL: proteolysis.

*) After 6, 8 and 5 days of storage of A, B and C samples.

Table S2

<table>
<thead>
<tr>
<th>Storage Temperature (°C)</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (r) (TDPC × PA)</td>
<td>CC (r) (TDPC × PL)</td>
<td>CC (r) (TDPC × PA)</td>
</tr>
<tr>
<td>2</td>
<td>0.35‡</td>
<td>0.42‡</td>
<td>0.43‡</td>
</tr>
<tr>
<td>4</td>
<td>0.53‡</td>
<td>0.52‡</td>
<td>0.46‡</td>
</tr>
<tr>
<td>6</td>
<td>0.68‡</td>
<td>0.62‡</td>
<td>0.60‡</td>
</tr>
<tr>
<td>8</td>
<td>0.81‡</td>
<td>0.82‡</td>
<td>0.80‡</td>
</tr>
<tr>
<td>10</td>
<td>0.87*</td>
<td>0.86*</td>
<td>0.85*</td>
</tr>
<tr>
<td>12</td>
<td>0.90*</td>
<td>0.91*</td>
<td>0.90*</td>
</tr>
</tbody>
</table>

After 6, 8 and 5 days of storage of A, B and C samples.

Correlation coefficient; TDPC: Thermoduric psychrotrophic count; PA: protease activity; PL: proteolysis.

*) After 6, 8 and 5 days of storage of A, B and C samples.

Multiple samples were analysed with SD ±1.5 (n = 9)
Table S3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Storage Temperature (°C)</th>
<th>Observed SLQ£</th>
<th>Observed SLS†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before HTST (days)</td>
<td>After HTST¥</td>
<td>Before HTST (days)</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>9*</td>
<td>&gt;10*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6§</td>
<td>&gt;10*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5§</td>
<td>8*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4‡</td>
<td>5§</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2‡</td>
<td>4‡</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1‡</td>
<td>2‡</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>10*</td>
<td>&gt;10*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8*</td>
<td>&gt;10*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6§</td>
<td>7*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4§</td>
<td>8*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2‡</td>
<td>5§</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1‡</td>
<td>4‡</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>8§</td>
<td>10*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5§</td>
<td>7§</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4‡</td>
<td>6§</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3‡</td>
<td>4‡</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2‡</td>
<td>3‡</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1‡</td>
<td>2‡</td>
</tr>
</tbody>
</table>

*‡§Means significance levels by MANOVA (SPSS Windows Ver 21) * P < 0.001; § P < 0.05; ‡ P > 0.05.
£SLQ: Storage life in quality aspect: time to reach psychrotrophic proteolytic count (PPrBC) of 5 × 10^4 cfu/mL.
†SLS: Storage life in safety aspect: time to reach thermoduric psychrotrophic count (TDPC) of 1 × 10^4 cfu/mL.
¥HTST: High temperature short time pasteurisation: 75 ± 0.5 °C for 15 s heat-treatment.

Multiples samples were analysed with SD ±2.1 (n = 9).
Fig. S1

(A) Total Plate Count (TPC) vs Storage time (Days) for different samples.

(B) Psychrotrophic bacterial count (PBC) vs Storage time (Days) for different samples.

(C) TPC and PBC vs Storage time (Days) for different samples.