1 **Microbiological Quality of Raw Milk Attributable to Prolonged Refrigeration**

2 **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 ℃, 4 ℃, 6 ℃, 8 ℃, 10 ℃ and 12 ℃ 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 ℃ 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 ℃. Similar trends in BC, PA and PL were observed during the extended storage of raw milk at 4 ℃. Both PA and PL showed strong correlation with the psychrotrophic proteolytic count (PPrBC: at ≥ 4 ℃) and thermoduric psychrotrophic count (TDPC: at ≥ 8 ℃) 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 ℃) and combination of heating and refrigeration (≤ 4 ℃) 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 ⁰C 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 ⁰C 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 ⁰C and 7 ⁰C; optimum temperature is in the range of 25-35 ⁰C; and maximum temperature can be as high as 45 ⁰C. In addition, some thermoduric psychrotrophs are able to withstand temperatures as high as 72-74 ⁰C (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

79 higher protease expression can produce this level of protease activity within a few hours under

80 suboptimal storage conditions (Renner, 1988).

81 The relationship between psychrotrophs and milk quality has been widely investigated (Oliveira *et al.*,

82 2015; Marchand *et al.*, 2009a). To date, limited evidence has been found associating the effect of

83 storage conditions with the growth of psychrotrophic bacteria, their proteolytic potential and

84 deterioration of milk proteins due to proteolysis (Haryani *et al.*, 2003; O'Connell *et al.*, 2016; Griffiths

85 *et al.*, 1987). Changes in storage conditions are also associated with the microbial composition in the

86 corresponding samples (Hantsis-Zacharov & Halpern, 2007; Lafarge *et al.*, 2004; von Neubeck *et al.*,

87 2015). However, the experimental data demonstrating the relationship between microbial counts and

88 proteolysis in raw milk is not well established, due to the distinct variation in the proteolytic potential

89 and heat-resistance of those proteolytic enzymes produced by raw milk microbiota (Dogan & Boor,

90 2003; Marchand *et al.*, 2009b). Hence, the current study investigated the effects of microbiological

91 quality and associated proteolysis on storage life of raw milk under different refrigeration conditions

92 for a prolonged period with a focus on psychrotrophic proteolytic counts (PPrBC). The effect of high-

93 temperature short-time pasteurisation (HTST) of raw milk prior to the UHT processing on

94 microbiological and proteolytic parameters was also evaluated.

95

96 **Materials and Methods**

97 *Raw milk samples*

98 Raw milk samples from three commercial farms (designated as A, B and C) were provided by a

99 commercial UHT milk processor in Victoria, Australia. These samples were selected from seven

100 potential samples to represent high quality (A: 2.3×10^4 cells/mL) medium quality (B: 5.3×10^5

101 cells/mL) and poor quality (C: 6.7×10^6 cells/mL) raw milk based on Bactoscan counts as well as

102 statistics of the respective commercial processor (Vithanage *et al.*, 2014). Three representative

103 samples were collected directly from the bulk milk tank at each of the farms under aseptic conditions

104 and delivered to the laboratory on ice (at 4-5 ºC) within 2-3 h of the milking procedure. A volume (500

105 mL) of the samples were transferred into a sterile Erlenmeyer flask (1 L) under aseptic conditions and

106 stored under various experimental conditions (as described below). Samples were analysed daily,

107 commencing from day 0, representing three biological (three separate samples of milk from each bulk

108 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

139   through 0.45 µm syringe filter (Minisart® Regenerated Cellulose; Sartorius, Victoria, Australia) and the

140   filtrate was used for protease assays. The same procedure was used for obtaining the TCA-soluble

141   peptides for in the peptide analysis.

142

143   *Determination of protease activity*

144   Protease activity in the raw milk samples stored under different storage conditions was determined

145   using the Protease Fluorescent Detection Kit (Sigma-Aldrich, Castle Hill, Australia) according to the

146   manufacturer's instructions. The fluorescence intensity due to release of trichloroacetic acid (TCA)-

147   soluble fluorescent peptides was determined using a spectrofluorophotometer (POLARstar Omega;

148   BMG LABTECH, Mornington, Victoria, Australia) with excitation at a wavelength of 485 nm and the

149   emission at a wavelength of 535 nm in duplicate. The increase in fluorescence intensity obtained due

150   to hydrolysis of the protein was expressed as relative fluorescence units (RFU/mL). Thermolysin

151   (Sigma-Aldrich, Castle Hill, Australia) was used as the positive control, and it was also used to

152   generate a standard curve (0-25 ng) when determining the detection limit (ng/mL) (Cupp-Enyard,

153   2009).

