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Examining the interrelationship between milk storage and production conditions on milk and dairy products quality

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BSc. Food Engineering

This thesis is submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy in Food Science and Technology

Supervisor: Prof. Alan L. Kelly

Teagasc Supervisor: Dr. David Gleeson

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Conference contributions

Paludetti, L. F., Kelly, A. L., O'Brien, B. & Gleeson, D. (2018). Control of chlorate and trichloromethane residue levels in bulk tank milk. In: 69th Annual Meeting of the European Federation of Animal Science, August 2018, Dubrovnik, Croatia.

Paludetti, L. F., Kelly, A. L. & Gleeson, D. (2018). Microbial quality of milk from grass-based production system and its impact on milk powder quality. In: 27th European Grassland Federation General Meeting, June 2018, Cork, Ireland.

Paludetti, L. F., O'Brien, B., Kelly, A. L. & Gleeson, D. (2018). Iodine levels in bulk tank milk produced by dairy cows during the mid- and late-lactation periods. In: British Society of Animal Science Annual Conference, Dublin 2018, Ireland.

Paludetti, L. F., Kelly, A. L. & Gleeson, D. (2018). The effect of milk cooling rate on milk microbiological quality and composition. In: British Society of Animal Science Annual Conference, Dublin 2018, Ireland.

Paludetti, L. F., Kelly, A. L. & Gleeson, D. (2017). Monitoring residues in milk from farm to milk powder manufacture. In: IDF World Dairy Summit, October 2017, Belfast, Northern Ireland.

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Paludetti, L. F., Kelly, A. L. & Gleeson, D. (2016). Influence of storage time on the microbiological load of raw milk destined for UHT processing. In: IuFost 2016 – 18th World Congress of Food Science and Technology, August 2016, Dublin, Ireland.

Peer reviewed research articles

Paludetti, L. F., Kelly, A. L., O'Brien, B. & Gleeson, D. (2019). Monitoring residue concentrations in milk from farm and throughout a milk powder manufacturing process. *Journal of Dairy Research, 86,* 341-346.

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Paludetti, L. F., Kelly, A. L., O'Brien, B., Jordan, K. & Gleeson, D. (2018). The effect of different pre-cooling rates and cold storage on milk microbiological quality and composition. *Journal of Dairy Science, 101,* 1921-1929.

Paludetti, L. F., Jordan, K., Kelly, A. L. & Gleeson, D. (2018). Evaluating the effect of storage conditions on milk microbiological quality and composition. *Irish Journal of Agricultural and Food Research, 57,* 52-62.

List of Abbreviations

α_S-CN α_S-casein

α-lac α-lactalbumin

β-CN β-casein

β-Lg A β-lactoglobulin A

β-Lg B β-lactoglobulin B

κ-CN κ-casein

γ-CN γ-casein

a30 curd firmness at 30 min

ala alanine

arg arginine

asp Aspartic acid

BAC 12 Benzyldimethyldodecylammonium chloride

BAC 14 Benzyldimethyltetradecylammonium chloride

BAC 16 Benzyldimethylhexadecylammonium chloride

BT Bulk tank

C Control

CHLO Chlorate

CS Cream silo

CT Collection tankers

cys cysteine

DDAC Didecyldimethylammonium chloride

DIM Days in milk

DP Double-stage plate cooler

FAA Free-amino acids

FDM fat-in-dry matter

glu Glutamic acid

gly glycine

GW Ground water

his histidine

ile isoleucine

IMF Infant milk formula

k20 curd-firming time

leu leucine

LPC Laboratory pasteurisation count

LIP Lipolytic bacterial count

LPC-PBC Thermoduric-psychrotrophic bacterial count

lys lysine

met methionine

MFGM Milk fat globule membrane

MNFS Moisture in non-fat substances

N Total nitrogen content

NCN Non casein nitrogen

NCP Non-casein protein

NP No plate cooler

NPN Non protein nitrogen

P1 0.15 U/L

P4 0.60 U/ L

P10 1.50 U/ L

P10 neg 1.50 U/ L (non-inoculated supernatant)

para-κ-CN para-κ-casein

PBC Psychrotrophic bacterial count

PCHLO Perchlorates

pH 4.6-SN/TN Nitrogen content of pH 4.6 fractions of cheeses

phe phenylalanine

pro proline

PROT Proteolytic bacterial count

QAC Quaternary ammonium compound

RCT Rennet coagulation time

REC_{FAT} Fat recovery

REC_{PROT} Protein recovery

RSMP Reconstituted skim milk powder

S/M salt-in-moisture

SCC Somatic cell count

ser Serine

SMP Skim milk powder

SMS Skim milk silo

SP Single-stage plate cooler

SRC Sulphite-reducing Clostridia

TBC Total bacterial count

TCM Trichloromethane

THERM Thermophilic bacterial count

thre Threonine

tryp tryptophan

TS total solids

tyr tyrosine

val valine

WMS Whole milk silo

Y% Cheese yield

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work. I have exercised care to ensure that the work is original, and does not, to the best of my knowledge, breach any law of copyright, and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

_____ Date: 4th November 2019

Lizandra Fregonezi Paludetti

For my parents, Lucia and Abilio,

My sister, Mayara and

My beloved grandparents,

Laurinda and Arnaldo.

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Summary

The Irish dairy industry is currently expanding its market worldwide and exploring opportunities to diversify its portfolio of products. In this scenario, processors have to ensure the continuous production of high quality raw milk for the manufacture of dairy products, given that the quality requirements in the international market are becoming more stringent. The studies presented in this thesis provided information that can aid dairy suppliers and processors on improving the quality of raw milk and dairy products.

Firstly, the impact of different refrigerated storage conditions and pre-cooling routines was determined on the microbiological load and composition of raw milk. Those quality parameters were minimally affected when milk was stored at 2 °C for over 72 h, while increasing the temperature to 4 °C resulted in major increases in the bacterial numbers and limited storage time. However, the initial microbiological load of milk has a greater impact on the maintenance of its quality during storage than temperature. Appropriate cow management and sanitation are the primary critical practices to ensure the production of high quality raw milk.

The use of single-stage and double-stage plate coolers did not aid on decreasing bacterial growth in milk during 72 h of storage. The blending temperatures within each bulk tank decreases after each milking, due to the increase in the volume of milk stored at 3 °C. As a result, the maintenance of low temperatures within the bulk tank is more efficient on reducing bacterial growth in milk than pre-cooling rates. However, the use of single-stage plate coolers can aid on reducing the energy usage at farms. Double stage plate coolers are

recommended to farmers that already use an ice bank system to cool milk within bulk tanks.

The second part of this research investigated the impact of production season on the microbiological load in raw milk and its influence on the quality of milk powder, by enumerating different types of microorganisms throughout the manufacture of this product. The microbiological quality of the milk produced during late-lactation was inferior compared to the milk supplied during mid-lactation. This result highlighted again the importance of high hygienic standards at farms to ensure low bacterial levels in raw milk supplied to processors. This is highly important during late-lactation, as it coincides with the period of housing herds due to winter conditions. In addition, the incidence of thermoduric and thermophilic bacteria is higher during that period and, as a result pasteurisation was less efficient on reducing microbiological levels in late-lactation milk. This could have implications on the safety of milk powder, as some pathogenic microorganisms survive high temperatures.

The concentration of residues originated from cleaning products (chlorate, perchlorate, trichloromethane and quaternary ammonium compounds) were also monitored in milk throughout the same manufacturing process. Results indicated that production season can also have an influence on the cleaning practices adopted at farms, as higher levels of those residues were observed in late-lactation milk. Concentrations could have also been influenced by the lower levels of milk stored in bulk tanks in late-lactation. This study also provided further understanding regarding the dynamics of residue levels throughout the manufacturing stages. Chlorate was highly concentrated in milk powder after evaporation and spray-drying of milk. The levels of chlorate allowed in raw milk

supplied to manufacturers could be adjusted by also accounting for those increases during processing, in order to ensure that the levels in the final product are within specifications. Trichloromethane concentrated in the cream portion separated from milk as expected.

Finally, the influence of a thermo-resistant protease, which is produced by a *Pseudomonas fluorescens* strain, was determined on the cheese-making properties of milk and quality of Cheddar cheese. The increase in activity level of the added protease affected the formation of curd, as the hydrolysis of β- and α_S-caseins increased. However, the same levels of activity had a minimal impact on the composition of milk during 48 h of storage, as well as on the manufacture and quality of Cheddar cheese. Possibly, the low storage temperatures and pH values obtained during manufacture affected the activity of the protease.

This thesis highlighted the major influence that microbiological quality and composition of raw milk have on its processing properties and impact on dairy products and, unveiled factors that can aid in controlling different quality aspects of milk at the farm and during processing.

Chapter 1

Literature review

Declaration: This chapter was written by Lizandra F. Paludetti, with corrections and comments from Prof. Alan L. Kelly from University College Cork, and Dr. David Gleeson from Teagasc Moorepark.

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1. Introduction

Ireland is one of the largest dairy exporter nations, exporting 85% of its dairy output worldwide. Irish milk is destined for production of a wide range of dairy products and nutritional ingredients, such as: butter, cheddar cheese, infant formula, yogurts, whey protein based products and dairy-based drinks (More, 2009). Since the abolition of milk quotas (2015) and launch of the Food Harvest 2020 programme, Irish milk production has been continuously increasing (DAFM, 2010; Lapple and Hennessy, 2012). While milk production expands, Irish milk quality maintenance is essential to meet specific requirements for dairy products and hold market share. Those specifications concern the microbiological load, composition and level of contaminants in milk, which can be determinants of its processability, nutritional value, as well as dairy products quality and safety (Malek dos Reis, 2013).

Several factors throughout the production chain could affect the quality of milk and dairy products. Livestock management, sanitation practices, milk cooling rates and storage conditions (time and temperature) on-farm can have a direct impact on the microbiological and compositional quality of raw milk. Enzymes produced by spoilage bacteria can cause compositional changes in milk, affecting its physico-chemical properties (e.g., pH and viscosity) and functional properties important for the manufacture of several dairy products (e.g., emulsifying ability, foaming ability and renneting properties). The seasonal character of the milk production system in Ireland is another determinant of milk microbiota. For example, the levels of thermoduric and thermophilic bacteria in milk tend to be higher during the winter periods, when livestock remain indoors, compared to the grazing periods; requiring appropriate management during those

different seasons. The decrease in milk collection frequency and consequent extended bulk tank storage are among the future challenges for dairy suppliers to produce high quality milk. In this scenario, it is important to determine optimum storage conditions that will minimally impact the bacterial load and technological value of milk. Prolonged milk storage will also potentially result in the increase of costs associated with energy usage for cooling, which will also be impacted by changes in the metering system. Therefore, investigations regarding the optimum balance between production costs and quality, including alternatives that decrease energy usage and efficiently cool milk, are necessary.

The quality of the bulk raw milk delivered to dairy processors is the main determinant of the final product quality; therefore, production practices adopted by each individual dairy supplier on-farm can have a major effect on the microbiological and compositional quality of the final product (Nada *et al.*, 2012). For example, the efficiency of thermal treatments to reduce the levels of spoilage and pathogenic bacteria in milk could be affected (Griffiths *et al.*, 1988a). Also, the activity of thermo-resistant enzymes produced by those bacteria can reduce the shelf life and cause sensorial defects in the final product, such as gelation in UHT milk. On the other hand, the indigenous microflora of milk can be an important determinant of flavour and texture development during ripening of cheese (Callon *et al.*, 2005; Fox *et al.*, 2017). For example, Tomasino *et al.* (2018) compared Cheddar cheeses made using raw milk and low-temperature long-time pasteurised milk, which were collected from different regions. The authors observed differences in the flavour compounds during ripening of those cheeses, which was attributed to differences in the microbiota in the milk collected from different regions. Further studies are necessary to understand how different bacterial

enzymes act (i.e., hydrolysis of specific caseins) and at what levels they would have a positive or negative effect on cheese sensorial characteristics.

Additionally, in the recent years, dairy processors have been focusing on monitoring the levels of residues from sanitation products in milk, such as trichloromethane (TCM), chlorate (CHLO), perchlorates (PCHLO) and quaternary ammonium compounds (QACs), which can negatively impact human health and the commercialisation of Irish products. At farm level, sanitation practices are the main contributors to residues levels in milk; therefore, the control of concentrations of those agents is targeted on-farm, as there is no processing technology to reduce them. The establishment of appropriate guidelines regarding milk equipment washing routines and use of chlorine-based products are currently required in the dairy industry, as well as investigations about the dynamics of residues concentrations throughout manufacturing processes, to understand the effect of processing parameters.

1.1. Milk quality parameters

Quality can be defined as the degree to which a set of inherent characteristics meet requirements. The dairy industry has to ensure the production of high-quality dairy products by ensuring the safety, nutritional value and specific sensorial characteristics of those products. Several factors throughout the milk production chain could affect those quality parameters, such as the microbiological load, composition and possible contaminants in milk.

The following topics will be discussed:

- main spoilage bacteria types that could be detected in milk throughout the production chain (mesophilic, psychrotrophic, lipolytic, proteolytic, thermoduric and thermophilic bacteria);
- pathogenic bacteria of most concern in the dairy industry (*Bacillus cereus* and sulphite-reducing *Clostridia* groups);
- factors that could affected the composition of milk;
- influence of somatic cells on milk quality;
- factors influencing iodine levels in milk and;
- TCM, CHLO, PCHLO and QACs residues in milk.

1.1.1. Bacteria types

Milk is a suitable culture medium for the growth of several types of microorganisms. The region, environment, sanitation practices, production and processing conditions are some of the factors that can influence the great variation of microorganisms in milk. Some of the main types of spoilage bacteria that could be found in milk, as well as their possible sources and products that they could be

present are shown in Table 1.1. The following sections will discuss the presence of mesophilic, psychrotrophic, lipolytic, proteolytic, thermoduric and thermophilic bacteria, as well as pathogenic bacteria (*Bacillus cereus* and bacteria from the *Clostridia* genera).

Table 1.1. Microorganisms associated with possible spoilage of milk and milk products (Walstra *et al.*, 2006).

Organism	Source	Heat resistant	Spoilage
Spore formers			
Bacillus cereus	Feed, dung, soil, dust	+	Sweet curdling, bitty cream in pasteurised milk and cream
Bacillus subtilis	Feed, dung, soil, dust	+	
Bacillus stearothermophilus	Feed, soil	+	Spoil sterilised milk
Clostridium tyrobutyricum	Soil, silage, dung	+	Spoil evaporated milk Late blowing in cheese
Coliforms			
Escherichia coli	Faeces, milking utensils, contaminated water	-	Spoil milk and cheese
Klebsiella aerogenes		-	Spoil milk
Lactic acid bacteria			
Lactobacillus species	Milking utensils, parlor	-	Sour milk
Lactobacillus lactis		-	Sour milk
Streptococcus thermophiles		+	Sour milk
Psychrotrophs			
e.g., *Pseudomonas*	Milking utensils, cold-stored milk	-	Hydrolyse protein and fat in cold-stored milk
Thermoduric bacteria			
e.g., *Micrococcus* species	Milking utensils	+	Can grow in pasteurised products
Yeasts	Dust, milking utensils	-	Spoil cheese, butter, sweetened condensed milk
Molds	Dust, dirty surfaces, feed	-	Spoil cheese, butter, sweetened condensed milk

1.1.1.1. Mesophilic bacteria

Aerobic mesophilic bacteria in raw milk could include bacteria of technological importance (i.e., lactic acid bacteria for the production of cheese) or spoilage bacteria, which include Gram-positive and Gram-negative strains that could have considerable effects on the quality of milk and dairy products (Cousin, 1982; Coppola *et al.*, 2008). Different bacterial groups could contribute to milk spoilage depending on the sanitary conditions, season and geographical origin; consequently, the microbiological ecology of raw milk is very complex (Ercolini *et al.*, 2009). For example, Ercolini *et al.* (2009) identified spoilage bacteria from the groups *Pseudomonas, Staphylococcus, Bacillus, Rodococcus, Staphylococcus, Lactococcus, Corynebacterium* and *Carnobacterium* in milk produced in southern Italy.

Mesophilic bacteria are enumerated in milk to monitor and to infer the sanitary conditions during the production, collection and handling of raw milk (Harding, 1995; Robinson, 2002). The test used is called total bacterial count (TBC) and can be undertaken using various methods: standard plate count, Petrifilm aerobic count, plate loop count, and automated bacteria counting (Lachowsky *et al.*, 1997; Laird *et al.*, 2004). Samples are usually incubated at 32 ˚C for 48 h. The growth of those bacteria can be affected by the storage conditions (temperature and time) or inadequate sanitisation of the milking equipment, as residual milk on the surface of milking or processing equipment are environments rich in nutrients and are favourable for the growth of microorganisms. High levels of TBC in milk are also associated with inappropriate teat preparation pre-milking, unclean milking and housing environment or mastitic cows. Appropriate

cow management and hygienic milking conditions on-farm are vital to ensure low TBC levels in raw milk (Coorevits *et al.*, 2008; O'Connell *et al.*, 2013).

According to the European Regulation EC No 853/2004, TBC in raw milk should be less than 100,000 cfu/ mL (5.00 $\log_{10}$ cfu/ mL; plate count at 30 ˚C) when milk is destined for dairy products manufacture. However, some Irish dairy processors apply a TBC limit of 50,000 cfu/ mL (4.70 $\log_{10}$ cfu/ mL), establishing price incentives for farmers when levels are lower than that limit. The Regulation EC No 853/2004 also established that immediately prior to processing, the TBC of raw bovine milk should be lower than 300,000 cfu/ mL (5.48 $\log_{10}$ cfu/ mL), while processed raw milk (after pasteurisation) destined for dairy products manufacture should have a TBC lower than 100,000 cfu/ mL (5.00 $\log_{10}$ cfu/ mL) at 30 ˚C.

1.1.1.2. Psychrotrophic bacteria

Psychrotrophic bacteria grow at refrigeration temperatures (7 ˚C or less), while their optimum and maximum growth temperatures are above 15 and 20 ˚C, respectively (Frank and Yousef, 2004; Moyer and Morita, 2007). Gram-negative or Gram-positive psychrotrophic bacteria can be found in milk. The Gram-negative group includes *Pseudomonas*, *Aeromonas*, *Serratia*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Enterobacter* and *Flavobacterium* bacteria; the Gram-positive group includes *Bacillus*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Streptococcus*, *Staphylococcus* and *Lactobacillus* (Cousin, 1982; Shah, 1994; Sorhaug and Stepaniak, 1997). The most common psychrotrophs that are found in raw milk belong to the genus *Pseudomonas spp.* (Ercolini *et al*., 2009).

The level of psychrotrophic bacterial count (PBC) in milk is influenced by the health status and hygiene of dairy cows, hygiene of the milking and housing environment, udder preparation, milking machines sanitisation and milk storage conditions (Cousin, 1982; Chambers, 2002; Cempirkova, 2007). According to Griffiths (2010), the PBC level in raw milk at collection should have a TBC:PBC ratio of 6:1. Therefore, considering that the TBC European threshold limit is 100,000 cfu/ mL, PBC level should be approximately 16,700 cfu/ mL (4.22 $\log_{10}$ cfu/ mL). Additionally, psychrotrophic bacteria represents up to 70% to 90% of the bacterial population in raw milk after 2 to 3 days of storage (Mottar, 1989; Sorhaug and Stepaniak, 1997; Skeie, 2007).

Psychrotrophs represent a major problem for the dairy industry, as several factors throughout the production chain can contribute to their growth, such as: low temperatures applied during milk storage, low frequency of raw milk collection from dairy farms (two or three times per week) and further storage of milk at low temperatures in the dairy plant (Sorhaug and Stepaniak, 1991; Champagne *et al.*, 1994; Shah, 1994). Psychrotrophs are capable of producing heat resistant extracellular proteases and lipases, which could survive pasteurisation (e.g., 72 ˚C for 15 s) or even ultra-high temperature processing (UHT; 138 ˚C for 2 s or 149 ˚C for 10 s) (Cousin, 1982; Lopez-Fandino *et al.*, 1993). Muir (1996) suggested that raw milk with a PBC higher than 5×10^{6} cfu/ mL should be rejected for processing, due to the possible high levels of lipases and proteases in that milk. Populations of psychrotrophs ranging from 10^6 to 10^7 cfu/ mL can produce sufficient amounts of extracellular enzymes to cause defects in milk that are detectable by sensory tests. Lipases and proteases hydrolyse triglycerides and proteins, respectively, causing sensorial defects and alteration of

the physico-chemical properties of dairy products (Walstra *et al.*, 2006). Lipolysis could result in flavour defects, for example, in cream, butter, cheese and UHT products; while proteolysis could be associated with bitterness in milk, gelation of UHT sterilised milk and could contribute for cheese yield reduction (Cousin, 1982; Braun *et al.*, 1999).

Strains from the genus *Pseudomonas spp.* represent not more than 10% of the microbiota in fresh raw milk; however, they become one of the main and most frequently identified populations in raw milk over storage. Consequently, most of the heat-resistant peptidases produced during storage are secreted by *Pseudomonas spp* (Marchand *et al.*, 2009; Baur *et al.*, 2015). Most of the psychrotrophic proteases, as well as proteases produced by *Pseudomonas*, are metalloproteases, which preferentially hydrolise κ-casein, then β-casein and then α_{S1}-casein (Decimo *et al.*, 2014). The heat-resistant peptidase alkaline metallopeptidase (AprX) is widespread among *Pseudomonas spp.* and are produced during the late exponential or early stationary growth phase of the bacteria, generally at bacterial count of 10^7 to 10^8 cfu/ mL (Stoeckel *et al.*, 2016). Therefore, the production of AprX is determined by the storage time, temperature, and the pseudomonads numbers. As those proteases exhibit a high thermal stability, they can remain active after heat treatments, such as UHT treatments. The thermostability of that protease could be due to the presence of zinc, calcium and the absence of S-S bridges in the protein. The activity of the protease AprX is optimal at a pH value between 7 and 9 and at a temperature range between 30 and 45 ˚C (Martins *et al.*, 2015). Zhang *et al.* (2018) shown that increasing concentrations of AprX resulted in increasing hydrolysis, mainly of κ-casein, and gelation in UHT milk. Bagliniere *et al.* (2013) used milk with 0.2 mg/ L of AprX

to produced UHT milk. Proteolysis of caseins increased over storage (increased NCN and NPN fractions) and the destabilisation of UHT milk was visual after only 8 days of storage. The authors also found the enzyme to hydrolyse all casein, with a preference for β-casein. The protease was produced by inoculating a complex medium with a *Pseudomonas fluorescens* strain. The medium was centrifuged, the supernatant was dialysed and after size-exclusion chromatography was used to obtain the protease. Stuknyte *et al.* (2016) observed that the thermo-resistant protease produced by *Pseudomonas fluorescens PS19* preferrely hydrolyse β- and κ-casein, when incubated at 7 or 22 ˚C over 6 days and 96 h, respectively. The majority of the current studies regarding AprX focused on identifying strains that may produce this protease, optimum conditions for production and characterisation of its activity. Also, most of those studies focused on evaluating the activity of thermo-resistant proteases from *P. fluorescens* on raw milk or UHT milk (Mateos *et al.*, 2015; Caldera *et al.*, 2016; Meng *et al.*, 2018). Further studies are still necessary to determine the impact that those proteases could have on dairy products, especially in manufacturing processes that involve heat-treatments.

1.1.1.3. Lipolytic bacteria

The microorganisms classified as lipolytic produce extracellular lipases, which are capable of hydrolysing milk fat (triacylglycerols) into mono- and di-glycerides, fatty acids and water (Deeth, 2006). As milk cold storage is favourable for the growth of psychrotrophic bacteria, the dairy industry has particular interest in the lipases produced by psychrotrophic bacteria due to their heat stability. Consequently, lipolysis could happen in a variety of dairy products, such as UHT

milk, milk powder, butter and cheese, during storage (Fitz-Gerald and Deeth, 1983; Shelley *et al.*, 1987). The main problems caused by lipolysis in milk are undesirable flavour production and altered functionality. Short- and medium-chain length free fatty acids have strong flavours, described as rancid, butyric, bitter, unclean and astringent. Lipolysis in milk also causes loss of foaming and creaming ability and could increase churning time during butter manufacture (Shelley *et al.*, 1987). The main lipolytic bacteria species that could be found in dairy products are *Bacillus*, including *B. cereus, B. polymyxa, B. licheniformis, B. circulans, B. subtilis, b. laterosporus* and *B. coagulans* (Matta and Punj, 1999). Other species, such as *Pseudomonas* also produce lipases when milk is stored at low temperatures (Deeth, 2006).

1.1.1.4. Proteolytic bacteria

Proteolytic bacteria produce extracellular enzymes that hydrolyse proteins into peptides and amino acids, or also have the ability to survive heat treatments, such as pasteurisation. The majority of proteolytic bacteria found in milk are psychrotrophs, which produce proteases in the late exponential or stationary phase of growth (Kohlman *et al.*, 1991; Chen *et al.*, 2003). The proteolytic bacteria count (PROT) in milk is useful as an indicator of inadequate handling and storage of raw or processed milk (Frank and Yousef, 2004; Pinto *et al.*, 2006). Psychrotrophs proteinases preferentially degrade the milk proteins of great technological importance: κ-, α_{s1}- and β- caseins (Gebre-Egziabher *et al.*, 1980). This results in defects in dairy products, such as bitterness in milk, gelation of UHT milk and reduced yields of cheese (Datta and Deeth, 2003). The main proteolytic psychrotrophic bacteria species that could be found in milk belong to

the *Pseudomonas* and *Bacillus* groups (Chen *et al.*, 2003; Martins *et al.*, 2006). Proteases produced by bacteria from the *Bacillus* group are resistant to heat and cleaning reagents, and consequently can survive several processing conditions and affect dairy products (Griffiths and Phillips, 1990).

1.1.2. Heat Resistant Bacteria

1.1.2.1. Thermoduric bacteria

Thermoduric bacteria are microorganisms capable of surviving in environments at temperatures higher than their maximum growth temperatures. The vegetative cells or spores of those microorganisms are capable of surviving heat treatments (i.e., pasteurisation) and multiplying, resulting in hydrolysis of protein or lipids in dairy products. Consequently, the quality and shelf life of those products is affected (Hayes and Boor, 2001; Frank and Yousef, 2004).

Thermoduric bacteria have their origin in the environment, occurring in feed, forage, bedding, dust, faeces and soil. Therefore, appropriate udder preparation prior to milking is essential to avoid the transference of high levels of those bacteria into milk; which could consequently contaminate milking machine and bulk tank surfaces (Gleeson et al., 2013; Doyle *et al.*, 2015). The main sources of milk contamination during the grazing and housing period are the soil and faeces or bedding material, respectively (Christiansson *et al.*, 1999; Magnusson *et al.*, 2007). Other sources of contamination of raw milk could be biofilms, mineral deposits on cracks or imperfections in the rubber parts of the equipment. Dairy suppliers should regularly inspect the milking equipment to identify deposits of organic matter and to ensure that the equipment is being appropriately cleaned (Buehner *et al.*, 2014).

The determination of thermoduric bacteria in milk can be performed by a test called a laboratory pasteurisation count, which consists of heating milk samples to 63 °C for 30 minutes and, after plating, samples are usually incubated at 32 ˚C for 48 h (Frank and Yousef, 2004). The following species of thermoduric bacteria have been reported to survive laboratory pasteurisation (63 ˚C for 30 minutes): *Microbacterium*, *Micrococcus*, *Bacillus* spores, *Clostridium* spores, *Alcaligenes*, *Streptococcus*, *Lactobacillus* and bacteria of the coryneform group (Chambers, 2002; Frank and Yousef, 2004; Hayes and Boor, 2001).

Irish dairy companies require a thermoduric bacterial count of less than 500 cfu/ mL in raw milk destined for processing. Buehner *et al.* (2014) assessed the levels of thermoduric bacteria in raw milk from 10 dairy farms in the eastern part of South Dakota, observing an average of 2.76 and 2.61 $\log_{10}$ cfu/ mL, during summer and winter, respectively. The authors observed a high variability in the levels of thermodurics on the 10 farms (summer: 1.46 to 4.45 $\log_{10}$ cfu/ mL; winter: 1.81 to 3.50 $\log_{10}$ cfu/ mL), suggesting that individual cleaning practices have a significant effect on the presence of thermodurics in milk. Some bacterial strains that belong to the enterococci genus are thermo-resistant and could be spoilage microrganisms, presenting a health risk to humans. McAuley *et al.* (2012) reported that strains of *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans* and *Enterococcus hirae*, isolated from six milk silos, were heat resistant to pasteurisation (63 ˚C for 30 min).

The two thermoduric bacteria of major concern for the dairy industry are: bacteria that belong to the *Bacillus cereus* group and the sulphite-reducing *Clostridia* group. Both are spore-forming bacteria and food-borne pathogens (Gleeson et al., 2013). Thermoduric spore-formers and their spores are of concern

during the manufacture of dried products due to the concentration effect. Currently, there are no international standards set for acceptable spore concentrations in milk powders. Buehner et al. (2015) measured thermoduric bacteria in milk powder from three different North American companies and found levels ranging from 2.8 ± 0.22 to 3.5 ± 0.15 $\log_{10}$ cfu/ g. Scott *et al.* (2007) evaluated milk powders from 18 different countries, reporting that 92% of the bacteria isolates were classified as *Geobacillus stearothermophilus*, *Bacillus licheniformis*, or *Anoxybacillus flavithermus*.

1.1.2.2. Thermophilic bacteria

Thermophilic bacteria are those that grow in environments at temperatures higher than 50 °C, and can grow abundantly at temperatures between 60℃ to 70℃ (Bergey, 1919). Therefore, temperatures applied in thermal treatments during dairy processing (e.g., pasteurisation, evaporation) are favourable for the growth of this type of bacteria (Murphy *et al.*, 1999). Initial thermophile contaminants possibly enter a dairy manufacturing process via raw milk in the form of endospores, which can originate from the farm environment: silage, bedding materials and livestock manure (McGuiggan *et al.*, 2002; Vissers *et al.*, 2007b; Quiberoni *et al.*, 2008). Thermophilic vegetative cells or spores can attach to equipment surfaces and germinate once the conditions are favourable (high water activity and temperatures), resulting in the formation of biofilms (Chen *et al.*, 2004b). Levels of thermophiles in raw milk are usually low (less than 10 cfu/ mL); however, it is essential to ensure appropriate sanitisation of the processing line to avoid the formation of those biofilms, which could contaminate the subsequent dairy products (McGuiggan *et al.*, 2002; Burgess *et al.*, 2010).

Therefore, the quantification of thermophiles in milk is an indicative test of hygienic conditions on-farm or within the processing plant (Abdul-Hadi *et al.*, 2014).

Thermophilic spore-forming bacteria are a concern in UHT products, as some strains have the ability to survive the temperatures applied during such process and subsequently grow under the ambient storage conditions of the product (Scheldeman *et al.*, 2006). Consequently, those bacteria can result in quality defects such as off-flavours and body defects (e.g., gelation). Proteolysis or lipolysis caused by thermophilic bacteria can also result in functional and flavour defects in dairy products such as milk powder, pasteurised milk, buttermilk or whey (Murphy *et al.*, 1999; Scott *et al.*, 2007; Abdul-Hadi *et al.*, 2014). The bacteria strains belonging to the *Bacillus* genus are the main thermophilic bacteria that can be found in milk. Some examples of thermophilic *bacilli* are: *Anoxybacillus flavithermus, Geobacillus spp., Streptococcus thermophilus, Bacillus licheniformis, Bacillus coagulans* and *Bacillus subtilis* (Crielly *et al.*, 1994; Flint *et al*., 2001; Ronimus *et al.*, 2003). Thermophilic bacteria are also related to pathogenic bacteria such as *Clostridium botulinum* and *Bacillus cereus*, which produce toxins that could cause severe foodborne diseases.

1.1.3. Pathogenic bacteria

1.1.3.1. Bacillus cereus

Bacillus cereus group, or *Bacillus cereus sensu lato* group, consists of seven closely related species, which cannot be distinguished by 16sRNA gene sequencing. Those bacteria are rod-shaped, motile, endospore-forming, aerobe-to-facultative and Gram-positive (Kumari and Sarkar, 2016), and also have

mesophilic and psychrotrophic strains (Svensson *et al.*, 2004). As *Bacillus cereus* strains possess spore-forming and psychrotrophic properties, those bacteria are able to survive both milk refrigerated storage conditions and heat treatments (Coghill and Juffs, 1979; Svensson *et al.*, 2006). The spores of those bacteria can germinate in dairy products during storage and produce enterotoxins and emetic toxins, which could cause diarrheal and emetic gastrointestinal syndromes, respectively (Di Pinto *et al.*, 2013). Enterotoxins are heat-labile, while emetic toxins are heat-stable (temperatures up to 90 ˚C) and can survive in a pH range of 2 to 11 (ICMSF 1996; Granum and Lund, 1997; Clavel *et al.*, 2004). Both toxins can be produced at low temperatures (Christiansson *et al.*, 1999).

One of the main concerns of the Irish dairy industry is the contamination of infant formula with *Bacillus cereus*, as their spores germinate and produce toxins when the product is reconstituted for consumption (Di Pinto *et al.*, 2013). Haughton *et al.* (2010) reported that 24% of infant formula samples (representing 10 leading brands in Ireland), contained *Bacillus cereus*. After reconstitution and storage over 24 h, *Bacillus cereus* strains were detected in 35% of the samples at levels of 10^3 cfu/ g. The European regulation EC 1441/2007 states that four out of five samples of infant formula produced in a commercial processing plant should have less than 50 cfu/ g of that microorganism, while the fifth sample can have levels between 50 and 500 cfu/ g.

