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Evaluating the effect of storage conditions on milk microbiological quality and composition

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Abstract

In this study, the effect of storage temperature (2 or 4°C) on the composition of milk and microbiological load was investigated over 96 h. Milk samples were collected from farm bulk milk tanks after one complete milking and stored at 2 or 4°C over 96 h. Total bacterial count (TBC), psychrotrophic bacterial count (PBC) and proteolytic bacterial count (PROT) were affected by storage time and temperature and varied significantly between farms ($P < 0.05$). The levels of TBC, PBC and PROT bacterial count increased from 4.37 to 6.15 log cfu/mL, 4.34 to 6.44 log cfu/mL and 3.72 to 4.81 log cfu/mL, respectively, when the milk was stored for 96 h at 2°C. The milk samples stored at 4°C had higher increases in these bacterial counts after 72 h in comparison to milk samples stored at 2°C. The casein fraction content was lower in milk samples stored at 4°C, which could be due to high levels of PROT bacteria or enzyme activity in these samples. Milk stored for 96 h at 2°C has less impact on composition or processability parameters compared to milk stored at 4°C.

Keywords

cold storage • dairy microbiology • proteolysis • raw milk quality

Introduction

The Food and Agriculture Organization of the United Nations (OECD/FAO, 2016) reported that the demand for milk and milk products is increasing worldwide, mainly due to rising incomes, population growth and changes in diets in developing countries; according to their report, milk production is expected to increase by 20% by 2025 worldwide. This expansion could result in the extension of milk storage time on farms beyond the current 48 h period practiced for most of the year in some countries. On considering prolonging storage of milk on farms or within the processing plant, it is necessary to evaluate how extended storage of milk at low temperatures could affect milk quality. Milk composition and microbiological load are important factors to consider when evaluating quality, due to their influence on milk processability, nutritional quality, dairy product quality and safety (Malek dos Reis *et al*., 2013). The most relevant bacterial groups for determining milk quality are counts of mesophilic bacteria, psychrotrophic bacteria, lipolytic (LIP) bacteria, proteolytic (PROT) bacteria, thermoduric bacteria [laboratory pasteurisation count (LPC)) and thermoduric-psychrotrophic bacteria (LPC-PBC).

Total bacterial count (TBC) and psychrotrophic bacterial count (PBC) are laboratory tests that allow for quantification of mesophilic and psychrotrophic bacteria (growth temperature of ≤7°C; Frank and Yousef, 2004) in milk, respectively. These tests are used to assess or monitor the sanitary and storage conditions during production, collection and handling of raw milk (Harding, 1995; Robinson, 2002). Hygienic milking conditions are vital to ensure high initial microbiological quality; however, milk storage conditions (i.e., temperature) can also influence bacterial growth. Some psychrotrophic bacterial strains can be classified as LIP or PROT bacteria, which can increase during milk cold storage, producing lipases and proteases, the action of which could affect milk functionality and also result in defects in dairy products such as rancidity and bitter flavours (Muir, 1996). Bacteria of the *Pseudomonas* genus are considered as one of the predominant psychrotrophic groups in raw milk with a high spoilage potential (De Jonghe *et al*., 2011; Machado *et al*., 2015). Thermoduric and thermoduric-psychrotrophic bacteria are capable of surviving thermal treatments (i.e., pasteurisation), while the latter can also grow at low temperatures; consequently, they are capable of multiplying during different processing stages (Robinson, 2002; Fromm and Boor, 2004; Barbano *et al*., 2006). These bacteria originate in the environment and could be present in feed, forage, bedding material, dust, faeces and soil, and, once in contact with cow's teat skin, could contaminate milk (Gleeson *et al*., 2013).

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Regarding milk composition, milk contains components of technological and nutritional importance (Walstra *et al*., 2005). Milk fat is a high-value component, important for the manufacture of dairy products such as butter and cheese. Fat hydrolysis caused by lipases can result in undesirable flavours (i.e., rancid, butyric and bitter), as well as loss of functional properties of milk such as foaming and creaming ability during manufacture of butter (Shelley *et al.,* 1987).

Milk proteins play critical roles in the physical stability and rheological properties of milk products. The main change in the protein system during cold storage is the migration of β-casein to the serum phase, which may impact on cheese production, resulting in losses of fat and curd fines in whey, prolonged clotting times and poor rennetability (Walstra *et al*., 2005). Proteolysis may also occur during cold storage, albeit likely slowly, due to endogenous enzymes (from psychrotrophic bacteria) or indigenous bovine enzymes. Indigenous proteinases in milk such as plasmin preferentially hydrolyse β-casein, α_{s1}-casein and α_{s2}-casein (Crudden *et al*., 2005), resulting in defects in dairy products, such as bitterness in milk, gelation of ultra-high temperature processing (UHT) milk and reduction in yields of cheese (Datta and Deeth, 2003). Proteolysis and lipolysis can also be caused by indigenous enzymes in milk associated with somatic cells; several studies have reported that milk quality decreases with the increasing somatic cell count (SCC) in milk and consequent increased activity of lipases and proteases (Santos *et al*., 2003; Barbano *et al*., 2006; Wickstrom *et al*., 2009).

