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Microbiological Quality of Raw Milk Attributable to Prolonged Refrigeration Conditions

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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 ºC, 4 ºC, 6 ºC, 8 ºC, 10 ºC and 12 ºC 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 ºC 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 ºC. Similar trends in BC, PA and PL were observed during the extended storage of raw milk at 4 ºC. Both PA and PL showed strong correlation with the psychrotrophic proteolytic count (PPrBC: at ≥ 4 ºC) and thermoduric psychrotrophic count (TDPC: at ≥ 8 ºC) 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 (S_{LQ}), and storage life for safety (S_{LS}) 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 ºC) and combination of heating and refrigeration (≤ 4 ºC) significantly extended the S_{LQ} and S_{LS} 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 thermoduric 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 ºC 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 ºC 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 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 Δ 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, ε 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 × 10^4 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_{LQ}) was defined depending on the PPrBC counts, hence time to reach PPrBC of 5.0 × 10^4 cfu/mL was defined as S_{LQ} (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_{LQ} 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 °C 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_{LQ} ($P < 0.05$), 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_{LQ} and S_{LS}) was observed when raw milk was stored at 2 ºC, while storage life was significantly reduced when it was stored at ≥ 8 ºC (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 ºC) and TDPC (≥ 8 ºC) 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 ºC, and TDPC ≥ 1×10^4 cfu/mL at ≥ 8 ºC 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 ºC storage. The TDPC with similar initial counts can increased to ≥ 1×10^4 cfu/mL within 5-9 days at 8 ºC. At 4 ºC, the PPrBC counts reached the corresponding levels within 5-8 days storage and less than 2 days of storage at ≥ 8 ºC. Thus, 2 ºC is highly recommended as a storage temperature, while temperatures below 6 ºC can be recommended

288 for the purpose of pre-processing storage of raw milk, depending on the initial bacterial counts and
289 the duration of storage.
290 Interestingly, some of the growth curves of bacteria exhibited a double-sigmoidal shape at ≥ 8 ˚C. It
291 can be speculated that an increasing growth rate and production of antimicrobial metabolites under
292 elevated temperature conditions may result in antagonistic effects within the mixed microbial
293 population (Ma *et al.*, 2014; Vine *et al.*, 2004). The fluctuation in the microbial counts also
294 accompanied by slight fluctuation in the protease activity and proteolysis. This is possibly related to
295 the balance between production and utilisation of small peptides by indigenous microbiota or due to
296 the presence of artefacts especially in FITC method (Haryani *et al.*, 2003).
297 The extended storage of raw milk under various refrigeration conditions resulted in significant diversity
298 in the raw milk microbiota. For example, storage temperatures below 4 ºC resulted in an increase in
299 the level of *Pseudomonas* spp. and some *Bacillus* spp. with simultaneous reduction in the enteric and
300 miscellaneous NF-GNB isolates. However, the counts of isolates that belong to family *Bacillaceae*
301 and *Enterobacteriaceae* were significantly increased above 8 ºC storage. Among the thermoduric
302 psychrotrophic isolates, species belong to *B. cereus* group was predominantly isolated especially ≥ 8
303 ºC. *B. cereus* is known to produce emetic type toxin under refrigeration conditions that can cause
304 public health concerns when the isolates reach 1×10^3 cfu/mL (Christiansson *et al.*, 1989). Most
305 importantly, the spores produced by these isolates are able to withstand pasteurisation and UHT
306 processing (Champagne *et al.*, 1994). According to FSANZ guidelines, the counts of *P. fluorescens*
307 and *B. cereus* in premium quality raw milk are required to be maintained below 10^7 cfu/mL and 10^5
308 cfu/mL, respectively (FSANZ, 2014). These two genera are considered as the major cause of concern
309 in commercial milk processing. Additionally, the diversity of raw milk microbiota can be affected by
310 seasonal differences, for example, psychrotolerant PPrBC, PBC and TDPC appear to increase during
311 the winter months, while thermoduric counts representing mesophilic bacteria were at their highest
312 during the summer months (Marchand *et al.*, 2009a; Vithanage *et al.*, 2016).
313 In the present study, sample B showed significantly lower protease activity and proteolysis. This can
314 be related to the diversity of psychrotolerant bacteria in the respective sample. Previously, we
315 observed that sample B comprised psychrotrophic isolates with limited proteolytic potential (Vithanage
316 *et al*., 2016). Dogan and Boor (2003) also observed variation in the proteolytic potential even within
317 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_{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 (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 ºC 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