Other studies have reported the contamination of pasteurised milk with bacteria from the *Bacillus cereus* group. Salustino *et al.* (2009) have identified *Bacillus cereus* in pasteurised milk and post-pasteurisation equipment surfaces, which were: storage tanks, packaging machines, levelling tanks and package-forming tube surfaces. The contamination during or post-pasteurisation may occur

due to the presence of heat resistance spores in raw milk or by milk recontamination, due to inadequately sanitized surfaces (Boor, 2001; Shaheen *et al.*, 2010; Bermudez-Aguirre *et al.*, 2012). *Bacillus cereus* spores have the ability to adhere and form biofilm onto equipment surfaces, which is potential source of contamination of processed milk and finished dairy products (Shaheen *et al.*, 2010). The biofilm protects spores and vegetative cells against cleaning agents and some spores could develop resistance to antimicrobial agents (Ryu and Beuchat, 2005).

Bacillus cereus bacteria are widely distributed in the environment. During the grazing period, the main sources of contamination are soil, feed and manure, which contaminate the cows' udder through direct contact (Griffiths and Phillips, 1990; Magnusson *et al.*, 2007). Contamination of cows' udder and teats, in addition to the absence of or inadequate teat preparation prior to milking, results in the contamination of the milking equipment and raw milk.

Traditional culture methods have been unable to differentiate *Bacillus cereus* from *Bacillus cereus* group species (*B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. weihenstephanensis* and *B. anthracis*) (FDA, 2012). Only presumptive colonies of *Bacillus cereus* can be identified using those methods and additional phenotypic analyses are necessary to identify the species within the *Bacillus cereus* group. According to O'Connell (2015), the use of BACARA agar (Biomerieux, Hampshire, UK) is more accurate for the detection of *B. cereus* than mannitol egg yolk polymyxin agar (MYP).

1.1.3.2. Sulphite-reducing Clostridia

Sulphite-reducing *Clostridia* have the ability to reduce sulphite to sulphide under anaerobic conditions. The main sources of *Clostridia* organisms on-farm are soil, feed and manure, where their spores are naturally present (Vissers *et al.*, 2007a; Drean *et al.*, 2015). Milk can be contaminated with spores that contaminated teats during milking (Aureli and Franciosa, 2002). The European Union has not stated limits for the presence of *Clostridia* strains or spore-formers in raw milk or dairy products (Lindstrom *et al.*, 2010). The pathogenic species *Clostridium perfringens* and *Clostridium botulinum* are SRCs and are the bacteria of most relevance to the dairy industry, mainly in the infant formula manufacturing process, due to their capacity of producing toxins responsible of causing severe foodborne diseases (Doyle *et al.*, 2015).

Clostridium perfringens is an anaerobic, Gram-positive spore-forming bacteria, which optimum growth temperature ranges from 43 to 45 ˚C. Some strains are capable of growing at lower and higher temperatures, extending the temperature growth range (15 to 50 ˚C). *Clostridium perfringens* grows at a pH 6 to 7; which includes the pH of milk (6.8). The spores of this microorganism can survive great extremes of pH (lower than 5.0) and water activity (Fernandes, 2009). The vegetative cells are not capable of surviving temperatures higher than 50 °C, being eliminated during heat treatments (Fernandes, 2009). Additionally, vegetative cells cannot grow at refrigeration temperatures within the range of 0 and 10 ˚C, which include temperatures applied during milk storage (Fernandes, 2009). However, spores of *Clostridium perfringens* present a wide variety of heat resistance levels, with D values at 95 ˚C that could range from 17.6 to 64 min (Wrigley, 1994; Labbe and Juneja, 2006; Fernandes, 2009). Thus, these spores

survive thermal treatments and consequently contaminate dairy products. *Clostridium perfringens* type A strains produce an enterotoxin responsible for causing gastrointestinal disease (i.e., diarrhoea, cramps, nausea and vomiting (Kokai-Kun *et al.*, 1994; Andersson *et al.*, 1995).

Clostridium botulinum is a strictly anaerobic bacterium, with optimum growth temperature ranging from 20 to 45 ˚C. Those microorganisms are classified in four main groups; however, the most common organisms responsible for food-borne botulism belong to the proteolytic (I) and non-proteolytic (II) groups (Peck, 2005). Several researchers have reported that the minimum and maximum growth temperatures of bacteria from group I is approximately 10 and 50 °C, respectively (Johnson, 2000; Hinderink *et al.*, 2009). Group II is composed of psychrotrophic strains, which grow and produce toxins at temperatures as low as 3 °C (Kim and Foegeding, 1993; Lund and Peck, 2000). Therefore, the botulin toxin is produced at different stages of the milk production, from cold storage, throughout processing stages (e.g., heat treatment) and during dairy product storage. However, all toxins produced by *C. botulinum* are heat-labile, and is inactivated when heating milk to 80 °C for 20 to 30 min, to 85 °C for 5 min, or to 90 °C for a few seconds (Fernandes, 2009).

Vegetative cells of *Clostridium botulinum* are not heat-resistant; however, the spores from both Groups I and II are heat-resistant and are capable of surviving heat processes employed in the food industry (Fernandes, 2009; Silva and Gibbs, 2010). Temperatures higher than 100 °C are required to eliminate spores from strains from Groups I, while spores from Group II can be eliminated at temperatures lower than 100 °C (Lindstrom *et al.*, 2010). After processing, when conditions are favourable (e.g., storage temperature, pH, water activity),

surviving spores can germinate and grow to attain high numbers and cause foodborne diseases or spoilage (Silva and Gibbs, 2010). *Clostridium botulinum* outbreaks in dairy have been reported in Argentina (17 outbreaks), Australia (1), Iran (1), Italy (1), Kenya (1), Turkey (1), United Kingdom (2) and United States (9). The mean fatality rate of those outbreaks was 17.9%, some related to the consumption of commercial milk or dairy products or even home-prepared dairy products. Factors that could be the causes include: unsafe formulation, inadequate fermentation, insufficient thermal processing, post-process contamination and lack of quality control (Lindstrom *et al.*, 2010).

1.1.4. Milk composition

Storage and processing conditions can result in physical, chemical and biochemical changes in milk composition (Table 1.2). Some of those changes to the milk components of high technological value (fat and protein) will be discussed in the following sections.

Table 1.2. Typical composition of bovine milk (Walstra *et al.*, 2006).

Component	Average content in milk (% w/w)
Water	87.1
Lactose	4.6
Fat	4.0
Protein	3.3
- casein	2.6
Minerals	0.7
Organic acids	0.17
Miscellaneous	0.15

1.1.4.1. Fat

The lipid content in bovine milk consists of a mixture of neutral glycerides (triglycerides, diglycerides and monoglycerides), corresponding to 98 % of milk fat, while the other 2% consists of free fatty acids (i.e. phospholipids, glycolipids, glycosylceramides, sterols, carotenoids and vitamin A) (Table 1.3). The lipid phase of milk is distributed in the form of droplets (≤ 1 to approximately 1 μm in diameter) that are surrounded by an amphiphilic membrane. This milk fat globule membrane (MFGM) is composed mainly by proteins and lipids, as well as carotenoids, vitamin A and minerals (iron and copper) (Walstra *et al.*, 2006).

Table 1.3. The classes of lipids in milk (Adapted from Jenness & Walstra, 1984; MacGibbon & Taylor, 2006).

Lipid Class	Average Amount (%, w/w)
Triacylglycerols	98.3
Diacylglycerols	0.3
Monoacylglycerols	0.03
Free fatty acids	0.1
Phospholipids	0.8
Sterols	0.3
Carotenoids	Trace
Fat-soluble vitamins	Trace
Flavour compounds	Trace

According to the National Milk Agency report (2018), the average fat in raw milk supplied during 2018 was 4.14%. The fat content of Irish milk can vary from approximately 3.3% during summer months to 4.1% during winter months. Bovine milk contains 3% to 5% w/ w fat, which can vary depending on the phenotype of the cow, season and lactation stage. Those factors can also

considerably influence the fatty acid profile in milk, which in turn may affect the rheological and crystallisation properties of milk fat (Shi *et al.*, 2001). Those properties have an important role in the manufacture of several dairy products, such as butter, whipped cream and ice cream. For example, the milk fatty acid composition could influence the melting properties, microstructure, yield, texture and flavour of cheese (Guinee *et al.*, 1999). In butter manufacture, the three-dimensional crystalline network formed by milk fat determines the yield stress and viscoelasticity of the final product (Palmquist *et al.*, 1993).

The structure and composition of fat globules could be also affected by low and high temperatures applied during milk storage and processing. According to Lindmark-Mansson and Akesson (2000), lipids in milk would be more prone to autoxidation when milk is stored at low temperatures, as the activity of an antioxidative enzyme (i.e., superoxide dismutase) decreases in that condition. Agglutination of the fat globules is another phenomenon that may occur at low temperatures due to the adsorption of a protein called agglutinin on the globules surface, resulting in aggregation. The floccules thus formed rise, as fat has a lower density then milk plasma, forming a cream layer. Therefore, the regular agitation of milk during cold storage is necessary to avoid fat separation and maintain a homogenous milk composition. Milk cold storage could also lead to irreversible migration of some components of the MFGM to the milk plasma, such as phospholipids, proteins, xanthine oxidase and copper (Walstra *et al.*, 2006). Patton *et al.* (1980) stored milk at 2 – 4 ˚C for 24 h and observed an increase of 18% on the skim milk phospholipids content. The MFGMs are considered efficient emulsifiers and can influence the microstructure and flavour of ripened cheese and

also have a high water-holding capacity, resulting in greater cheese yield in some cases (Kanno *et al.*, 1991; Goudedranche *et al.*, 2000; Lopez *et al.*, 2007).

Furthermore, thermal degradation of lipids in milk is generally not observed during processing, as temperatures greater than 200 ˚C are required to promote non-oxidative decomposition of fatty acids. However, temperatures greater than 70 ˚C could result in the denaturation of the proteins in the MFGM. The removal of phospholipids from the MFGM could also occur at elevated temperatures (Tamime, 2007).

When in sufficient numbers, spoilage microorganisms in milk could produce lipases, which hydrolyse lipids into fatty acids and partial glycerides. Those compounds are responsible for sensory changes in dairy products, such as off-flavours (rancid, butyric, bitter, unclean, soapy or astringent), and could also result in a lower foaming ability, reduction of shelf life, reduction in solubility, wettability, and flow characteristics of milk powder (Kirst, 1986; Ray *et al.*, 2013). Psychrotrophic bacteria, such as *Pseudomonas*, produce heat-stable lipases, and therefore are not inactived during HTST and to UHT (approximately 140 ˚C for 4s) treatments. Their effects are observed in dairy products (i.e., UHT milk, butter, cheese, milk powders) after a certain period of storage (Deeth, 2006). Additionally, the MFGM does not appear to be a barrier to those lipases to access the fat in intact fat globules (Fitz-Gerald & Deeth, 1983; Deeth, 2006). Decimo *et al.* (2014) tested 80 psychrotrophic bacterial strains, isolated from Italian bulk milk tanks, regarding their ability to produce lipases and proteases. The authors reported that the majority of the strains (59) presented lipolytic activity at different incubation temperatures (7, 22 and 30 ˚C) when bacterial levels reached a range of 4.0 to 6.0 $\log_{10}$ cfu/ mL. Most of those strains belong to the

Pseudomonas spp. genus (*P. fluorescens, P. fragi, P. aeruginosa, P. fulva, P. libanensis, P. mosselii, P. putida, P. rhodesiae, P. teatrolens, P. chlororaphis*).

1.1.4.2. Proteins (caseins)

According to the National Milk Agency report (2018), the average protein content in raw milk supplied during 2018 was 3.48%. O'Brien *et al.* (1999) reported that the protein content of Irish milk can vary from 3.2% during summer months to 3.6% during winter. Approximately 75% to 80% of the milk proteins are classified as caseins, which precipitate at pH 4.6. The caseins are phosphoproteins, denominated α_{s1}-, α_{s2}-, β- and κ-caseins, and are presented in milk as micelles, (Figure 1.1). Almost all casein is present in roughly spherical particles (40 to 300 nm in diameter), which comprise approximately 10^4 casein molecules. The casein micelles also contain inorganic matter, mainly calcium phosphate, small quantities of some other proteins (i.e., proteose peptone) and certain enzymes. The micelles also hold a significant amount of water, giving a hydrodynamic voluminosity of about 4 mL/ g of dry casein. The core of the micelle consists of roughly equal amounts of α_s- and β-casein, with small amounts of κ-casein, while the outer layer consists of about equal amounts of κ- and α_s-casein. The surface area is mainly composed of κ-casein. That "hairy layer" consists of the C-terminal end of κ-casein and is hydrophilic and negatively charged. This layer is essential in providing colloidal stability (Walstra *et al.*, 2006). Some models consider that the micelle is built of mixed-composition submicelles (12 to 15 nm in size), as shown in Figure 1.1. Nanoclusters of calcium phosphate and protein moieties are also present in the micelle structure.

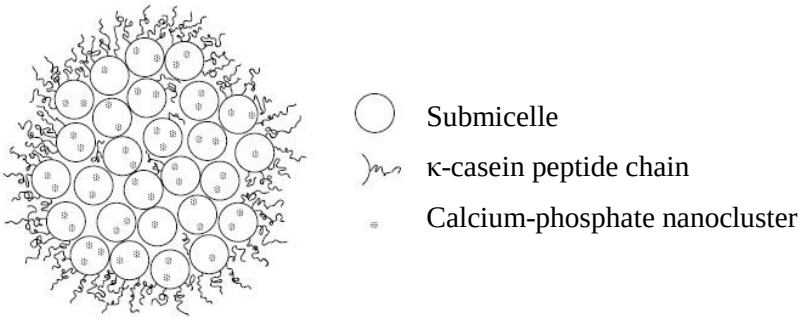


Figure 1.1. Cross section through a tentative model of a casein micelle (Walstra *et al.*, 2006).

A casein micelle and its surroundings are constantly exchanging components (Figure 1.2). Mineral compounds, such as calcium, phosphate and citrate, are exchanged rapidly; while casein can diffuse in and out of each micelle. Temperature, pH and calcium activity are factors that could influence the dynamic equilibrium in the micelles.

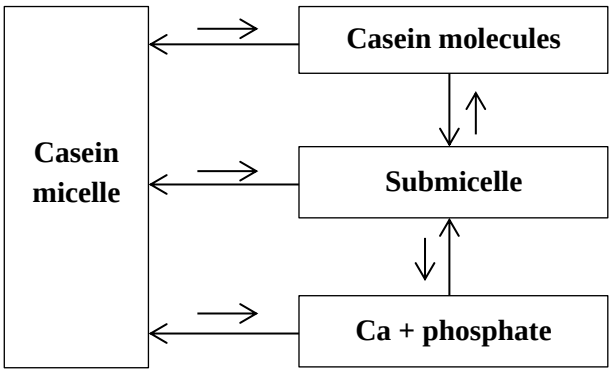


Figure 1.2. Outline of the main dynamic equilibria between casein micelles and milk serum (Walstra *et al.*, 2006).

When milk is stored at low temperatures, the β-casein is the most affected. At temperatures below 5 ˚C, the hydrophobic bonds responsible for the β-casein binding become weaker, resulting in their dissolution into milk serum phase (Creamer *et al.*, 1977; Walstra *et al.*, 2006). Davies and Law (1983) reported that,

after 48 h of milk storage at 4℃, approximately 60% of the micellar β-casein migrates to the serum phase. Other caseins, such as α_s-casein, dissociate into the serum phase but in lower quantities. Consequently, these caseins can be converted into γ-casein and protease-peptones by plasmin and bacterial proteinases, respectively (Eigel *et al.,* 1979; Snoeren and Van Riel, 1979; Walstra *et al.* 2006). Additionally, the association of Ca^{2+} ions with α_{s1}-caseins decreases with decreasing temperature, resulting in the dissolution of part of the colloidal calcium phosphate and causing a weaker binding of individual casein molecules in the micelles (Walstra *et al.,* 2006). These changes occur slowly, taking 24 h to be observed, however, they are reversible when milk is heat up to temperatures between 20 ˚C and 40 ˚C (Creamer *et at.,* 1977; Davies and Law, 1983; Walstra *et al.,* 2006). Even if those changes are reversible, it is still questionable whether the resulting casein micelles are identical to the original micelles.

Changes in the micelles can affect milk properties, which determine physical stability of milk products during heat treatment, concentrating and storage, as well as rheological properties of acidified and concentrated milk products (Walstra *et al.,* 2006). For example, the high β-casein concentrations in the serum have a technological impact on several dairy products. In the case of cheese manufacture, some effects are loss of fat and curd fines in whey, as well as prolonged clotting times. Consequently, a weak curd structure and low cheese yields are obtained (Ali *et al.*, 1980). Also, the colloidal stability of the micelles is greater and therefore milk presents poor rennetability during cheese making (Walstra *et al*., 2006).

High temperatures (above 100 ˚C) could also result in the destabilisation of the micelles by promoting the dissolution of part of κ-casein. This effect also

depends on the pH of the medium, no dissolution occurs below pH 6.2 and complete dissolution can happen at pH 7.2. This effect could be partly due to the increased effect of entropy at high temperatures and/ or due to the absence of serine phosphate in part of the κ-casein chain, resulting in weak bonds of that casein with colloidal phosphate. Furthermore, another effect of high temperatures is the association of serum proteins with casein micelles during heat denaturation, e.g., association of β-lactoglobulin with κ-casein, which is irreversible on cooling. Changes in the micelle structure may also occur due to decreases in pH. Some of those changes are: solubilisation of the colloidal phosphate, association of hydrogen ions with acid and basic groups of the proteins, increased Ca^{2+} ion activity and increased hydrophobicity of casein (Walstra *et al.*, 2006).

Proteases from spoilage bacteria, as well as indigenous proteases, hydrolyse proteins in milk. Consequently, the changes in protein structure promoted by those enzymes in milk may affect the manufacture of dairy products (i.e., rennet coagulation during cheese production), resulting in sensorial defects (i.e., bitterness) or affecting product stability (i.e., gelation in UHT milk).

1.1.5. Somatic cell count

Somatic cell count (SCC) is an indicator of udder health, milk microbiological and technological quality (Smith, 2002; Piccinini *et al.*, 2006). Somatic cells arise mostly from the immune system and comprised of different cell types, for example, lymphocytes, macrophages, polymorphonuclear leucocytes and some epithelial cells (Sordillo *et al.*, 1997; Pillai *et al.*, 2001). Increases in SCC could be due to a response of the immune system to an intramammary infection (e.g., mastitis) or due to the late stage of lactation

(Leitner *et al.*, 2006; Merin *et al.*, 2008). On farm, several factors can influence SCC in milk, such as animal species, milk production level, lactation stage and management practices (Rupp *et al.*, 2000). The European Directive 92/46 (1992) states that milk with a SCC over 4 x 10^5 cells/ mL is considered unfit for human consumption and should not be used for further processing. Financial incentives are offered by some Irish dairy processors when farmers produce milk with SCC lower than 200,000 cells/ mL.

A range of indigenous enzymes can be released into milk due to lysis, leakage or secretion from the somatic cells: lipases (e.g., lipoprotein lipase), oxidases (e.g., catalase and lactoperoxidase), glycosidases (e.g., lysozyme) and proteases (e.g., cathepsins, elastase and collagenase) (Kelly and Fox, 2006; Li *et al.*, 2014). Those enzymes are active at a large range of pH and could have a negative impact on milk composition, affecting the quality and yield of dairy products (Santos *et al.*, 2007; Li *et al.*, 2014; Cinar *et al.*, 2015). Additionally, the types, quantity and activity of those enzymes can be influenced by the different levels and types of somatic cells in milk, leading to different levels of proteolysis and lipolysis (Li *et al.*, 2014). Litwinczuk *et al.* (2011) observed a negative relationship between casein content in milk and SCC, when studying milk from four breeds with four different levels of SCC, ranging from levels lower than 1 x 10^5 cells/ mL to 1 x 10^6 cells/ mL. Malek dos Reis *et al.* (2013) reported that each increase of 1 x 10^5 cells/ mL in SCC reduced the lactose and non-fat solids content of milk by 0.02%. Forsback *et al.* (2010) observed that milk with SCC levels greater than 1 x 10^5 cells/ mL had lower α- and β- casein contents then in milk with SCC lower than 1 x 10^5 cells/ mL. Some studies have reported that milk storage temperature or time had no effect or resulted in small variations on SCC

(Sierra *et al.*, 2006; Erdem *et al.*, 2012; O'Connell *et al.*, 2016); however, further studies are necessary to determine the effects of milk storage conditions on the stability of somatic cells, such as lysis.

Prior to processing, the mixing of many milk volumes might minimise the effects of high levels of SCC of milk from individual milk suppliers; however, it is still essential to control SCC on-farm to avoid the possible negative effects on milk technological properties (Kelly *et al.*, 2009). Geary *et al.* (2013) reported that each $\log_{10}$ increase in SCC was positively correlated with non-protein nitrogen (NPN, 0.006%), whey protein (0.041%), and negatively with lactose (-0.14%) and casein levels as a percentage of total protein (-0.96%); therefore, high SCC could negatively impact the final yield of dairy products. During cheese manufacture, for example, high SCC could contribute to increased casein loss into whey and increased curd formation time (Leitner *et al.*, 2008; Forsback *et al.*, 2011).Geary *et al.* (2013) applied a meta-analysis across several studies to investigate the relantionship between SCC and cheese processing characteristics. The authors reported that fat and protein recovery, as well as the protein content of cheese, significantly descrease as SCC levels increase in milk. Ubaldo *et al.* (2015) used milk with different SCCs to produced Mozzarella cheese and observed that the milk with the highest SCC had higher pH, lower lactose contents and higher proteolysis levels compared to the milk with lower SCC. However, those differences did not affect the sensorial characteristics of the cheese produced.

1.1.6. Residues

1.1.6.1. Trichloromethane

Trichloromethane ($CHCl_3$, TCM) is a by-product formed when residual chlorine comes into contact with milk. In Ireland, 77% of detergent steriliser products used for cleaning milking equipment are chlorine-based, as these are considered the most effective and economical; however, the misuse of those products could lead to the formation of TCM in milk (Gleeson and O'Brien, 2011). When the cleaning solvent is not completely rinsed from the equipment surface, TCM is formed in the milk that subsequently comes in contact with those surfaces (Ryan *et al.*, 2012). The International Agency for Research on Cancer states that TCM could be possibly carcinogenic to humans (ICAR, 1999).

Detergent steriliser products should contain less than 3.5% of chlorine, and should be used as recommended by the manufacturers (Gleeson and O'Brien, 2011). Gleeson and O'Brien (2010) reported that farms using cleaning products which contain a high chlorine content (> 8%) are more likely to have high levels of TCM residues in milk. Other recommendations to avoid TCM formation are to drain pipelines after milking and from bulk tanks after collection and rinse out each milking cluster with 14 L of clean water (O'Brien, 2009; Ryan *et al.*, 2013). Resch and Guthy (2000) observed that the lack of rinsing milking equipment results in an increase in the TCM concentration in the detergent solution and in the milk subsequently passing through the milking machine. Additionally, the recycling of the detergent solution could result in further TCM concentration increase in milk. Gleeson and O'Brien (2010) concluded that the main factors influencing TCM levels in Irish raw milk are: incorrect use of chlorine-based

cleaning products, insufficient rinse water volume, reuse of rinse water and use of chlorine for udder preparation prior to milking. According to Ryan *et al.* (2013), sanitisation practices on-farm are the key to controlling TCM levels in milk.

The European Union has no regulations regarding TCM levels in foodstuffs; however, some countries have determined limits of that substance in certain dairy products. For example, the German importers require that lactic butter should contain less than 0.030 mg/ kg (Verordnung über Höchstmengen an Schadstoffen in Lebensmitteln, 2003). In order to achieve those levels in butter, Irish processors apply a limit of 0.015 mg/ kg of TCM in milk. Therefore, it is economically important to Ireland to achieve those TCM levels required by markets, in order to maintain a competitive position.

Fat-based products contaminated with TCM are the main concern for the dairy industry, especially for the Irish lactic butter export market. Hubbert *et al.* (1996) reported that chlorinated hydrocarbons, such as TCM, accumulate in fat-rich portions. During cream or butter manufacture, milk cream is concentrated, consequently the TCM content in the final product increases (Resch and Guthy, 1999). Since 2007, TCM levels in Irish butter have been gradually reduced from 0.07 mg/kg to 0.03 mg/ kg, targeting the limit determined by the German market, which is one of the main Irish butter importers. Corrective measures have been applied at farm level by monitoring sanitisation practices, constantly visiting and advising farmers, and also performing routine screening for TCM in both tankers and individual suppliers' milk (Jordan *et al.*, 2012). According to Ryan *et al.* (2013), regular farm visits are essential to ensure fast and immediate resolution of milk quality issues.

1.1.6.2. Chlorate and perchlorate

Chlorine gas, dioxide or hypochlorite may be used for the sanitisation of drinking water or water for food production, while chlorine-based detergents (e.g., sodium hypochlorite) are used for the sanitisation of milking or processing equipment due to their bactericidal properties and efficiency (Gates *et al.*, 2009; Garcia-Villanova *et al.*, 2010; Gleeson and O'Brien, 2011, McCarthy *et al.*, 2018). However, the decomposition of chlorine compounds result in the production of oxyhalide species (ClO^- and ClO_2^-) that react and form chlorate (ClO_3^-, CHLO). Further reactions of CHLO with those oxyhalides result in the formation of perchlorate (PCHLO) (Gordon and Tachiyashiki, 1991). Laboratory tests on animals demonstrated that the primary targets of CHLO toxicity in the organism are the thyroid gland and the haematological system (EFSA, 2015).

At farm level and in the processing plant, the same precautions to avoid TCM formation are applied to avoid milk contamination with CHLO or PCHLO when using chlorine-based products. Additionally, chlorine-based detergents should be purchased often and stored under cool dark conditions (for periods no longer than 3 months) (O'Brien, 2016).

Water destined for food production or equipment sanitisation could also contain CHLO (McCarthy *et al.*, 2018). Gleeson (2016) measured CHLO levels in water from Irish industries in different water schemes and reported concentrations varying from a minimum of 0.058 mg/ L to a maximum of 0.340 mg/ L. Fenelon (2016) reported CHLO levels in mains water utilised by dairy processors varied from 0.059 to 0.246 mg/ L. In both studies, CHLO levels were in accordance to the threshold limit determined by the World Health Organisation (0.7 mg/ L). Nevertheless, both studies highlighted the importance of using water with low

CHLO levels or chlorine free, as water is a potential contributor to increasing CHLO levels throughout the dairy production chain.

The European regulation (EC) no 396/2005, which states the maximum residue levels of pesticides in or on food, have not established a threshold for CHLO or PCHLO. The Commission Directive 2006/141/EC proposed a default limit of 0.0100 mg/ kg for foodstuff. That limit is applied for reconstituted infant formula, corresponding to approximately 0.0814 mg/ kg of CHLO in non-reconstituted infant formula. Some Irish dairy processors require that CHLO levels in skim milk or whey powders should be 0.050 mg/ kg.

Chlorate levels in infant formula are a concern to the dairy industry, as infants have a lower tolerance to intoxication compared to adults. Milk is evaporated and concentrated during the manufacturing process of milk powder; consequently, the CHLO content per gram of product increases. In contrast to TCM, CHLO is highly water-soluble and ionic; therefore, in process involving cream separation, CHLO will remain in skimmed milk (Smith and Taylor, 2011).

Kettlitz *et al.* (2016) measured chlorate levels in several European food products. Out of the skim and whole milk powder products tested, 97% contained more than 0.0100 mg/ kg (range: 0.0100 to 0.6800 mg/ kg), while 87% of the whey powder and milk protein concentrates tested contained levels ranging from 0.0100 to 16.50 mg/ kg. Those groups had the highest percentage of positive samples among all the dairy products tested.

1.1.6.3. Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) have the basic structure NR_4^+, a nitrogen atom linked to four alkyl groups, which could vary in structure and

complexity. Quaternary ammonium compounds molecules possessing long alkyl chains are known to be effective as antimicrobial agents and are useful for the disinfection of containers and surfaces (Xian *et al.*, 2016). The main primary QACs that may be identified in milk and dairy products are: benzyldimethyldodecylammonium chloride (BAC 12), benzyldimethyltetradecylammonium chloride (BAC 14), benzyldimethylhexadecylammonium chloride (BAC 16) and didecyldimethylammonium chloride (DDAC) (Reuter, 2015).

In the European Union (EU), DDAC and BAC are allowed for use in the food industry, as they are believed to be environmentally benign because they do not bio-accumulate and have a short degradation half-life (Bassarab *et al.*, 2011). However, since studies reported QAC toxicity for different organisms (e.g., fish, algae and protozoan) and effects on human health, its use had to be re-evaluated (Kreuzinger *et al.*, 2007; Voorde *et al.*, 2012; Chen *et al.*, 2014; Zhang *et al.*, 2015). Currently, there are no specific limits for QACs in foodstuffs; however, it was recommended by the European Union Reference Laboratory (EURL; EU 396/2005) that the general residue limit of 0.0100 mg/ kg should be applied.

In the dairy industry, products employed for the sanitisation of processing equipment, collection tankers and silos could contain QACs. On farm, teat sanitising wipes or dips, as well as disinfectant agents for milking equipment could contain QACs, as those compounds could aid in preventing mastitis or in producing milk with low bacterial levels. Eventually, if those products are not appropriately removed from the surfaces after application, they may come into contact with milk (Xian *et al.*, 2016). Some QACs (e.g., benzalkonium) could

have adverse effects on human health, such as causing asthma, skin allergy or eye irritation (Voorde *et al.*, 2012).

The European Food Safety Authority (EFSA) published a report on levels of BACs and DDAC in several food products, which were collected from 16 member states and Norway (EFSA, 2013). BACs and DDAC were detected in 12% of the dairy products tested, corresponding to the highest percentage compared to other food groups tested. DDAC was detected in ice cream (3.64 mg/ kg), in butter and other churned products (concentrations varying from 0.010 to 0.030 mg/ kg) and in unprocessed milk (concentrations varying from 0.01 to 0.03 mg/ kg). BACs were detected in ice cream (1.4 mg/ kg), churned dairy products (1.1 mg/ kg) and cheese (concentrations varying from 0.010 to 0.070 mg/ kg).

1.1.6.4. Iodine

Iodine is a micronutrient essential for humans and animals for the production of thyroid hormones thyroxine and triiodothyronine, responsible for key metabolic processes and the development of body and brain (Leung and Braverman, 2014). According to the World Health Organisation (WHO, 1996; WHO 2007), the recommended daily iodine intake is 90 µg for infants between 0 and 6 years, 120 µg for infants between 6 to 12 years, 150 µg for adolescents (above 12 years) and adults, and 250 µg for pregnant and lactating women. Excesses of iodine in the organism could result in thyroid dysfunction in vulnerable patients (infants or elders), such as hyperthyroidism or hypothyroidism (Leung and Braverman, 2014). One of the most important iodine sources for humans is bovine milk. The main factors that could influence the iodine content in milk are: animal feeding (e.g., supplementation with iodine-containing mineral-

feed), teat dipping, as well as factors throughout milk processing (Borucki Castro *et al.*, 2012; Flachowsky *et al.* 2014).

The EFSA (2005) reported that the average iodine content in bulk tank milk samples from several European studies varied predominately between 100 and 200 µg/ L of milk. Those concentrations were suitable to meet the required iodine daily intake defined by WHO for children, adolescents and adults. Iodine intake is the most important influencing factor on the iodine content in milk. Rations supplemented with iodine are used in order to meet the iodine requirements for food-producing animals. The British Agricultural Research Council (1980) and the US National Research Council (NRC, 2001) recommend that the daily iodine intake by dairy cows should be 10 mg. Dairy cows with deficiency require a higher iodine supplementation (Flachowsky *et al.*, 2014). Sources of iodine in cows' diet can be forage, ration or nutritive additives. The iodine content of forage could vary according to the location, concentrations in the soil or vegetation (Suttle, 2010). In relation to rations, the iodine content could vary from 10 to 40 mg/ kg in rations usually commercialised by Irish dairy companies. The nutritive additives approved by the EU-Legislative (2005) for feed supplementation are: sodium iodide (NaI), potassium iodide (KI), calcium iodate hexahydrate ($Ca(IO_3)_2 \cdot 6H_2O$) and anhydrous calcium iodate ($Ca(IO_3)_2$). Incorrect feeding management, such as utilisation of rations with higher levels of iodine than required or overfeeding cows, could result in high concentrations of iodine in milk. Borucki Castro *et al.* (2012) reported a linear effect of dietary iodine content on milk iodine concentration ($P<0.0001$), when providing feed with 0.3, 0.6 and 0.9 mg iodine/ kg dry matter. Franke *et al.* (2009) used iodine supplements in feed at different concentrations (0, 0.5, 1, 2, 3, 4 and 5 mg/ kg of

dry matter) and also observed a linear relationship between iodine concentration in feed and that in milk.