On farms, milk is added to bulk tanks at least twice every day; therefore, the last volume of milk added to the tank remains stored for a shorter period of time. Hence, any significant effect caused by enzyme activity, bacterial growth, storage temperature and time on the quality of milk over 96 h may not be detected due to the addition of fresh milk (Perko, 2011; Reche *et al*., 2015; O'Connell *et al*., 2016). Therefore, the present study focused on analysing bulk tank milk from the first complete herd milking, produced under different farm management conditions.

The aim of this study was to investigate the effect of milk storage temperature and time on the quality of raw milk by evaluating the microbiological load and composition when milk was stored under controlled laboratory conditions at 2 or 4°C over 96 h.

Materials and methods

Sample collection

Milk samples were collected from bulk milk tanks of four autumn-calving dairy farms in the Cork region (Ireland) during the indoor period. The indoor period represents the first 150 days of lactation, after which cows are managed outdoors on grass. The farms were labelled as W, X, Y and Z, and the bulk tanks had milk only from the first milking. This milk was stored for <4 h, and therefore, samples analysed on the first day are referred to as 0 h samples. After agitation (1 min), one milk sample (1 L) was collected from the top of each bulk tank using a sterilised jug, transferred to a sterile bottle and transported to the laboratory at <4°C within 3 h. The samples were subdivided immediately after manual agitation to avoid unequal fat distribution due to fat separation in the original sample (Tamime, 2007). Each sample was subdivided into twenty 30 mL sterile bottles, which corresponded to four milk samples for each storage time (0, 24, 48, 72 and 96 h). In all, 10 bottles from each sample were stored at 2°C, while the other 10 bottles were stored at 4°C. At 0, 24, 48, 72 and 96 h, one sample from each temperature was analysed in duplicate for bacterial counts, composition (fat, protein, lactose and total solid contents) and SCC. In addition, two extra milk samples out of the 1 L were separated for each farm and were stored at 2 and 4°C for 0 and 96 h, respectively, in order to quantify casein and nitrogen fractions and to obtain peptide profiles.

Microbiological analysis

Raw milk samples were tested in duplicate every 24 h for a range of bacterial groups. All the microbiological analyses were performed in accordance with the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004). TBC, PBC, LPC and LPC-PBC were measured using Petrifilm, a ready to use medium (3 M; Technopath, Tipperary, Ireland), in accordance with the procedures described by Laird *et al*. (2004). The samples tested for LPC and LPC-PBC were pasteurised at 63°C for 35 min, allowing extra time for samples to reach the required temperature (Frank and Yousef, 2004). Afterwards, the samples were cooled to 10°C in iced water before testing. The samples tested for TBC and LPC were incubated for 48 h at 32°C (Laird *et al*., 2004), while samples tested for PBC and LPC-PBC were incubated for 10 days at 7 ± 1°C (Frank and Yousef, 2004). The number of bacterial colonies present was counted using a Petrifilm plate reader.

LIP and PROT bacterial counts were performed by spread plating 100 µL of the appropriate dilutions on tributyrin agar with added glyceryl tributyrate (Sigma Aldrich, Dublin, Ireland) and on calcium caseinate agar with added skim milk powder (Merck, Darmstadt, Germany), respectively. The agar plates were incubated at 37°C for 48 h for both methods. LIP bacterial colonies were identified as colonies surrounded by a clear zone in a turbid medium, while the PROT colonies were identified as colonies surrounded by a clear zone in an opaque medium.

Composition and SCC

Raw milk sample composition and SCC were measured using a Fossomatic FC (Foss Electric, HillerØd, Denmark). Fat,

protein, lactose and total solid percentages were quantified. Raw milk samples were also analysed in duplicate to quantify the non-protein nitrogen (NPN), non-casein nitrogen (NCN) and total protein content (N) using the Kjeldahl method [methods 20-4 (IDF, 2001), 29-1 (IDF, 2004a) and 20-3 (IDF, 2004b), respectively], using a Tecator Digestor Auto and Kjeltec 8400 distiller (Foss Electric). Milk samples stored for 0 and 96 h at 2 or 4°C were selected for these analyses.

High-performance liquid chromatography (HPLC) was used to quantify the casein content (in triplicate) and to obtain peptide profiles. To quantify the casein content, an aliquot of 200 µL of each milk sample was diluted in 3,780 µL of dissociating buffer (7 M urea and 20 mM Bis-tris propane, pH 7.5), to which 20 µL/mL of mercaptoethanol was added before filtering through a 0.22-µm filter. The method described by Mounsey and O'Kennedy (2009) was applied to perform gradient elution and peak detection. The HPLC equipment used was an Agilent 1200s system (Agilent Technologies, Santa Clara, CA, USA) with a quaternary pump and a multi-wavelength detector. The separation of the milk protein fractions was performed in the reversed-phase mode using an Agilent Poroshell 300SB C18 column (2.1 mm × 75 mm; Agilent Technologies).

The peptide profiles were obtained for samples, which showed significant differences in the casein content after 96 h. Samples stored at 0 and 96 h had their non-protein fraction extracted using trichloroacetic acid, according to the extraction procedure described in the IDF method 20-4 (Determination of Nitrogen Content) (IDF, 2001). To obtain a clear chromatogram, the extracts were not diluted but were filtered using 0.45 µm syringe cellulose filters (Ø 25 mm, Chromafil Xtra RC-45/25). The separation of milk peptides was performed in the reverse-phase mode using an Agilent Zorbax 300SB C8 column (4.6 mm ID × 150 mm; Agilent Technologies). The gradient elution and peak detection methodology was an adaption of the methodology of Rohm *et al*. (1996). The same HPLC equipment for quantification of caseins was used in this analysis, in which 50 µL samples were injected (in duplicate) onto the column and the flow rate was 0.50 mL/min.