348 milk can be achieved by thermisation at 75 ºC for 15 s (instead of 65 ºC) followed by 2 ºC storage.
349 However, profiling of individual species with higher spoilage potential using rapid and reliable
350 screening would be more informative and will be the focus of future studies. This would allow for the
351 production of superior quality dairy products with extended shelf life that can be distributed to wider
352 geographical regions, benefitting commercial milk processing.

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Caption of Tables

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 5×10^4 cfu/mL and TDPC reach 1×10^4 cfu/mL under different storage conditions.

Caption of Figures

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.

564
565
566 **Table 1**
567

Microorganisms	**% of isolates**					
(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

568 *NF-GNB: Non-Fermenting Gram Negative Bacilli with 75% of *Acinetobacter* and *Stenotrophomonas* spp.
569 [£]Approximately 76% of the isolates from family *Enterobacteriaceae* were belong to *Hafnia* and *Serratia*.
570 [§]85% of this genera was belong to *P. fluorescens.*
571 [¥]GPB: Gram positive Bacilli; 80% of the GPB was belong to *B. cereus* and *M. lacticum.*
572 [‡]GPC: Gram Positive Cocci mainly *Streptococci* and *Staphylococci* spp.

Table 2

Sample	Storage Temperature (ºC)	Time[€,†] (days)	PPrBC (log cfu/mL)	TDPC (log cfu/mL)	Protease activity (RFU/mL[¤])	Protease concentration (ng/mL[Ø])	DH[¥] (proteolysis) (%)
A	2	9[€], >10[†]	4.68	2.87	$1.2\times10^{2,‡}$	5.0[‡]	3.4[‡]
	4	6[€], >10[†]	4.67	2.97	$2.8\times10^{3,§}$	9.3[§]	12.1[‡]
	6	5[€], 8[†]	4.69	4.06	$3.9\times10^{4,§}$	12.1[§]	18.2[§]
	8	4[€], 5[†]	4.70	4.01	$4.4\times10^{4,*}$	13.3[*]	35.2[*]
	10	2[€, †]	4.71	4.02	$5.0\times10^{4,*}$	15.1[*]	48.5[*]
	12	1[€, †]	4.73	4.01	$4.3\times10^{5,*}$	15.9[*]	52.3[*]
B	2	10[€], >10[†]	4.69	3.05	$9.8\times10^{1,‡}$	2.4[‡]	2.5[‡]
	4	8[€], >10[†]	4.69	3.32	$1.0\times10^{2,‡}$	3.5[‡]	8.4[‡]
	6	6[€], 7[†]	4.69	4.06	$2.9\times10^{3,§}$	5.4[‡]	10.4[‡]
	8	4[€, †]	4.68	4.06	$3.4\times10^{4,§}$	10.6[§]	23.3[§]
	10	2[€, †]	4.67	4.07	$3.4\times10^{4,*}$	11.7[*]	37.1[*]
	12	1[€]	4.73	4.08	$3.8\times10^{4,*}$	12.9[*]	42.2[*]
C	2	8[€], 10[†]	4.69	4.02	$2.8\times10^{3,§}$	9.3[§]	5.8[‡]
	4	5[€], 9[†]	4.68	4.06	$4.0\times10^{4,§}$	11.9[§]	15.1[§]
	6	4[€], 5[†]	4.69	4.05	$5.3\times10^{4,*}$	13.2[*]	21.3[*]
	8	3[€, †]	4.70	4.07	$5.5\times10^{4,*}$	15.6[*]	45.2[*]
	10	2[€, †]	4.71	4.05	$5.5\times10^{5,*}$	17.1[*]	53.5[*]
	12	1[€, †]	4.67	4.06	$6.2\times10^{5,*}$	18.7[*]	58.2[*]

[*,§,‡]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; [Ø]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).