Teat disinfectants containing iodine are still common for teat disinfection pre- or post-milking. However, the correct application of those products is required to avoid significant increases in the iodine content in milk. Iodine-based teat disinfectants can be absorbed through the teat skin and transferred to milk during synthesis; milk can also be contaminated when in contact with the teat skin surface (Conrad and Hemken, 1978; Rasmussen *et al.*, 1991). Borucki Castro *et al.* (2012) observed that the iodine concentration in milk, following use of a teat disinfectant pre-milking containing 0.5% of iodine, was significantly higher (252 µg/ kg) than the iodine concentration in milk produced when not using a teat disinfectant (164 µg/ kg). Also, in that study, 1% iodine-based teat disinfectant was applied post-milking, resulting in significantly higher iodine concentrations in milk (218 µg/ kg) compared to the control group. The use of iodine-based teat disinfectants pre-milking should be avoided, due to the difficulty in completely removing the product. When applying such products post-milking, teats should be washed and dried prior to the next milking.

The iodine concentrations in milk could also be affected throughout dairy processing. Norouzian *et al.* (2009 and 2011) verified in two studies that pasteurisation could decrease the iodine content in milk, registering losses between 17.6% and 53.1%. The reduced concentrations could be associated with sublimation of the iodine, as the majority of iodine in milk is inorganic. The iodine content in infant formula is a concern in the dairy industry, as the component is important for the metabolism and central nervous system development in infants. EU regulation 2016/127 specifies that the minimal and the

maximum iodine content in infant formula should be 15 µg/ 100 kcal and 29 µg/ 100 kcal, respectively. The International Council for Control of Iodine Deficiency Disorders (ICCIDD) recommends that the iodine content in infant formula should be 5 µg/ dL milk to 10 µg/ dL for full term and 20 µg/ dL for premature babies (Delange *et al.*, 1993), in order to prevent iodine deficiency.

1.2. Influence of farm factors on the quality of raw milk

1.2.1. Milk production season and lactation stage

The main milk production system on Irish dairy farms is pasture-based seasonal-calving, which coordinates milk production peak with grass growth peak (summer months) (Dillon *et al.*, 1995), while a small proportion of cows are calved in autumn to maintain a fresh milk supply throughout the year. Therefore, on the majority of Irish dairy farms, the early and mid-lactation stages coincide with spring and summer months (from March to August), period during which cows are grazing outdoors; while the late-lactation stage coincides with autumn and winter months (September – February), the period during which cows are housed indoors. Changes in dairy herd environment due to seasonality can result in the exposure of animals to distinct microbial communities that could be transferred to raw milk, which may affect the technological value of milk and safety of dairy products. Doyle *et al.* (2017) reported that the habitat has a great influence on the raw milk and teat microbiota. For example, in that study, bacteria from the genera *Ruminococcus*, *Eremococcus*, *Ruminococcaceae Incertae Sedis* and *Corynebacteriales* were more prevalent in milk produced during the indoors period, while bacteria from the genera *Pseudomonas*, *Acinetobacter* (spoilage-associated genera) and *Lactococcus* were more prevalent in milk produced during

the outdoors period. In both periods, regardless of the environment, the teat skin was identified as the greatest contributor to the raw milk microbiota. Some of those bacterial strains have already been detected in dairy products, such as cheese, in other studies (Beresford *et al.*, 2001; Quigley *et al.*, 2013b; O'Sullivan *et al.*, 2015), highlighting the impact of farm management and animal grazing practices on the microbiota of dairy products. Therefore, hygiene practices (e.g., teat disinfection prior to milking, sanitation of milking equipment), husbandry practices, herd health and herd housing should be appropriately managed to ensure the production of raw milk of high microbiological quality throughout the different seasons and lactation stages (Vacheyrou *et al.*, 2011).

The continuous production of high quality milk during winter months is a challenge for dairy farmers, and, as a consequence, the maintenance of high quality dairy products during that period is also a challenge. During the housing period, additional care is necessary regarding cow management, as the probability of milk contamination with thermoduric, thermophilic and spore-forming bacteria (e.g., *Bacillus cereus* and sulphite-reducing *Clostridia*) is higher during that period (O'Connell, 2015). Additionally, during the winter months, cows are more prone to acquire intramammary infections (mastitis) due to being indoors and environmental conditions (e.g., temperature, humidity), which could result in increases in the SCC in milk (Olde Riekerink *et al.*, 2007). Bacteria can be present in feed, bedding material, dust and livestock manure (McGuiggan *et al.*, 2002; Vissers *et al.*, 2007b; Quiberoni *et al.*, 2008); therefore, the dairy cow housing should be a clean, dry and comfortable environment for livestock. Cubicles should be cleaned twice daily and new bedding should be applied as necessary to maintain a dry lie at all times. Also, removal of manure should be as frequently as

possible (e.g., automatic scrapers, slats) to avoid teat contamination (O'Brien, 2009).

Furthermore, milk composition varies significantly throughout lactation, mostly due to cows physiological changes. The fat and protein content increase in late-lactation, while the lactose content decreases and milk yield is lower (Audist *et al.*, 1995; Henao-Velasquez *et al.*, 2014). Changes in milk composition can also occur due to the season, which affects diets of dairy herds. Grass is less available during autumn and winter and, therefore, the use of supplements such as concentrates and forages is required. For example, Reid *et al.* (2015) reported that increased dietary levels of crude protein can increase the amount of protein degraded in the rumen; consequently, large amounts of ammonia can be produced and converted into urea in the liver. Some urea diffuses into the milk, which is measured as milk urea nitrogen, which can adversely affect milk properties for processing (e.g., heat stability).

1.2.2. Milk cooling and cold storage

1.2.2.1. Influence of storage temperature and time on milk quality

During milking, milk is collected at approximately 35 °C and the microbiological load or enzymatic activity could increase rapidly if it were not immediately refrigerated to temperatures lower than 6 ˚C during storage and prior to processing (Walstra *et al.*, 2006). Therefore, by reducing the growth of microorganisms and enzymatic reactions, milk cooling aids on preserving the composition and consequently the technological properties of milk during cold storage. In Ireland, raw milk is usually stored in farm bulk tanks (BTs) for up to 24 or 48 h prior to collection and transport to processing plants. During winter

months, milk could be collected after 72 h of storage, as that time of the year usually coincides with the late-lactation period and cows produced less milk compared to early and mid-lactation periods. On-farm and prior to processing, milk is usually stored at temperatures lower than 6 ˚C (3 to 4 ˚C), as recommended by the European regulation EC no 853/2004.

The growth of some microorganisms is not completely ceased during cold storage, as some bacterial species have an optimum growth at low temperatures (i.e., psychrotrophs or psychrophiles) (Quigley *et al.*, 2013a). O'Connell *et al.* (2016 and 2017) reported that raw milk with low initial bacterial levels (approximately 2,000 cfu/ mL) can be stored at 2 or 4 ˚C for up to 96 h with minimal effects on the microbiological quality, composition and functional properties. While the TBC in milk stored at 2 or 4 ˚C were approximately 4,000 cfu/ mL after 96 h, the authors observed a significant increase in levels when storing milk at 6 ˚C for periods greater than 48 h (74,000 cfu/ mL). After storing milk at 6 ˚C for 96 h, the authors also observed a significant increase in PBC (from 741 to 38,904 cfu/ mL) compared to 2 and 4 ˚C (from 794 to approximately 2,500 cfu/ mL). Other studies have also reported a negative impact of storage temperatures higher than 4 ˚C on milk microbiological quality (Banks *et al.*, 1988; Griffiths *et al.*, 1988b; Vithanage *et al.*, 2017). Some of those studies are laboratory-based and reported increases in the bacterial counts in milk exceeding 1×10^6 cfu/ mL after 48 h at temperatures ≥ 4 ˚C; however, the addition of fresh milk to farm BTs after each milking was not included in their experimental design. The differences in bacterial numbers between laboratory-based and farm-based studies may be due to a dilution effect, which may account for lower

bacterial levels in milk. Therefore, the addition of fresh milk throughout storage is likely to limit the rate of deterioration of milk quality.

Milk is collected from dairy farms by insulated tankers, which will keep milk temperature at approximately 4 ˚C. As milk quality deteriorates over time, milk is usually not stored in silos for longer than 24 h prior to processing. Rasolofo *et al.* (2010) observed considerable increases in PBC in milk stored at 4 and 8 ˚C after 168 (7 days) and 72 h, respectively. After 72 h at 4 ˚C, PBC was similar to initial levels (approximately 6,000 cfu/ mL). Prolonged refrigerated storage of milk may have a negative impact on dairy products quality, as microorganisms, such as psychrotrophs, can produce heat-resistant enzymes that may still be active after pasteurisation and UHT treatments (Cousin, 1982; Lopez-Fandino *et al.*, 1993). Differently, moderate levels of these microbial enzymes may be beneficial for cheese manufacture due to the development of desirable flavours and aromas (Vithanage *et al.*, 2016).

The microbiological load of milk over storage is also influenced by the initial bacterial levels in milk. The microbiological load could increase considerably after 24 h of storage when initial levels are higher than 10,000 cfu/ mL (Guinot-Thomas *et al.*, 1995; Vithanage *et al.*, 2017; Ribeiro *et al.*, 2018). Therefore, good hygiene practices, appropriate cow management and sanitation of milking equipment are essential factors to produce milk of good microbiological quality.

1.2.2.2. Pre-cooling systems

Bulk tanks equipped with a cooling jacket, in which refrigerant liquid or chilled water circulate, are the conventional refrigeration systems used on Irish

dairy farms. As milk is obtained from cows at approximately 35 ˚C, the temperature gradient between milk and cold storage temperature in the BTs (2 to 4 ˚C) is large and consequently some time is necessary until milk reaches the set point temperature. Pre-cooling systems are used to cool raw milk rapidly prior to cooling within BTs and are also an alternative to reduce energy requirements and costs on-farm. Plate heat exchangers are the equipment usually used for that purpose and consist of a heat transfer device that is used for transfer of internal thermal energy between two fluids available at different temperatures, separated by a heat transfer surface (Thulukkanam, 2013). Plate heat exchangers comprise of stainless steel plates in a sandwich arrangement, in which milk and cooling water flow in parallel or opposite directions through the spaces between alternate plates (Wang *et al.*, 2007; Figure 1.3). The equipment may have one cooling stage, in which well water (approximately at 15 ˚C) is used, or two stages, in which well and chilled water (approximately at 0 ˚C) are used in the first and second stages, respectively (Murphy *et al.*, 2013). Due to the large surface area of the plates, heat exchange between water and milk occurs rapidly and aids in reducing the temperature gradient between fresh milk and milk that is already stored in the BTs. The efficiency of plate cooler systems depends on the temperature difference between milk and water, flow rates of both fluids and size of the equipment. The number of plates should be determined according to the milk flow rate (Thulukkanam, 2013). Milk should be filtered prior to entering the plate cooler to avoid residues accumulations in the space between plates. A water filter might also be necessary to also avoid the accumulation of foreign matter in the equipment. Filter socks can be fitted in the milk or water line for that purpose.

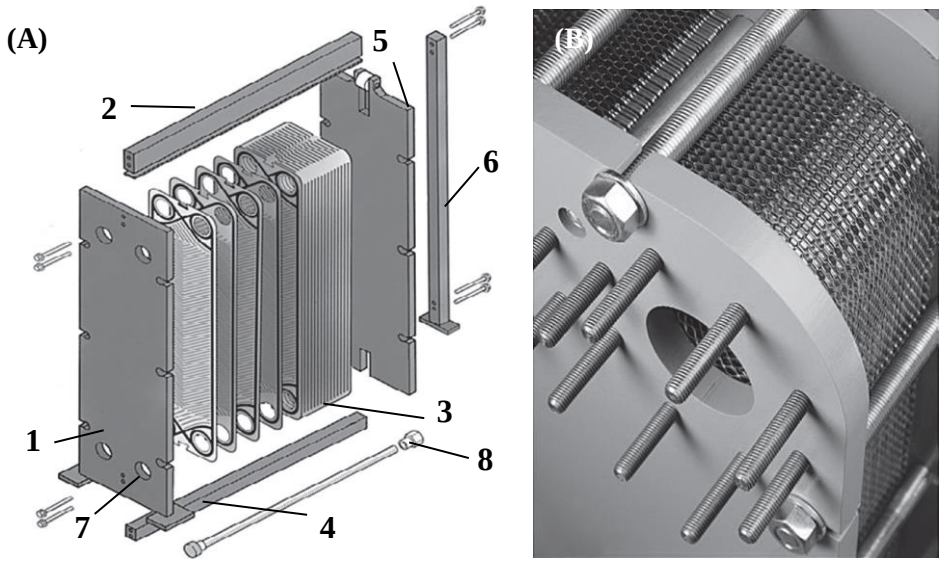


Figure 1.3. (A) Construction of a plate heat exchanger (1 – fixed frame plate; 2 – top carrying bar; 3 – plate pack; 4 – bottom carrying bar; 5 – movable pressure plate; 6 – support column; 7 – fluid port; 8 – tightening bolts) and **(B)** closer view of assembled plates. (Thulukkanam, 2013).

Rapidly cooling milk to low temperatures may prevent further increases in the PBC. According to Murphy *et al.* (2013), pre-cooling and gradually refrigerating milk to temperatures between 2 to 4 °C within 30 min after milking greatly reduces the possibility of significant bacterial growth. Hartmann *et al.* (2008) reported that pre-cooling milk aided in reducing TBC in raw milk during storage. Additionally, another advantage of rapidly cooling milk is that collection and transport to the processing plant can take place right after milking.

1.2.2.3. Energy requirements and water usage

Milk production in Ireland has been increasing considerably since the abolition of the milk quotas in 2015; however, with milk price being volatile in a now open market, farmers have the challenge to produce more milk at lower costs with a lower overall environmental footprint (Shine *et al.*, 2018b). Concurrently,

electricity costs might increase on-farm, due to the implementation of a dynamic electricity pricing system. In 2009, the Irish Government adopted a National Energy Efficiency Action Plan 2009-2020 (NEEAP) in order to achieve a national goal of 20% improvement in energy efficiency by 2020. The proposed smart metering system (Figure 1.4) would result in higher rates during peak periods of consumption (currently from 17:00 to 19:00 h) (EirGrid, 2012). Upton *et al.* (2013) reported that the peak electricity consumption on 22 Irish dairy farms occurred during the time intervals when demand on the grid was the highest; therefore, in a dynamic electricity-pricing system, those times would correspond to periods of higher electricity costs. Also, according to that study, electricity made up to 12% of total energy demand per litre of milk, from cradle to farm gate (Upton *et al.*, 2013; Shine *et al.*, 2018b). Therefore, alternatives for optimising energy usage on-farm may contribute to improve the cost competitiveness of Irish dairy products (Upton *et al.*, 2013).

Figure 1.4. Smart metering plan proposed by the National Energy Efficiency Action Plan (Upton *et al.*, 2010).

Milk cooling is the main contributor to electricity usage on Irish dairy farms (31% of total electricity consumption) and over 60% of milk cooling electricity consumption currently occurs on the most expensive day-rate tariff (Upton *et al.*, 2013). A strategy to reduce the energy costs is to use plate heat exchangers to pre-cool milk and finish cooling within bulk tanks. Pre-cooling systems aid with reducing the temperature gradient between the bulk tank set point and milk entering the tank; therefore, the energy consumption of the bulk tank to cool milk is reduced. Shine *et al.* (2018b) surveyed 58 Irish dairy farms regarding energy consumption during milking and reported that the average (± SD) energy usage when using a pre-cooling system (10.54 ± 2.55 Wh/ L) was lower than when not pre-cooling milk (12.68 ± 5.20 Wh/ L). A second strategy to reduce the energy consumption during peak times would be to decouple the milk cooling load from milking times by shifting the load to off-peak periods. Ice builder systems could be used for that purpose and cold energy can be generated when electricity is cheaper. Upton *et al.* (2013) observed that ice builder systems ran on day tariffs for 30% of their operating times, while direct expansion systems (bulk tanks) used 70% day tariff electricity.

Milk production costs might also increase due to the monetisation of public supply and waste water in Ireland, as outlined in the Water Services Act 2013. Most of Ireland's dairy farm water is supplied by groundwater boreholes and an average of 7.42 L_{water}/ L_{milk} are consumed on farms (Upton *et al.*, 2013; Shine *et al.*, 2018b). Water demand is expected to increase with milk production, which may result in water shortages during periods of little rainfall and higher demand for public water supply. Water consumption can be influenced by factors such as herd size, number of milking units, use and type of pre-cooling systems or

bulk tank size. In the study of Shine *et al.* (2018b) the water consumption within the milking parlour corresponded to 33% of total dairy farm water consumption. In the parlour, pre-cooling systems had the highest water consumption (1.81 L_{water}/ L_{milk}), followed by wash down (1.31 L_{water}/ L_{milk}) and sanitation (0.15 L_{water}/ L_{milk}). Some alternatives to reduce water consumption are: recycle the water used for pre-cooling, re-utilise that water for washdown or as drinking water for livestock and monitor water usage during wash cycles (Murphy *et al.*, 2016a; Shine *et al.*, 2018a).

1.3. Influence of factors throughout the production chain on the quality of dairy products

1.3.1. Cheddar cheese

Cheese is one of the main dairy commodities exported by Ireland and, according to a report from the International Dairy Federation (IDF, 2012), Irish cheese production in 2011 was approximately 180 x 10^3 tonnes, and is expected to reach more than 200 x 10^3 tonnes by 2020. Global production of cheese is approximately 19 x 10^6 tonnes per year and has increased at an average annual rate of approximately 4% over the past 30 years. The consumption of cheese *per capita* has also been increasing consistently over many years, some reasons for this are a positive dietary image of the product, convenience and flexibility in use, and the great diversity of flavours and textures (Fox *et al.*, 2017).

1.3.1.1. Cheddar cheese manufacture

Cheddar cheese is classified as a rennet-coagulated hard cheese type. Hard cheeses usually have a moisture content ranging from 30 to 45% and are subjected

to high pressure during manufacturing to give a hard, uniform and close texture (Fox *et al.*, 2017). The traditional protocol for the manufacture of Cheddar cheese is shown in Figure 1.6.

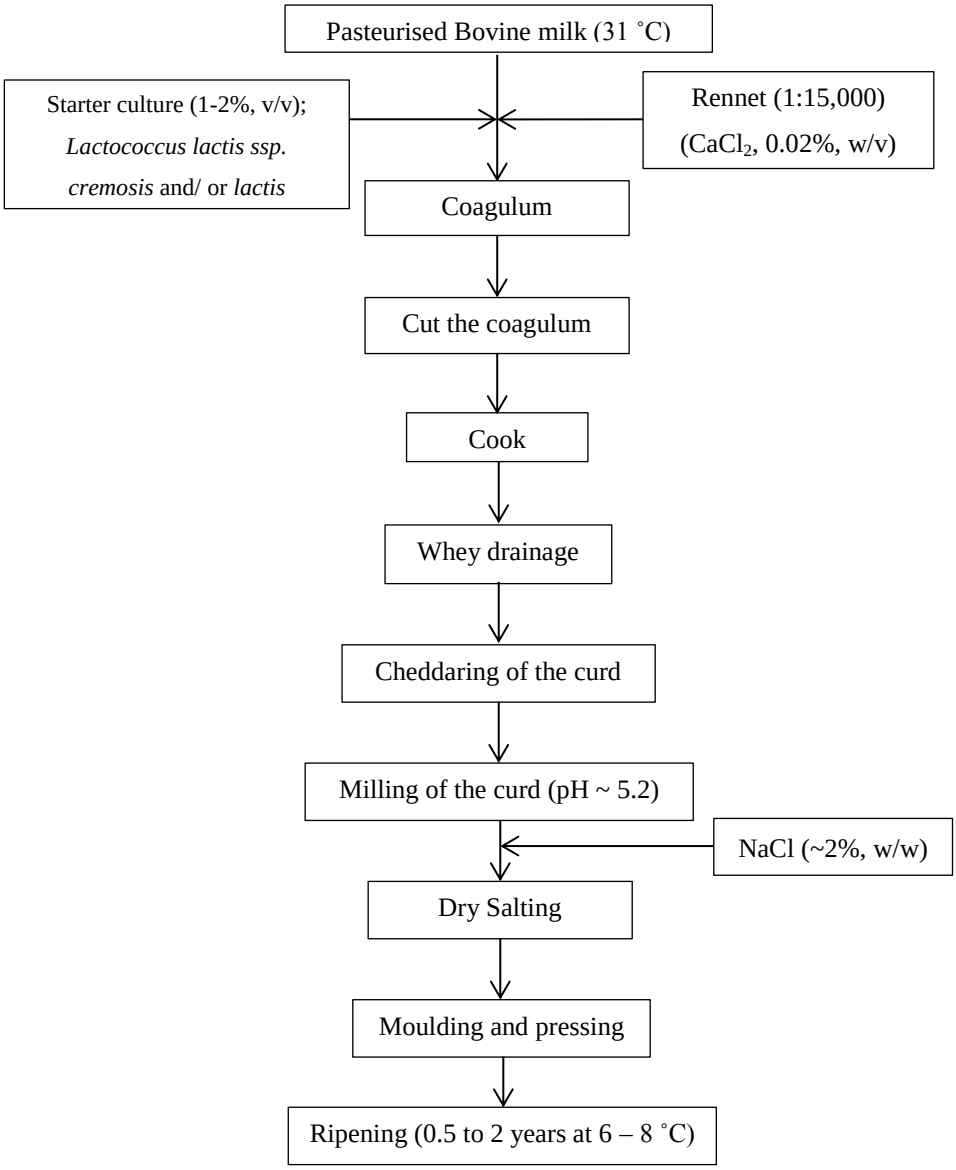


Figure 1.6. Traditional protocol for the manufacture of Cheddar cheese (Fox *et al.*, 2017).

On an industrial scale, Cheddar cheese is usually produced using bovine milk that is pasteurised at 72 ˚C for 15 seconds. Milk is also standardised to a casein: fat ratio of 0.67 to 0.72:1.00 to compesate for seasonal variations in milk

composition, as well as to optimise cheese yield and to maintain a constant cheese composition. The protein and fat contents in milk can be adjusted by the addition of concentrated non-fat milk solids (i.e., skim milk powder or condensed milk) or skim milk or by the removal of cream. Milk is heated to approximately 30 ˚C and then the starter culture and rennet are added. The main starter cultures used are *Lactococcus ssp. lactis* and *Lactococcus lactis ssp. cremosis*; however, sometimes mixed and undefined cultures could be also used. Starter cultures are responsible for the production of lactic acid during cheese-making, which results in the acidification of curd and whey. The decrease in pH promotes rennet activity, and aids the expulsion of whey from the curd, thus reducing the moisture content of the cheese, which helps to prevent the growth of undesirable bacteria in the cheese. Starter cultures are also important in developing flavour during ripening of cheese.

The coagulation of milk is promoted by the addition of proteases to the milk, which could be rennet or, currently most commonly, fermentation-produced chymosin (FPC). Rennet includes proteases produced in the stomachs of ruminants and contains approximately 60 to 70 RU (rennet units)/ mL. Chymosin represents more than 90% of the milk clotting activity of calf rennet, while the remaining activity is due to pepsin (Fox *et al.*, 2017). Currently, FPC represents 70 to 80 % of the total rennet market. The three main commercial FPC used are: Maxiren (secreted by *K. marxianus var. lactis*, Gist Brocades, The Netherlands), ChyMax secreted by *A. niger* or *E. coli* (Hansen, Denmark) (Jacob *et al.*, 2011). Those proteases hydrolyse the Phe_{105}-Met_{106} bond of κ-casein, releasing a caseinomacropeptide (CMP) into the aqueous medium. After this, the rennet-altered micelles (paracasein) aggregate in the presence of Ca^{2+}, entrapping fat

globules and milk serum in a network. The coagulum is cut into small pieces (approximately 6 mm cubes), cooked to 39 to 40 ˚C over 30 minutes, and held at this temperature for about 1 h. Once a certain pH is reached, the whey is drained and the curds are “cheddared”. During the cheddaring process, the curd blocks are piled on top of each other, with regular turning and stacking of the curd blocks. The cheddaring process allows time for further decreases in the pH (e.g., from 6.1 to 5.4), thus solubilising some colloidal calcium phosphate, as well as assisting in whey drainage as the curds are subjected to gentle pressure. During this process, the curds granules fuse and the texture changes from soft and friable to tough and pliable.

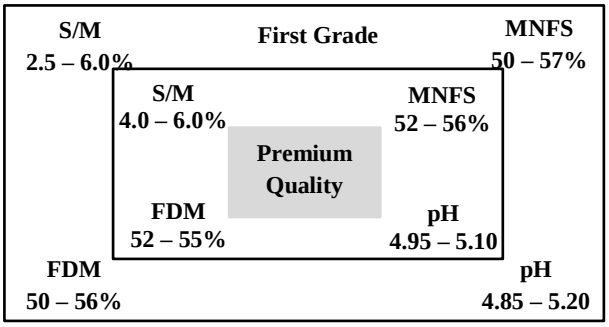
The curds are milled and dry-salted when the pH is approximately 5.4. During the “mellowing” period, the salt dissolves in moisture on the surface of the curd. Finally, the curds are moulded and pressed overnight at up to 200 kN/ m^2.

Cheddar is then stored at 6 to 10 ˚C for a period ranging from 3 to 4 months to 2 years to ripen (Walstra *et al.*, 2006; Fox *et al.*, 2017). The primary biochemical changes that occur during ripening include the metabolism of residual lactose, lactate and citrate, as well as lipolysis and proteolysis. The secondary changes include the metabolism of fatty acids and of amino acids, responsible for the development of flavour (McSweeney, 2004). The characteristics of the manufacturing process, such as starter culture type, level of residual coagulant activity and composition of cheese (i.e., moisture, NaCl contents and pH), are the main determinants of those changes in cheese during ripening. The complex set of biochemical reactions is responsible for changes in flavour, aroma and texture in cheese, which are unique characteristics for each type of cheese (Fox *et al.*, 2017).

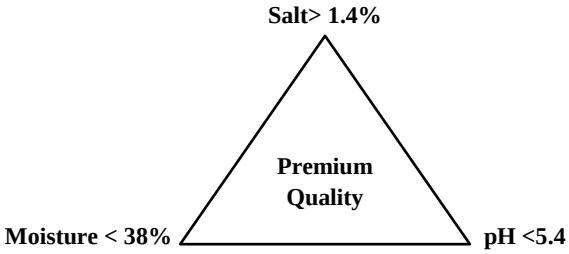
1.3.1.2. Composition of Cheddar cheese

The quality of Cheddar cheese is usually graded according to its composition for commercial purposes. Cheddar grading systems mainly specify the moisture content, NaCl concentration [expressed as salt-in-moisture (%S/M)], pH, moisture in non-fat substances (MNFS; ration of protein to moisture) and percentage fat-in-dry matter (FDM). Several studies have attempted to investigate the relationship between Cheddar cheese quality and composition (Gilles and Lawrence, 1973; Fox, 1975; Pearce and Gilles, 1979; Figure 1.7). It has been concluded that the moisture content, % S/M and pH are the key determinants of cheese quality; however, studies disagree regarding the relative importance of those parameters. The only grading scheme in commercial use appears to be that proposed by Gilles and Lawrence (1973), which is applied in New Zealand for young (14 day) Cheddar cheese (Figure 1.7). In the United Kingdom, there are legal limits in relation to the maximum moisture (39.0%) and minimum % FDM (50.0%) for full-fat Cheddar cheese (Lawrence and Gilles, 1980). Lawrence *et al.* (2004) also suggested ranges of S/M, MNFS, FDM and pH for first-grade and second-grade young Cheddar cheese (14 day), which are shown in Figure 1.8.

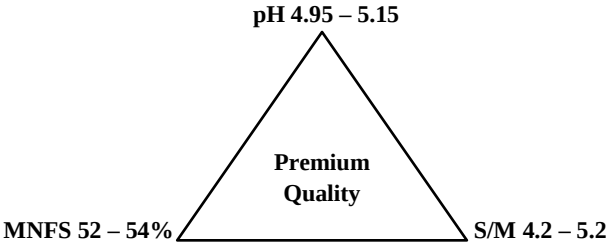
According to Fox *et al.* (2017), composition is not a good predictor of Cheddar cheese quality, as its quality can be evaluated considering its compositional, functional, sensory and safety aspects. Additionally, factors such as the microflora, activity of indigenous milk enzymes, relatively small variations in cheese composition, and probably other unknown factors, are also determinants of Cheese quality and should be taken into account during production.



Gillles and Lawrence (1973): Composition of cheese was determined at 14 days and related to quality of mature Cheddar cheese.



Fox (1975): Relationship between the quality and composition of 10-week-old Cheddar cheese.



Pearce and Gilles (1979): Composition of cheeses was determined at 14 days and related to quality of Cheddar cheese.

Figure 1.7. Relationships between composition and the quality of mature Cheddar cheese (moisture-in-non-fat substances [MNFS]; fat-in-dry matter [FDM], and salt-in-moisture [S/M]) (Redrawn from Fox *et al.*, 2017).

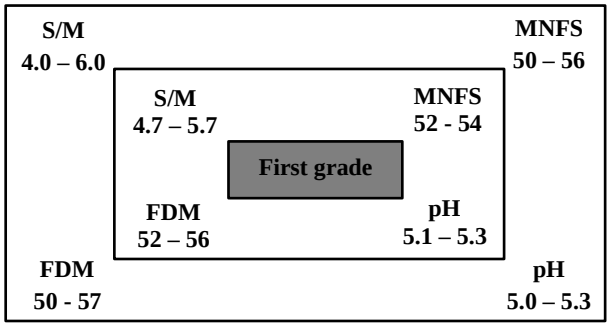


Figure 1.8. Suggested ranges of salt-in-moisture (S/M), moisture-in-non-fat-substance (MNFS), fat-in-dry-matter (FDM), and pH for first-grade and second-grade Cheddar cheese (Redrawn from Lawrence *et al.*, 2004).

1.3.1.3. Factors that influence Cheddar cheese quality

The milk supply quality is critical to maintain the consistent production of high quality Cheddar throughout the year (Murphy *et al.*, 2016b). Three aspects of raw milk quality must be considered: microbiological, enzymatic and chemical (Fox *et al.*, 2017). According to Murphy *et al.* (2016b), it is difficult to determine a bacterial count limit for raw milk to ensure no negative effects on cheese quality or yield, due to the natural variation in the enzyme activities of milk microflora (Murphy *et al.*, 2016b). For example, some studies suggested that levels higher than 1 x 10^6 cfu/ mL of psychrotolerant bacteria in raw milk could cause flavour defects in cheese and reduce yields, due to the production of heat-stable proteases and lipases (Cousin, 1982; Fairbairn and Law, 1986; Mottar, 1989; Champagne *et al.*, 1994; Sorhaug and Stepaniak, 1997). However, some authors observed different results; for example, Leitner *et al.* (2008) produced cheese using BT milk from 15 farms stored for 0, 24 and 48 h at 4 ˚C. After 48 h, TBC levels increased from 68 x 10^3 to 24 x 10^6 cfu/ mL and the cheese curd yield decreased by 7%; however, this decrease was not significantly correlated with bacterial count increase. According to the review published by Murphy *et al.* (2016b), the

use of raw milk with bacterial counts lower than 1 x 10^5 cfu/ mL is unlikely to negatively affect cheese manufacture and quality. In countries with a developed dairy industry, the TBC of milk supplied is usually lower than 2 x 10^4 cfu/ mL, which may increase during transport and storage in the factory; however, numbers are reduced after thermisation (65 ˚C x 15 s) or pasteurisation (72 ˚C x 15 s) (Fox *et al.*, 2017). The pasteurisation of milk used for cheese production is the main reason for the low incidence of pathogens in this product. Other contributing factors would be the decrease in pH and water content during manufacture. The Food Safety Authority of Ireland (2004) reported that none of 512 retail cheeses tested contained salmonella, one sample contained *Listeria monocytogenes* (5.7 x 10^3 cfu/ g), three samples had *Escherichia coli* (10^4 to 10^5 cfu/ g) and 16 samples had *Staphylococcus aureus* levels higher than 10^4 cfu/ g.

Even though bacterial numbers can be considerably reduced in cheese milk after heat treatments, the control of those is still required due to the potential production of proteases or lipases. Those could be heat-resistant and not eliminated during pasteurisation, and consequently affecting milk cheese-making properties and cheese quality parameters (flavour and texture) over ripening. Most of those heat-resistant enzymes are produced by psychrotrophic bacteria (Mottar, 1989; Sorhaug and Stepaniak, 1997; Skeie, 2007). Mankai *et al.* (2012) observed low dry matter content of Gouda cheeses produced from milk stored at 4 ˚C for 48, 72 and 96 h, which was possibly related to psychrotrophic proteases responsible for losses of protein in whey. The authors also observed a negative relationship between milk storage time and cheese yield, which decreased as the milk used was stored for longer. The same was observed by Shah (1994) and Boulares *et al.* (2011), who associated decreases in cheese yield with enzymes

produced by Gram-negative psychrotrophs. Proteolysis is also a major contributing factor associated with the development of textural and rheological properties of cheese (Lawrence *et al.*, 2004). Novella-Rodriguez *et al.* (2004) and Mankai *et al.* (2012) observed that the proteolysis level was higher for cheeses made with milk stored for 48 h and 96 h, respectively, whatever the stage of ripening. Those results were associated with physicochemical changes and increased in enzymatic activity in milk over storage. Furthermore, growth of citrate-metabolising *lactobacilli* is responsible for open texture or slit openness in Cheddar cheese, due to the production of CO_2 from citrate. Non-starter *lactobacilli* bacteria (e.g., *Lactobacillus casei*, *plantarum* and *brevis*) at levels higher than 10^7 cfu/ g can result in the production of gas and putrid off-flavour development. Thermo-resistant strains, such as *Streptococcus thermophiles*, could accumulate in pasteurisers not properly sanitised and numbers could increase to 10^8 cfu/ g in cheese in the early ripening stages, causing unclean flavours in cheese (Fox *et al.*, 2017).