Statistical analysis

Least square means for the main effects of storage time, temperature, farm, and their interaction were calculated using the MIXED procedure in SAS 9.3 (SAS Institute, 2016). The milk samples from the farms were the experimental units. The response variables were TBC, PBC, LPC, LPC-PBC, PROT bacterial count, LIP bacterial count, protein content, fat content, lactose content, total solid content, SCC, casein fractions (α_{s1}-casein, α_{s2}-casein, κ-casein and β-casein; α-lactalbumin; β-lactoglobulin A and B) and nitrogen fractions (N, NPN and NCN). The fixed effects included in each model were storage time (0, 24, 48, 72 and 96 h), farm milk samples (W, X, Y or Z) and temperature (2 or 4°C). Residual checks were made to ensure that the assumptions of the analysis were met. Where appropriate, log transformation was used to correct distributional issues. The Tukey's test (at 5% error probability) was used to compare the means for all variables. The correlations between TBC and PBC were assessed by applying Pearson's correlation coefficient using the CORR (correlation) procedure (SAS, 2016). The GLM (generalised linear model) procedure was used to determine the regression relationship between protein content and PROT bacteria.

Results

TBC

TBC was affected by storage time ($P < 0.001$), storage temperature ($P < 0.01$) and farm ($P < 0.001$), as well as by the interaction between temperature and time ($P < 0.05$; Table 1). Differences in initial TBC, as well as differences in the bacterial growth rates, were observed between milk samples (Figure 1A.1 and A.2). For example, the initial TBC in milk

Table 1. The significance of the main effects of time, temperature, farm and the interaction between time and temperature and between farm and time on the total bacterial count (TBC), psychrotrophic bacterial count (PBC), lipolytic (LIP) bacterial count, proteolytic (PROT) bacterial count, thermoduric bacterial count [laboratory pasteurisation count (LPC)] and thermoduric-psychrotrophic bacterial count (LPC-PBC) of the milk samples from all farms

Bacterial counts	P value				
	Time	Temperature	Time × temperature	Farm	Farm × time
TBC	<0.001	<0.01	<0.05	<0.001	0.37
PBC	<0.0001	<0.001	<0.001	<0.001	0.11
LIP	<0.0001	0.17	0.01	0.15	<0.05
PROT	<0.05	<0.01	<0.05	<0.001	0.86
LPC	0.71	0.13	0.50	<0.05	0.59
LPC-PBC	0.40	0.12	0.66	0.14	0.72