154

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

156   Separation of TCA-soluble peptides was performed on a reversed-phase HPLC (Varian Analytical

157   Instruments, Walnut Creek, CA, USA) equipped with C-18 monomeric column (5 µm, 300A, 250 mm x

158   4.6 mm; Grace Vydac, Hesperia CA, USA) at 35°C and a UV/Vis detector at 214 nm according to the

159   method described by Datta & Deeth (2003), with some modifications. A volume (50 µL) of TCA-

160   soluble peptides was injected and the peptides were eluted by a linear gradient from 100% to 0% of

161   solvent A (0.1% trifluoroacetic acid (TFA) in Milli-Q water) in solvent B (0.1% TFA in 90%, v/v HPLC-

162   grade acetonitrile in Milli-Q water) over 40 min at a flow rate of 0.75 mL/min in duplicate.

163

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

165   The extent of proteolysis was also determined using the modified OPA method (Zarei et al., 2012) in

166   duplicate. A volume (5 µL) of TCA-soluble peptides was mixed with 245 µL of OPA reagent (Thermo

167   Fisher Scientific, Victoria, Australia) in microtiter plates and the absorbance was determined using a

168   spectrofuorophotometer (POLARstar Omega; BMG LABTECH, Mornington, Victoria, Australia) with a

169   wavelength of 340 nm in duplicate. The degree of hydrolysis (DH %) was calculated based on the

170   following formula (i.e., equation 1) (Slattery & Fitzgerald, 1998).

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

172   where Δ A is the difference between the absorbance of test sample and un-hydrolysed sample at 340

173   nm, M is the molecular mass of the test protein (Da), d is the dilution factor, ε is the molar extinction

174   coefficient at 340 nm (6000 L/mol/cm), c is the protein concentration (g/L) and N is the total number of

175   peptide bonds per protein molecule.

176

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

178   Raw milk samples from all three farms were heated at 75 ± 0.5 ºC for 15 s in a shaking oil bath

179   (Ratek, Boronia, Victoria, Australia), excluding the come up time (Griffiths et al., 1987). Following heat

180   treatment, the samples were aseptically transferred into 1 L sterile Erlenmeyer flasks and stored

181   under different temperature at 2 ºC, 4 ºC, 6 ºC, 8 ºC, 10 ºC and 12 ºC for 10 days. The enumeration of

182   bacteria and analysis of protease activity and proteolysis was conducted as described before (n = 9).

183

184   *Data processing and statistical analysis*

185   The analysis was conducted in triplicate. Correlation coefficients and significance levels (MANOVA) of

186   the tested sets (TPC; PBC; PPrBC; TDPC) were calculated using the SPSS software for Windows

187   (Version 21 software; IBM Corp. in Armonk, NY). *P* < 0.05 was considered statistically significant.

188

189   **Results**

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

191   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)

192   log cfu/mL, respectively. Similarly, the initial PBC in the corresponding samples were in the following

193   order; A: 2.66 (±1.11); B: 2.87 (±1.01); C: 4.85 (±1.21) log cfu/mL. Interestingly, the PPrBC counts

194   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

195   (±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)

196   log cfu/mL and 3.61(±0.11) log cfu/mL, respectively.

197

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

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

200   growth pattern with different growth rates when stored under different refrigerated conditions (Fig. S1;

201   Fig. 1). The growth curves of PPrBC, TDPC of sample A, B and C showed a double-sigmoidal shape

202   (Fig. 1). However, Storage of raw milk at 2 ºC storage showed significant inhibition of the PPrBC and

203   TDPC. Storage temperatures of ≥ 4 ºC resulted in significant increases in PPrBC, whereas TDPC

204   showed significant increases in growth rate at ≥ 8 ºC ($P < 0.05$) (Fig.1).

205

206   *Diversity of raw milk microbiota under refrigerated conditions*

207   The predominant microorganisms isolated were *Pseudomonas*, *Bacillus*, and *Microbacterium* and, to

208   a lesser extent, members of the family *Enterobacteriaceae* (Table 1). The most predominant genera

209   found in refrigerated raw milk were *Pseudomonas* (mainly *Pseudomonas fluorescens)* and *Bacillus*

210   (*Bacillus cereus*, *Bacillus weihenstephensis* and *Bacillus circulans*). This diversity varied depending

211   on the sample and temperature tested. For example, the level of enteric, non-fermenter Gram

212   negative bacilli (NF-GNB), Gram positive cocci and Gram positive bacillus were higher at

213   temperatures ≥ 8 ºC (Table 1).