High levels of SCC could also have a negative impact in cheese manufacture. According to Politis and Ng-Kwai-Hang (1988), increasing SCC incrementally from 10^5 to levels higher than 10^6 cells/ mL resulted in linear increases in the percentages of milk fat (1%) and protein (3%) lost in whey, and reductions in Cheddar cheese yield. Also, adverse effects of SCC higher than 5 x 10^5 cells/ mL on the rennet coagulation of milk, cheese yield and recovery protein and fat to cheese have also been reported in other studies (Auldist and Hubble 1998; Barbano *et al.*, 1991; Auldist *et al.*, 1996; Klei *et al.*, 1998; Srinivasan and Lucey, 2002). Somatic cells counts lower than 3 x 10^5 cells/ mL are recommended for cheese manufacture (Fox *et al.*, 2017).

Residues from cleaning and disinfecting agents are also a concern in cheese manufacture. According to Singh and Ghandi (2015), levels around 5 ppm of QACs in dairy products could inhibit lactic acid bacteria, which could affect the formation of the coagulum and increase the milling time in preparation of Cheddar cheese. The Federal Institute of Risk Analysis in Berlin (2012) has reported that the QAC compound, DDAC, is frequently detected in dairy products. The maximum concentration of that compound reported in cheese was 0.235 mg/ kg, which is higher than the European limit (0.0100 mg/ kg). Some types of cheeses are immersed in brine solutions prepared with disinfected water, which could contain residues of chlorine-based sanitisers, TCM and non-volatile haloacetic acids (Grellier *et al.*, 2010; Rahman *et al*., 2010; Cardador *et al.*, 2016). Proper sanitation on-farm and within factories are required to avoid high levels of those contaminants in dairy products.

1.3.2. Infant formula

Ireland supplies 10% of all global infant milk formula (IMF), which accounts for 35% of all Irish dairy exports and is valued at around 35 billion euros in retail sales per year (DAFM, 2012; Allen, 2016). As a product of high commercial value destined for infants, it is of extreme importance to ensure high quality and safety. Infant milk formula is manufactured based on the nutritional profile of human milk, in order to satisfy the nutritional requirements of new-borns until they are introduced to appropriate complimentary food (European Commission, 2006; Codex Alimentarius, 2007). There are three types of IMF manufacturing process: wet-mixing, dry-blending, or a combination of wet-

mixing and dry-blending (McSweeney, 2008). A generalised wet process for the manufacture of powdered IMF is shown in Figure 1.5.

In the first IMF production stage, specific powder and/ or liquid ingredients are combined with milk and/ or water to obtain the desired macro-composition. The raw materials that are commonly used in IMF are listed in Table 1.4. The bacterial and chemical residues levels in raw milk, or in the powdered ingredients produced from such milk, are important determinants of the quality of the dairy product that will be manufactured. Some of the pathogenic bacteria of most concern in the infant formula production are *Salmonella spp.*, *Clostridium botulinum* and *perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter spp.*, *Staphylococcus aureus* and bacteria from the *Bacillus cereus* group (Scott *et al.*, 2015). Those microorganisms could be all present in the cows' environment and therefore farm-level control of those numbers through appropriate cow management and udder preparation is essential. The chemical hazard of concern in IMF processing would be the levels of CHLO and iodine in the ingredients. There is no technology available to reduce the levels of those residues in milk, highlighting the importance of appropriate sanitation practices and feed management on-farm. The same is valid within the factory, as residual chlorine on the surface of processing equipment or water with high levels of chlorine could increase residues levels in milk.

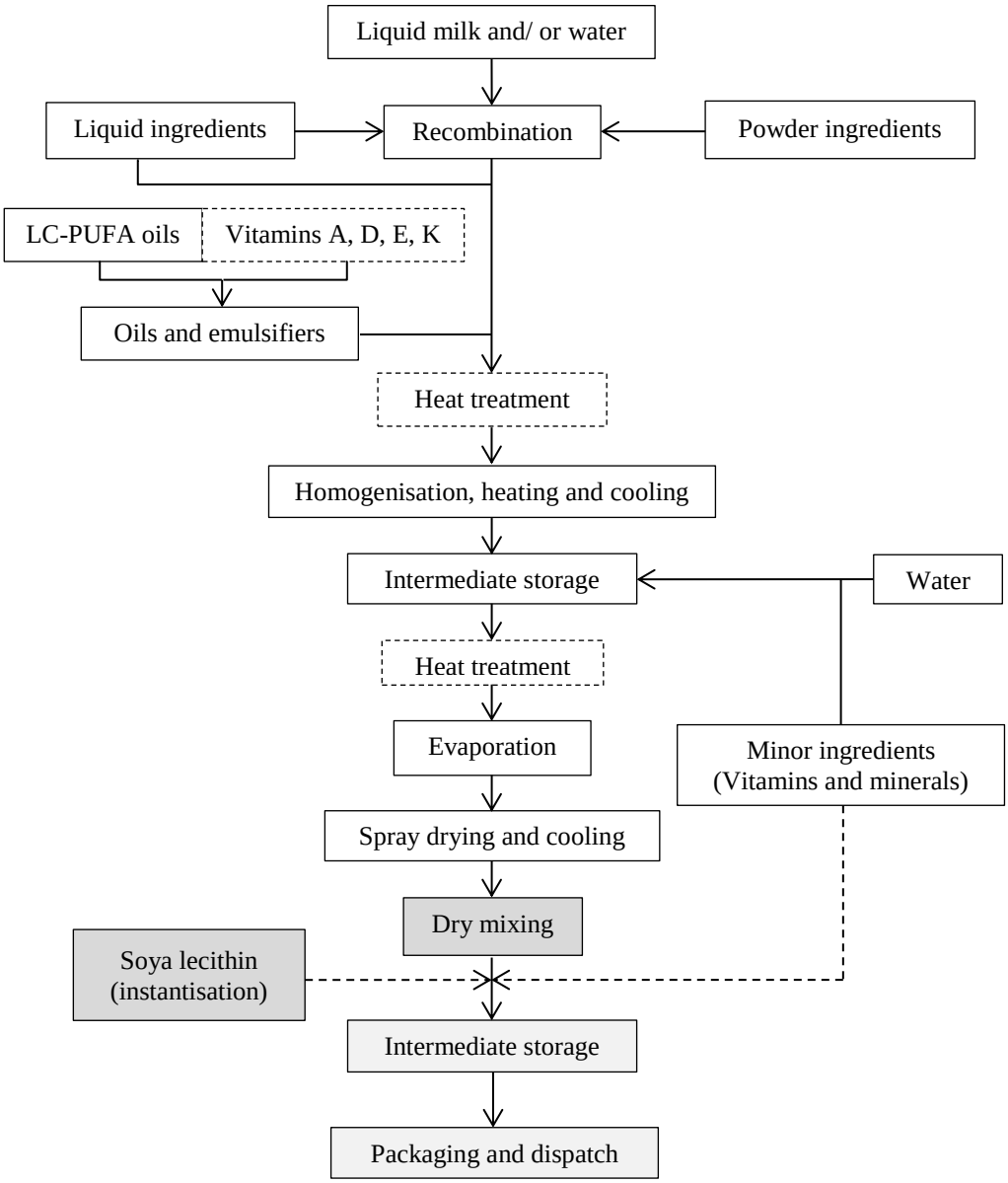


Figure 1.5. Generalised powdered IMF manufacturing process (grey filled areas indicate alternative processing options) (Adapted from Sorensen *et al.*, 1992; Montagne *et al.*, 2009).

In relation to other ingredients, oils (such as polyunsaturated fatty acids) and fat-soluble vitamins (such as A, D, E and K) are usually incorporated into the wet-mix prior to homogenisation to prevent their separation with the fat fraction. Heat-sensitive ingredients, such as minerals and other vitamins, are added after heat treatment (Murphy, 2015).

Table 1.4. Typical raw materials for IMF (Murphy, 2015).

Casein Source	Skim milk powder (SMP), evaporated skim milk, acid casein, sodium/ calcium/ potassium caseinate, milk protein concentrate
Whey Source	Demineralised whey powder, whey protein concentrates, whey protein isolates, hydrolysed whey ingredients
Alternative protein source	Soy milk, soy protein isolate, locust bean seed protein, amino acids
Oils	Soy, corn, safflower, sunflower, rapeseed, palm, copra, structured lipids
Carbohydrates	Lactose, starch, sucrose, corn syrup, corn syrup solids
Major minerals	Calcium carbonate, calcium phosphates, dibasic magnesium phosphate, potassium citrate, magnesium chloride
Minor minerals	Potassium iodide, ferrous sulphate, manganese sulphate, copper sulphate, zinc sulphate
Vitamins	A, D, E, K, B1, B2, B6, B12, niacin, folic acid, pantothenic acid, biotine, choline, inositol
Functional ingredients	Soy lecithin, mono- and di-glycerides

The positioning of heat-treatment in the process is flexible, and could be placed prior to or post homogenisation, as indicated in Figure 1.5. Aggregates of fat globules are formed during heat treatments and therefore it is beneficial to place that step prior to homogenisation when producing whole milk powder destined for IMF manufacture (McSweeney *et al.*, 2004). The milk powder used or produced during the production of IMF can be classified as low-, medium- or high-heat powder according to the heat treatment applied. Typical heat treatments are 70 - 72 ˚C for 15 s for low-heat milk powder, and 120 ˚C for 60 - 120 s, or 90

˚C for 300 s high-heat milk powder (Kelly *et al.*, 2003). The heat treatment stage is a critical control point as it has to ensure the reduction of pathogenic bacteria numbers to safe levels. Commission Regulation (EC) No 2073/2005 establishes the microbiological criteria for foodstuff, including IMF. According to that regulation, the food safety criteria applicable to IMF requires absence of *Salmonella spp.* and *Cronobacter spp.* in 25 and 10 g of 30 sample units, respectively. The presence of *Salmonellae* indicates survival or contamination after processing and it is used as an indicator of hazards in dried milk and IMF (WHO, 1992). Also, according to the EC regulation, the process hygiene criteria applicable to IMF requires absence of *Enterobacteriaceae* in 10 g of 10 sample units, while four samples should have presumptive *Bacillus cereus* levels lower than 50 cfu/ g and the fifth sample can have levels between 50 and 500 cfu/ g. Even though bacterial levels can be considerably reduced after pasteurisation, it is critical to control them at farm level, as the effects of high bacterial numbers in milk cannot be addressed with processing technology. Most importantly, the levels of thermoduric, thermophilic and spore-forming bacteria should be controlled, as those microorganisms can survive heat treatments and most of the pathogens of concern in IMF manufacture belong to those bacterial groups. Therefore, if pathogens are initially present in high numbers, there is the risk of not reducing them to safe levels (Cho *et al.*, 2018; Zou and Liu, 2018).

After the heat treatment, the wet-mix is evaporated and powder is produced by spray-drying (Montagne *et al.*, 2009). The temperature gradient in an evaporator system ranges typically from 70 ˚C in the first effect to 45 ˚C in the last effect (Murphy, 1999). After this, powder is generally produced using two or three stage spray dryers. Those dryers consists of a large drying chamber (stage 1,

at 180 to 200 ˚C) in which the bulk of water is removed, followed by supplementary drying using an internal fluidised bed (stage 2, at 50 to 60 ˚C) and/ or an external fluidised bed (stage 3, at 20 to 30 ˚C) (Montagne *et al.*, 2009). The evaporation and spray-drying processes may also contribute to further decreases in the bacterial levels in the product, due to the high temperatures applied during those operations. However, thermoduric and thermophilic bacteria can survive those temperatures and levels could concentrate in the final product. Griffiths et al. (1988b) enumerated thermoduric bacteria at several stages of a milk powder manufacturing process (pasteurisation, evaporation and spray drying) and in the milk powder produced, and reported that numbers of thermoduric bacteria did not decrease throughout the processing stages.

Although most of the bacteria can be eliminated after heat treatment, many species of psychrotroph, mesophilics, thermoduric and thermophilic bacteria produce heat-stable lipases and proteases, which can retain activity in powdered dairy products that could be used as ingredients during the manufacture of infant formula (Chen *et al.*, 2003). The presence of these enzymes could affect the quality of those powdered ingredients, by affecting their physico-chemical, functional and sensory properties. Celestino *et al.* (1997) reported that reconstituted UHT milk powder manufactured using raw milk stored for 4 days before processing had rancid and bitter flavours compared to UHT milk powder produced using fresh raw milk, possibly due to differences in the bacterial levels and enzymatic activity between the milk volumes used. Oliveira *et al.* (2000) observed that whole milk powder produced from raw milk containing high levels of psychrotrophs (1.8×10^7 cfu/ g) presented higher insolubility indexes. Both

studies indicate that microbiological quality of raw milk has a major impact on the quality of the final powder product.

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Experimental objectives

Considering the gaps in knowledge and current challenges of dairy suppliers and processors, the major objective of this study was to investigate how production conditions on-farm influence raw milk quality and processability, and how the quality of milk can impact on the quality of dairy products. The studies were conducted in commercial and research dairy farms, in a commercial milk powder processing plant, and in a pilot cheese processing plant, which ensured that milk was produced and processed according to typical farm and industrial conditions. The work is presented in the experimental chapters as outlined in Figure 1.8.

The specific objectives of this thesis were:

- Determine the effect of different storage temperatures and time on the microbiological and compositional quality of raw milk from different farm bulk tanks;
- Investigate the effect of different pre-cooling rates on milk microbiological quality and composition, as well as on energy requirements. This study was conducted in a manner that mimicked on-farm milk production conditions: morning and evening milkings, similar milk storage conditions, and use (or not) of precooling systems;
- Investigate the conjoined impact of seasonal production conditions of individual dairy suppliers (sanitation practices and storage conditions) on the quality of the bulk milk used for the production of milk powder and its effect on the final product quality. The

quality aspects investigated were microbiological load and concentration of residues from sanitation products and concentrations of iodine;

- Understand the dynamics of changes in residues (TCM, CHLO, QACs) from sanitation products throughout milk powder processing;
- Investigate the effect of thermo-resistant protease produced by *Pseudomonas fluorescens*, the main psychrotroph present in milk, on the cheese-making properties of milk and Cheddar cheese quality parameters.

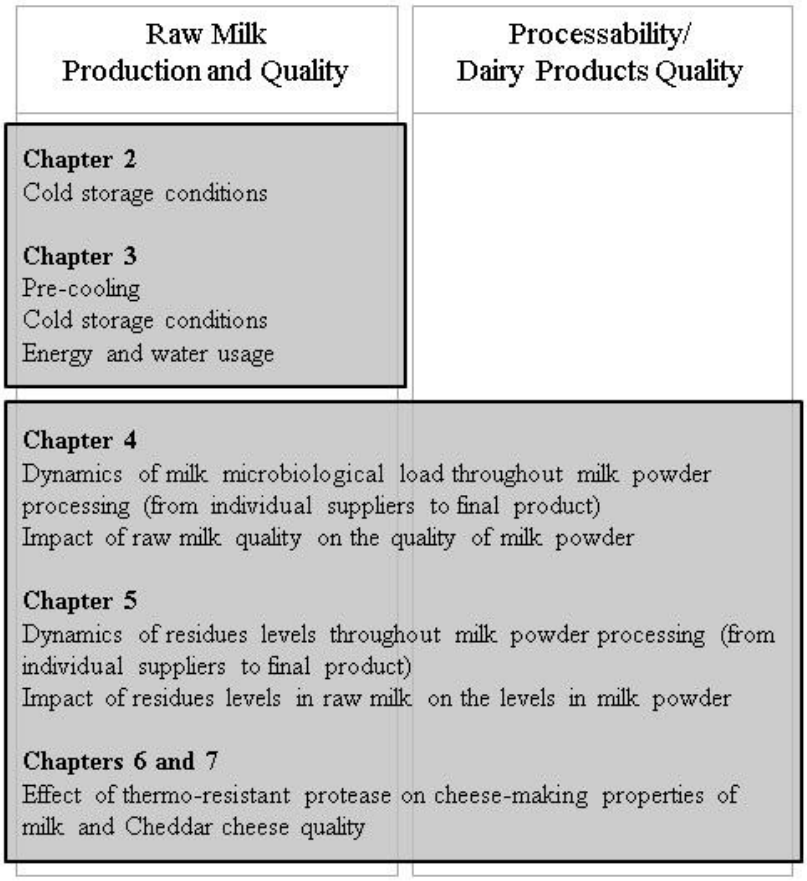

Figure 1.8. Structure of experimental chapters and topics investigated in each study.

Chapter 2

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

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Declaration: Milk collection, microbiological analysis and determination of milk composition (nitrogen fractions, casein fractions and peptide profiles) were conducted by Lizandra Paludetti at Teagasc, Moorepark Food Research Centre. Dr. Kieran Jordan gave guidance regarding the experimental design and microbiological analysis. All experimental data were analysed and the chapter written by Lizandra Paludetti, with corrections and comments from Prof. Alan L. Kelly from University College Cork, Dr. David Gleeson and Dr. Kieran Jordan from Teagasc Moorepark.

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_{10} cfu/ mL, 4.34 to 6.44 log cfu/mL and 3.72 to 4.81 log_{10} 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.

2.1. 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).

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, αs1-casein and αs2-casein (Crudden *et al*., 2005), resulting in defects in dairy products, such as bitterness in milk, gelation of ultra-high temperature processing (UHT) processing 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.

2.2. Materials and methods

2.2.1. 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.

2.2.2. 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.

2.2.3. 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 nitrogen 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 5 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 multiwavelength 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.

2.2.4. 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 (αS1-casein, αS2-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.

2.3. Results

2.3.1. 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, were observed between milk samples (Figure 2.1.A.1 and A.2). For example, the initial TBC in milk samples from farms W and Z were similar (3.93 ± 0.06 $\log_{10}$ cfu/ mL and 3.88 ± 0.06 $\log_{10}$ cfu/ mL, respectively), and these samples had a similar TBC after 96 h when stored at 2 °C (Figure 2.1.A.1); however, samples stored at 4 °C had different TBCs after 72 h, corresponding to 5.84 ± 0.06 $\log_{10}$ cfu/ mL and >7.00 $\log_{10}$ cfu/ mL, respectively (Figure 2.1.A.2).

2.3.2. 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 2.1) but no interaction between farm and time ($P > 0.05$; Table 2.1). Similar to the TBC results, differences between the initial PBC levels, as well as differences in the bacterial growth over 96 h, between the farm milk samples were observed (Figure 2.1.B). For example, samples from farms W and Z had similar initial PBC (3.76 ± 0.07 $\log_{10}$ cfu/ mL and 3.80 ± 0.09 $\log_{10}$ cfu/ mL, respectively); however, after 48 h, sample W had a PBC >7.00 $\log_{10}$ cfu/ mL,

while sample Z reached that level after 96 h (Figure 2.1.B.2). In this study, TBC was correlated with PBC, $r(40) = 0.90983$, $P < 0.0001$.

2.3.3. 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 2.1). 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 2.2.A and B). Only PROT bacterial count was significantly affected by temperature ($P < 0.01$; Table 1), as shown in Figure 2.2.A and B. The initial LIP bacterial counts were similar between farms ($P > 0.05$), while the PROT bacterial count had a significant variability ($P < 0.0001$; Table 1). The increase in LIP and PROT bacteria varied among farm milk samples when stored at 4 °C (Figure 2.1.C.2 and D.2).

Table 2.1. 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 counts (TBC), psychrotrophic bacterial count (PBC), lipolytic bacterial count (LIP), proteolytic bacterial count (PROT), thermoduric bacterial count [laboratory Pasteurisation Count (LPC)] and thermoduric-psychrotrophic bacterial count (LPC-PBC) of the milk samples from all farms.

Bacterial Counts	**Significance**				
	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

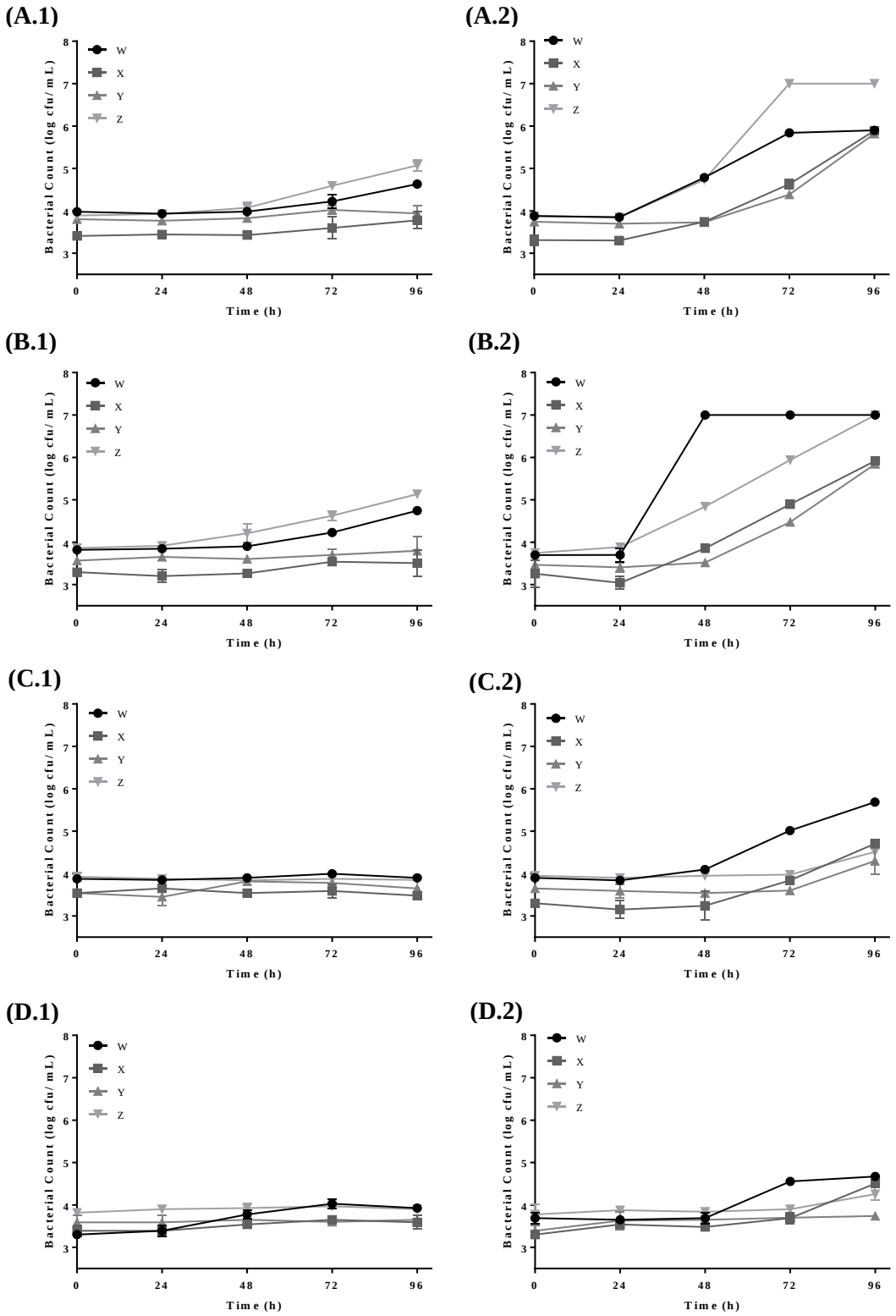

Figure 2.1. (A) Total bacterial count (TBC), **(B)** psychrotrophic bacterial count (PBC), **(C)** proteolytic (PROT) bacterial count and **(D)** lipolytic (LIP) bacterial count over 96 h for milk samples W, X, Y and Z stored at **(1)** 2 ˚C or **(2)** 4 ˚C.

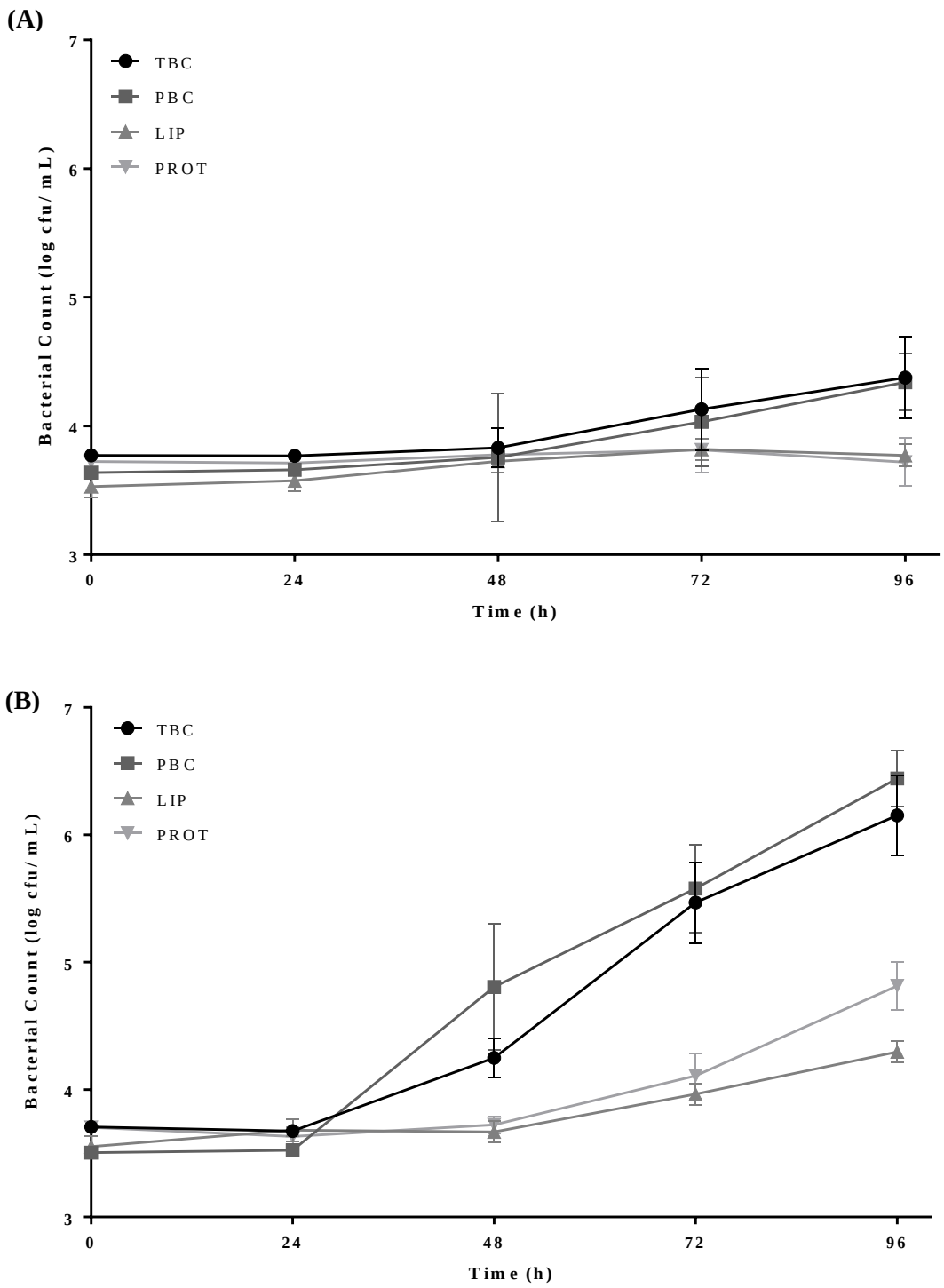

Figure 2.2. Total bacterial count (TBC), psychrotrophic bacterial count (PBC), lipolytic (LIP) bacterial count and proteolytic (PROT) bacterial count over 96 h for milk samples from four dairy farms (W, X, Y and Z) stored at **(A)** 2 ˚C and **(B)** at 4 ˚C.

2.3.4. 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 2.1). However, the LPC was significantly different between farms (P < 0.05; Table 2.1), with initial counts varying from 2.11 to 2.64 lo_{10}g cfu/ mL (128–445 cfu/ mL). The LPC-PBC was not affected by time, temperature, farm or their interaction (P > 0.05; Table 2.1). The LPC-PBC levels varied from 0 to 1.40 log_{10} cfu/ mL (25 cfu/ mL).

2.3.5. 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), αS1-casein (P < 0.05), αS2-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 2.3.A and B indicated decreases in the casein content in the milk samples from farms W and Z. The αS1-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 2.4.A and B indicated an increase in the concentrations of peptides in samples W and Z.

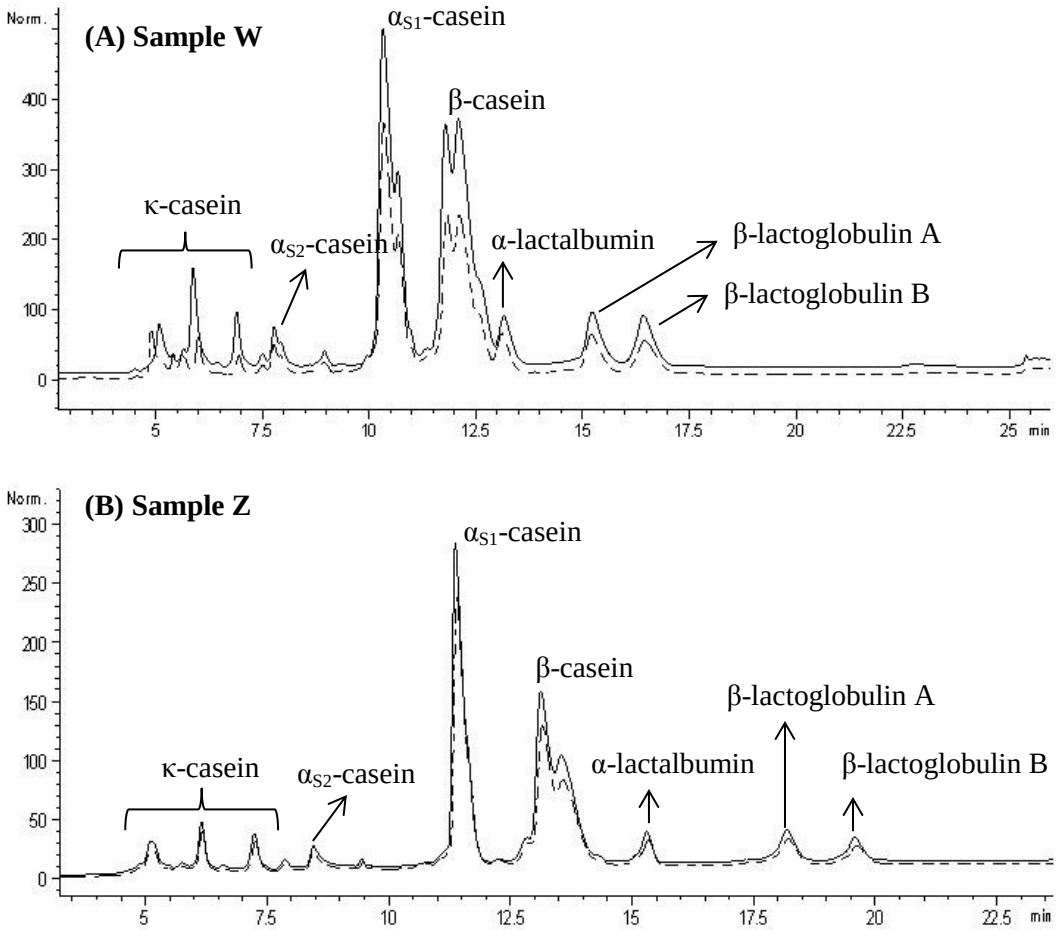

Figure 2.3. Separation of bovine milk proteins by reversed-phase high-performance liquid chromatography (HPLC). Chromatograms of samples **(A)** W and **(B)** Z stored at 4 ˚C are shown. Full line (-) shows the 0 h sample; dashed line (---) shows the 96 h sample.