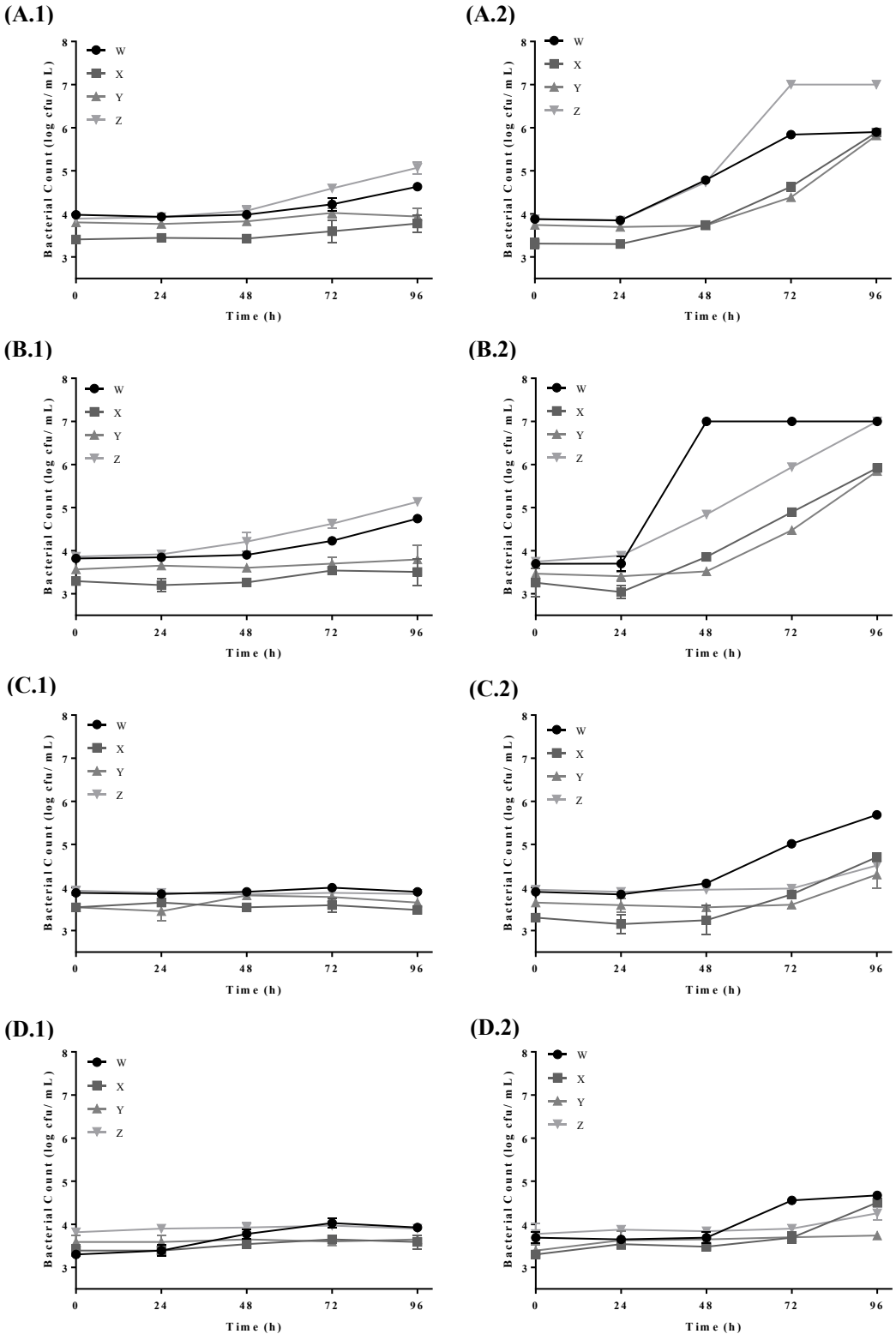

Figure 1. (A) Total bacterial count (TBC), **(B)** psychrotrophic bacterial count (PBC), **(C)** proteolytic (PROT) bacterial count and **(D)** lipolytic (LIP) bacterial count over 96 h for milk samples W, X, Y and Z stored at **(1)** 2°C or **(2)** 4°C.

samples from farms W and Z were similar (3.93 ± 0.06 log cfu/mL and 3.88 ± 0.06 log cfu/mL, respectively), and these samples had a similar TBC after 96 h when stored at 2°C (Figure 1A.1); however, samples stored at 4°C had different TBCs after 72 h, corresponding to 5.84 ± 0.06 log cfu/mL and >7.00 log cfu/mL, respectively (Figure 1A.2).

PBC

The PBC was significantly affected by farm ($P < 0.001$), time ($P < 0.0001$) and temperature ($P < 0.001$); there was an interaction between time and temperature ($P < 0.001$; Table 1) but no interaction between farm and time ($P > 0.05$; Table 1). Similar to the TBC results, differences between the initial PBC levels, as well as differences in the growth rates over 96 h, between the farm milk samples were observed (Figure 1B). For example, samples from farms W and Z had similar initial PBC (3.76 ± 0.07 log cfu/mL and 3.80 ± 0.09 log cfu/mL, respectively); however, after 48 h, sample W had a PBC >7.00 log cfu/mL, while sample Z reached that level after 96 h (Figure 1B.2). In this study, TBC was correlated with PBC, $r(40) = 0.90983$, $P < 0.0001$.

LIP and PROT bacterial counts

The LIP and PROT bacterial counts were significantly affected by storage time ($P < 0.0001$ and $P < 0.05$, respectively; Table 1) and by the interaction between time and temperature ($P = 0.01$ and $P < 0.05$, respectively; Table 1). Similar to TBC and PBC, storage at 2°C resulted in lower increases in LIP and PROT bacterial counts over 96 h in comparison to samples stored at 4°C (Figure 2A and B). Only PROT bacterial count was significantly affected by temperature ($P < 0.01$; Table 1), as shown in Figure 2A and B. The initial LIP bacterial counts were similar between farms ($P > 0.05$), while the PROT bacterial count had a significant variability

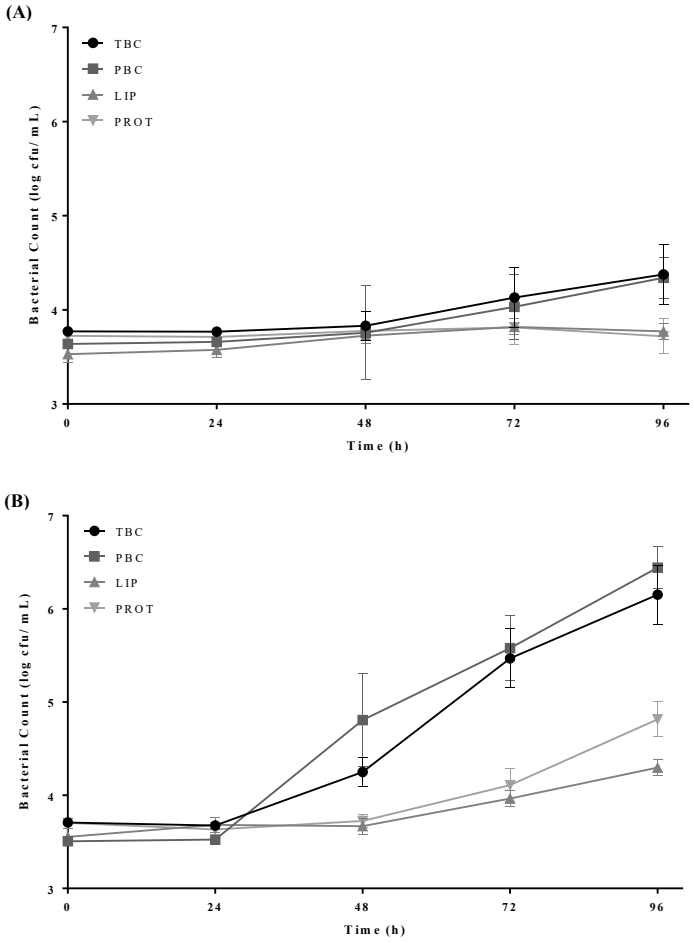

Figure 2. Average of the total bacterial count (TBC), psychrotrophic bacterial count (PBC), lipolytic (LIP) bacterial count and proteolytic (PROT) bacterial count over 96 h for milk samples from four dairy farms (W, X, Y and Z) stored at **(A)** 2°C and **(B)** 4°C.