214

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

216   The initial protease activities (PA) of A, B and C raw milk samples were 404.5 (±4.76), 257 (±2.82)

217   and 604.3 (±5.13) RFU/mL. Consequently, the initial proteolysis (PL) that has been denoted by

218   degree of hydrolysis (%DH) of each samples was in the following ascending order; B: 0.88 (±0.51) %,

219   A: 1.32 (±1.02) % and C: 2.42 (±1.13) %.   A significant increase in PA and PL (denoted by %DH) was

220   apparent at storage conditions ≥ 6 ºC ($P < 0.05$) (Fig. 2). Even the standard refrigeration condition (4

221   ºC) showed significant increase in PA and PL during the extended storage of raw milk (10 days) and

222   this was observed after 6, 8 and 5 days in A, B and C samples, respectively ($P < 0.05$) (Fig. 2; Fig.

223   3). In contrast, 2 ºC storage resulted in significant reduction in the PA and DH in all three raw milk

224   sample ($P < 0.0001$) (Fig. 2).

225

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

227   An increase in protease activity and proteolysis were observed when the PPrBC counts reached 5.0 ×

228   10^4 cfu/mL at all temperature conditions, except for 2 ºC (Table 2; Fig. 2). However, the corresponding

229 protease activity and proteolysis varied as function of temperature (Table 2; Fig. 2). For example, the

230 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

231 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

232 were equivalent to 9.3 ng/mL, 3.5 ng/mL and 11.9 ng/mL as calculated using thermolysin as the

233 positive control by the FITC method, respectively (Table 2; Fig. 2). The proteolysis of the samples,

234 denoted by DH %, were 12.1%, 8.4% and 15.1%. In contrast, at 6 ºC with similar PPrBC (ranging

235 from $5.2\text{-}5.4 \times 10^4$ cfu/mL), the protease activities in the samples were 3.9×10^4 RFU mL^{-1}, 2.9×10^3

236 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

237 18.2%, 10.4% and 21.3%, representing farms A, B and C, respectively (Table 2; Fig. 2).

238 Interestingly, the correlation coefficients (r) between PPrBC and PA/PL were highly significant (r ≥

239 0.90, $P < 0.0001$; at ≥ 4 ºC), when PPrBC reached 5.0×10^4 cfu/mL (Table S1). This correlation was

240 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

241 correlation coefficients between PBC and PA and/or PL was significant (r ≥ 0.82-0.95, $P < 0.05$),

242 however, the TPC showed poor correlation with PA/PL (r = 0.55-0.62, $P > 0.05$) (data not shown).

243

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

245   Besides the significant correlation in increase in PA and PL with PPrBC, both parameters appear to

246 vary depending on the temperature condition. Therefore, the storage life in the aspect of raw milk

247 quality (S_{LQ}) was defined depending on the PPrBC counts, hence time to reach PPrBC of 5.0×10^4

248 cfu/mL was defined as S_{LQ} (Table S3). However, the storage life in the aspect of raw milk safety (S_{Ls})

249 was dependent on the counts of pathogenic thermoduric psychrotrophs such as *B. cereus* and the

250 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

251 significant correlation with initial counts ≥ 4 ºC and ≥ 8 ºC storage, respectively (Table S1; S2).

252

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

254 Heating of raw milk samples at 75 °C for 15 s followed by storage at different refrigeration conditions

255 resulted in a significant reduction of PPrBC ($P < 0.05$) (Table S3). This consequently decreased the

256 PA and PL with concomitant increased in the S_{LQ} ($P < 0.05$), especially the temperature conditions ≤ 8

257 ºC storage (Table S3). In contrast, the S_{Ls} showed only slight increase ($P > 0.05$). The most

258   significant increase in storage life (both S_{LQ} and S_{LS}) was observed when raw milk was stored at 2 ℃,

259   while storage life was significantly reduced when it was stored at ≥ 8 ℃ (Table S3).