595 **Fig. 1**
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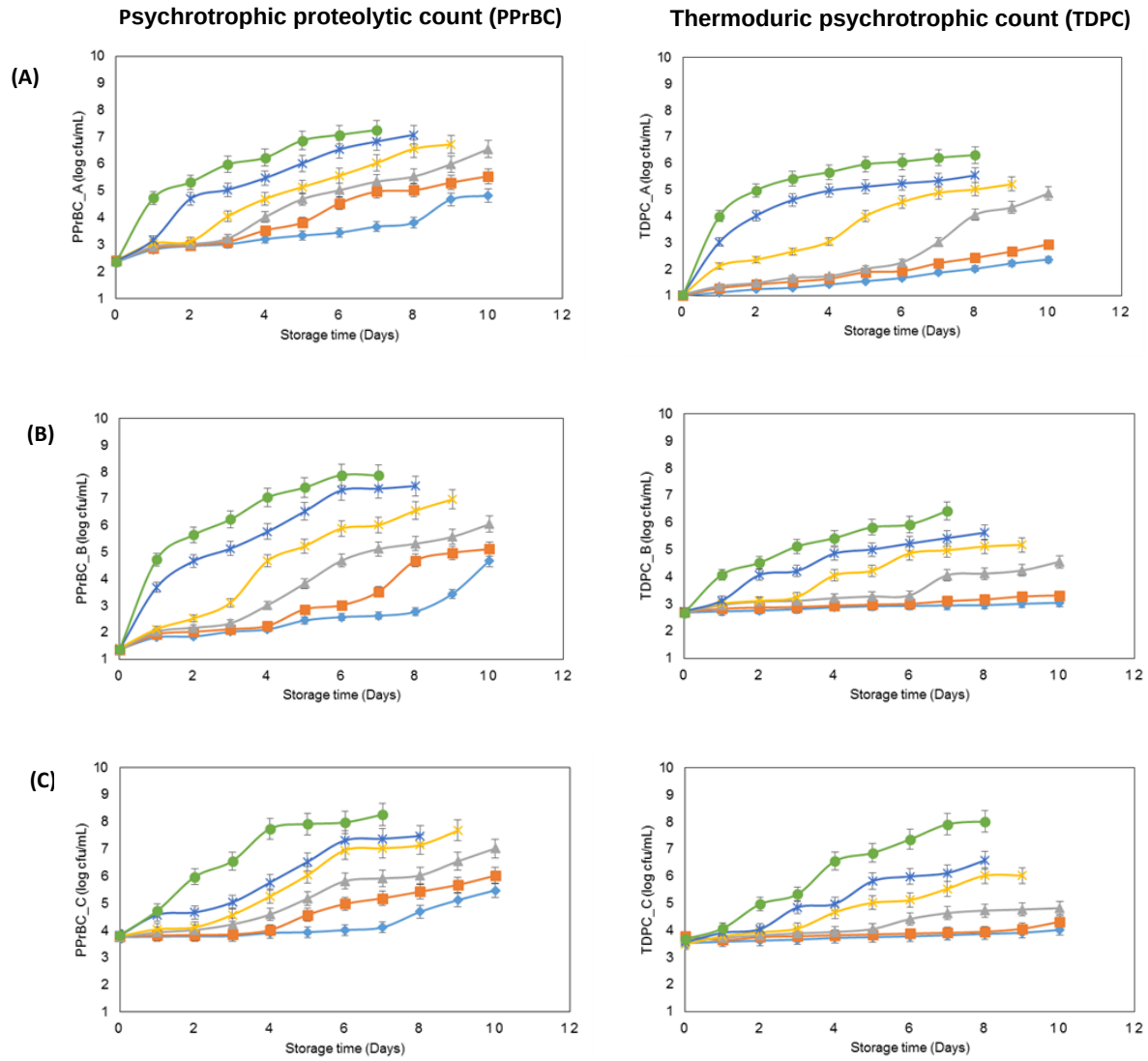