(P < 0.0001; Table 1). The growth rates of LIP and PROT bacteria varied among farm milk samples when stored at 4°C (Figure 1C.2 and D.2).

Thermoduric bacterial count and thermoduric-psychrotrophic bacterial count

The LPC was not affected by storage time (P = 0.71), temperature (P = 0.13) or their interaction (P = 0.50; Table 1). However, the LPC was significantly different between farms (P < 0.05; Table 1), with initial counts varying from 2.11 to 2.64 log cfu/mL (128–445 cfu/mL).

The LPC-PBC was not affected by time, temperature, farm or their interaction (P > 0.05; Table 1). The LPC-PBC levels varied from 0 to 1.40 log cfu/mL (25 cfu/mL).

Composition

The fat, protein and total solid contents of the milk samples were affected by storage time (P < 0.001, P < 0.0001 and P < 0.001, respectively), which decreased by 0.04%, 0.01% and 0.07% after 96 h, respectively. The lactose content remained the same over 96 h. The composition of milk samples was not affected by storage temperature. The fat (P < 0.05), protein (P < 0.0001), lactose (P < 0.001), total solids (P < 0.001), κ-casein (P < 0.05), α_{S1}-casein (P < 0.05), α_{S2}-casein (P < 0.01), β-lactoglobulin A (P < 0.01) and β-lactoglobulin B (P < 0.001), total casein (P < 0.05), N (3.03%–3.30%) and NPN (0.026%–0.028%) contents (P < 0.01) varied between farm milk samples. The NCN content was similar between farms (0.10 ± 0.003%, P > 0.05).

Statistical analysis did not indicate significant changes in casein and nitrogen fractions over time or at different temperatures (P > 0.05, data not shown). The chromatograms presented in Figure 3A and B indicated decreases in the casein content in the milk samples from farms W and Z. The α_{S1}-casein and β-casein contents decreased in sample Z, as well as in sample W, with a decrease in k-casein content after 96 h. The chromatograms in Figure 4A and B indicated an increase in the concentrations of peptides in samples W and Z.

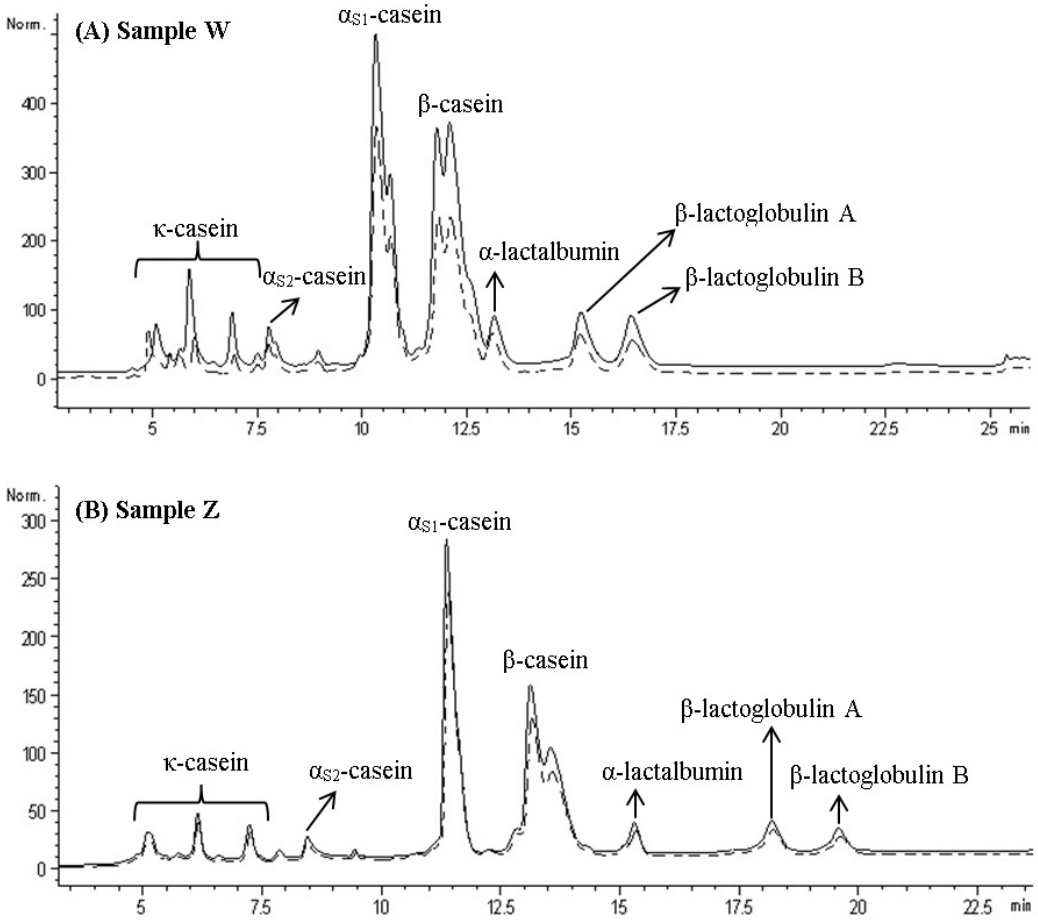

Figure 3. Separation of bovine milk proteins by reversed-phase high-performance liquid chromatography (HPLC). Chromatograms of samples **(A)** W and **(B)** Z stored at 4°C are shown. Full line (-) shows the 0 h sample; dashed line (– –) shows the 96 h sample.