260

261   **Discussion**

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

263   TDPC, possibly related to the different the farm management systems and hygienic protocols used

264   during the milking process of these farms (Cempírkova, 2007; Srairi *et al.*, 2009). Interestingly, the

265   PPrBC was higher in sample A compared to sample B. This may result in significantly greater

266   protease activity and proteolysis in the corresponding sample, regardless its lower TPC, compared to

267   sample B. Furthermore, proteolysis and protease activity showed a more significant correlation with

268   PPrBC (≥ 4 ℃) and TDPC (≥ 8 ℃) than that with TPC and PBC in raw milk. This indicates that PPrBC

269   and TDPC are the most important quality criteria that can be incorporated into the guidelines for the

270   production of high quality milk and dairy products. Moreover, the maximum production of proteolytic

271   enzymes and subsequent proteolysis was observed when PPrBC counts were above ≥ 5×10^4 cfu/mL

272   at ≥ 4 ℃, and TDPC ≥ 1×10^4 cfu/mL at ≥ 8 ℃ and those limits were used for predicting storage life

273   of raw milk with respect to both quality and safety. Thus, according to the results of the present study,

274   it can be speculated that production of UHT milk requires PPrBC counts below 5×10^4 cfu/mL and

275   TDPC of 1×10^4 cfu/mL for shelf life extension and product safety. This is consistent with a PPrBC

276   count of 4.5×10^4 cfu/mL representing the threshold with respect to milk quality (Silveira *et al.*, 1999;

277   Vyletelova *et al.*, 2000b). Similarly, the TDPC comprising significantly higher numbers of *B. cereus*

278   can be a food safety concern when it reaches 1.0×10^4 cfu/mL (Valik *et al.*, 2003). In contrast, several

279   other studies determined the relationship between proteolysis with slightly higher bacterial counts in

280   the range of 10^6-10^7 cfu/mL (O'Connell *et al.*, 2016; Haryani *et al.*, 2003; Griffiths *et al.*, 1987).

281   However, Gillis et al. (1985) also demonstrated significant decrease in proteolysis and bitter peptide

282   production with raw milk microbiota less than 10^4 cfu/mL.

283   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

284   elevated PA and PL within 4-7 days at 6 ℃ storage. The TDPC with similar initial counts can

285   increased to ≥ 1×10^4 cfu/mL within 5-9 days at 8 ℃. At 4 ℃, the PPrBC counts reached the

286   corresponding levels within 5-8 days storage and less than 2 days of storage at ≥ 8 ℃. Thus, 2 ℃ is

287   highly recommended as a storage temperature, while temperatures below 6 ℃ 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 ⁰C 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 ⁰C storage. Among the thermoduric

302   psychrotrophic isolates, species belong to *B. cereus* group was predominantly isolated especially ≥ 8

303   ⁰C. *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

318 family extracellular protease, referred to as AprX (EC 3.4.24.40), while *Bacillus* spp. produce serine

319 family proteases known as thermolysin (EC 3.4.24.27), substilisin (EC 3.4.21.62) (Bach *et al.*, 2001;

320 Machado *et al.*, 2013; Marchand *et al.*, 2009b; Dufour *et al.*, 2008). Expression of the genes encoding

321 these proteases was shown to be regulated by incubation temperature (Morita *et al.*, 1997; Burger *et*

322 *al.*, 2000). Alternatively, differences in proteolysis can be related to the characteristics of proteolytic

323 enzymes such as their cold-active nature, specificity and temperature-dependence (McKellar, 1989).

324 The growth of spoilage bacteria in raw milk can be controlled by thermisation (at 65 °C for 15 s),

325 followed by storing of the heated milk under refrigeration conditions (Griffiths *et al.*, 1987;

326 Stadhouders, 1982). In contrast to these earlier studies, the current study used heating of raw milk at

327 75 °C for 15 s, which is typically used in HTST pasteurisation. This practice is often used upon

328 receiving raw milk at dairy processing plants prior to UHT treatment. This resulted in significant

329 reduction (1-log) in PPrBC counts, but not TDPC, however resulted in significant decrease in protease

330 activity. This in turn showed significantly higher S_{LQ}, but no significant difference in S_{LS}. Thus, the

331 knowledge of number and diversity of psychrotrophic proteolytic bacteria in raw milk can be used for

332 appropriate production of milk and dairy products (Vithanage *et al.*, 2016; Anzueto, 2014). Similarly,

333 reliable control of raw milk isolates with higher proteolytic potential would be important for the

334 extension of raw material storage with concomitant increase in flexibility of the manufacturing process

335 (Griffiths *et al.*, 1987).