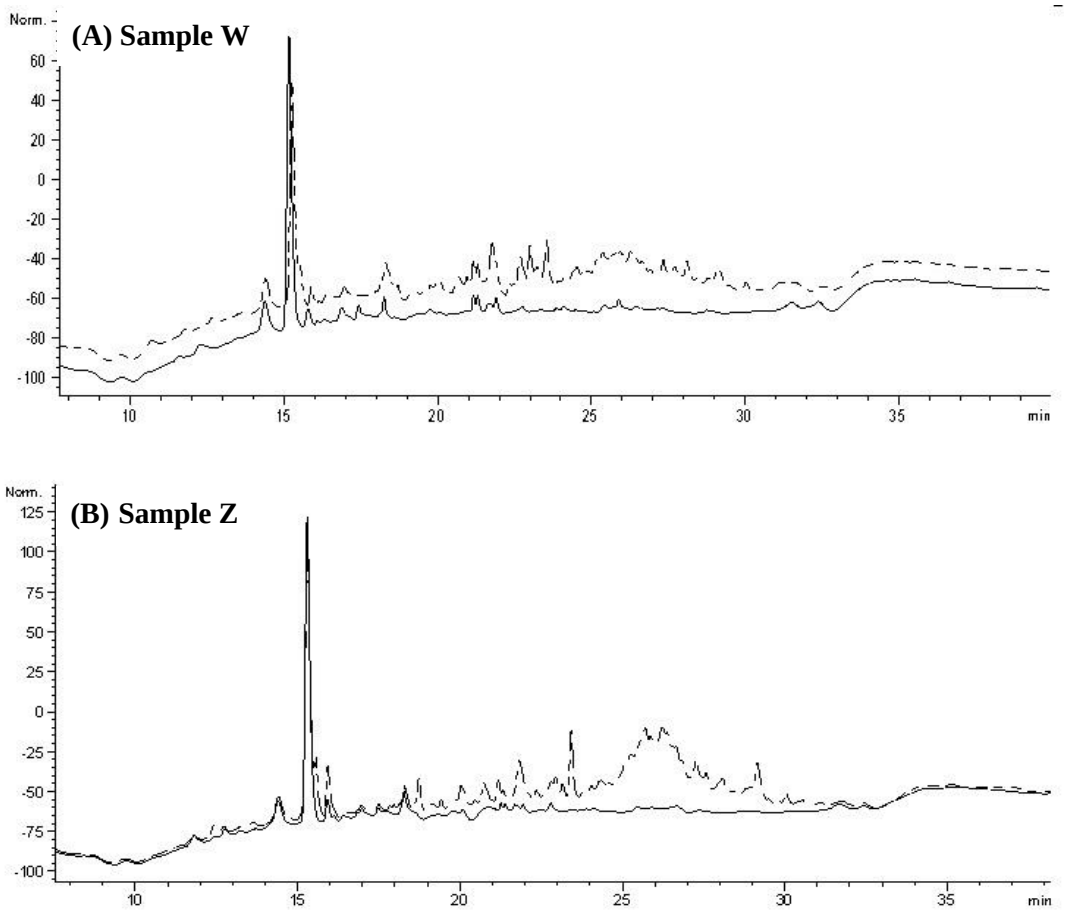

Figure 2.4. Separation of bovine milk peptides by reversed-phase high-performance liquid chromatography (HPLC). Chromatograms of samples **(A)** W and **(B)** Z stored at 4 ˚C are shown. Full line (-) shows the 0 h sample; dashed line (---) shows the 96 h samples.

2.3.6. 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 $62 \pm 4.1 \times 10^3$ cells/mL, $78 \pm 6.2 \times 10^3$ cells/mL, $77 \pm 4.2 \times 10^3$ cells/mL and $214 \pm 7.9 \times 10^3$ 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.

2.4. Discussion

2.4.1. TBC

According to European Regulation EC No 853/2004 (2004), TBC should be less than 5.00 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ cfu/ mL; Figure 2.2.A); however, milk stored at 4 °C reached a TBC of 5.47 ± 0.32 $\log_{10}$ cfu/ mL after 72 h (Figure 2.2.B). 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 $\log_{10}$ 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 2.1.A.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).

2.4.2. 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 $\log_{10}$ cfu/ mL. After 96 h, samples stored at 2 °C had a PBC over that limit (4.34 ± 0.22 log cfu/mL; Figure 2.2.A), 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 2.2.B). 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 $\log_{10}$ 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 $\log_{10}$ cfu/mL after 96 h, the difference between the average PBC of these samples and the European threshold (4.22 $\log_{10}$ 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 2.1) could be due to differences in practices on each of the farms, which lead to different contamination levels. The different bacterial increases 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.

2.4.3. 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 $\log_{10}$ 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 $\log_{10}$ cfu/ mL; PROT bacterial count: 3.72 ± 0.19 $\log_{10}$ cfu/ mL). However, PROT bacterial count reached 4.81 ± 0.19 $\log_{10}$ 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 $\log_{10}$ cfu/ mL) (Figure 2.2.A and B).

The LIP and PROT bacterial growth were affected by storage conditions and varied among farm milk samples (Figure 2.1.C.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 increases 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 $\log_{10}$ cfu/ mL, and counts after 48 h were 3.56 and 4.28 $\log_{10}$ 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.

2.4.4. 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 $\log_{10}$ 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.

2.4.5. 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, αS1-casein and β-casein contents in milk samples stored at 4 °C, affecting the total casein content

(Table 2.2). 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 2.3.A and B. The chromatograms presented in Figure 2.4.A 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 2.4.A 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 2.4.A 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 $\log_{10}$ cfu/ mL, and sample W had the highest level of PROT bacteria after 96 h (5.68 ± 0.01 $\log_{10}$ cfu/ mL). According to Lewis and Deeth (2009), when levels of psychrotrophic bacteria in milk reach 6.00 $\log_{10}$ cfu/ mL, the production of lipases and proteases begins. When the levels of PROT bacteria reach 4.65 $\log_{10}$ 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).

2.4.6. 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.

Table 2.2. Contents of casein fractions in samples stored at 2 ˚C or 4 ˚C, for 0 h and 96 h.

Temperature (˚C)	Time (h)†	Casein fractions (mg/ mL)						
		κ-casein	**α_{S1}-casein**	**α_{S2} -casein**	**β - casein**	**α-lactalbumin**	**β - lactoglobulin A + B**	**Total Casein**
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

†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).

2.5. Conclusion

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

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Chapter 3

The effect of different pre-cooling rates and cold storage on milk microbiological quality and composition

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Declaration: The experiment was conducted by Lizandra Paludetti, as well as the microbiological analysis, peptide profiles analysis and assessment of electricity at Teagasc Moorepark Dairy Parlor and Food Research Centre. Dr. Bernadette O'Brien and Dr. Kieran Jordan gave guidance on the planning the experimental design. All experimental data were analysed and the chapter written by Lizandra Paludetti, with corrections and comments from Prof. Alan L. Kelly from University College Cork, Dr. David Gleeson, Dr. Bernadette O'Brien and Dr. Kieran Jordan from Teagasc Moorepark.

Abstract

The objective of this study was to measure the effect of different milk cooling rates, before entering the bulk tank, on the microbiological load and composition of the milk, as well as on energy usage. Three milk precooling treatments were applied before milk entered 3 identical bulk milk tanks: no plate cooler (NP), single-stage plate cooler (SP), and double-stage plate cooler (DP). These precooling treatments cooled the milk to 32.0 ± 1.4 °C, 17.0 ± 2.8 °C, and 6.0 ± 1.1 °C, respectively. Milk was added to the bulk tank twice daily for 72 h, and the tank refrigeration temperature was set at 3 °C. The blend temperature within each bulk tank was reduced after each milking event as the volume of milk at 3 °C increased simultaneously. The bacterial counts of the milk volumes precooled at different rates did not differ significantly at 0 h of storage or at 24-h intervals thereafter. After 72 h of storage, the TBC of the NP milk was 3.90 ± 0.09 log10 cfu/ mL, whereas that of the precooled milk volumes were 3.77 ± 0.09 (SP) and 3.71 ± 0.09 (DP) log10 cfu/ mL. The constant storage temperature (3 °C) over 72 h helped to reduce bacterial growth in milk; consequently, milk composition was not affected and minimal, if any, proteolysis occurred. The DP treatment had the highest energy consumption (17.6 ± 0.5 Wh/ L), followed by the NP (16.8 ± 2.7 Wh/ L) and SP (10.6 ± 1.3 Wh/ L) treatments. This study suggests that bacterial count and composition of milk are minimally affected when milk is stored at 3 °C for 72 h, regardless of whether the milk is precooled; however, milk entering the tank should have good initial microbiological quality. Considering the differences between bacterial counts, however, the use of the SP or DP precooling systems is recommended to maintain low levels of bacterial counts and reduce energy consumption.

3.1. Introduction

Milk cooling and refrigerated storage are necessary after milking to reduce bacterial growth. Milk leaves the udder at approximately 35 °C, which is a favorable temperature for bacterial growth (Walstra *et al.*, 2006). Thus, the microbial load could increase rapidly if milk is maintained at that temperature. According to Holm *et al.* (2004), cooling milk rapidly (below 6 °C) is necessary to avoid the multiplication of microorganisms, especially psychrotrophs, which can grow at refrigeration temperatures but have optimal and maximal growth temperatures at >15 and 20 °C, respectively (Moyer and Morita, 2007). Thus, the precooling of milk (before it enters the bulk tank) could further reduce the bacterial growth. A further possible benefit of precooling milk is the reduction of energy costs on-farm (Murphy *et al.*, 2013).

The equipment used to precool milk consists of plate heat exchangers incorporating stainless steel plates in a sandwich arrangement, in which milk and cooling water flow in opposite directions through the spaces between alternate plates (Wang *et al.*, 2007). This system may have 1 or 2 cooling stages, in which well water and well and chilled waters are used in the first and second stages, respectively. O'Connell *et al.* (2016) observed only a minimal increase in milk bacterial count over time when fresh milk from each milking event was precooled using a single-stage plate cooler before being added to the bulk milk tanks twice daily.

Total bacterial count (TBC) is the main test used by milk processors to assess milk microbiological quality and it quantifies aerobic mesophilic bacteria in milk. In conjunction with TBC, the psychrotrophic bacterial count (PBC) is used to assess the hygiene quality of milk and is an indicator of hygiene

conditions on-farm (Harding, 1995; Robinson, 2002). Milk cooling reduces the growth of mesophilic and psychrotrophic bacteria, the optimum growth temperatures of which are between 20 and 45 °C and <7 °C, respectively (Frank and Yousef, 2004; Willey *et al.*, 2008). Thermoduric and thermophilic bacteria are the other relevant groups of bacteria that are measured in milk. These bacteria are important because they can survive thermal treatments such as those frequently applied in dairy processing to reduce bacterial numbers (e.g., pasteurization; Murphy *et al.*, 1999; Robinson, 2002). The main sources of those bacteria are in the cows' environment, because their vegetative cells and spores can be present in feed, forage, bedding material, dust, feces and soil (Scheldeman *et al.*, 2005; Gleeson *et al.*, 2013). *Clostridium perfringens* and *Clostridium botulinum* are the pathogenic thermoduric bacteria of most relevance to the dairy industry because of their heat-resistant spores and toxins (Wrigley, 1994; Fernandes, 2009).

Some mesophilic, psychrotrophic, thermoduric, and thermophilic bacterial strains have the ability to produce lipases and proteases. These enzymes hydrolyze fat and protein, resulting in sensorial defects and altering the physico-chemical properties and processability of milk (Chen *et al.*, 2003; Deeth, 2006). Lipolytic activity produces flavors described as rancid and bitter (Deeth, 2006) and can, for example, result in loss of foaming and creaming ability during butter manufacture (Shelley *et al.*, 1987). Celestino *et al.* (1997) reported that reconstituted UHT milk powder manufactured using 4-d-old raw milk had rancid and bitter flavours compared with UHT milk powder produced using fresh raw milk, probably due to bacterial protease and lipase activity. Therefore, the control of bacterial numbers

in milk helps to preserve milk functionality, allowing the production of a range of dairy products in accordance with specific quality parameters.

The aim of this study was to investigate the effect of precooling milk at different rates on the microbiological quality and composition of milk, as well as on energy usage. This study was conducted in a manner that mimicked on-farm milk production conditions: morning and evening milkings, similar milk storage conditions, and use or not of precooling systems.

3.2. Materials and methods

3.2.1. Experimental Design

This experiment was carried out in the dairy parlor at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Cork, Ireland. Springcalving dairy cows (n = 210) were milked in a 30-unit side-by-side milking parlor, twice daily over two 3-wk periods, with milking commencing at 0700 and 1430 h. Period 1 extended from June 13 to July 2, 2016, and period 2 extended from July 25 to August 13, 2016.

Before milking, cows' teats were washed and disinfected with chlorhexidine foam teat cleaner (Deosan Teatfoam Advance AG104, Sealed Air, Johnson Diversey Ltd., Dublin, Ireland) and dried using individual paper towels. The milk was transferred from clusters through 16-mm (internal diameter) milk tubes to a mid-level milk line (72 mm, internal diameter), with a milk lift of 1.5 m. The milk was collected in a receiver jar and pumped through a 48-mm stainless steel pipe, using a variable speed milk pump, to the bulk milk tanks (Figure 1). Once the milk flow rate dropped to 0.2 kg/ min, clusters were automatically removed, with a delay time of 20 s. A system to individually wash

and disinfect each cluster between each individual cow milking (Cluster Cleanse, Dairymaster, Causeway, Kerry, Ireland) was used. After each milking, the milking equipment was rinsed with water (14 L per milking unit), followed by a hot (75 °C) liquid detergent sterilizer wash (Liquid Gold, Dairymaster) circulated for 8 to 10 min in the milk line. Following this, the milking equipment was rinsed twice, and the final rinse contained peracetic acid (0.3–0.5% concentration). An acid-descale (Extrastrong descaler, Dairymaster) was incorporated into the wash regime before the detergent cycle once a week.

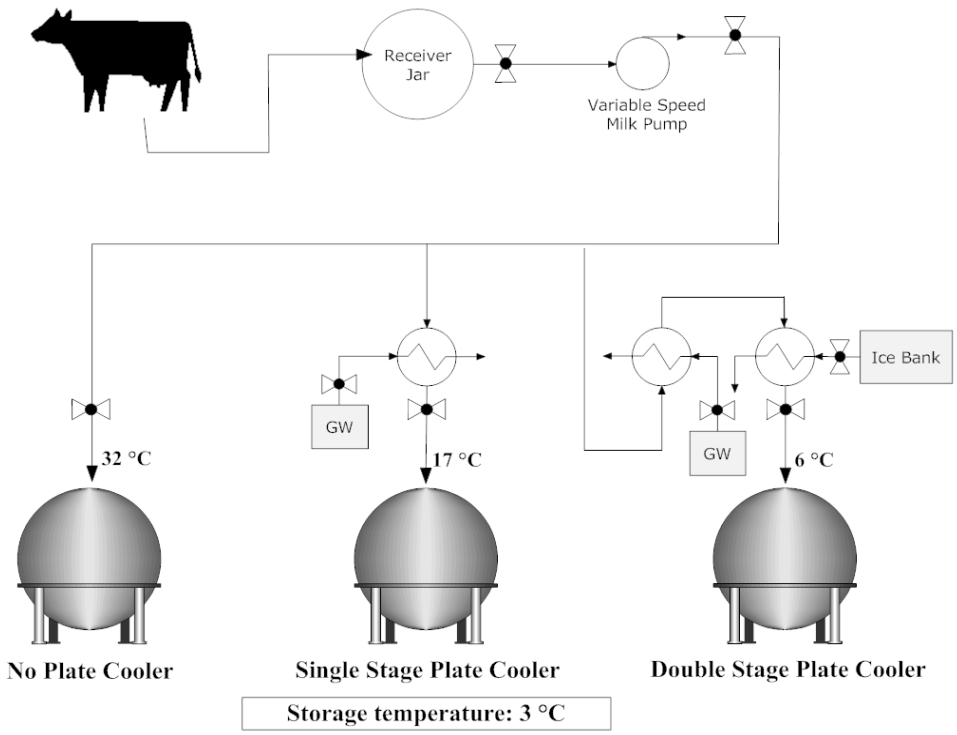

Figure 3.1. Experimental design: three pre-cooling systems: no plate cooler, single-stage plate cooler and double-stage plate cooler (GW: ground water).

The volume of milk collected during each milking was distributed equally into 3 identical bulk milk tanks. The milk line for each bulk tank was fitted with shut-off valves, which were used to control the milk flow rate and guarantee an

equal distribution of milk to the tanks. Each bulk tank had capacity of 4,000 L (Swiftcool, Dairymaster) and was fitted with a 5.5-Hp condensing unit. A screen on the front of each tank displayed the milk temperature, time, and milk volume. The milk was cooled to 3 °C within the tanks and stored for up to 72 h from once the first milking entered the tank. Approximately 800 and 500 L of milk were added to each bulk milk tank during the morning and afternoon milkings, respectively. At the end of each 72-h storage period, the milk was collected and the bulk milk tanks were washed using a hot detergent/sterilizer wash (50 °C). This was followed by a cold-water rinse and an additional rinse containing peracetic acid. An acid-descale wash product was used at every third wash.

Before entering the bulk tanks, the milk underwent 1 of 3 precooling treatments: no precooling (NP), single-stage (SP), or double-stage (DP) plate cooling (Figure 3.1). In the NP treatment, the ground water line was closed; therefore, no precooling was undertaken in that treatment. In the SP treatment (37 plates), the milk exchanged heat with ground water at approximately 15 °C. In the DP treatment (45 plates), the milk was cooled in 2 stages; in the first stage, ground water was used (at approximately 15 °C) and in the second stage, ice water (at approximately 0 °C) was used. Ice water was produced in an ice bank, a system with external melting ice on a coil thermal storage unit with an inline coil array. In the NP, SP, and DP treatments, the milk entered the bulk tanks at average temperatures of 32 ± 1.4 °C, 17 ± 2.8 °C, and 6 ± 1.1 °C, respectively. The temperature and volume of milk in each tank was recorded by an integrated system (Swiftcool, Dairymaster) and transmitted via Global System for Mobile communications (GSM) technology to a computer over the two 3-wk trial periods.

3.2.2. Milk sampling

During each milking, a milk sample was collected from the milk line using a sterile Durham flask surrounded by ice to assess the quality of milk entering the tanks. After the initial morning milking, duplicate milk samples (30 mL) were collected from each bulk tank once the milk temperature within each tank reached 3 °C, corresponding to 0-h samples (one milking). The subsequent samples (24, 48, and 72 h) were collected before the addition of each subsequent morning milk on subsequent days, when the bulk tanks contained milk from 2, 4, and 6 milkings, respectively. Before sample collection, the milk was agitated at 24 rpm for 1 min, and samples were collected from the top viewing inlet using sterilized sample dippers. Samples were transported to the laboratory in ice boxes, delivered within 30 min of collection, and analyzed. One sample from each tank was used for microbiological analysis and the other for compositional analysis and SCC.

3.2.3. Microbiological analysis

Immediately on delivery to the laboratory, raw milk samples collected every 24 h were tested in duplicate for a range of bacteria. All the microbiological analyses were performed according to the Standard Methods for the Examination of Dairy Products (Wehr and Frank, 2004). The TBC, PBC, thermoduric (laboratory pasteurization count, LPC), and thermophilic (THERM) bacterial counts were estimated using Petrifilm aerobic count plates, a ready-to-use medium (3M, Technopath, Tipperary, Ireland). Samples tested for LPC were pasteurized at 63 °C for 30 min, with an additional 5 min that allowed time for the samples to reach the required temperature (Frank and Yousef, 2004); after heating, the samples were cooled to 10 °C in iced water before testing. Samples

tested for TBC and LPC were incubated for 48 h at 32 °C (Laird *et al.*, 2004; Pantoja *et al.*, 2009; O'Connell *et al.*, 2016), whereas samples tested for PBC and THERM were incubated at 7 ± 1 °C for 10 d and at 55 °C for 48 h, respectively (Frank and Yousef, 2004). We are aware that using Petrifilm plates at 7 °C or 55 °C is outside the validated range. However, a pretrial experiment for THERM indicated that, at the same dilution, plate count agar plates were uncountable due to bacterial colonies spreading over the surface of agar plates, whereas Petrifilm plates were countable (data not shown). In other studies, Petrifilm plates have been used for PBC at 7 °C (Ramsahoi *et al.*, 2011). The number of bacterial colonies was assessed using a Petrifilm Plate Reader (3M, Technopath). The lipolytic bacterial count (LIP) was performed by spread-plating 100 µL of the appropriate dilutions on tributyrin agar with added glyceryl tributyrate (0.01 mL/mL of agar prepared; Sigma Aldrich, Dublin, Ireland). The proteolytic bacterial count (PROT) was estimated by spread-plating 100 µL of the diluted sample on calcium caseinate agar with added skim milk powder (2.5 mg/mL of agar; Merck, Darmstadt, Germany). For both methods, samples tested were incubated at 37 °C for 48 h. Lipolytic bacteria colonies were identified as colonies surrounded by a clear zone in a turbid medium, whereas proteolytic bacteria colonies were identified as colonies surrounded by a clear zone in an opaque medium.

The sulphite-reducing *Clostridia* count (SRC) was assessed by pour-plating 1 mL of diluted sample in iron sulphite agar and incubating plates under anaerobic conditions for 72 h at 37 °C, in accordance with ISO standard 15213 (ISO, 2003). Presumptive SRC colonies were identified as black colonies.

3.2.4. Composition and SCC

Raw milk samples collected every 24 h had their composition (fat, protein, lactose, and TS contents) and SCC determined using a Fossomatic FC (Foss Electric, Hillerød, Denmark) within 24 h after arrival in the laboratory.

3.2.5. Peptide profiles

Milk samples were collected from the bulk milk tanks at 0 and 72 h to obtain the peptide profiles. Trichloroacetic acid (TCA) was used to extract the nonprotein fraction of the milk samples, according to the extraction procedure described in IDF method 20-4 (IDF, 2001). The extracts were not diluted but were filtered using 0.45-µm syringe cellulose filters (25 mm diameter, Chromafil Xtra RC-45/25, Macherey-Nagel, Dublin, Ireland). The HPLC equipment used was an Agilent 1200s system (Agilent Technologies, Santa Clara, CA), with quaternary pump and multi-wavelength detector. A Zorbax 300SB column (4.6 mm internal diameter × 150 mm; Agilent Technologies) was used to perform the separation of milk peptides. The gradient elution and peak detection methodology was an adaptation of the methodology of Rohm *et al.* (1996). Samples were injected onto the column (50 µL) in duplicate and the flow rate was 0.50 mL/min.

3.2.6. Assessment of electricity consumption

The energy consumption of each treatment, expressed in Watt-hours (Wh), was measured as the energy usage of each bulk milk tank when each of the precooling treatments was applied. For the DP treatment, the energy usage of the ice bank was also considered. Energy usage was assessed using energy analyzers

(EM24 DIN) and Digi Connect wireless WAN cellular routers (Carlo Gavazzi Automation SpA, Lainate, Italy), which measured and transmitted the energy data, respectively. The cumulative energy usage was recorded every 1 min using the software program Powersoft (Carlo Gavazzi Automation SpA). To determine electricity costs associated with milk cooling, the data on electricity use was combined with day and night tariffs [day tariff was 0.16 €/ kWh (60%); night tariff was 0.07 €/ kWh (40%)], similarly to the procedure described by Upton *et al.* (2013).

3.2.7. Statistical analysis

This study was carried out following a Latin square design with repeated measures, in which samples were collected every 24 h, and each bulk tank (n = 3) received a different precooling treatment (NP, SP, DP) in each week (n = 3). Each Latin square was conducted over two 3-wk periods.

Least squares means for the main effects of period, week, storage time, and precooling system, as well as the interaction between storage time and precooling system, were calculated using the MIXED procedure in SAS 9.3 (SAS Institute Inc., Cary, NC). The fixed effects included in each model were period (1 and 2), week (1, 2, and 3), precooling system (NP, SP, and DP), and storage time (0, 24, 48, and 72 h). Repeated-measures models were used to account for correlations between time points. Tank within week was considered the experimental unit. The response variables were TBC, PBC, LPC, THERM, PROT, LIP, SRC, SCC, and fat, protein, lactose, and TS contents. 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 test (at 5% error probability) was used to compare the means for all variables.

3.3. Results and Discussion

3.3.1. Microbiological analysis

During the first milking occasion on the first day of each trial week, a milk sample was collected from the milk line before distribution of the milk to each bulk tank. The average (± SD) TBC of those milk samples was 3.35 ± 0.29 $\log_{10}$ cfu/ mL, indicating that milk of good microbiological quality was produced. The TBC least squares means of milk samples from each bulk tank collected at 0 h (after first milking) was 3.54 ± 0.05 $\log_{10}$ cfu/ mL. The similarity between the 2 TBC levels for those samples indicated that the precooling treatments did not affect the microbiological load and that milk of good microbiological quality entered each tank. The average (±SD) TBC of the milk line over 72 h of storage and the 2 trial periods was 3.55 ± 0.26 $\log_{10}$ cfu/ mL. Good hygiene practices (e.g., teat preparation, individual cluster cleaning between milkings, and equipment wash routines) contributed to the high quality of the milk entering the bulk milk tanks.

The TBC levels at 0 h for NP, SP, and DP were 3.55, 3.57, and 3.50 ± 0.09 $\log_{10}$ cfu/ mL, respectively; the PBC least squares means were 3.11, 3.04, and 3.07 ± 0.11 $\log_{10}$ cfu/ mL; the LIP least squares means were 3.24, 3.26, and 3.28 ± 0.10 $\log_{10}$ cfu/ mL; and the PROT least squares means were 3.20, 3.14, and 3.24 ± 0.07 $\log_{10}$ cfu/ mL, respectively (Figure 3.2). The differences in the time required to cool the milk to 3 °C (within the bulk tanks) were expected to affect those initial bacterial counts of the milk volumes; however, the bacterial counts were

not significantly different ($P > 0.05$). The NP, SP, and DP treatments precooled the milk to average (±SD) temperatures of 32.0 ± 1.4 °C, 17.0 ± 2.8 °C, and 6.0 ± 1.1 °C, respectively; and the average time taken to cool milk to 3 °C within the bulk milk tanks on the first morning milking on each week was approximately 2 h, 1 h, and 20 min, respectively. Given the low initial bacterial counts, the difference in these bulk tank-cooling times was not sufficient to result in different bacterial levels at 0 h.

The different precooling treatments also did not affect any of the bacterial counts over the storage time up to 72 h ($P > 0.05$, Table 3.1). The volume of milk stored at 3 °C increased in each bulk tank after each milking, resulting in a decrease in the blend temperature within the tanks over time. After the first 2 milking occasions, the milk volume produced at subsequent milkings blended with a higher volume of milk previously cooled to 3 °C; consequently, the milk was cooled faster than that from the first 2 milking events. Therefore, the maintenance of low temperatures within the bulk tanks did not allow for significant increases in bacterial numbers in the milk; consequently, the precooling system had no significant effect on bacterial counts over time. However, after 72 h, we observed a difference between the bacterial counts in milks subjected to different precooling treatments (Figure 3.2). After 72 h, TBC and PBC (least squares means) in milk that was not precooled were 3.90 ± 0.09 and 3.38 ± 0.11 $\log_{10}$ cfu/ mL, respectively. The SP milk precooled had TBC and PBC (least squares means) of 3.77 ± 0.09 and 3.28 ± 0.11 $\log_{10}$ cfu/ mL, and that precooled using DP had similar TBC and PBC: 3.71 ± 0.09 and 3.25 ± 0.11 $\log_{10}$ cfu/ mL, respectively.

Storage time affected TBC, PBC, and PROT ($P = 0.004$, $P < 0.001$, and $P = 0.03$, respectively, Table 3.1), which were 3.54 ± 0.05, 3.07 ± 0.06, and 3.19 ± 0.04 $\log_{10}$ cfu/ mL at 0 h; and 3.79 ± 0.05, 3.30 ± 0.06, and 3.36 ± 0.04 $\log_{10}$ cfu/ mL after 72 h, respectively (least squares means across precooling treatments). The increases in TBC and PBC were not considered biologically relevant because both were well below the European thresholds determined in document EC no 853/2004 [European Commission, 2004; TBC: 5.00 $\log_{10}$ cfu/ mL (1.00×10^5 cfu/ mL); PBC: 4.22 $\log_{10}$ cfu/ mL (16,700 cfu/ mL)] and typical TBC limits applied by some milk processors [e.g., 4.70 $\log_{10}$ cfu/ mL (5.00×10^4 cfu/ mL)]. The least squares means of PROT and LIP levels at 72 h (3.36 ± 0.04 $\log_{10}$ cfu/ mL and 3.34 ± 0.06 $\log_{10}$ cfu/ mL, respectively) were also not considered relevant as both were well below the limit suggested by Vyletelova *et al.* [2000; 4.65 $\log_{10}$ cfu/ mL (44,668 cfu/ mL), for each]; those authors suggested that LIP and PROT should be below this level to avoid the production of heat-resistant hydrolytic enzymes when milk is destined for dairy product manufacture, because such enzyme activities could result in loss of milk functional properties and sensory defects.

The PBC was different between the 2 trial periods (1 and 2; $P = 0.002$, Table 3.1), whereas TBC and LIP varied between weeks ($P = 0.02$ and $P = 0.005$, respectively, Table 3.1). Variations in bacterial population in milk at different periods could be related to the cows' health status (e.g., mastitis) or different bacteria strains present in the cows' environment (e.g., feed; Lafarge *et al.*, 2004).

Table 3.1. The significance of the main effects of period (2 x 3 weeks), week (6 weeks), storage time (72 h) and pre-cooling systems (no plate cooler, single-stage plate cooler and double-stage plate cooler), as well as the interaction between storage time and pre-cooling system on the total bacterial counts (TBC), psychrotrophic bacterial count (PBC), lipolytic bacterial count (LIP), proteolytic bacterial count (PROT), thermoduric bacterial count (LPC) and thermophilic bacterial count (THERM) of the milk samples.

Bacterial Counts	**Significance**				
	Period	**Week**	**Storage Time**	**Pre-cooling system**	**Pre-cooling system* Storage Time**
TBC	0.23	0.02	0.004	0.61	0.93
PBC	0.002	0.18	<0.001	0.68	0.99
LIP	0.08	0.005	0.05	1.00	0.96
PROT	0.30	0.05	0.03	0.77	0.92
LPC	0.82	0.42	0.20	0.71	0.38
THERM	0.79	0.08	0.70	0.12	0.69

The LPC and THERM counts did not differ between periods and weeks, and storage time and precooling systems did not affect their levels ($P > 0.05$, Table 3.1). At 0 and 72 h, the least squares means of LPC were 0.80 and 0.83 ± 0.11 $\log_{10}$ cfu/mL, whereas THERM counts were 0.85 and 0.64 ± 0.13 $\log_{10}$ cfu/mL, respectively. A typical industry LPC specification can range from 2.70 to 3.00 $\log_{10}$ cfu/mL (500 to 1,000 cfu/mL), although there are no European legislation thresholds or dairy processor specifications for thermophilic bacteria in milk. According to Byrne and Bishop (1991), some species of Micrococcus do not grow well on Petrifilm, although those authors concluded that Petrifilm aerobic count plates are a suitable alternative to agar plates for determination of LPC. The SRC levels varied between 0 and 1 $\log_{10}$ cfu/mL (10 cfu/mL), indicating a low level of contamination with those organisms. Because of the low incidence, we

could not determine the influence of storage and production conditions on these bacteria.

The milk volumes stored in the 3 tanks had low bacterial growth, indicating that the storage temperature was effective in preventing an increase in bacterial numbers in the milk over the storage period. O'Connell *et al.* (2016) stored milk in bulk milk tanks for over 96 h at 2 or 4 °C and observed similar results to this study. In that study, milk stored at 2 or 4 °C for over 72 h had average TBC, PBC, PROT, and LIP of 3.58, 3.11, 2.94, and 2.91 $\log_{10}$ cfu/ mL, respectively. As well as the storage temperature, the initial microbial load of the milk will influence the microbial load over storage (Guinot-Thomas *et al.*, 1995). Therefore, milk entering the tank has to be of high microbiological quality to obtain bacterial counts similar to those obtained after the storage period in the present study. Thus, to minimize bacterial growth in milk during storage, it is important that appropriate cleaning practices (for milking equipment and cows) be carried out during milking.

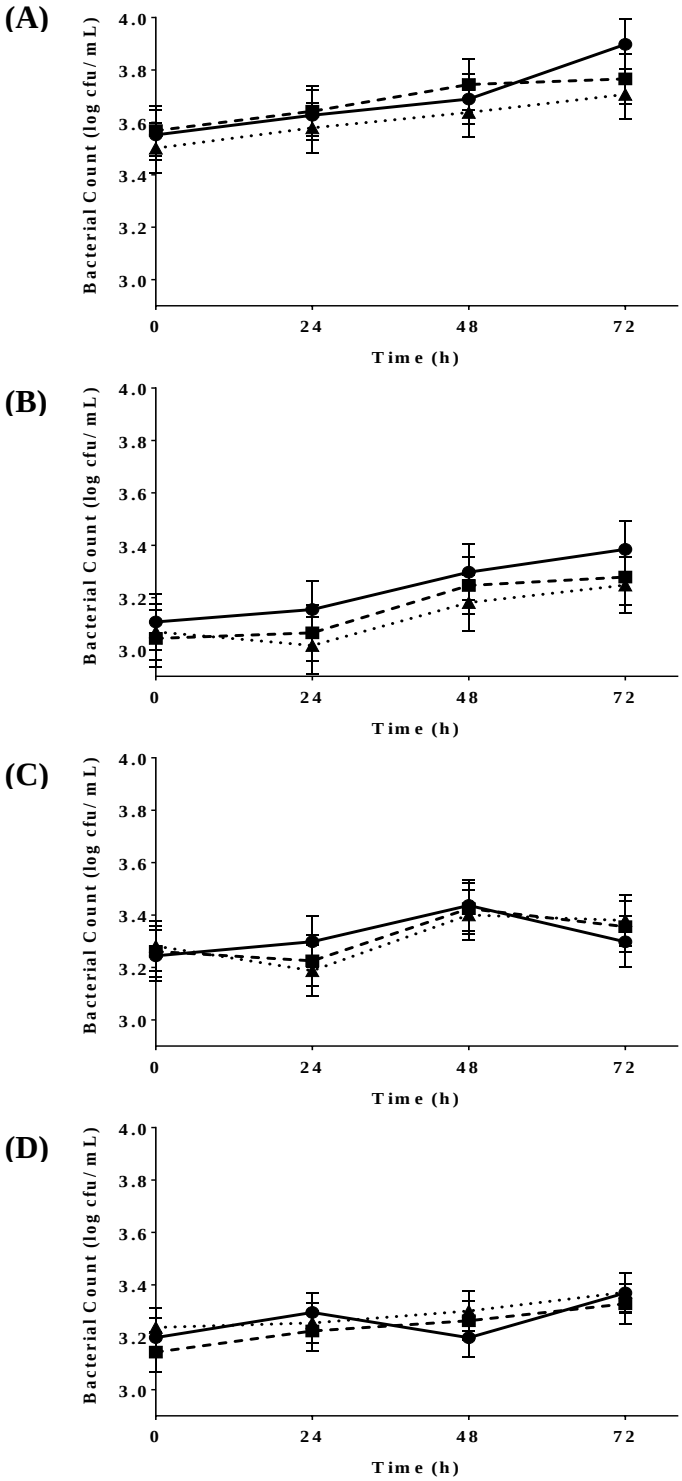

Figure 3.2. Effect of storage time and different pre-cooling systems [-●- no plate cooler (NP); -■- single stage plate cooler (SP); -▲- double stage plate cooler (DP)] on the (A) total (TBC), (B) psychrotrophic (PBC), (C) lipolytic (LIP) and (D) proteolytic (PROT) bacterial counts in milk stored for 72 h.