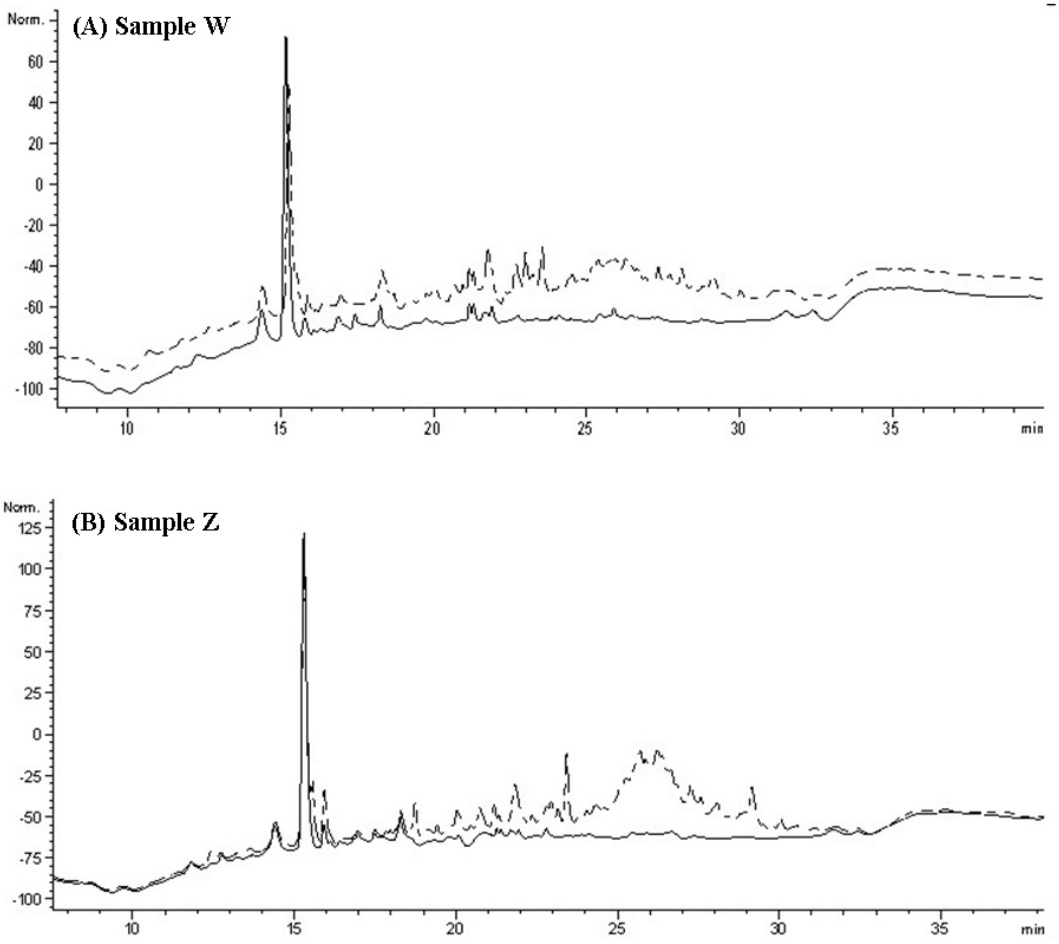

Figure 4. Separation of bovine milk peptides by reversed-phase high-performance liquid chromatography (HPLC). Chromatograms of samples **(A)** W and **(B)** Z stored at 4°C are shown. Full line (-) shows the 0 h sample; dashed line (– –) shows the 96 h sample.

SCCs

SCCs were different between farm milk samples ($P < 0.001$). The average (s.d.) SCC of the farms W, X, Y and Z were 62 ± 4.1 × 10^3 cells/mL, 78 ± 6.2 × 10^3 cells/mL, 77 ± 4.2 × 10^3 cells/mL and 214 ± 7.9 × 10^3 cells/mL, respectively. The levels of SCC were significantly affected by storage time ($P < 0.01$) but not by temperature ($P > 0.05$). The least square means for both temperatures (2 and 4°C) were 96,000 cells/mL.

Discussion

TBC

According to European Regulation EC No 853/2004 (2004), TBC should be less than 5.00 log cfu/mL (1.00 × 10^5 cfu/mL) when milk is destined for manufacture of dairy products. However, some milk processors apply a lower TBC limit (e.g., 4.70 log cfu/mL or 5.00 × 10^4 cfu/mL) for raw milk at the farm level. According to Pantoja *et al*. (2012), when the TBC of raw milk is <5.00 log cfu/mL, it is assumed that pasteurisation will reduce TBC to safe levels, destroying all pathogenic and most non-pathogenic bacteria present in milk. After 96 h, samples stored at 2°C had a TBC lower than this limit (4.37 ± 0.32 log cfu/mL; Figure 2A); however, milk stored at 4°C reached a TBC of 5.47 ± 0.32 log cfu/mL after 72 h (Figure 2B). Therefore, applying the legislation and industry criteria, milk stored at 4°C would be unsuitable for processing after 72 h of storage, while milk stored at 2°C could have the storage period extended to 96 h and remained suitable for processing. This information could be relevant for the extended storage of milk on farms, as well as within a dairy plant, where milk is stored in silos prior to processing.