336 Although the current study used raw milk representing various quality levels, a large-scale analysis

337 would provide a more comprehensive understanding of the effect of storage conditions on raw milk

338 quality. However, these results are in general agreement with the results of large scale studies

339 (O'Connell *et al.*, 2016).

340 In conclusion, storage temperature, time and initial counts can affect microbiological quality of raw

341 milk, in which PPrBC and TDPC are good indicators than other microbiological criteria for predicting

342 the quality and safety of raw milk. It is important to determine a particular predictive model to estimate

343 the PPrBC and TDPC in samples for improving the quality and reducing large-scale wastage of raw

344 milk. Thus, PPrBC and TDPC data can be used to evaluate specific on-farm technological

345 requirements when deciding on quality-dependent incentive schemes for raw milk suppliers.

346 Additionally, deep cooling of raw milk at 2 ºC may be a reliable alternative for dairy farms when raw

347 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 ⁰C for 15 s (instead of 65 ⁰C) followed by 2 ⁰C 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

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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.

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565
566 **Table 1**
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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.

573   **Table 2**

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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	$1.2 \times 10^{2,‡}$	5.0[‡]	3.4[‡]
	4	6[€], >10[†]	4.67	2.97	$2.8 \times 10^{3,§}$	9.3[§]	12.1[‡]
	6	5[€], 8[†]	4.69	4.06	$3.9 \times 10^{4,§}$	12.1[§]	18.2[§]
	8	4[€], 5[†]	4.70	4.01	$4.4 \times 10^{4,*}$	13.3[*]	35.2[*]
	10	2[€†]	4.71	4.02	$5.0 \times 10^{4,*}$	15.1[*]	48.5[*]
	12	1[€†]	4.73	4.01	$4.3 \times 10^{5,*}$	15.9[*]	52.3[*]
B	2	10[€], >10[†]	4.69	3.05	$9.8 \times 10^{1,‡}$	2.4[‡]	2.5[‡]
	4	8[€], >10[†]	4.69	3.32	$1.0 \times 10^{2,‡}$	3.5[‡]	8.4[‡]
	6	6[€], 7[†]	4.69	4.06	$2.9 \times 10^{3,§}$	5.4[‡]	10.4[‡]
	8	4[€†]	4.68	4.06	$3.4 \times 10^{4,§}$	10.6[§]	23.3[§]
	10	2[€†]	4.67	4.07	$3.4 \times 10^{4,*}$	11.7[*]	37.1[*]
	12	1[€]	4.73	4.08	$3.8 \times 10^{4,*}$	12.9[*]	42.2[*]
C	2	8[€], 10[†]	4.69	4.02	$2.8 \times 10^{3,§}$	9.3[§]	5.8[‡]
	4	5[€], 9[†]	4.68	4.06	$4.0 \times 10^{4,§}$	11.9[§]	15.1[§]
	6	4[€], 5[†]	4.69	4.05	$5.3 \times 10^{4,*}$	13.2[*]	21.3[*]
	8	3[€†]	4.70	4.07	$5.5 \times 10^{4,*}$	15.6[*]	45.2[*]
	10	2[€†]	4.71	4.05	$5.5 \times 10^{5,*}$	17.1[*]	53.5[*]
	12	1[€†]	4.67	4.06	$6.2 \times 10^{5,*}$	18.7[*]	58.2[*]