3.3.2. Composition and SCC

The average (±SD) fat, protein, lactose, and TS contents of the sample collected from the milk line after the first milk occasion (first day of each trial week) were 4.52 ± 0.26, 3.58 ± 0.09, 4.76 ± 0.18, and 13.36 ± 1.93%, respectively. After the first morning milking, the milk samples (0 h) precooled at different rates had average (±SD) fat, protein, lactose, and TS contents similar to those in the milk line sample: 3.49 ± 0.09, 3.63 ± 0.06, 4.81 ± 0.06, and 12.53 ± 0.10% (NP treatment); 3.48 ± 0.16, 3.62 ± 0.05, 4.82 ± 0.06, and 12.52 ± 0.17% (SP treatment); and 4.14 ± 0.22, 3.59 ± 0.07, 4.76 ± 0.08, and 13.12 ± 0.21% (DP treatment), respectively. These results, compared with the milk line results, indicate that the precooling treatments did not affect the milk composition as would have been expected. The differences in the fat contents noted could be due to fat distribution when sampling.

The precooling treatments had no effect on milk composition ($P > 0.05$), and storage time did not affect fat, protein, or TS content ($P > 0.05$). After the 2 milking occasions on the first day, the contents of fat, protein, lactose, and TS (least squares means) were 4.41 ± 0.06, 4.59 ± 0.08, 5.78 ± 0.05, and 13.35 ± 0.07%, and after 72 h (6 milkings) were 4.44 ± 0.06, 4.58 ± 0.08, 5.79 ± 0.05, and 13.41 ± 0.07%, respectively. The protein contents (least squares means) were different in the 2 periods (period 1: 3.55 ± 0.002%; period 2: 3.63 ± 0.002%, $P = 0.0001$) and between weeks ($P = 0.02$), ranging from 3.54 to 3.72%. The lactose content was also different between periods (period 1: 4.82 ± 0.002%; period 2: 4.74 ± 0.002%, $P = 0.007$). As fresh milk was transferred to the tanks every day, the composition of milk stored within the bulk tanks may have varied according to the content of components in the fresh milk added to the tank on each milking

occasion. Those variations in milk composition could be related to cows' physiology or days in milking (Linn, 1988). Also, the interval between milkings can affect milk composition, influencing the TS content of milk collected during the morning and afternoon (Ayadi *et al.*, 2004).

The SCC between periods were statistically different (P = 0.003); however, there was a marginal difference of 36.6 × 103 cells/mL between periods 1 and 2, which is probably not biologically relevant. Furthermore, SCC in both periods (period 1: 115.9 × 10^3 cells/mL; period 2: 152.5 × 10^3 cells/mL) were below the European Union legislation threshold (400 × 10^3 cells/mL).

3.3.3. Peptide profiles

High-performance liquid chromatography was performed to determine whether precooling treatments would result in different peptide profiles after 72 h of storage, thus indicating proteolysis. The chromatograms presented in Figure 3.3.A, B, and C are an average of the chromatograms obtained for all milk samples precooled using the NP, SP, and DP systems, respectively, over the 2 periods. The chromatograms indicated no difference between the initial peptide concentrations in milk volumes precooled at different rates (0 h), no increase in concentrations over time, and no appearance of peaks that characterize proteolysis. We also noted in those chromatograms the absence of peaks after 20 min, indicating the lack of plasmin action, which hydrolyses β-, αS1-, and αS2-caseins into peptides and proteose-peptones (Crudden *et al.*, 2005). Therefore, the application of different precooling treatments did not affect proteolysis levels in the milk.

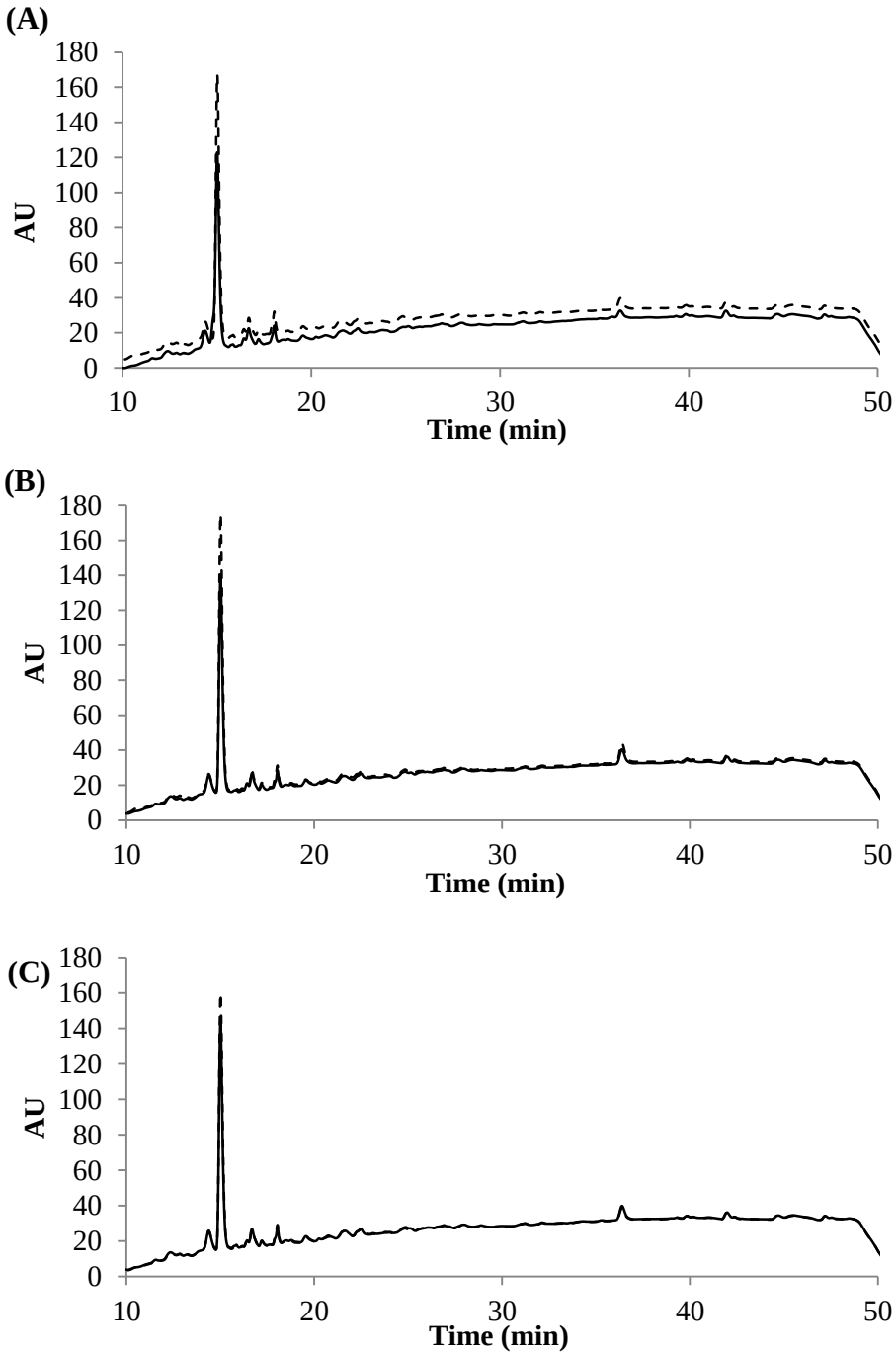

Figure 3.3. Separation of bovine milk peptides by reversed-phase HPLC. Chromatograms of samples pre-cooled using (A) no plate cooler (NP), (B) single stage plate cooler (SP) and (C) double stage plate cooler (DP) stored at 3 ˚C are shown. Black line: 0 h; grey dashed line: 72 h. (AU: absorbance units).

The peptide peak at 15 min (unknown) is the only peptide whose concentration varied over 72 h and we observed only small differences between treatments for that peak. However, the variation in the concentration of this peptide could be caused by the addition of fresh milk to the tanks, the composition of which could vary, as previously mentioned. The low levels of proteolysis observed might be due to the low levels of proteolytic bacteria in the milk, which did not reach a level sufficient for significant production of proteolytic enzymes (4.65 $\log_{10}$ cfu/mL; Vyletelova *et al.*, 2000). The low storage temperature applied over the storage period could have been effective in reducing the growth of proteolytic bacteria and is far from the optimum temperature for most enzymes (Kelly and Fox, 2006).

3.3.4. Energy consumption

When the NP and SP treatments were used, the average energy usage (± SD) of the bulk tanks was 16.8 ± 2.7 and 10.6 ± 1.3 Wh/ L of milk, respectively. The energy usage for the NP treatment was higher than that for the SP treatment because milk entered the bulk tank at a higher temperature (32.0 ± 1.4 °C) and the compressor running time was longer to achieve the required storage temperature. In the NP and SP treatments, an average (± SD) of 129 ± 10 min and 57 ± 22 min were necessary to cool milk to 3 ˚C, respectively. For the DP treatment, the average energy usage (±SD) of the bulk milk tank and ice bank was 4.0 ± 0.5 and 13.6 ± 0.2 Wh/ L of milk, respectively (total energy usage was 17.6 ± 0.5 Wh/ L of milk). When this treatment was used, milk was cooled faster when compared to the other treatments (21 ± 3 min).

Considering the similar bacterial counts between the precooling treatments and the energy usage of each treatment over the 72-h storage period, the SP system would achieve low levels of bacterial counts over storage time and lower energy usage rates compared with the DP treatment. Energy usage was higher for the DP system than for the SP system because of the energy requirements to produce ice. However, this system could be recommended for farms in which an ice bank system is already being used for cooling milk within the bulk tank.

The energy consumption of each treatment is within the ranges reported by Shine *et al.* (2018), who surveyed 58 Irish commercial dairy farms regarding energy consumption at milking. In that study, the average (±SD) energy usages reported were 12.68 ± 5.20, 10.54 ± 2.55, and 14.94 ± 5.45 Wh/ L for NP, SP, and DP systems, respectively. The variation in results between studies could be due to the age of the bulk tanks, the size of the tanks, and how they were installed. The ice bank energy usage in the present study was similar to average usage reported in a survey of 25 Irish commercial dairy farms (13.0 Wh/ L; Murphy *et al.*, 2013) and similar to the average value reported by Upton *et al.* (2013; 19.2 Wh/ L, range: 16.0–21.8 Wh/ L).

In this study, the calculated cost related to milk cooling when using NP, SP and DP (with ice bank) was €0.0021, €0.0014 and €0.0022/ L of milk, respectively. Those results are similar to the average estimated cost reported by Upton *et al.* (2013) (€0.0016), who monitored the electricity consumption on 22 Irish dairy farms. In that study, the average cost was calculated considering farms that did or did not use a pre-cooling system. Also, if the water charges are fully implemented in Ireland by 2020, water consumption will be another contributing factor to the expenses related to milk production on-farm. In a previous plan

established by the Irish government in 2015, the rate would be €1.85 per 1,000 L of water when using one public water service. According to Shine *et al.* (2018), pre-cooling has the highest water consumption in Irish dairy parlors (1.81 L_{water}/ L_{milk}). In the present study, the water consumption was measured when using the SP and DP treatments; however, the ratio water to milk was high in both periods (Period 1 – SP: 3.1 and DP: 9.2 L_{water}/ L_{milk}; Period 2 – SP: 16.9 and DP: 10.2 L_{water}/ L_{milk}) and is not a appropriate representation of the actual water consumption on commercial dairy farms.

3.4. Conclusion

The microbiological load of milk precooled at different rates did not differ statistically at 0 h or over the 72 h of storage, indicating no significant difference between the precooling treatments. No technologically relevant variations were observed in milk composition, and no considerable enzymatic activity was observed, possibly because of the good microbiological quality of the milk. This study suggests that the bacterial count and composition of milk are minimally affected when milk is stored at 3 °C for 72 h whether the milk is precooled or not; however, milk entering the tank should have good initial microbiological quality. Regarding energy usage, the SP treatment required less energy than the other treatments to maintain an equivalent microbiological load in milk. Considering that the milk volumes undergoing the SP and DP treatments had the lowest bacterial counts over 72 h of storage, it may be beneficial and economical to incorporate the DP system on farms that already use an ice bank bulk milk tank and SP system on other farms. Precooling good quality milk with an SP or DP

system and subsequent storage at 3 °C for 72 h can maintain good microbiological and compositional quality of milk with reduced energy consumption.

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Chapter 4

Microbiological quality of milk from farms to milk powder manufacture: an industrial case study

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Abstract

The experiments reported in this research paper aimed to track the microbiological load of milk throughout low-heat skim milk powder (SMP) manufacturing process, from farm bulk tanks to final powder, during mid- and late-lactation (spring and winter, respectively). In the milk powder processing plant studied, low-heat SMP was produced using only the milk supplied by the farms involved in this study. Samples of milk were collected from farm bulk tanks (mid-lactation: 67 farms; late-lactation: 150 farms), collection tankers (CTs), whole milk silo (WMS), skim milk silo (SMS), cream silo (CS) and final SMP. During mid-lactation, the raw milk produced on-farm and transported by the CTs had better microbiological quality than the late-lactation raw milk [e.g., total bacterial count (TBC): 3.60 ± 0.55 and 4.37 ± 0.62 $\log_{10}$ cfu/ mL, respectively]. After pasteurisation, reductions in TBC, psychrotrophic (PBC) and proteolytic (PROT) bacterial counts were of lower magnitude in late-lactation than in mid-lactation milk, while thermoduric (LPC – laboratory pasteurisation count) and thermophilic (THERM) bacterial counts were not reduced in both periods. The microbiological quality of the SMP produced was better when using mid-lactation than late-lactation milk (e.g., TBC: 2.36 ± 0.09 and 3.55 ± 0.13 cfu/ g, respectively), as mid-lactation raw milk had better quality than late-lactation milk. The bacterial counts of some CTs and of the WMS samples were higher than the upper confidence limit predicted using the bacterial counts measured in the farm milk samples, indicating that the transport conditions or cleaning protocols could have influenced the microbiological load. Therefore, during the different production seasons, appropriate cow management and hygiene practices (on-farm and within the factory) are necessary to control the numbers of different bacterial

groups in milk, as those can influence the effectiveness of thermal treatments and consequently affect final product quality.

4.1. Introduction

Bovine milk is used to produce a wide range of dairy products and nutritional ingredients. Each dairy product has to conform with specific quality parameters determined by regulatory authorities and international markets, which could be related to safety, nutritional value, physical and sensory characteristics. Bacterial numbers in milk are one of the main factors that can impact those parameters, and their control throughout processing is essential to achieve dairy products of high quality (Kable *et al*., 2016).

The first stage of the milk supply chain is the farm, where factors such as cow management, stage of lactation and equipment cleaning protocols can affect bacterial numbers in milk (O'Connell *et al*., 2015). A variety of microorganisms could grow in milk, including: mesophilic, psychrotrophic, lipolytic, proteolytic, thermoduric and thermophilic bacteria, as well as pathogenic bacteria. Huck *et al.* (2008) observed that some spore-forming bacteria (*Bacillus*, *Paenibacillus and Sporosarcina*) were identified throughout the processing stages of fluid milk production, from the farm to the packaged product, suggesting that multiple potential entry points for those bacteria into milk are at the farm. Therefore, the production of raw milk under appropriate hygienic conditions is critical to control bacterial numbers, as thermal treatments during dairy processing cannot always completely reduce the bacterial load.

Several studies have focused on quantifying and identifying bacterial types in raw milk on-farm and their effect on dairy products (Barbano *et al*., 2006; Quigley *et al*., 2013a; Murphy *et al*., 2016). However, the combined influence of farm practices, storage conditions, transport and processing conditions on the microbiological quality of final product is not well understood and further

investigations are necessary Kable *et al.* (2016) reported that the microbiota in collection tankers (CTs) can be highly diverse and differ according to season. This diversity may be attributed to contributing on-farm factors, such as cattle skin, bedding, feed, human handling, milking equipment, and on-site bulk tanks used for storage. Thus, each individual supplier could impact differently on the levels of different bacterial groups in the milk within CTs that collect milk from multiple farms.

When milk is collected from farm bulk tanks, it is still prone to further increases in bacterial populations, which can arise due to inappropriate equipment sanitation, favourable storage conditions or processing parameters for rapid bacterial multiplication (Teh *et al.*, 2011; Cherif-Antar *et al.*, 2016). Therefore, dairy processors have to adopt good manufacturing practices and monitor several critical control points throughout the manufacturing processes to guarantee food safety and conformity with legislation or specifications. For example, one of the challenges regarding equipment sanitation concerns heat-resistant spore-forming bacteria. These bacteria can develop cleaning-resistant biofilms on the interior surfaces of pipelines or equipment, enabling cross-contamination of finished products (Jindal *et al*., 2016). Processing parameters can also have an impact on bacterial load, especially thermal treatments. For example, the temperature programme and holding time during pasteurisation should be appropriate to reduce the microbial load and the number of viable pathogens in milk (Tucker, 2015).

The objective of this study was to monitor the microbiological quality of milk throughout the processing of low-heat skim milk powder (SMP), from individual farm bulk tanks to the final powder produced, during mid- and late-

lactation periods. This study will aid in determining the association between the quality of milk and subsequent SMP produced, as well as the impact of processing parameters on milk and SMP quality. To our knowledge, this is the first such study that has tracked milk quality from individual farms to final product.

4.2. Materials and methods

4.2.1. Milk collection and skim milk powder manufacture

This study was conducted on commercial dairy farms and in a milk powder processing plant, which produced SMP only using the milk supplied by the farms involved in this study. This experiment was conducted during the mid- and late-lactation periods (May 2016 and December 2016, respectively), which corresponded to spring and winter in Ireland. During those periods, cows were grazing outdoors and housed indoors, respectively. The dairy farms involved in this study were located in the Kilkenny and Waterford regions of Ireland. During mid-lactation, 67 Irish dairy farms supplied sufficient milk to the factory to undertake the manufacturing process; during late-lactation, 150 dairy farms were necessary, due to the lower milk yield per cow during that period. The raw milk harvested during mid- and late-lactation were stored within the bulk tanks for an average (± SD) of 44 ± 11 h (range: 2 to 52 h) and 70 ± 19 h (range: 24 to 217 h) prior to tanker collection, at 3.1 ± 0.7 °C (range: 0.9 to 4.5 °C) and 3.3 ± 1.2 °C (range: 0.5 to 9.5 °C), repectively. The average (± SD) milk volume collected from each farm was 4,418 ± 3,066 L and 1,786 ± 1,905 L, during mid- and late-lactation, respectively. Each collection tanker (CT, n = 11) collected milk from approximately 6 and 14 farms in mid- and late-lactation, respectively; and the temperature in the CTs ranged from 3.7 to 4.2 °C. A total of 296,003 L and

267,932 L of milk were transported to a commercial SMP factory during mid- and late-lactation, respectively. Those volumes were stored in a whole milk silo (WMS) within the factory for approximately 5.5 h (time between the transference of the first CT milk and the eleventh CT milk to the silo), at an average (± SD) temperature of 4.6 ± 0.2 °C, and agitated for 1 min every 29 min. Subsequently, the milk was pasteurised by applying a HTST treatment (75 ˚C, 25 s). After pasteurisation, the cream was separated and stored in the cream silo (CS), while the skim milk (0.075% of cream) was stored in the skim milk silo (SMS). The skim milk was concentrated from 9% w/w to 52% w/w of total solids in a triple effect evaporator. Afterwards, the evaporated milk underwent spray-drying process and the average moisture content of the skim milk powder (SMP) produced was 3.2 ± 0.2% w/w. Approximately 22,000 kg of low-heat SMP were produced during both lactation periods that this study was carried out. The commercial processing plant in which this experiment was carried out detains further details regarding the processing parameters.

4.2.2. Sampling procedure

During mid- and late-lactation, 300-mL samples were collected from the top inlet of the 67 and 150 farm bulk tanks, respectively, using sterilised sample dippers. On arrival at the processing plant, 300-mL samples were collected from the top inlet of each CT (n = 11) using sterilised dippers. Samples (300 mL) were also collected from the top and bottom sampling ports of both WMS and SMS using industrial syringes. Additionally, in late-lactation, cream samples (300 mL) were collected from the top and bottom of the CS using industrial syringes, as that cream was produced only using the milk supplied by the 150 farms. All silo

samples were collected after the whole milk, skim milk or cream was completely transferred to the respective silos. Additionally, three 25-kg SMP bags were collected within the factory at the start, middle and final stages of the spray-dryer run, giving a total of 9 bags. Powder samples were reconstituted using deionised water (1:10 dilution).

All samples collected in mid-lactation and samples from the factory collected during late-lactation (CT, WMS, CS and SMS samples) were transported in cooling boxes (< 4 °C), within 6 h, and analysed in the milk quality laboratory in Teagasc Moorepark (Fermoy, Co. Cork, Ireland). Due to the high number of farm milk samples collected in late-lactation, those samples were analysed at the laboratory in the factory.

In relation to the low-heat SMP samples, 100 g were taken from the top, middle and bottom of each bag; these were mixed to obtain a representative 300-g samples from each bag. These powders were reconstituted using deionised water (1:10 dilutions) and sub-divided into 30-mL sterile bottles for microbiological analysis. Those samples were also analysed at the milk quality laboratory in Teagasc Moorepark.

A schematic drawing of the SMP manufacturing process is shown in Figure 4.1, as well as the sampling points.

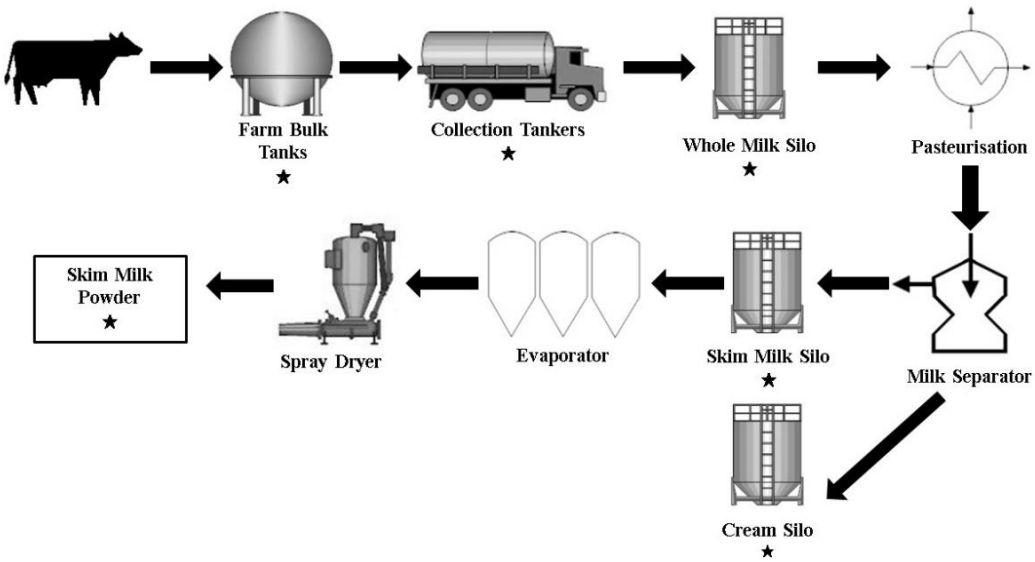

Figure 4.1. Milk supply chain and manufacturing process for conversion to low-heat skim milk powder, conducted in the mid- and late-lactation periods. The sampling points are indicated with a ★.

4.2.3. Microbiological analysis

All samples collected during mid-lactation and the CT, WMS, CS, SMS and SMP samples collected during late-lactation were tested in duplicate for a range of bacterial species. All the microbiological analyses were performed according to the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004). Total (TBC), psychrotrophic (PBC), thermoduric (Laboratory Pasteurisation Count - LPC) and thermophilic (THERM) bacterial counts were measured using Petrifilm aerobic count plates (ready to use media; 1 mL of diluted sample on each plate) (3M, Technopath, Tipperary, Ireland), in accordance with the procedures described by Laird *et al*. (2004). The LPC test consisted of pasteurising the milk samples at 63 ˚C for 35 min, including time to allow samples to reach the required temperature (Frank and Yousef, 2004); afterwards, the samples were cooled to 10 ˚C using iced water before testing. Samples tested for TBC and LPC were incubated for 48 h at 32 ˚C, while samples tested for

THERM were incubated for 48 h at 55 ˚C. The Petrifilms corresponding to the PBC test were incubated for 10 days at 7 ± 1 ˚C (Frank and Yousef, 2004). The authors are aware that using Petrifilm at 7 or 55 ˚C is outside the validated temperature range for that media. However, a pre-trial experiment for THERM indicated that, at the same dilution, plate count agar plates were uncountable due to bacterial colonies spreading over the surface of agar plates, whereas Petrifilm plates were countable (data not shown). Regarding PBC, other studies have been using Petrifilm for that test at 7 ˚C (Ramsahoi *et al*., 2011). A Petrifilm Plate Reader (3M, Technopath, Tipperary, Ireland) was used to assess the number of bacterial colonies.

The proteolytic bacterial count (PROT) test consisted of spread plating the diluted sample (100 µL) on calcium caseinate agar with added skim milk powder (Merck, Darmstadt, Germany). Plates were incubated at 37 ˚C for 48 h. Proteolytic bacterial colonies were identified as colonies surrounded by a clear zone in an opaque medium.

The TBC of the 150 farm milk samples collected during late-lactation were analysed within the factory using a MilkoScan FT2 system (Foss Electric, Hillerød, Denmark).

4.2.4. Statistical analysis

The statistical analyses were performed using the software SAS 9.3 (SAS Institute, 2016). The bacterial counts means (TBC, PBC, PROT, LPC and THERM) of each CT were predicted using the volume and bacterial count measured in the milk of all farms that supplied each CT. The same bacterial counts were predicted for the WMS using the volume and bacterial counts

measured in the milk of all CTs that supplied that silo. Those predictions were calculated as volume weighted means with estimated confidence interval. The actual bacterial counts measured in each CT and WMS samples were compared to the respective confidence interval for those predicted means of the bacterial counts. Agreement plots were also used to check for bias in the relationship between actual and predicted bacterial count means. There were insufficient numbers of samples from the factory (WMS, SMS and SMP samples) to determine the statistical differences between the bacterial counts measured in those samples. Therefore, only numerical differences between those samples were reported in this research paper to indicate the possible variations in bacterial load throughout the process. This study was performed once during each mid- and late-lactation periods.

4.3. Results

4.3.1. Mid-lactation study

The mean bacterial counts (TBC, PBC, PROT, LPC and THERM) of the samples from the farm bulk tanks, CTs, WMS, SMS and samples of SMP, which were collected during the mid-lactation period, are shown in Table 4.1. Small increases were observed when comparing all mean bacterial counts of the farm bulk tanks and CTs (Table 4.1). Pronounced increases in the TBC, PBC and PROT were observed in the WMS samples when compared to the CT samples (Table 4.1). The mean TBC, PBC and PROT were lower in the SMS samples compared to the WMS samples; however, the LPC and THERM levels were not different between them (Table 4.1).

The comparisons between the actual bacterial counts of each CT sample with the respective confidence interval for the predicted means, which were calculated considering the volume and bacterial count of each farm's milk supplied to each CT, are shown in Table 4.2. The TBC, PBC, PROT, LPC and THERM of two, three, one, two and four CT samples, respectively, were not within the respective confidence intervals (Table 4.2).

The comparisons between the actual bacterial counts of the WMS samples and the respective confidence interval for the predicted means, which were calculated considering the volume and bacterial count of each CT milk supplied to the silo, are shown in Table 4.3. The mean TBC, PBC, PROT and THERM of the WMS samples were not within the respective confidence intervals (Table 4.3).

4.3.2. Late-lactation study

The mean bacterial counts (TBC, PBC, PROT, LPC and THERM) of the samples from the farm bulk tanks, CTs, WMS, CS, SMS and samples of SMP, that were collected during late-lactation period, are shown in Table 4.1. The mean TBC of the CT samples was higher than the mean TBC of the farm milk samples (Table 4.1). The mean TBC, PBC and PROT of the WMS samples were higher than the CT samples means (Table 4.1). The mean TBC, PBC and PROT of the SMS samples were lower compared to the WMS samples, while their LPC and THERM levels were similar (Table 4.1).

The comparisons between the actual mean TBC measured in each CT sample with the respective confidence interval for the predicted means, which were calculated considering the volume and TBC of each farm milk supplied to each CT, are shown in the supplementary Table 4.4. The mean TBC of nine CT

samples (1, 3, 5, 6, 7, 8, 9, 10 and 11) were not within the respective confidence intervals (Table 4.4).

The comparisons between the actual bacterial counts of the WMS samples with the respective confidence interval for the predicted means, which were calculated considering the volume and bacterial count of each CT milk supplied to the silo, are shown in Table 4.3. The mean TBC, PBC and PROT of the late-lactation WMS samples were not within the respective confidence intervals (Table 4.3).

Table 4.1. Mean (± SD) total bacterial count (TBC), psychrotrophic (PBC), proteolytic (PROT), thermoduric (LPC – Laboratory pasteurisation count) and thermophilic (THERM) bacterial counts of the samples collected from the farm bulk tanks, collection tankers (CTs), whole milk silo (WMS), cream silo (CS), skim milk silo (SMS) and samples of skim milk powder (SMP) from the mid- and late-lactation periods.

Mid-Lactation Bacterial counts (log_{10} cfu/ mL)	**Farm bulk tanks† (n=67)**	**CT† (n=11)**	**WMS (n=2)**	**CS‡ (n=2)**	**SMS (n=2)**	**SMP‖ (n=9)**
TBC	3.60 ± 0.55 (2.65 to 4.90)	3.90 ± 0.40 (3.22 to 4.62)	5.89 ± 0.02		2.61 ± 0.20	2.36 ± 0.09 (2.26 to 2.50)
PBC	3.54 ± 0.65 (2.70 to 6.00)	3.70 ± 0.53 (2.74 to 5.97)	6.00 ± 0.00		2.00 ± 0.00	1.21 ± 0.15 (1.00 to 1.40)
PROT	3.50 ± 0.56 (3.00 to 5.10)	3.66 ± 0.29 (3.30 to 4.30)	5.72 ± 0.62		2.00 ± 0.00	1.36 ± 0.30 (1.00 to 1.70)
LPC	1.35 ± 0.33 (1.00 to 2.60) ¶	1.44 ± 0.28 (1.00 to 1.98)	1.58 ± 0.17		1.69 ± 0.07	2.45 ± 0.08 (2.30 to 2.51)
THERM	1.43 ± 0.47 (1.00 to 2.52) ¶	1.62 ± 0.35 (1.00 to 2.47)	2.02 ± 0.14		1.85 ± 0.10	3.63 ± 0.11 (3.50 to 3.79)
Late-lactation Bacterial counts (log_{10} cfu/ mL)	**Farm bulk tanks†,§ (n=150)**	**CT† (n=11)**	**WMS (n=2)**	**CS (n=2)**	**SMS (n=2)**	**SMP‖ (n=9)**
TBC	4.37 ± 0.62 (3.60 to 7.16)	5.12 ± 0.53 (4.32 to 5.96)	5.84 ± 0.09	2.32 ± 0.09	5.00 ± 0.00	3.56 ± 0.08 (3.44 to 3.69)
PBC		5.25 ± 0.58 (4.15 to 5.97)	5.80 ± 0.04	1.15 ± 0.21	5.00 ± 0.00	2.07 ± 0.10 (1.90 to 2.19)
PROT		4.09 ± 0.72 (3.30 to 5.95)	4.68 ± 0.40	4.27 ± 0.27	2.52 ± 0.35	2.18 ± 0.26 (2.00 to 2.54)
LPC		2.60 ± 0.23 (2.35 to 2.99)	2.55 ± 0.03	2.33 ± 0.01	2.61 ± 0.17	3.51 ± 0.09 (3.33 to 3.62)
THERM		2.72 ± 0.19 (2.51 to 2.98)	2.74 ± 0.06	4.54 ± 0.01	2.63 ± 0.04	3.58 ± 0.09 (3.41 to 3.69)

n = number of samples analysed in duplicate, ranges are given between parentheses. †Weighted means calculated considering the volumes and bacterial counts of each farm or CT sample. ‡Cream samples were not collected during mid-lactation. §Only TBC was measured in the late-lactation farm milk samples. ‖Bacterial counts in log_{10} cfu/ g. ¶Weighted means calculated not considering the samples in which those bacteria were not detected.