In a farm scenario, the addition of fresh milk to the bulk milk tank at least twice a day could result in bacterial counts different from bacterial counts reported for milk from a first milking only, stored for the same amount of time (Perko, 2011). While the present study could indicate that the storage of milk at 4°C

should be limited to 48 h, O'Connell *et al*. (2016) demonstrated that milk stored in farm bulk tanks at the same temperature for 96 h (fresh milk added twice daily) had minimal deterioration of microbiological quality (3.68 log cfu/mL). However, the present study determines the effects possibly caused by enzyme activity or bacterial growth that would not be detected when fresh milk is added to the tank every day.

The differences in initial TBC observed between milk samples were considered relevant, indicating that samples had different microbiological qualities. Guinot-Thomas *et al*. (1995) suggested that bacterial counts are a reflection of the hygiene and sanitation practices at the farm level. Even though some of these initial TBCs were similar, the bacteria in the milk samples appeared to have different growth rates, as observed when comparing milk samples from farms W and Z that were stored at 2 and 4°C (Figure 1A.1 and A.2, respectively). These differences could be due to differences in the make-up of the milk microbiota, considering that there are a variety of strains within the mesophilic bacterial group that can survive and grow at different temperatures (Hantsis-Zacharov and Halpern, 2007).

PBC

According to Griffiths (2010), the PBC limit in raw milk at the collection point should be in accordance with the ratio of 6:1 (TBC:PBC). Therefore, based on the EU limit for TBC (5.00 log cfu/mL), the PBC limit should be approximately 4.22 log cfu/mL. After 96 h, samples stored at 2°C had a PBC over that limit (4.34 ± 0.22 log cfu/mL; Figure 2A), while samples stored at 4°C were over that limit after 48 h (4.80 ± 0.50 log cfu/mL), reaching a PBC of 6.44 ± 0.22 log cfu/mL after 96 h (Figure 2B). However, after 96 h, samples stored at 2 and 4°C may still be suitable, for example, for UHT, where milk is heated to a temperature >135°C, with a holding time of 2–5 s. Muir (1996) suggested that raw milk with a PBC of 6.70 log cfu/mL should be rejected for UHT milk production, as high levels of psychrotrophic counts result in faster milk spoilage, which is due to the production of heat-resistant enzymes (Machado *et al*., 2017). Considering that the samples stored at 2°C had a PBC level considerably lower than 6.70 log cfu/mL after 96 h, the difference between the average PBC of these samples and the European threshold (4.22 log cfu/mL) can be considered to be not biologically relevant.

The differences in the initial PBC levels between the farm milk samples ($P < 0.001$; Table 1) could be due to differences in practices on each of the farms, which lead to different contamination levels. The different growth rates observed over 96 h were probably due to variation in microbiota between samples. Similarly, Vithanage *et al*. (2016) observed that the same milk samples stored at different temperatures (2, 4, 6, 8 or 10°C) showed significant differences in their microbiota and bacterial counts over time.

The TBC of raw milk is normally used as a major quality indicator by milk processors, while PBC is not considered as a quality parameter of raw milk. However, considering the positive correlation between TBC and PBC as well as that refrigerated storage conditions are favourable for the growth of psychrotrophic bacteria, it should perhaps be considered as a quality indicator. Hantsis-Zacharov and Halpern (2007) also observed a correlation between TBC (mesophilic bacterial count) and PBC that increased or decreased in a similar range in different seasons when milk was collected from bulk tanks, also indicating similar dynamics for the two bacterial groups.

LIP and PROT bacterial counts

According to Vyletelova *et al*. (2000), when milk is destined for manufacture of dairy products, PROT and LIP bacterial counts in milk should be less than 4.65 log cfu/mL. Milk samples stored at 2°C for over 96 h would be in accordance with this limit (LIP bacterial count: 3.77 ± 0.08 log cfu/mL; PROT bacterial count: 3.72 ± 0.19 log cfu/mL). However, PROT bacterial count reached 4.81 ± 0.19 log cfu/mL after 96 h at 4°C, which is above the suggested limit, while LIP bacterial count was still below the limit (4.30 ± 0.08 log cfu/mL) (Figure 2A and B).

The LIP and PROT bacterial growth rates were affected by storage conditions and varied among farm milk samples (Figure 1C.1 and C.2 and D.1 and D.2). This result highlights again the significance of differences in milk sample microbiota and their subsequent growth during storage. Celestino *et al*. (1996) also reported different growth rates of PROT and LIP bacteria in samples stored at 4°C over 48 h; initial PROT and LIP bacterial counts were 2.78 and 3.90 log cfu/mL, and counts after 48 h were 3.56 and 4.28 log cfu/mL, respectively. The increased rates are different on comparing this study to that of Celestino *et al*. (1996), probably due to differences in initial microbiota.