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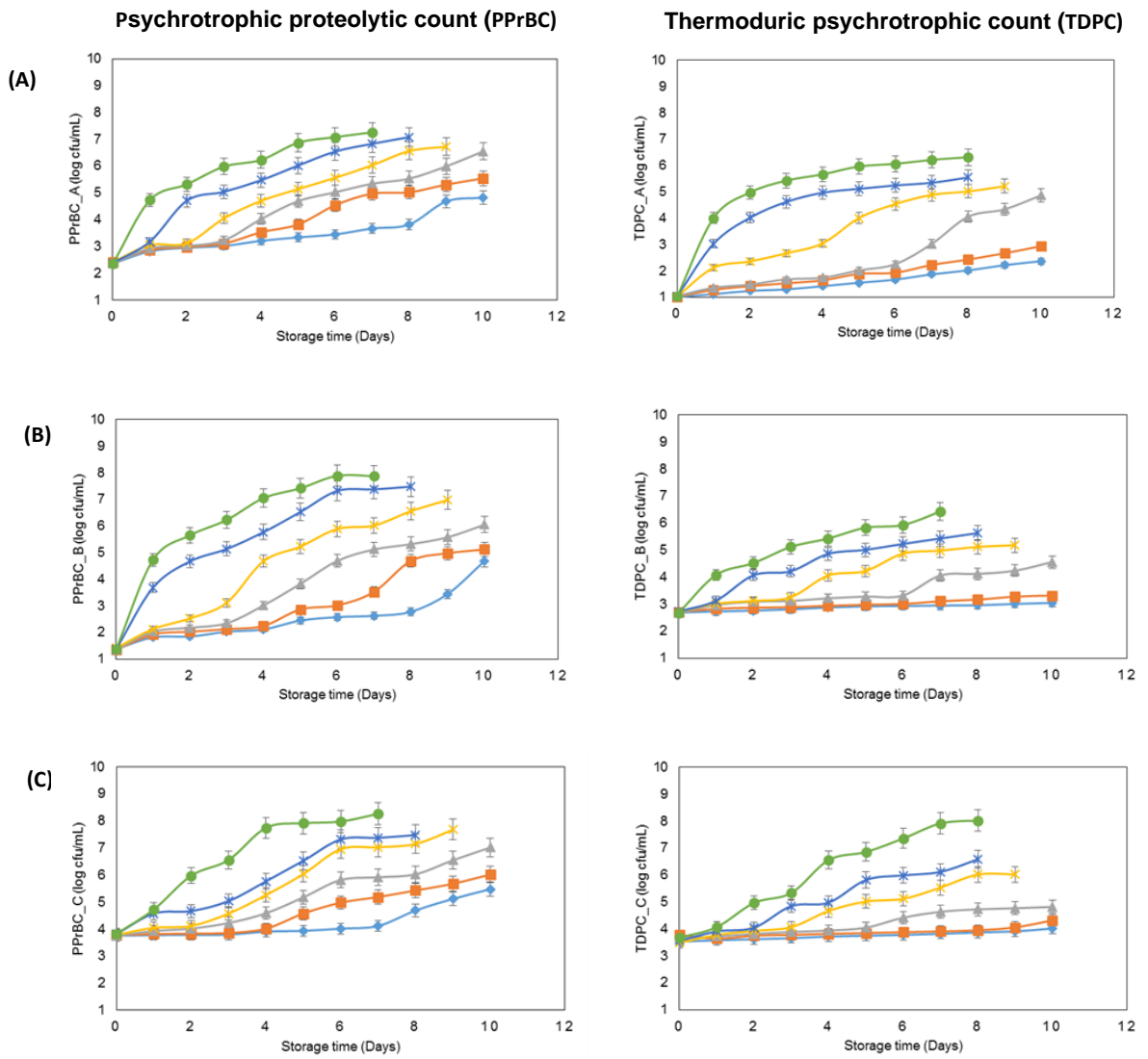
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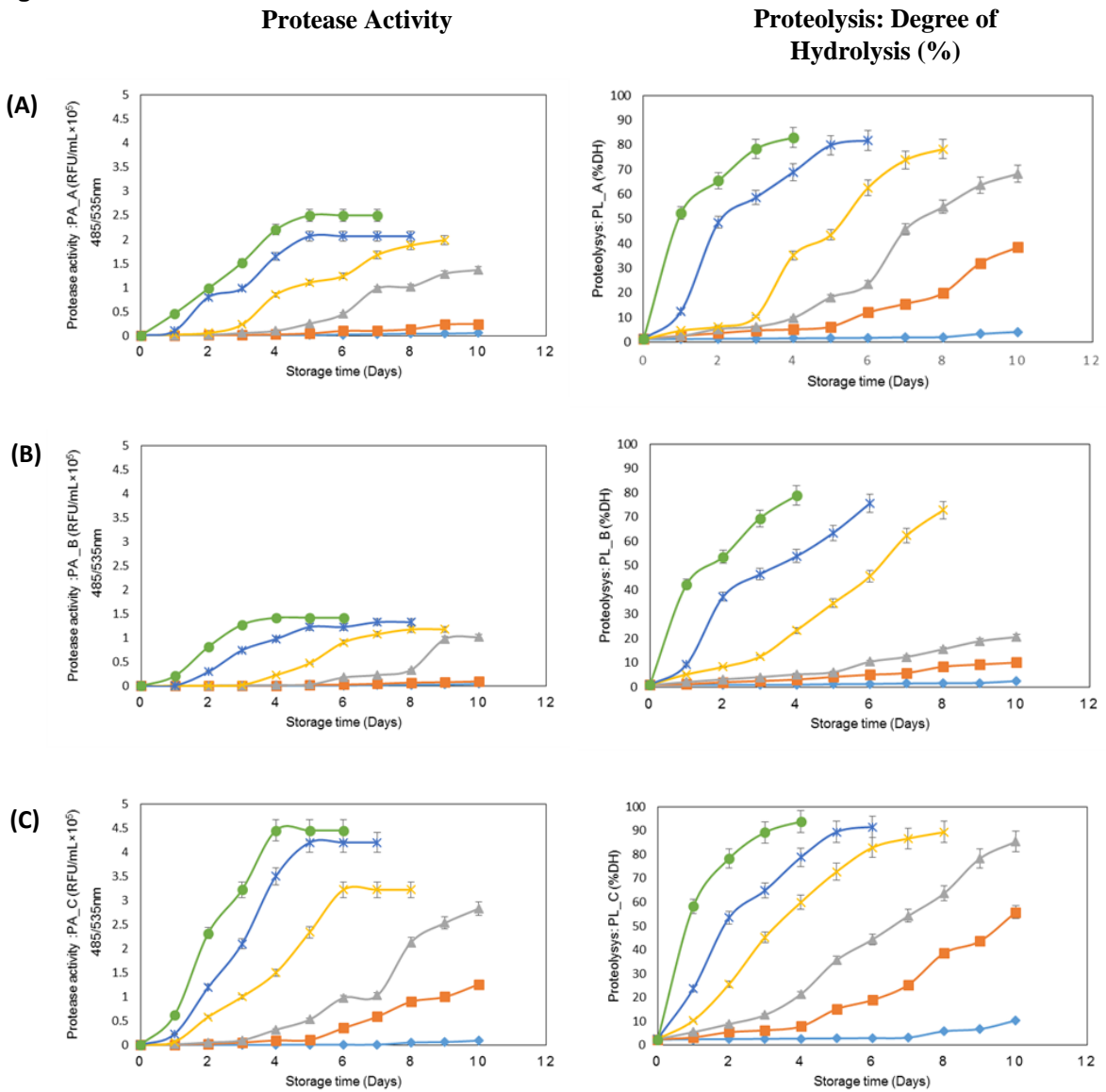
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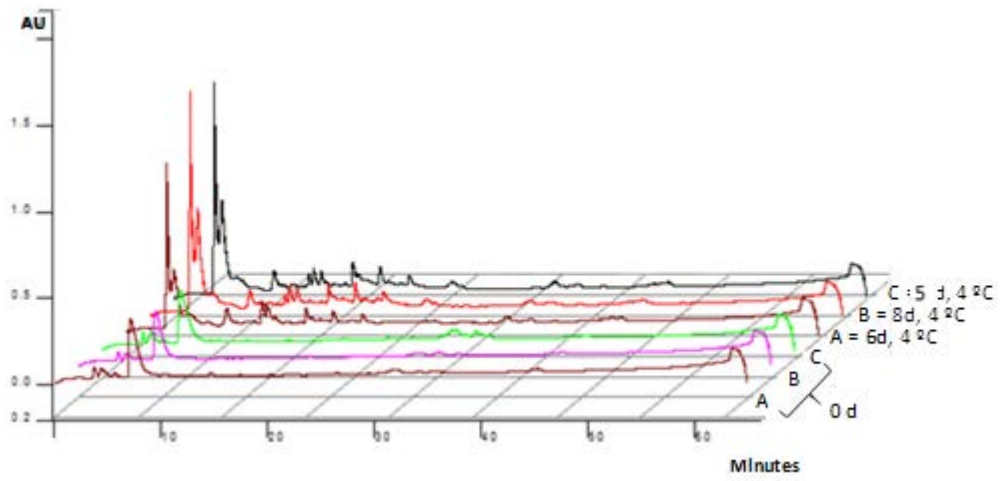**Psychrotrophic proteolytic count (PPrBC)**　　　　**Thermoduric psychrotrophic count (TDPC)**

599 **Fig. 2**

Protease Activity | Proteolysis: Degree of Hydrolysis (%)

(A)

(B)

(C)

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622 **Fig.3**
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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‡
4œ	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 (°C)	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 °C 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)