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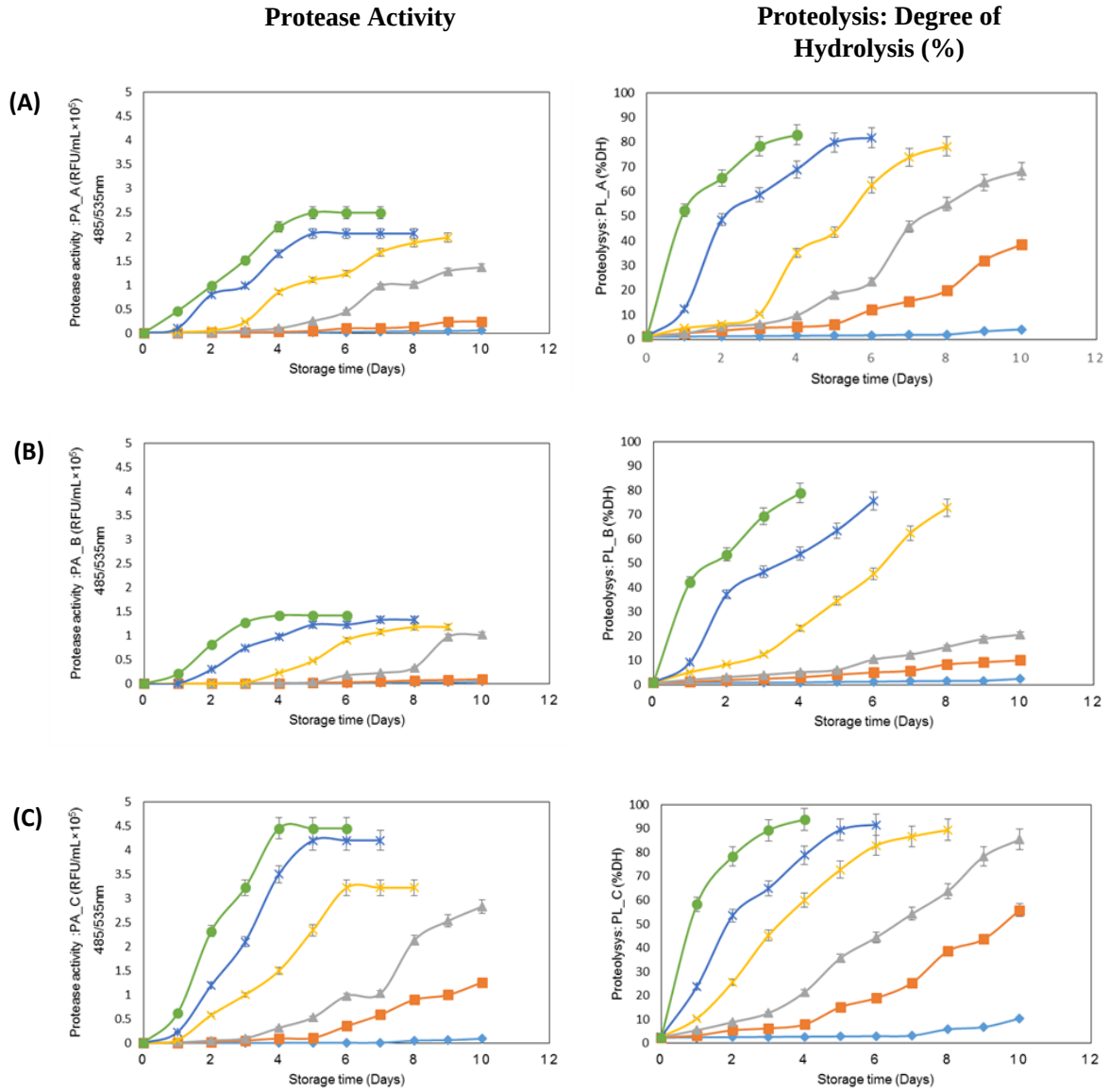
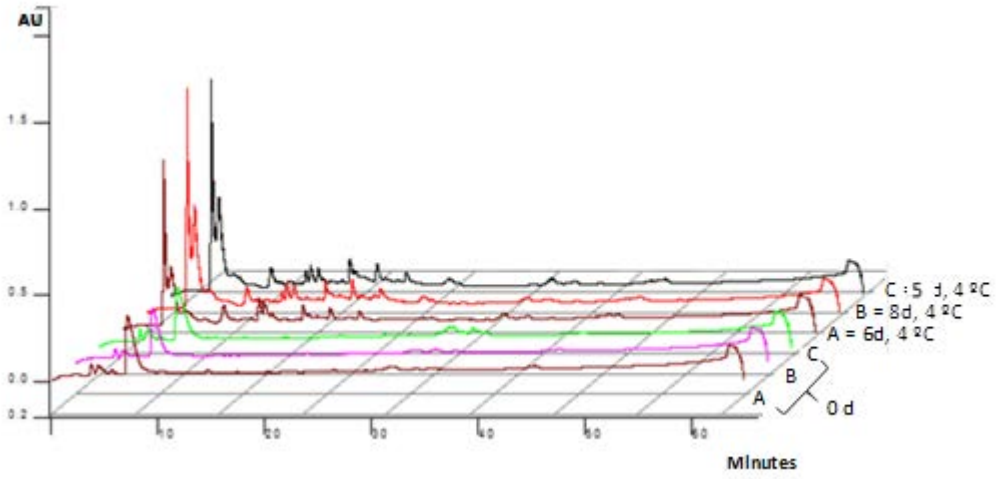
Fig. 2