Thermoduric bacterial count and thermoduric-psychrotrophic bacterial count

Statistical analysis indicated that LPC was not affected by time, temperature or their interaction, suggesting that thermoduric strains present in the samples could not grow at low temperatures. The initial LPC levels in the farm milk samples were below a typical industry LPC specification, which ranged from 2.70 to 3.00 log cfu/mL (500 to 1,000 cfu/mL). Griffiths *et al*. (1988) also observed no significant increase in the LPC of milk stored for 72 h at 2°C. Different levels of thermoduric bacteria between farm milk samples suggest that the contamination level depends on the environmental and milking conditions on farms (Gleeson *et al*., 2013).

The low levels of LPC-PBC indicated that the milk samples were not considerably contaminated with this bacterial group. This result could be related to the hygiene practices adopted

at the farms in this study, which may have prevented high levels of contamination. Similarly, Celestino *et al*. (1996) reported no significant increase in psychrotrophic spore-former count in milk stored at 4°C for 48 h.

Composition

The decreases in the fat, protein and total solid contents are not considered technologically relevant (Guinee *et al*., 2000). The variations in milk composition between farms can be related to cow diet, breed, physiology and environment (Linn, 1988).

The milk protein content measured includes the casein fraction, the whey protein fraction and the NPN fraction. The activity of enzymes in milk during storage could decrease the percentage of protein and increase the fraction of NPN in milk (i.e., amino acids and peptides) (Verdi *et al*., 1987). Hence, in order to detect possible changes in the proportions of these proteins over time and at different temperatures, casein and nitrogen fractions were quantified. Even though casein and nitrogen fractions did not vary significantly over storage time, technologically relevant changes were observed in the κ-casein, α_{s1}-casein and β-casein contents in milk samples stored at 4°C, affecting the total casein content (Table 2). Milk samples from farms W and Z showed the greatest decreases in the total casein content: 4.86 and 1.34 g/L, respectively (data not shown), as also observed in the chromatograms in Figure 3A and B. The chromatograms presented in Figure 4A and B indicated protein breakdown in both samples after 96 h, through appearance of peptides. Datta and Deeth (2003) suggested that early eluting peptide peaks in HPLC chromatograms, produced using similar methods, are possibly related to bacterial proteolysis. However, in this study, the chromatograms from samples W and Z show the appearance and/or increase in peaks after 20 min (Figure 4A and B), which are possibly characteristics of plasmin action (authors' unpublished data). The peaks areas between 20 and 28 min increased 2.9 and 3.2 times in the 96 h chromatograms for samples W and Z, respectively, in comparison to the 0 h chromatograms (Figure 4A and B). The low temperatures applied during bulk tank milk storage are far from the optimum temperature for most enzymes; however, during a long storage period, products of these enzyme activities could accumulate (Kelly and Fox, 2006).

The decrease in the casein fraction and whey protein content and increase in the peptide content in samples W and Z could also be related to the increase in the PROT bacterial population, which was statistically correlated with the protein content ($P < 0.0001$). Milk samples W and Z had the highest levels of PBC after 96 h, which were >7.00 log cfu/mL, and sample W had the highest level of PROT bacteria after 96 h (5.68 ± 0.01 log cfu/mL). According to Lewis and Deeth (2009), when levels of psychrotrophic bacteria in milk reach 6.00 log cfu/mL, the production of lipases and proteases begins. When the levels of PROT bacteria reach 4.65 log cfu/mL, proteases are also produced (Vyletelova *et al*., 2000). The PBC and PROT bacterial count of the other two milk samples (X and Y) stored at 4 and 2°C are below these levels, which could be the reason why casein fractions and whey protein levels did not vary (data not shown).

SCC

All SCCs of the farm milk samples were below the EU legislation threshold (400 × 10^3 cells/mL), also suggesting that cow management on these farms was appropriate (Smith, 2002; Piccinini *et al*., 2006). The marginal difference in SCC between 96 h (89,000 cells/mL) and 0 h (98,000 cells/mL) is probably not relevant, and levels remained below the EU threshold during storage.

Conclusions

Mesophilic, psychrotrophic, LIP and PROT bacterial counts in milk are influenced by storage temperature, which consequently can influence the storage time of this milk. The initial microbiological counts in milk are influenced by farm management practices, which may impact on the milk bacterial growth during storage and possibly limit storage time. The results regarding proteolysis levels highlight the importance of considering PBC as an important milk quality parameter, due to the capacity of psychrotrophs to produce proteases. According to this study, milk could be stored at 2°C for 96 h with minimal quality deterioration, while storage at 4°C would limit storage time to 48 h for processing of milk. In

Table 2. Contents of casein fractions in samples stored at 2 or 4°C for 0 and 96 h

Temperature (°C)	Time (h)[1]	κ-casein (mg/mL)	α_{s1}-casein (mg/mL)	α_{s2}-casein (mg/mL)	β-casein (mg/mL)	α-lactalbumin (mg/mL)	β-lactoglobulin A + B (mg/mL)	Total casein (mg/mL)
2	0	4.74	11.43	1.68	10.90	0.85	3.58	33.20
	96	4.66	11.43	1.72	10.93	0.86	3.48	33.08
4	0	4.69	11.39	1.69	10.89	0.83	3.46	32.96
	96	4.40	11.12	1.64	10.12	0.85	3.44	31.65

[1]There is no statistical difference in the contents of casein fractions between 0 and 96 h and between samples stored at 2 or 4°C ($P > 0.05$).

conclusion, careful management of milk storage temperature and time is critical to improvement of quality of dairy products.

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