Table 4.2. Comparison of mean total (TBC), psychrotrophic (PBC), proteolytic (PROT), thermoduric (laboratory pasteurisation count – LPC) and thermophilic (THERM) bacterial counts measured in each collection tanker (CT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) during mid-lactation and those predicted (± standard error; S.E.) from the combined farm samples in each CT.

Bacterial counts	CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean CT bacterial count (log_{10} cfu/ mL)	Predicted bacterial count (weighted means; S.E.)† (log_{10} cfu/ mL)	95% CI‡		Mean CT bacterial counts covered by predicted C.I.
							LCL	UCL	
TBC									
	1	4	23771	5,943 ± 1,271	3.99	3.93 ± 0.09	3.64	4.23	Yes
	2	5	26503	5,301 ± 2,385	4.38	3.7 ± 0.27	2.95	4.45	Yes
	3	6	29122	4,854 ± 1,763	3.90	3.82 ± 0.32	2.98	4.65	Yes
	4	6	23780	3,963 ± 2,683	4.18	3.64 ± 0.23	3.06	4.22	Yes
	5	8	27585	3,448 ± 2,214	3.88	3.51 ± 0.19	3.05	3.97	Yes
	6	7	28628	4,090 ± 1,208	4.15	3.57 ± 0.2	3.08	4.06	No
	7	7	27188	3,884 ± 2,064	4.62	3.87 ± 0.33	3.06	4.67	Yes
	8	7	28470	4,067 ± 2,437	3.64	3.9 ± 0.08	3.71	4.09	No
	9	2	27147	13,574 ± 11,312	3.22	3.03 ± 0.07	2.2	3.86	Yes
	10	5	25248	5,050 ± 3,877	3.45	3.27 ± 0.13	2.93	3.62	Yes
	11	10	28561	2,856 ± 1,764	3.54	3.35 ± 0.12	3.08	3.62	Yes

†Weighted means were calculated considering the volume of milk supplied by each farm.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 4.2. Continuation.

Bacterial counts	CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean CT bacterial count (log_{10} cfu/ mL)	Predicted bacterial count (weighted means; S.E.)† (log_{10} cfu/ mL)	95% CI‡		Mean CT bacterial counts covered by predicted C.I.
							LCL	UCL	
PBC									
	1	4	23771	5,943 ± 1,271	3.99	3.61 ± 0.28	2.71	4.51	Yes
	2	5	26503	5,301 ± 2,385	3.52	3.36 ± 0.18	2.86	3.87	Yes
	3	6	29122	4,854 ± 1,763	4.04	3.83 ± 0.33	2.97	4.68	Yes
	4	6	23780	3,963 ± 2,683	3.56	3.51 ± 0.11	3.22	3.8	Yes
	5	8	27585	3,448 ± 2,214	3.74	3.36 ± 0.25	2.76	3.95	Yes
	6	7	28628	4,090 ± 1,208	3.80	3.45 ± 0.1	3.21	3.69	No
	7	7	27188	3,884 ± 2,064	5.97	4.11 ± 0.54	2.78	5.45	No
	8	7	28470	4,067 ± 2,437	3.60	3.97 ± 0.12	3.67	4.28	No
	9	2	27147	13,574 ± 11,312	2.74	3.04 ± 0.04	2.48	3.6	Yes
	10	5	25248	5,050 ± 3,877	3.23	3.35 ± 0.17	2.48	3.6	Yes
	11	10	28561	2,856 ± 1,764	3.51	3.29 ± 0.11	3.04	3.55	Yes

†Weighted means were calculated considering the volume of milk supplied by each farm.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 4.2. Continuation.

Bacterial counts	CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean CT bacterial count (log_{10} cfu/ mL)	Predicted bacterial count (weighted means; S.E.)† (log_{10} cfu/ mL)	95% CI‡		Mean CT bacterial counts covered by predicted C.I.
							LCL	UCL	
PROT									
	1	4	23771	5,943 ± 1,271	3.70	3.71 ± 0.15	3.24	4.17	Yes
	2	5	26503	5,301 ± 2,385	3.70	3.61 ± 0.41	2.48	4.73	Yes
	3	6	29122	4,854 ± 1,763	3.65	3.68 ± 0.27	2.98	4.38	Yes
	4	6	23780	3,963 ± 2,683	3.98	3.61 ± 0.28	2.9	4.33	Yes
	5	8	27585	3,448 ± 2,214	3.74	3.41 ± 0.15	3.05	3.76	Yes
	6	7	28628	4,090 ± 1,208	3.30	3.67 ± 0.24	3.08	4.26	Yes
	7	7	27188	3,884 ± 2,064	4.30	4.03 ± 0.26	3.39	4.67	Yes
	8	7	28470	4,067 ± 2,437	3.40	3.33 ± 0.09	3.1	3.56	Yes
	9	2	27147	13,574 ± 11,312	3.84	3.06 ± 0.12	1.52	4.61	Yes
	10	5	25248	5,050 ± 3,877	3.30	3.05 ± 0.05	2.9	3.2	No
	11	10	28561	2,856 ± 1,764	3.40	3.37 ± 0.1	3.14	3.6	Yes

†Weighted means were calculated considering the volume of milk supplied by each farm.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 4.2. Continuation.

Bacterial counts	CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean CT bacterial count (log_{10} cfu/ mL)	Predicted bacterial count (weighted means; S.E.)† (log_{10} cfu/ mL)	95% CI‡		Mean CT bacterial counts covered by predicted C.I.
							LCL	UCL	
LPC									
	1	4	23771	5,943 ± 1,271	1.54	1.21 ± 0.06	1.01	1.42	No
	2	5	26503	5,301 ± 2,385	1.18	1.35 ± 0.13	0.99	1.71	Yes
	3	6	29122	4,854 ± 1,763	1.00	1.07 ± 0.3	0.3	1.84	Yes
	4	6	23780	3,963 ± 2,683	1.48	1.34 ± 0.07	1.16	1.52	Yes
	5	8	27585	3,448 ± 2,214	1.98	0.79 ± 0.25	0.21	1.38	No
	6	7	28628	4,090 ± 1,208	1.30	1.24 ± 0.32	0.45	2.02	Yes
	7	7	27188	3,884 ± 2,064	1.60	1.12 ± 0.20	0.62	1.62	Yes
	8	7	28470	4,067 ± 2,437	1.18	0.96 ± 0.18	0.51	1.41	Yes
	9	2	27147	13,574 ± 11,312	1.70	0.48 ± 0.95	0	12.56	Yes
	10	5	25248	5,050 ± 3,877	1.70	1.44 ± 0.1	1.17	1.71	Yes
	11	10	28561	2,856 ± 1,764	1.30	1.26 ± 0.08	1.09	1.44	Yes

†Weighted means were calculated considering the volume of milk supplied by each farm.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 4.2. Continuation.

Bacterial counts	CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean CT bacterial count (log_{10} cfu/ mL)	Predicted bacterial count (weighted means; S.E.)† (log_{10} cfu/ mL)	95% CI‡		Mean CT bacterial counts covered by predicted C.I.
							LCL	UCL	
THERM									
	1	4	23771	5,943 ± 1,271	1.30	0.65 ± 0.34	0	1.73	Yes
	2	5	26503	5,301 ± 2,385	1.00	1.41 ± 0.19	0.88	1.94	Yes
	3	6	29122	4,854 ± 1,763	1.74	0.87 ± 0.32	0.03	1.7	No
	4	6	23780	3,963 ± 2,683	1.00	1.08 ± 0.35	0.17	1.99	Yes
	5	8	27585	3,448 ± 2,214	1.00	0.19 ± 0.15	0	0.56	No
	6	7	28628	4,090 ± 1,208	1.84	1.55 ± 0.33	0.73	2.37	Yes
	7	7	27188	3,884 ± 2,064	1.70	0.7 ± 0.3	0	1.44	No
	8	7	28470	4,067 ± 2,437	1.40	1.4 ± 0.12	1.12	1.69	Yes
	9	2	27147	13,574 ± 11,312	2.47	0.51 ± 1.0	0	13.15	Yes
	10	5	25248	5,050 ± 3,877	1.95	0.73 ± 0.25	0.05	1.42	No
	11	10	28561	2,856 ± 1,764	1.48	0.92 ± 0.28	0.28	1.55	Yes

†Weighted means were calculated considering the volume of milk supplied by each farm.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 4.3. Comparison of mean total (TBC), psychrotrophic (PBC), thermoduric (laboratory pasteurisation count – LPC) and thermophilic (THERM) bacterial counts measured in the whole milk silo (WMS) during mid- and late-lactation and those predicted (± standard error; S.E.) from the combined collection tanker (CT) samples.

Stage of lactation	Bacterial count (log_{10} cfu/ mL)	Mean (± SD) bacterial count (WMS)	Predicted bacterial count (weighted means; S.E.)†	95% CI‡		Mean CT bacterial counts covered by predicted C.I.
				LCL	UCL	
Mid-lactation						
	TBC	5.89 ± 0.02	3.9 ± 0.13	3.62	4.18	No
	PBC	6.00 ± 0.00	3.7 ± 0.17	3.33	4.08	No
	PROT	5.72 ± 0.62	3.66 ± 0.09	3.45	3.87	No
	LPC	1.58 ± 0.17	1.46 ± 0.09	1.27	1.65	Yes
	THERM	2.02 ± 0.14	1.64 ± 0.11	1.39	1.88	No
Late-lactation						
	TBC	5.84 ± 0.09	5.1 ± 0.17	4.73	5.47	No
	PBC	5.80 ± 0.04	5.25 ± 0.18	4.84	5.66	No
	PROT	4.68 ± 0.40	4.09 ± 0.23	3.58	4.6	No
	LPC	2.55 ± 0.03	2.61 ± 0.07	2.44	2.77	Yes
	THERM	2.74 ± 0.06	2.73 ± 0.06	2.59	2.86	Yes

Mean (± SD) volume of milk measured per tanker in mid- and late-lactation were 26,909 ± 1,902 L and 24,357 ± 3,768 L, respectively.
†Weighted means were calculated considering the volume of milk supplied by each tanker.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 4.4. Comparison of mean total bacterial counts (TBC) measured in each collection tanker (CT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) during late-lactation and those predicted (± standard error; S.E.) from the combined farm samples in each CT.

CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean TBC of each CT (log_{10} cfu/ mL)	Predicted TBC (weighted means; S.E.)† (log_{10} cfu/ mL)	95% CI‡		Mean TBC of each CT covered by predicted C.I.
						LCL	UCL	
1	15	25,743	1,716 ± 2,135	5.64	4.38 ± 0.16	3.95	4.66	No
2	7	19,853	2,836 ± 3,542	5.33	5.12 ± 0.32	4.35	5.89	Yes
3	8	23,460	2,933 ± 2,381	5.96	4.8 ± 0.34	4.0	5.6	No
4	13	24,221	1,863 ± 1,401	4.32	4.14 ± 0.08	3.96	4.33	Yes
5	10	24,274	2,427 ± 2,558	4.64	4.34 ± 0.12	4.06	4.61	No
6	14	24,729	1,766 ± 2,489	5.90	4.24 ± 0.25	3.71	4.77	No
7	19	28,583	1,504 ± 1,168	4.86	4.4 ± 0.08	4.23	4.56	No
8	27	28,322	1,049 ± 881	4.81	4.24 ± 0.08	4.08	4.4	No
9	18	27,606	1,534 ± 1,794	4.84	4.17 ± 0.11	3.93	4.4	No
10	8	15,774	1,972 ± 1,002	5.40	4.27 ± 0.13	3.95	4.59	No
11	13	25,367	2,306 ± 2,221	4.66	4.15 ± 0.06	4.02	4.29	No

†Weighted means were calculated considering the volume of milk supplied by each farm.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

4.4. Discussion

Production season or storage conditions can affect the bacterial counts of different types of microorganisms in milk, which can impact on the final quality of SMP. In mid-lactation, the mean TBC and PBC of the farm milk samples were below the European limits (EC no 853/2004): 5.00 and 4.22 $\log_{10}$ cfu/ mL, respectively. The TBC was also below the typical limit of 4.70 $\log_{10}$ cfu/ mL applied by some Irish milk processors (Table 4.1). The mean PROT of the farm samples was below the limit suggested by Vyletelova *et al.* (2000) (4.65 $\log_{10}$ cfu/ mL), at which proteolytic bacteria would produce high levels of heat-resistant proteases. The mean LPC of the mid-lactation farm milk samples was lower than the typical industry specifications, which can range from 2.70 to 3.00 $\log_{10}$ cfu/ mL. Thermoduric and thermophilic bacterial colonies were not detected in 8 and 24 farm milk samples, respectively. In mid-lactation, some individual farm milk samples had TBC, PBC, PROT and LPC higher than the specified limits. However, considering that the milk volumes from all farms would be blended for processing, the comparisons between the weighted mean bacterial counts and the known specifications for raw milk indicated that good quality milk was delivered to the factory for processing in mid-lactation.

The mean TBC of late-lactation farm bulk tank milk samples was also lower than the European and industrial limits; however, 49 farm samples had TBC above those specifications. Statistical comparisons between the mean TBC of the farm samples collected during mid- and late-lactation were not possible, as the group of farms involved in the mid- and late-lactation studies were different and samples from those groups were analysed in different laboratories; however, the figures gave an indication that lower quality milk was produced in late-lactation.

The variations in the counts of different bacterial types between lactation periods could be related to seasonal differences in bacterial strains in the environment, cow management, cows' health status (e.g., mastitis), on-farm hygiene practices, or milk storage conditions (Linn, 1988; Lafarge *et al*., 2004).

In mid-lactation, the mean TBC, PBC, PROT and LPC of the CT milk samples were below the limits determined by the European legislation, industry and literature cited; while in late-lactation, the mean TBC and PBC were higher than the European limits (Table 4.1). The TBC, PBC, PROT, LPC and THERM of the CTs milk were higher in late-lactation compared to mid-lactation, possibly due to the production of milk of inferior quality on-farm during that period. Also, the longer milk collection periods in late-lactation (approximately 8 h) could have contributed to the increased bacterial numbers in the CTs. The CT milk samples that had the bacterial counts higher than the upper confidence limit (mid-lactation: TBC, PBC, PROT, LPC and THERM; late-lactation: TBC; Tables 4.2 and 4.4) indicated that those bacterial numbers could have been influenced by the transport duration, CT cleaning protocol, temperature during transport or by the impact of individual farm suppliers (Kable *et al.*, 2016).

In both lactation periods, some of the bacterial counts measured in the WMS samples were higher than the respective upper confidence limits (mid-lactation: TBC, PBC, PROT and THERM; late-lactation: TBC, PBC and PROT; Table 4.3). The increase in those bacterial counts could be due to the conditions of the equipment in the milk transfer line (from the CT to the silo) (e.g., pump system and filters), non-effective silo clean-in-place routine, storage time or favourable storage temperature for the growth of some bacterial strains, or could

be a result of blending raw milk from different origins and levels of contamination (Pinto *et al*., 2006).

In mid- and late-lactation, the mean TBC of the WMS samples was higher than the limit determined for raw milk prior to processing (5.48 $\log_{10}$ cfu/ mL; EC no 853/2004). However, the temperature-time binomial applied during pasteurisation (75 ˚C, 25 s) reduced the TBC, PBC and PROT, as observed in the SMS samples (Table 4.1). In both lactation periods, pasteurisation was not efficient in reducing the LPC and THERM, when comparing the figures obtained for the WMS and SMS samples (Table 4.1), as those bacterial types are capable of surviving the temperatures applied in thermal treatments (Delgado *et al.*, 2013; Quigley *et al.*, 2013b). Thermoduric bacteria are able to survive pasteurisation temperatures (above 63 ˚C), while thermophilic bacteria are able to survive and grow at 55 ˚C or above (Frank and Yousef, 2004). The decreases in TBC and PBC after pasteurisation were of lower magnitude in late-lactation than in mid-lactation (Table 4.1), indicating that milk may contain higher numbers of heat-resistant bacteria strains during winter. Furthermore, in late-lactation, the THERM levels were higher in the CS samples compared to the WMS and SMS samples (Table 4.1). Given that cream separation occurred after pasteurisation, the relative abundance of thermophiles in pasteurised whole milk was possibly higher than prior to pasteurisation. Thermophilic bacteria could have migrated with the fat globules due to density (Graham, 2004) or the high levels could be related to the cleaning of the silos, as the persistence of thermophilic bacteria is related to the formation of biofilms (Burgess *et al*., 2010).

Mid-lactation raw milk had better microbiological quality than late-lactation milk; consequently, the SMP produced using mid-lactation milk had

lower bacterial counts than that made from late-lactation milk (Table 4.1). Laboratory-based studies indicated that when TBC in milk is higher than 5.00 $\log_{10}$ cfu/ mL, the solubility index of SMP can increase, as well as the free fat acid content, while the heat stability decreases (Muir *et al*., 1986; Celestino *et al*., 1997). In relation to thermoduric and thermophilic bacteria, there are no European limits determined for milk powder; however, the SMP produced using mid- and late-lactation milk had THERM levels in accordance to the North American dairy industry requirements (less than 4.00 $\log_{10}$ cfu/ g) (Wehr and Frank, 2004). Furthermore, it is likely that evaporation and spray-drying processes may have contributed to further reductions in TBC, PBC and PROT in the SMP in both periods.

This study highlights the importance of controlling bacterial levels in milk on-farm and during manufacturing, as processing parameters might not be able to reverse the negative effects of high bacterial levels; consequently, compromising the quality of dairy products. For example, when in sufficient numbers, certain bacteria strains can produce lipases and proteases, which could not be eliminated in pasteurisation and could affect essential technological properties of milk for dairy products manufacture (Muir, 1996; Barbano *et al*., 2006). Hygiene practices, cow management and processing parameters can affect the abundance of different bacterial types in milk; and therefore, those should be adequate to guarantee milk powder high quality and safety (Craven *et al*., 2010; Watterson *et al*., 2014).

4.5. Conclusion

This was the first study that monitored the quality of milk from farm bulk tank, through processing stages, to skim milk powder. The effects of milk quality

parameters on the quality of low-heat skim milk powder were observed, as well as how those parameters were affected throughout the manufacturing process. The good microbiological quality of the mid-lactation farm milk resulted in the production of milk powder with lower bacterial counts in contrast to the powder produced during late-lactation with milk of inferior quality. The season and stage of milk production has an influence on the abundance of different bacterial types in milk, which could impact the effectiveness of thermal treatments and consequently affect final product quality. Also, the differences in bacterial counts between production stages are indications of the growth potential of the bacteria in the milk, or even an indication of possible contamination sources in the specific production stage in which changes were observed. The results observed can aid industry in targeting sources of contamination throughout processing stages and practices to control bacterial numbers, in order to ensure the consistent production of safe high-quality dairy products throughout the year.

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Chapter 5

Monitoring residue concentrations in milk from farm and throughout a milk powder manufacturing process

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Declaration: The quantification of trichloromethane was carried out by Siobhan Murphy from Teagasc Moorepark. The quantification of chlorate, perchlorates and quaternary ammonium compounds was carried out by Martin Danaher, from Teagasc Ashtown. The quantification of iodine was performed by Norann Galvin, from Teagasc Moorepark. Dr. Bernadette O'Brien aided on planning the experimental design. All experimental data were analysed and the chapter written by Lizandra Paludetti, with corrections and comments from Prof. Alan L. Kelly from University College Cork, Dr. David Gleeson and Dr. Bernadette O'Brien from Teagasc Moorepark.

Abstract

The experiments reported in this research paper aimed to investigate differences in the levels of chlorate (CHLO), perchlorate (PCHLO), trichloromethane (TCM) and iodine residues in bulk tank (BT) milk produced at different milk production periods, and to monitor those levels throughout a skim milk powder (SMP) production chain (BTs, collection tankers [CTs], whole milk silo [WMS] and skim milk silo [SMS]). Chlorate, PCHLO and iodine were measured in SMP, while TCM was measured in the milk cream. The CHLO, TCM and iodine levels in the mid-lactation milk stored in the WMS were lower than legislative and industrial specifications (0.0100 mg/ kg, 0.0015 mg/ kg and 150 µg/ L, respectively); however, in late-lactation, those levels were numerically higher than the mid-lactation levels and specifications. Consequently, CHLO and iodine levels in SMP were numerically higher in late-lactation than in mid-lactation. Trichloromethane accumulated in the cream portion after separation. Perchlorate was not detected in any of the samples. Regarding iodine, the levels in mid-lactation reconstituted SMP were higher than that required by manufacturers (100 µg/ L), indicating that the levels in milk should be lower than 142 µg/ L. The higher residue levels observed in late-lactation could be related to the low milk volume produced during that period and changes in sanitation practices, while changes in feed management could have affected iodine levels. This study could assist in controlling and setting limits for CHLO, TCM and iodine levels in milk, ensuring premium quality dairy products.

5.1. Introduction

International markets are setting high specifications for milk and dairy product quality, including stringent guidelines on concentrations of residues that could occur in milk. Potential milk contaminants of most concern include chlorate (CHLO, ClO_3^-), perchlorate (PCHLO, ClO_4^-) and trichloromethane (TCM, $CHCl_3$), which arise as a consequence of sanitation with chlorine products, and quaternary ammonium compounds (QACs), which are present in certain sanitation products.

Chlorate and PCHLO were reported to result in thyroid dysfunctions (EFSA, 2015), while TCM could possibly be carcinogenic to humans (ICAR, 1999). There are a few studies available that have discussed contributing factors on-farm (Gleeson *et al.*, 2013; Ryan *et al.*, 2013); however, the dynamic of residue concentrations when subjected to different milk processing conditions is not fully understood. Sodium hypochlorite, chlorine gas or dioxide may be used for the sanitation of water, while chlorine-based detergents are used for the sanitation of milking or processing equipment. Chlorine products generally have good bactericidal properties and are widely used because of their effectiveness and low cost (Garcia-Villanova *et al.*, 2010). The decomposition of chlorine compounds results in the production of oxyhalide species (ClO^- and ClO_2^-), which react and form CHLO. Further reactions of CHLO with those oxyhalides result in the formation of PCHLO (Gordon & Tachiyashiki, 1991). Residual chlorine, CHLO or PCHLO on the surfaces of processing equipment can contaminate milk (Asami *et al.*, 2013). The contamination of infant formula with CHLO is a major concern due to the risk of intoxication in infants, which have lower tolerance than adults. The contact of chlorine with milk could also result in the formation of

TCM (Tiefel & Guthy, 1997). Chlorinated hydrocarbons accumulate in fat-rich fractions; therefore, products such as butter and cream could contain high concentrations of TCM if milk is contaminated with high levels (Hubbert *et al*., 1996).

Products containing QACs can also be employed for disinfection of processing equipment, tankers and silos. On-farm, some teat sanitizing wipes and dips or disinfectant agents used for bulk tanks could contain QACs. Those products should also be rinsed from equipment surfaces to avoid milk contamination (Xian *et al.*, 2016). The main primary QACs that may be identified in milk and dairy products are: benzyldimethyldodecylammonium chloride (BAC 12), benzyldimethyltetradecylammonium chloride (BAC 14), benzyldimethylhexadecyl ammonium chloride (BAC 16) and didecyldimethylammonium chloride (DDAC) (Reuter, 2015). In a report published by the European Food Safety Authority (EFSA, 2013), BACs (benzalkonium chlorides) and DDAC were present in 12% of the milk products tested, being the highest percentage in comparison to other food groups tested.

Excessive levels of residual iodine in raw milk are another concern in the Irish dairy industry, especially in the manufacture of infant formula. Iodine is an essential micronutrient for the synthesis of hormones by the thyroid gland (Leung & Braverman, 2014). Even though iodine is a nutrient of extreme importance to the human organism, the daily consumption of iodine at higher levels than recommended could result in dysfunctions of the thyroid gland. Bovine milk is one of the main sources of iodine for humans and its content depends on the daily iodine intake by dairy cows (Flachowsky *et al*. 2014). The US National Research Council (2001) recommends that the daily iodine intake per cow should be 10 mg,

which is the reference value applied in Ireland. The utilisation of rations with higher levels of iodine than required or overfeeding cows can result in excessive iodine concentrations secreted into milk. Over supplementation of Irish herds is of most concern during early and late-lactation and during winter milk production (O'Brien *et al*., 2013). O'Brien *et al.* (1999) recorded an average of 227 ug/ L iodine in Irish milk, while concentrations of 510 and 180 ug/ L were recorded for December and June, respectively. Those levels were not a food safety concern at the time; however, processors are currently requiring lower levels of iodine in raw milk destined for the production of infant formula, in order to meet requirements of the international market. Some Irish dairy processors require that raw milk should contain less than 150 µg/ L of iodine. Other iodine sources in milk include mineral-added water, boluses, mineral licks and grass (Magowan *et al.*, 2010). The use of iodine-based teat disinfectants can also contribute to iodine content in milk, as those products are absorbed through the teat skin if not completely removed prior to milking (Flachowsky *et al*. 2014).

The first objective of this study was to investigate changes in the CHLO, PCHLO, TCM, QACs and iodine levels throughout the milk production chain, from farm to dairy product, in two different milk production periods (mid- and late-lactation). Chlorate, PCHLO and iodine were measured throughout the production stages of skim milk powder (SMP), while TCM was measured throughout the production stages of milk and cream, which were destined for butter manufacture. The second objective was to investigate differences in residue levels in bulk tank (BT) milk produced during mid- and late-lactation. The milk used in this study was produced on commercial dairy farms and processed in a commercial SMP processing plant.

5.2. Material and methods

5.2.1. Sampling procedure at the farms and throughout a skim milk powder manufacture process

In Ireland a seasonal spring-calving production system is practiced, with all cows calving within a 10-week period approximately (February to April). This experiment was performed on one occasion during each of mid- (May; 80 DIM) and late-lactation (December; 290 DIM). The farms that supplied milk to the factory (mid-lactation: 67 farms; late-lactation: 150 farms), milk storage conditions on-farm, amount of milk produced, milk collection and the skim milk powder manufacturing process was the same as described in Chapter 4. A schematic drawing of the SMP manufacturing process is shown in Figure 5.1, as well as the sampling points during the mid- and late-lactation periods.

In mid-lactation, samples were collected at various points of the manufacturing process between the farm BTs and the SMP [BTs, collection tankers (CTs), whole milk silo (WMS), skim milk silo (SMS) and final SMP] and were tested for CHLO, PCHLO and iodine. In late-lactation, samples were collected at various points between the CTs and the SMP [CTs, WMS, cream silo (CS), SMS and final SMP] and were tested for CHLO, PCHLO and iodine. In both lactation periods, TCM was quantified in all samples, with exception of the SMP samples (Figure 5.1). Due to the high number of farms (150) necessary to supply sufficient milk volume to undertake the manufacturing process in late-lactation (December), it was not possible to undertake collection and analysis of all individual BT samples. The collection of samples and preparation of SMP samples for analysis were performed as described in Chapter 4.

5.2.2. Comparison between the residue levels in the same 67 farm bulk tanks in mid- and late-lactation (May and November)

The concentrations of CHLO, PCHLO, TCM, QACs and iodine residues were measured in raw milk produced on the same 67 dairy farms sampled in mid-lactation (May, 80 DIM) and in late-lactation period (November; 260 DIM), to investigate the effect of milk production period on residue levels. Milk samples were collected as described in Chapter 4.

5.2.3. Quantification of chlorate and perchlorate

The quantification of CHLO and PCHLO was performed by high-performance liquid chromatography coupled to tandem mass spectrometry (LC/ MS-MS) with ESI electrospray ionisation in negative mode (-ESI). The mid-lactation milk and SMP samples, as well as the 67 late-lactation farm BT samples, were analysed in the laboratory of Labor Friedle GmbH group (Labor Friedle GmbH, Von-Heyden-Straβe 11, D-93105, Tegernheim, Germany), while the late-lactation samples from the factory (CT, WMS, SMS and SMP samples) were analysed in Teagasc Ashtown (Dublin, Ireland). The methodologies used are based on the procedures described in the European Quick Polar Pesticides method (QuPPe) (EURL-SRM, 2015). In the present study, some of the milk samples were analysed by both laboratories and the results were statistically similar ($P>0.05$). The detection limit of CHLO and PCHLO in milk was 0.0010 mg/ kg and in SMP was 0.010 mg/ kg.

5.2.4. Quantification of trichloromethane

Trichloromethane was quantified in the milk using static head-space gas chromatography (HS-GC) with electron capture detector (ECD), fitted with a low thermal mass system (LTM) (Agilent 7890A, Agilent Technologies, Santa Clara, California, USA). The trichloromethane detection limit in this analysis was 0.0001 mg/ kg. The methodology applied was an adaption of the procedure of Resch & Guthy (1999). This analysis was performed in the Milk Quality laboratory in Teagasc Moorepark (Fermoy, Co. Cork, Ireland).

5.2.5. Quantification of iodine

Iodine was quantified in milk and reconstituted SMP samples using inductively coupled plasma mass spectrometry (ICP-MS), using an Agilent ICP-MS 7700x (Agilent Technologies, Santa Clara, California, USA). The methodology used was based on the procedures described in the standard method for the determination of iodine compounds in foodstuffs (BS EN 15111:2007, 2007). Standard solutions of Tellurium and 1% TMAH were used to obtain a calibration curve. The limit of detection was 1.31 µg/ L. The mid-lactation milk and SMP samples were analysed in Teagasc Moorepark (Fermoy, Co. Cork, Ireland), while the late-lactation milk and SMP samples were analysed in FBA laboratories (Capoquinn, Co. Waterford, Ireland). Those laboratories used the same methodology and samples analysed by both laboratories had statistically similar results ($P>0.05$).

5.2.6. Quantification of quaternary ammonium compounds

Quaternary ammonium compounds were quantified in milk and SMP samples using ultra–performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). An Acquity UPLC system coupled to a Quattro Premier Triple Quadrupole mass spectrometer (Waters Ireland, Dublin, Ireland) was used. The analyses were performed at the Food Safety department laboratory, in Teagasc Ashtown (Dublin, Ireland) and the methodology was based on the procedures described in the EURL method for the quantification of QACs (EURL-SRM, 2016). A solution of milk or reconstituted SMP (10 g) with an internal standard was prepared according to the QAC of interest. The following internal standard solutions were prepared for the analysis: 0.2 mg/ mL of BAC12 D7, BAC16 D7, BAC18 D7, BISC10 D6; 0.5 mg/ mL of BAC10 D7, BAC14 D7; and 1 mg/ mL of BAC8 D7, BISC8 D6. The solutions of sample and internal standard were allowed to stand for 15 min. After, 10 mL of acetonitrile were added and samples were agitated for 1 min using a Vortex. Sodium chloride (1 g) and magnesium sulphate (4 g) were added and samples were agitated again for 1 min and centrifuged at 35,000 rpm for 10 min at 20 ˚C. Magnesium sulphate ($MgSO_4$) (450 mg) and primary-secondary amine (PSA) (150 mg) (Supelclean PSA SPE bulk packing, Supelco, Bellefonte, PA, USA) were mixed to the supernatant (3 mL); and the mixture was agitated (1 min) and centrifuged at 2500 rpm for 10 min at 20 ˚C. Polytetrafluoroethylene filters (0.2 µm) were used to filter the extracts, which were collected in autosampler vials for UPLC-MS/MS analysis. Samples were injected into an Agilent Poroshell 120 SB-AQ (50 m x 2.1 mm x 2.7 µm), in which the flow rate of the eluents was 0.5 mL/ min. The eluent A consisted of ammonium formate buffer (5 mM) dissolved in a solution of

0.01% (w/w) formic acid; while eluent B consisted of ammonium formate buffer (5mM) dissolved in a solution of 0.01% (w/w) formic acid and methanol. The following gradient program was used (T_{min}/ % solution A): 0/ 90, 1.5/ 40, 8.5/ 15, 11/ 0.1, 13/ 0.1 and 13.1/ 90.

5.2.7. Statistical analysis

5.2.7.1. Influence of individual farm milk volumes on the residue concentration in each CT and influence of CT milk on the residue concentration in WMS

The statistical analyses were performed using the software SAS 9.3 (SAS Institute, 2016). In mid-lactation, the iodine and TCM concentrations of each CT were predicted using the volume and iodine or TCM concentrations measured in the milk of all farms that supplied each respective CT. In mid- and late-lactation, the iodine and TCM concentrations in the WMS were also predicted using the volume and iodine or TCM concentrations in the milk of all CTs that supplied that silo. Those predictions were calculated as volume-weighted means with estimated confidence intervals. The actual iodine or TCM concentrations measured in each CT and WMS samples were compared to the respective confidence interval for those predicted means. Agreement plots were also used to check for bias in the relationship between actual and predicted means. It was not possible to perform the same analyses with the CHLO, PCHLO, QACs results, due to the low number of samples in which those residues were detected.