Protease Activity
Proteolysis: Degree of Hydrolysis (%)
(A)
(B)
(C)
Protease activity :PA_A (RFU/mL×10⁶) 485/535nm
Protease activity :PA_B (RFU/mL×10⁶) 485/535nm
Protease activity :PA_C (RFU/mL×10⁶) 485/535nm
Proteolysys: PL_A (%DH)
Proteolysys: PL_B (%DH)
Proteolysys: PL_C (%DH)
Storage time (Days)

622 **Fig.3**
623



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

Table S1

Storage Temperature (ºC)	Sample A			Sample B			Sample C		
	CC (r) (PPrBC × PA[¤])	CC (r) (PPrBC × PL[¥])	CC (r) (initial PPrBC × S_{LQ}[£])	CC (r) (PPrBC × PA[¤])	CC (r) (PPrBC × PL[¥])	CC (r) (initial PPrBC × S_{LQ}[£])	CC (r) (PPrBC × PA[¤])	CC (r) (PPrBC × PL[¥])	CC (r) (initial PPrBC × S_{LQ}[£])
2	0.65[‡]	0.67[‡]	0.72[‡]	0.65[‡]	0.58[‡]	0.67[‡]	0.72[‡]	0.78[‡]	0.76[‡]
4[œ]	0.98[*]	0.97[*]	0.90[*]	0.83[‡]	0.81[‡]	0.87[§]	0.96[*]	0.94[*]	0.91[*]
6	0.99[*]	0.98[*]	0.95[*]	0.89[§]	0.86[§]	0.89[§]	0.99[*]	0.98[*]	0.92[*]
8	0.97[*]	0.98[*]	0.95[*]	0.91[*]	0.94[*]	0.93[*]	0.92[*]	0.96[*]	0.98[*]
10	0.95[*]	0.93[*]	0.90[*]	0.93[*]	0.92[*]	0.91[*]	0.98[*]	0.98[*]	0.96[*]
12	0.96[*]	0.95[*]	0.94[*]	0.96[*]	0.92[*]	0.93[*]	0.98[*]	0.98[*]	0.97[*]

[*,‡,§]Means significance levels by MANOVA (SPSS Windows Ver 21) [*] $P < 0.001$; [§] $P < 0.05$; [‡] $P > 0.05$.
CC: Correlation coefficient; PPrBC: Psychrotrophic proteolytic count; PA: protease activity; PL: proteolysis.
[£]S_{LQ}; Storage life in quality aspect: time to reach PPrBC of 5×10^4 cfu/mL.
[œ] After 6,8 and 5 days of storage of A, B and C samples.
[¤]Protease activity determined by relative fluorescence units/mL.
[¥]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).

Table S2

Storage Temperature (ºC)	Sample A			Sample B			Sample C		
	CC (r) (TDPC × PA[¤])	CC (r) (TDPC × PL[¥])	CC (r) (initial TDPC× S_{LS}[†])	CC (r) (TDPC × PA[¤])	CC (r) (TDPC × PL[¥])	CC (r) (initial TDPC× S_{LS}[†])	CC (r) (TDPC × PA[¤])	CC (r) (TDPC × PL[¥])	CC (r) (initial TDPC × S_{LS}[†])
2	0.35[‡]	0.42[‡]	0.43[‡]	0.32[‡]	0.38[‡]	0.47[‡]	0.52[‡]	0.51[‡]	0.50[‡]
4	0.53[‡]	0.52[‡]	0.46[‡]	0.54[‡]	0.56[‡]	0.52[‡]	0.56[‡]	0.54[‡]	0.53[‡]
6	0.68[‡]	0.62[‡]	0.60[‡]	0.65[‡]	0.66[‡]	0.63[‡]	0.75[‡]	0.72[‡]	0.70[‡]
8	0.81[§]	0.82[§]	0.80[§]	0.84[§]	0.83[§]	0.81[§]	0.88[*]	0.91[*]	0.93[*]
10	0.87[*]	0.86[*]	0.85[*]	0.90[*]	0.89[*]	0.88[*]	0.93[*]	0.92[*]	0.90[*]
12	0.90[*]	0.91[*]	0.90[*]	0.94[*]	0.93[*]	0.92[*]	0.95[*]	0.94[*]	0.93[*]

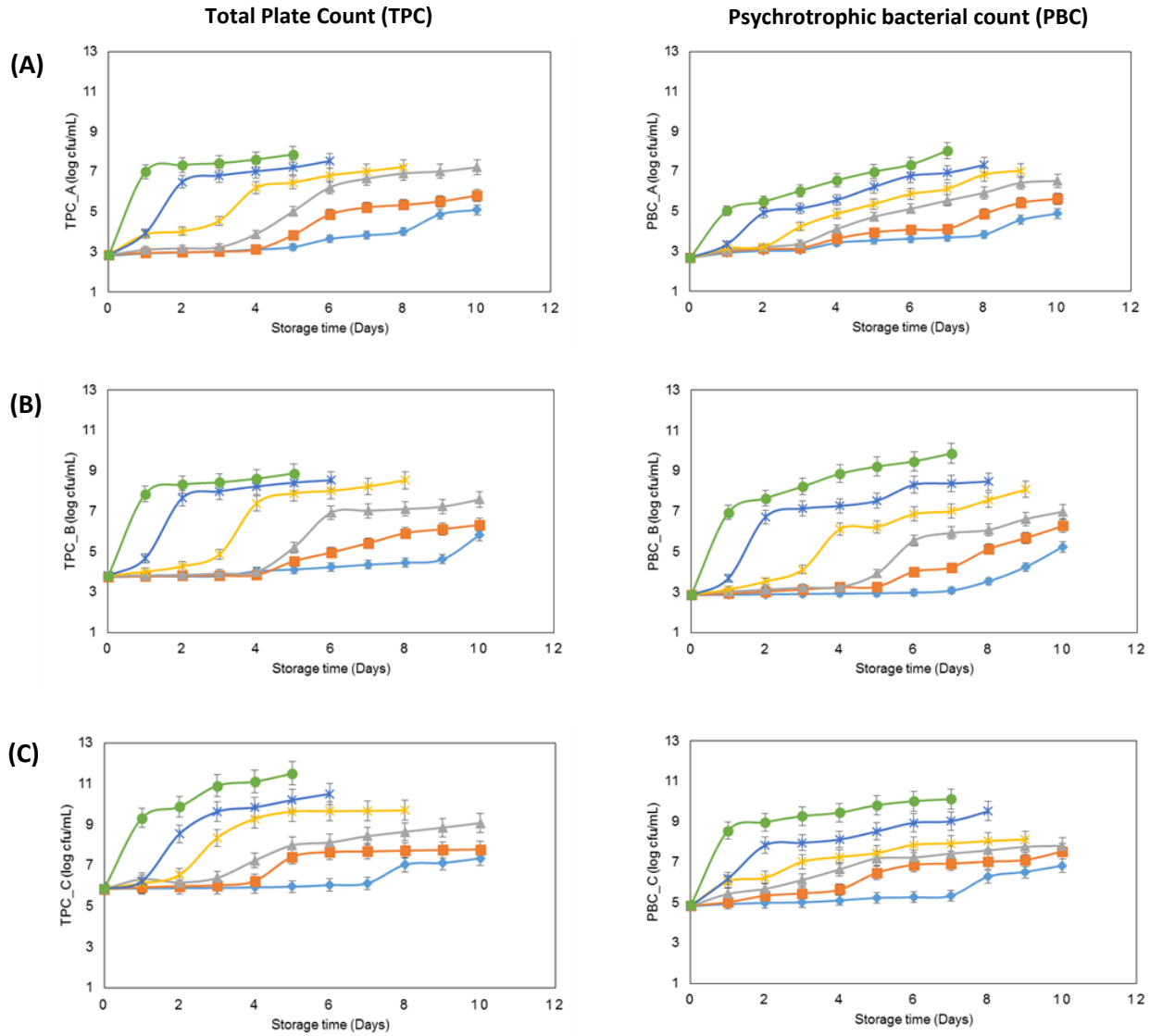
[*,‡,§]Means significance levels by MANOVA (SPSS Windows Ver 21) [*] $P < 0.001$; [§] $P < 0.05$; [‡] $P > 0.05$.
CC: Correlation coefficient; TDPC: Thermoduric psychrotrophic count; PA: protease activity; PL: proteolysis.
[†]S_{LS}; Storage life in safety aspect: time to reach TDPC of 1×10^4 cfu/mL.
[¤]Protease activity determined by relative fluorescence units/mL.
[¥]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).

Table S3

Sample	Storage Temperature (ºC)	Observed S_{LQ}[£]		Observed S_{LS}[†]	
		Before HTST (days)	After HTST[¥] (days)	Before HTST (days)	After HTST[¥] (days)
A	2	9*	>10*	>10*	>10*
	4	6[§]	>10*	>10*	10*
	6	5[§]	9*	8*	8*
	8	4[‡]	5[§]	5[§]	6[§]
	10	2[‡]	4[‡]	2[‡]	5[‡]
	12	1[‡]	2[‡]	1[‡]	3[‡]
B	2	10*	>10*	>10*	>10*
	4	8*	>10*	>10*	>10*
	6	6[§]	>10*	7*	>10*
	8	4[§]	8*	4*	8*
	10	2[‡]	5[§]	2[§]	6[§]
	12	1[‡]	4[‡]	1[‡]	3[‡]
C	2	8[§]	10*	10*	>10*
	4	5[§]	7[§]	9[§]	9[§]
	6	4[‡]	6[§]	7[§]	6[§]
	8	3[‡]	4[‡]	5[‡]	5[‡]
	10	2[‡]	3[‡]	3[‡]	3[‡]
	12	1[‡]	2[‡]	2[‡]	3[‡]

*,§,‡Means significance levels by MANOVA (SPSS Windows Ver 21) * $P < 0.001$; § $P < 0.05$; ‡ $P > 0.05$.
£S_{LQ}; Storage life in quality aspect: time to reach psychrotrophic proteolytic count (PPrBC) of 5×10^4 cfu/mL.
†S_{Ls}; 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 ºC for 15 s heat-treatment.
Multiples samples were analysed with SD ±2.1 (n = 9).

Fig. S1


Total Plate Count (TPC)
Psychrotrophic bacterial count (PBC)
(A)
(B)
(C)
TPC_A (log cfu/mL)
PBC_A (log cfu/mL)
TPC_B (log cfu/mL)
PBC_B (log cfu/mL)
TPC_C (log cfu/mL)
PBC_C (log cfu/mL)
Storage time (Days)