5.2.7.2. Comparison between the residue levels in the same 67 farm bulk tanks in mid- and late-lactation (May and November)

Differences between the adjusted least square means of the mid- and late-lactation milk samples, collected in May and November, were calculated using the MIXED procedure in SAS 9.3 (SAS Institute, 2016). The fixed effects included in each model were lactation period (mid- and late-lactation) and farms (numbered from 1 to 67). Farms were considered the experimental unit and the response variable was iodine or TCM. Residual checks were made to ensure that the assumptions of the analysis were met.

It was not possible to statistically determine the differences between CHLO, PCHLO and QACs levels measured in mid- and late-lactation milk samples, due to insufficient number of samples in which those residues were detected. McNemar's test was applied to compare the number of BT milk samples in mid- and late-lactation that had CHLO and TCM concentrations ≥ 0.0010 and 0.0015 mg/kg, respectively. The GLM procedure was used to determine the regression relationship between CHLO and TCM concentrations.

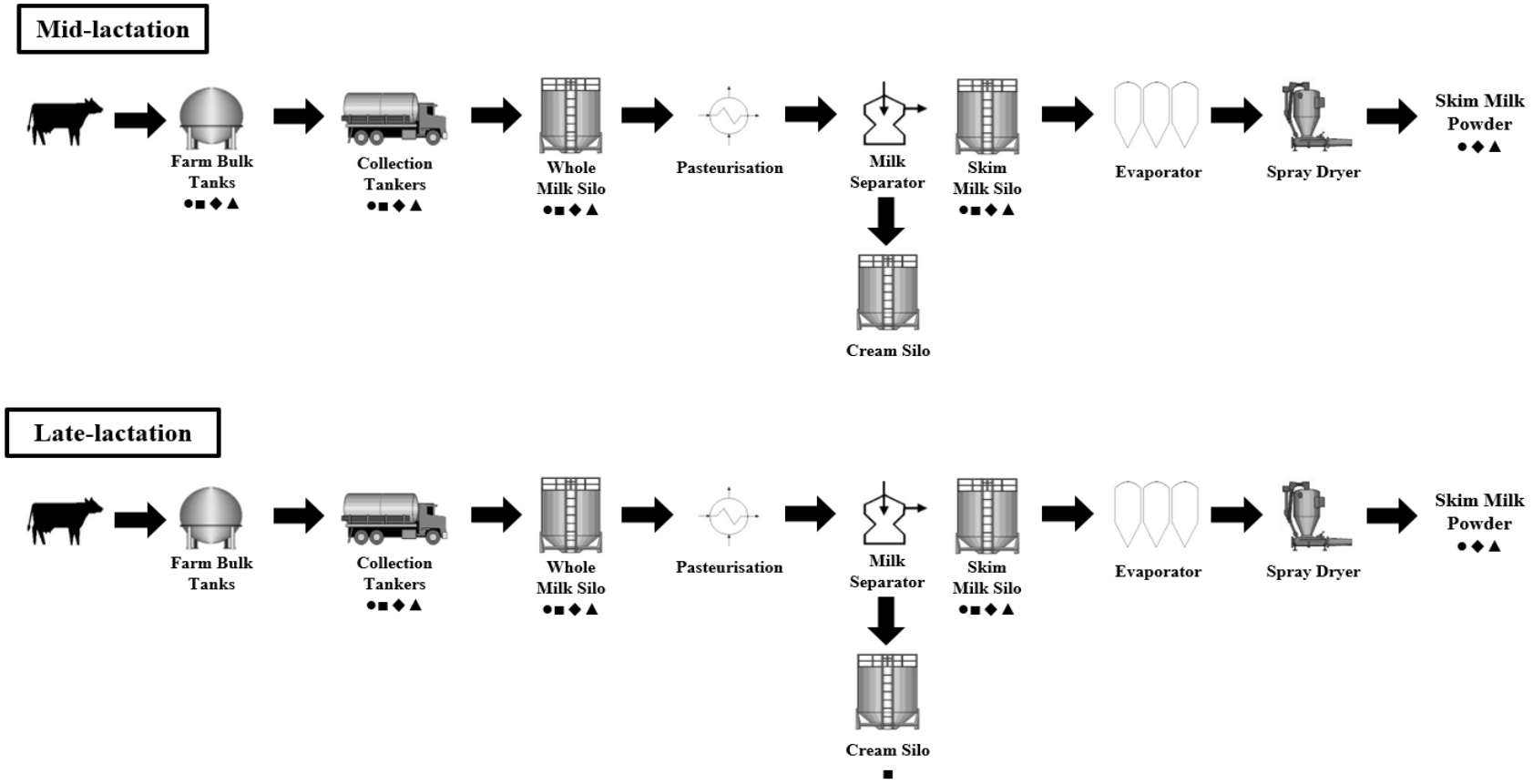

Figure 5.1. Milk supply chain and manufacturing process for conversion to low-heat skim milk powder, conducted in the mid- and late-lactation periods. The sampling points for chlorate (CHLO) and perchlorate (PCHLO), trichloromethane (TCM), quaternary ammonium compounds (QACs) and iodine are indicated with a ●, ■, ♦ and ▲, respectively.

5.3. Results

The mean CHLO, TCM and iodine concentrations of samples collected during mid- and late-lactation (May and December, respectively) throughout the milk powder production chain are shown in Table 5.1.

5.3.1. Chlorate and perchlorate

In mid-lactation (May), CHLO was detected in 14 of the 67 BT and 6 of the 11 CT samples. The weighted mean CHLO concentration was calculated at the basis of the milk volume supplied by those farms and CTs (Table 5.1). The volume-weighted mean CHLO concentrations of these farms and CTs were numerically similar.

In late-lactation (December), CHLO was detected in 6 of the 11 CT samples also, but the volume-weighted mean of those samples was higher compared to mid-lactation (Table 5.1).

In both mid- and late-lactation, the mean CHLO concentration in the WMS and SMS were numerically similar (Table 5.1).

The mean CHLO concentration of the SMP samples was higher in late-lactation (December) compared to mid-lactation (May) (Table 5.1). In both lactation periods, the CHLO concentration in powder increased approximately 50 times compared to the concentrations in SMS samples. In mid-lactation, the CHLO levels in the SMP samples decreased throughout the spray-dryer run. At the start, middle and end of the spray-drying process, the CHLO levels were: 0.0630 ± 0.0020, 0.0610 ± 0.0060 and 0.0470 ± 0.0020 mg/ kg, respectively. In contrast, the CHLO concentration of the late-lactation SMP samples did not vary

throughout the spray-dryer run (start: 0.124 ± 0.003 mg/ kg; middle: 0.129 ± 0.011 mg/ kg; end: 0.126 ± 0.006 mg/ kg).

Perchlorate was not detected in any of the mid- and late-lactation samples collected throughout the manufacturing process.

5.3.2. Trichloromethane

Trichloromethane was detected in all BT and CT samples collected in mid-lactation (May) and in all CT samples collected in late-lactation (December). The volume-weighted mean TCM concentration of those samples was calculated considering the milk volume supplied by each BT or CT (Table 5.1). The volume-weighted mean TCM concentration of the CT milk samples was higher in late-lactation compared to mid-lactation. In mid-lactation, the volume-weighted mean TCM concentrations of the milk samples from the BTs and CTs were numerically similar. The mean TCM concentrations of the milk samples from the CTs and WMS were also numerically similar in both mid- and late-lactation.

The comparisons between the actual TCM concentration and the respective confidence interval for the predicted means for each mid-lactation CT sample, are shown in Table 5.2. The TCM concentrations in all of the mid-lactation CT samples were within their respective confidence intervals. A similar comparison for the mid- and late-lactation WMS samples is shown in Table 5.3. The TCM concentration in the WMS samples were also within their respective confidence interval in mid- and late-lactation.

In both lactation periods, the mean TCM concentration decreased in the SMS samples compared to the WMS samples, as expected (Table 5.1).

5.3.3. Quaternary ammonium compounds

In mid-lactation, QACs were detected in 2 farm milk samples. One sample contained BAC 12 (10.5 µg/ kg), BAC 14 (2.00 µg/ kg) and BISC 10 (12.00 µg/ kg), while the second contained BAC 12 (5.00 µg/ kg). Quaternary ammonium compounds were also present in two CT milk samples; one had BISC 10 (7.1 µg/ kg), while the other had BAC 12 (7.4 µg/ kg) and BISC 10 (10.4 µg/ kg). No QACs were detected in the WMS, SMS and SMP samples.

In late-lactation, one CT sample had a QAC compound (BISC 10: 3.4 µg/ kg), while the WMS and CS samples had 3.4 and 7.3 µg/ kg of BISC 10, respectively. No QACs were detected in the SMS and SMP samples.

5.3.4. Iodine

In mid-lactation, the volume-weighted mean iodine concentration was numerically higher in the BT samples than in the CT samples. The volume-weighted mean iodine concentration of all of the CTs was numerically higher in late-lactation than in mid-lactation. In mid-lactation, the mean iodine concentrations in the CTs and WMS were similar, while in late-lactation, the mean concentration was numerically higher in the CTs compared to the WMS (Table 5.1). In both lactation periods, the iodine concentrations increased in SMS samples; and consequently, as levels were higher in late-lactation BT milk, the iodine concentration in SMP was higher in late-lactation than in mid-lactation.

The comparisons between the actual iodine concentrations of each mid-lactation CT sample with the respective confidence interval for the predicted means are shown in Table 5.4, while such comparison for the mid- and late-lactation WMS samples are shown in Table 5.5. All the iodine concentrations

measured in each mid-lactation CT sample were within the respective confidence intervals, as well as the WMS samples collected in mid- and late-lactation.

5.3.2. Comparison between the residue levels in the same 67 farm bulk tanks in mid- and late-lactation (May and November)

The number of BT samples in which CHLO was detected was significantly higher in late-lactation (32 out of the 67 samples) than in mid-lactation (14 out of the 67 samples) ($P<0.0001$). Also, in contrast to mid-lactation, 8 out of the 67 late-lactation BT samples contained 0.0010 mg/ kg of PCHLO.

The volume-weighted mean TCM concentration was significantly higher in late-lactation (0.0015 ± 0.0014 mg/ kg; range: 0.0003 to 0.0074 mg/ kg) than in mid-lactation (0.0009 ± 0.0008 mg/ kg; range: 0.0002 to 0.0043 mg/ kg) ($P<0.0001$).

In late-lactation, QACs were detected in two BT milk samples, however those were different to the BT samples in which QACs were detected in mid-lactation. One milk sample contained BAC 12 (3.4 µg/ kg) and BISC 10 (6.6 µg/ kg), while the other sample contained BISC 10 (3.2 µg/ kg).

The volume-weighted mean iodine concentrations of the BT samples in mid- and late-lactation (142.2 ± 129.2 and 119.7 ± 151.6 µg/ L, respectively) were not statistically different ($P = 0.63$).

Table 5.1. Mean (± SD) chlorate (CHLO), trichloromethane (TCM) and iodine concentrations in samples collected from the farm bulk tanks (BTs), collection tankers (CTs), whole milk silo (WMS), skim milk silo (SMS), cream silo (CS) and samples of skim milk powder (SMP) from the mid- and late-lactation periods.

	Mid-Lactation		
	CHLO (mg/ kg)	**TCM (mg/ kg)**	**Iodine (µg/ L)**
Farm BTs (n=67)†	0.0021 ± 0.0019 (0.0010 to 0.0070) ‡	0.0009 ± 0.0008 (0.0002 to 0.0043)	142.2 ± 129.2 (10.4 to 561.2)
CT (n=11)†	0.0020 ± 0.0010 (0.0010 to 0.0030) §	0.0009 ± 0.0003 (0.0006 to 0.0015)	134.2 ± 89.6 (58.3 to 390.8)
WMS (n=2)	0.0010 ± 0.0000	0.0009 ± 0.0000	135.5 ± 7.6
SMS (n=2)	0.0010 ± 0.0000	0.0002 ± 0.0000	142.1 ± 9.1
SMP (n=9)	0.0570 ± 0.0090 ‖		142.2 ± 10.0 (120.2 to 153.5)
	Late-Lactation		
	CHLO (mg/ kg)	**TCM (mg/ kg)**	**Iodine (µg/ L)**
CT (n=11)†	0.0410 ± 0.0554 (0.0020 to 0.1550) §	0.0020 ± 0.0007 (0.0010 to 0.0033)	437.6 ± 155.2 (225 to 709)
WMS (n=2)	0.0025 ± 0.0000	0.0018 ± 0.0000	419.0 ± 2.8
SMS (n=2)	0.0025 ± 0.0000	0.0005 ± 0.0000	450.0 ± 7.1
CS (n=2)		0.0190 ± 0.0000	
SMP (n=9)	0.1263 ± 0.0071‖		398.2 ± 22.8 (257 to 425)

n = number of samples; ranges are given between parentheses.

†Weighted means and standard deviations calculated considering the volumes of milk and residues concentrations of each farm or CT sample.

‡Weighted mean CHLO of the 14 bulk tank milk samples in which chlorate was detected.

§Weighted mean CHLO of the CT milk samples in which chlorate was detected (mid-lactation: 6 samples; late-lactation: 6 samples).

‖Results for non-reconstituted skim milk powder

Table 5.2. Comparison of mean trichloromethane (TCM) concentrations measured in each collection tanker (CT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) during mid-lactation and those predicted (± standard error; S.E.) from the combined farm samples in each CT.

CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean TCM concentration of each CT (mg/ kg)	Predicted TCM concentration (weighted means ± S.E.)† (mg/ kg)	95% CI‡		Mean TCM concentration of each CT covered by predicted C.I.
						LCL	UCL	
1	4	23771	5,943 ± 1,271	0.0015	0.0014 ± 0.0009	0.0000	0.0043	Yes
2	5	26503	5,301 ± 2,385	0.0008	0.0005 ± 0.0002	0.0000	0.0011	Yes
3	6	29122	4,854 ± 1,763	0.0012	0.0009 ± 0.0003	0.0001	0.0016	Yes
4	6	23780	3,963 ± 2,683	0.0012	0.0009 ± 0.0003	0.0002	0.0016	Yes
5	8	27585	3,448 ± 2,214	0.0008	0.0004 ± 0.0001	0.0002	0.0005	Yes
6	7	28628	4,090 ± 1,208	0.0011	0.0008 ± 0.0004	0.0000	0.0018	Yes
7	7	27188	3,884 ± 2,064	0.0006	0.0004 ± 0.0001	0.0002	0.0006	Yes
8	7	28470	4,067 ± 2,437	0.0007	0.0004 ± 0.0002	0.0001	0.0008	Yes
9	2	27147	13,574 ± 11,312	0.0010	0.0007 ± 0.00004	0.0002	0.0012	Yes
10	5	25248	5,050 ± 3,877	0.0007	0.0003 ± 0.0001	0.0000	0.0006	Yes
11	10	28561	2,856 ± 1,764	0.0008	0.0005 ± 0.0001	0.0003	0.0008	Yes

†Weighted means were calculated considering the volume of milk supplied by each farm or by each CT.

‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 5.3. Comparison of mean trichloromethane (TCM) concentrations measured in the whole milk silo (WMS) during mid- and late-lactation and those predicted (± standard error; S.E.) from the combined collection tankers (CTs) samples.

	Mean TCM concentration of the WMS (mg/ kg)	**Mean (± SD) volume measured per CT (L)**	**Predicted TCM concentration (weighted means ± S.E.)† (mg/ kg)**	**95% CI‡**		**Mean TCM concentration of WMS covered by predicted C.I.**
				LCL	**UCL**	
Mid-lactation	0.0009	26,909 ± 1,902	0.0007 ± 0.00009	0.0005	0.0009	Yes
Late-lactation	0.0018	24,357 ± 3,768	0.0019 ± 0.0002	0.0014	0.0024	Yes

†Weighted means were calculated considering the volume of milk supplied by each CT.

‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 5.4. Comparison of mean iodine concentrations measured in each collection tanker (CT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) during mid-lactation and those predicted (± standard error; S.E.) from the combined farm samples in each CT.

CT	Number of farms	Mean (± SD) volume measured per farm (L)	Total volume per CT (L)	Iodine concentration measured in each CT sample (µg/ L)	Predicted iodine concentrations (weighted means ± SE) (µg/ L)†	95% CI‡		Mean iodine concentration of each CT covered by predicted CI
						LCL	UCL	
1	4	5,943 ± 1,271	23,771	83.9	89.2 ± 21.8	19.8	158.6	Yes
2	5	5,301 ± 2,385	26,503	81.8	90.0 ± 23.8	23.9	156.2	Yes
3	6	4,854 ± 1,763	29,122	120.0	117.9 ± 45.6	0.6	235.3	Yes
4	6	3,963 ± 2,683	23,780	58.3	61.2 ± 8.5	39.3	83.7	Yes
5	8	3,448 ± 2,214	27,585	125.9	141.0 ± 27.8	75.4	206.7	Yes
6	7	4,090 ± 1,208	28628	138.4	144.1 ± 55.7	7.9	280.3	Yes
7	7	3,884 ± 2,064	27188	112.0	116.7 ± 15.7	78.4	155.1	Yes
8	7	4,067 ± 2,437	28470	76.3	82.9 ± 20.9	31.6	134.1	Yes
9	2	13,574 ± 11,312	27147	390.8	335.7 ± 91.6	0	1,500	Yes
10	5	5,050 ± 3,877	25248	202.9	282.7 ± 121.2	0	619.7	Yes
11	10	2,856 ± 1,764	28561	80.0	101.7 ± 12.1	74.3	129.1	Yes

†Weighted means were calculated considering the volume of milk supplied by each farm.

‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table 5.5. Comparison of mean iodine concentrations measured in the whole milk silo (WMS) during the mid- and late-lactation periods and those predicted (± standard error; S.E.) from the combined collection tankers (CTs) samples.

	Mean (± SD) iodine concentration of the WMS (µg/ L)	Mean (± SD) volume measured per CT (L)	Predicted iodine concentration (weighted means ± SE) (µg/ L)†	95% CI‡		Mean iodine concentration of the WMS covered by predicted CI
				LCL	UCL	
Mid-lactation	135.5 ± 7.6	26,909 ± 1,902	134.2 ± 28.3	71.0	197.3	Yes
Late-lactation	419.0 ± 2.8	24,357 ± 3,768	421.4 ± 50.5	308.8	534.0	Yes

†Weighted means were calculated considering the volume of milk supplied by each CT.

‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

5.4. Discussion

5.4.1. Residues related to the use of chlorine

Concentrations of CHLO and PCHLO were monitored throughout the production chain of SMP in mid- and late-lactation (May and December, respectively). In Europe, a default threshold limit of 0.0100 mg/ kg of CHLO and PCHLO is applied for milk (EC no 396/2005). In mid-lactation (May), the volume-weighted mean CHLO concentration in the 14 BTs and 6 CTs (in which CHLO was detected) were lower than that limit; however, in late-lactation (December), the mean CHLO concentration of the 6 CTs (in which CHLO was detected) was higher than the EC limit and higher than the volume-weighted mean concentration in mid-lactation.

In mid-lactation, the CHLO concentrations in each of the CTs could have been diluted as CHLO was not detected in 53 of the BTs. For example, CHLO was not detected in 4 CT milk samples, as only one of the BT milk volumes contributing to each of those CTs contained CHLO. Additionally, CHLO was not detected in most of the BT milk supplied to the 6 CTs in which CHLO was detected, indicating that the sanitation of those CTs could possibly have influenced the CHLO levels. In both mid- and late-lactation, as CHLO was not detected in most of the CT milk volumes, the CHLO concentrations could have also been diluted in the WMS; therefore, it is likely that the sanitation practices of the silos did not influence the CHLO levels. Consequently, the mean CHLO concentrations in the WMSs were lower than the EC limit of 0.0100 mg/ kg. However, as the milk supplied to the factory during late-lactation contained higher levels of CHLO than the mid-lactation milk, the CHLO levels in the WMS in late-

lactation were higher compared to mid-lactation; consequently, the CHLO levels in the SMP were higher in late-lactation than in mid-lactation.

In mid-lactation, the mean CHLO concentration of the SMP samples was lower than the limit applied by some Irish infant formula manufacturers (0.100 mg/ kg). The difference of 0.0016 mg/ kg between the mean CHLO concentration of the SMP samples collected at the end and start of the spray-drying run, indicated that the sanitation of the spray-dryer could have contributed to the CHLO levels in SMP. The interior surface of the spray-dryer could have contained residual CHLO, and the majority of that residue was transferred to the first batch of evaporated skim milk that entered the equipment. In late-lactation, the mean CHLO concentration of the SMP samples was higher than 0.100 mg/ kg, indicating that the CHLO level in the bulk milk stored in the WMS should had been lower than 0.0025 mg/ kg. Even though no variations in the CHLO concentration were observed in SMP samples collected throughout the spray-drying run in late-lactation, sanitation practices of that equipment could have also contributed to the increased CHLO levels in SMP. Additionally, the variations in CHLO concentrations throughout the spray-drying run that were observed in mid-lactation and not observed in late-lactation could be due to differences in the sanitation practices between production periods.

The concentrations of TCM were also monitored throughout the production chain of SMP in mid- and late-lactation (May and December, respectively). There are no European regulations that have defined a standard TCM limit for milk or dairy products; however, Irish dairy processors apply a limit of 0.0015 mg/ kg to milk destined for the production of lactic butter which should have less than 0.0300 mg/ kg of TCM, as required by the export market

(Ryan et al., 2013). In mid-lactation, the mean TCM concentrations of the BTs, CTs and WMS were all lower than that limit; while, in late-lactation, the mean TCM concentrations of the CTs and WMS were higher than that limit and higher than the concentrations in mid-lactation. The agreement between the TCM concentrations of each mid-lactation CT sample and the contributions of each BT milk volume supplied, as well as the agreement between the TCM concentrations of the WMS samples and the contributions of each CT in both lactation periods, indicated that the cleaning protocol of the CTs or WMS did not contribute to any increases in the TCM levels in milk (Tables 5.2 and 5.3).

In both lactation periods, the decrease in the TCM concentrations in the SMS in relation to the WMS was expected, due to the accumulation of TCM in the cream during separation (Hubbert *et al.*, 1996; Table 5.1). As the levels of TCM were higher in late-lactation milk, the TCM concentration in late-lactation cream was possibly higher than the levels expected in cream produced with mid-lactation milk.

The concentrations of CHLO and TCM were also monitored in the same 67 farm BTs in mid- and late-lactation (May and November, respectively) to investigate if those concentrations could differ in milk produced by the same farm during different production periods. None of the mid-lactation BT samples contained CHLO levels higher than 0.0100 mg/ kg (EC limit), while 5 late-lactation BT samples contained levels higher than that limit. In relation to TCM, the number of BT samples that contained levels greater than 0.0015 mg/ kg was significantly higher in late-lactation (21 BT samples; range: 0.0016 to 0.0074 mg/ kg) than in mid-lactation (7 BT samples, range: 0.0017 to 0.0043 mg/ kg) ($P = 0.002$). Those increases in the levels of those residues in late-lactation could be

related to changes in the sanitation practices on each farm. Chlorine detergent sterilisers should contain a maximum of 3.5% of chlorine and should be prepared and applied according to the manufacturer's instructions (Gleeson, 2016). According to Ryan et al. (2012), 14 L of rinse water per milking unit are recommended in order to totally remove the detergent solution, and the solutions should be rinsed immediately after the wash cycle. Additionally, the lower volume of milk produced per farm during late-lactation (1,683 ± 1,031 L) could have also contributed to the increase in CHLO or TCM levels during that period, as those residues could have been more concentrated. The presence of CHLO and TCM in milk was not correlated; therefore, if milk contains CHLO it will not necessarily contain TCM and *vice versa*. The contamination of milk with CHLO or TCM might be related to a combination of specific sanitation practices and further studies are necessary to determine them. In addition, the higher number of farms in late-lactation that supplied milk containing higher levels of CHLO or TCM indicated that extra care is required during that period for the production of milk powder or butter.

5.4.2. Quaternary ammonium compounds

The contamination of QACs in milk could be due to the use of sanitation products or teat disinfectants that contain those compounds (Danaher and Jordan, 2013). There are no specific limits for QACs in food matrices; however, the European Union Reference Laboratory (EURL) determined that the general QAC limit should be 0.010 mg/ kg (EU no 396/2005). In mid- and late-lactation, the QACs levels detected in BT, CT, WMS and CS samples were all below that limit.

In mid-lactation, the two CTs samples that had BISC 10 did not collected milk that contained that type of QAC, indicating that possibly sanitation products containing BISC 10 were used for the CTs cleaning; however, the product was not rinsed appropriately. Even though other CTs collected milk that had QACs, those were probably not detected due to a dilution effect. The same might explain why no QACs were detected in the WMS, SMS and SMP samples.

In late-lactation, the detection of BISC 10 in a CT, WMS and CS samples indicated again that the cleaning practices within the factory might have influence in the levels of that compound. Possibly, BISC 10 was separated with the cream, as it was not detected in the SMS and SMP samples.

When comparing the same 67 farms in mid- and late-lactation, the QAC levels detected in BT samples from both periods were below the limit determined by the European Union Reference Laboratory (0.010 mg/ kg). Changes in sanitation practices or teat disinfectants used could have made some samples to have detectable levels of QACs or not in mid- and late-lactation (Danaher and Jordan, 2013).

5.4.3. Iodine

Variations in the iodine concentrations were investigated throughout the production chain of SMP in mid- and late-lactation (May and December, respectively). The EFSA (2005) reported that the average iodine concentration in BT milk samples from several European studies was predominately between 100 and 200 µg/ L, which were suitable to meet the required iodine daily intake for children and adults. Some Irish dairy processors specify that the iodine levels in raw milk should be lower than 150 µg/ L to produce infant formula. In mid-

lactation, the mean iodine concentration of the BT, CT and WMS samples were all lower than that limit; while in late-lactation, the mean concentrations of the CT and WMS were higher than that limit.

Flachowsky *et al*. (2014) suggested that iodine could undergo sublimation throughout processing, as more than 90% of iodine in milk is in the inorganic form. Small decreases in the mean iodine concentration observed from the BTs to CTs (mid-lactation) and from the CTs to WMS (late-lactation) could be associated with the sublimation of iodine (Table 5.1). The actual iodine concentrations measured in each CT (Table 5.4) and WMS (Table 5.5) were in agreement with the contributions of each BT and CT, respectively. However, the actual concentrations of each CT and WMS were slightly lower than the predicted concentrations (Tables 5.4 and 5.5), indicating that possibly a small amount of iodine underwent sublimation during transport and storage, but not sufficient to be significant. Those small losses could have resulted in those decreases in the mean iodine concentrations shown in Table 5.1.

In mid-lactation, two CT samples had levels higher than 150 µg/ L (390.8 and 202.9 µg/ L). One of those CTs collected milk from two farms that supplied milk containing 289.1 and 516.0 µg/ L of iodine. The other CT collected milk from 5 farms; however, most of the volume collected was from one farm that supplied milk containing 561.2 µg/ L of iodine.

Therefore, it is important that individual milk suppliers control the iodine intake of their herds and correctly apply iodine-based teat disinfectants (US National Research Council, 2001; O'Brien *et al.*, 2013). In late-lactation, all of the CT samples had levels higher than 150 µg/ L, indicating that the iodine levels in BT milk were possibly higher in late-lactation than in mid-lactation. Those higher

levels could be due to the contribution of the increased number of farms (150) and also due to high levels of iodine in ration supplied to the cows when indoors.

In both lactation periods, the mean iodine concentrations increased in the SMS when compared to the WMS. Prior to pasteurisation and cream separation, milk permeate (details were not disclosure by the manufacture) is added to standardise the protein and lactose content in milk; therefore, that permeate could have contributed to an increase in the iodine content in the SMS.

The International Council for Control of Iodine Deficiency Disorders (ICCIDD; Delange *et al.*, 1993) specified that the iodine content in reconstituted SMP should be lower than 100 µg/ L. The mean iodine concentrations of the SMP produced in mid- and late-lactation were higher than that limit (Table 5.1). Therefore, in the case of the conditions of this study, the iodine levels in the bulk milk supplied to the factory should be lower than 142 µg/ L to produce SMP containing iodine levels within the specification. Also, as the iodine levels were higher in late-lactation compared to mid-lactation milk, the iodine content in reconstituted SMP was also higher in late-lactation than in mid-lactation.

In order to investigate variations in the levels iodine in BT milk during different production periods, the concentrations of such residue were also measured in the same 67 farm BTs in mid- and late-lactation (May and November, respectively). In mid- and late-lactation, 13 and 12 BT samples had iodine concentrations higher than 150 µg/ L, respectively. Questionnaires were completed on some of those farms, capturing information regarding animal feed. It was established that the majority of those farms were using concentrates from one manufacturer, which contained at least 10 and a maximum of 43 mg of iodine/ kg of ration. Therefore, the iodine intake from ration per cow on those

farms was likely to be higher than that recommended (10 mg per cow per day), as the average ration intake on those farms was 2.5 kg per cow per day. Other factors that were not included in the questionnaires could have contributed to iodine levels in milk such as grass, boluses, mineral-supplemented water and mineral licks. Furthermore, according to the questionnaires, five and two farms that were using iodine-based teat disinfectants supplied milk with iodine levels higher than 150 µg/ L, in mid- and late-lactation, respectively. O'Brien *et al*. (2013) also observed increases in the iodine levels in milk when applying those teat disinfectants post-milking. Those increases are associated with the absorption of iodine through the teat skin, particularly if pre-milking teat preparation is not being conducted.

5.5. Conclusion

Incorrect sanitation practices on-farm can result in increases in the CHLO or TCM levels in milk throughout the year, while the production of lower volumes of milk is an additional contributing factor in late-lactation; therefore, extra care is necessary during that period. Consequently, increases in the CHLO or TCM levels in milk result in increased residue levels in SMP or cream, respectively. Therefore, it is important to control the initial residue levels in milk destined for processing, especially considering that those could concentrate greatly after evaporation and spray-drying processes or cream separation. Appropriate sanitation practices should also be carried out within the processing plant to avoid increases in the residue levels throughout the processing stages. In relation to iodine, this study indicated that some Irish dairy herds are over supplemented with iodine, while the use of iodine-based teat disinfectants also contributed to high

levels in some BT samples. Also, the iodine content of the SMP produced in mid-lactation was not within the required specification, even though the WMS milk had lower iodine levels than specified, indicating that the levels in BT milk should be even lower. Finally, it is possible to calculate the expected residue levels in milk stored in the CTs or WMS based on the volumes and residue levels of milk supplied by each dairy farm, which could aid dairy processors to identify the stages that may have contributed to increases in those levels. This study highlights the importance of controlling the contributing factors on-farm and in the processing plant in order to maintain residues at safe and market-acceptable levels.

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Chapter 6

Effect of thermo-resistant protease of *Pseudomonas fluorescens* on cheese-making properties of milk and proteolysis

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Abstract

This study aimed to investigate the effect of different activity levels of a thermo-resistant protease, produced by *Pseudomonas fluorescens* (ATCC 17556), on the cheese-making properties of milk and proteolysis levels. Sterilised reconstituted skim milk powder was inoculated with the bacterial strains and, after incubation, centrifuged to obtain a supernatant containing protease. Raw milk was collected and inoculated to obtain a protease activity of 0.15, 0.60 and 1.5 U/ L of milk (treatments P1, P4 and P10, respectively). One sample was not inoculated (control) and non-inoculated supernatant was added to a fifth sample to be used as negative control (P10 neg). Samples were stored at 4 °C for 72 h. After 0, 48 and 72 h, the rennet coagulation properties and proteolysis levels were assessed. The protease produced was thermo-resistant, as there were no significant difference in the activity in the pasteurised (72 ˚C for 15s) and non-pasteurised supernatants. The chromatograms and electrophoretograms indicated that the protease preferably hydrolysed κ-casein and β-casein, and levels of proteolysis increased with added protease activity over storage time. The hydrolysis of α_S-CNs and major whey proteins increased considerably in milk samples P10. At 0 h, the increase in the level of protease activity decreased the rennet coagulation time (RCT) of the samples, possibly due to synergistic proteolysis of κ-casein into *para*-κ-casein. However, over prolonged storage, hydrolysis of β- and α_S-caseins increased in samples P4 and P10. The RCT of samples P4 increased over time and the coagulum became softer, while samples P10 did not coagulate after 48 h of storage. In contrast, the RCT of samples P1 decreased over time and a firmer coagulum was obtained, possibly due to a lower rate of hydrolysis of β- and α_S-caseins. Therefore, certain levels of action of the protease could actually improve

the rennet coagulation properties of milk. However, increased levels of protease could result in further hydrolysis of caseins, affecting the processability of milk over storage time.

Keywords: psychrotrophic bacteria, milk storage, rennet coagulation properties, thermoresistant protease.

6.1. Introduction

The storage of raw milk for extended periods at low temperatures (2 to 6 ˚C) on-farm has a significant impact on milk microflora, which becomes mostly composed of Gram-positive and Gram-negative psychrotrophic bacteria (Lafarge *et al.*, 2004). Increased levels of psychrotrophs in raw milk could negatively impact the manufacture of dairy products, as those bacteria have the ability to produce heat-resistant extracellular proteases and lipases. Those enzymes can survive thermal treatments, such as pasteurisation (e.g., 72 ˚C for 15 s) or even UHT processing (138 ˚C for 2 s or 149 ˚C for 10 s), decreasing yield and sensory quality of dairy products during storage (Cousin, 1982; Lopez-Fandino *et al.*, 1993). Muir (1996) suggested that raw milk with a psychrotrophic count higher than 5×10^{6} cfu/ mL should be rejected for processing, due to the possible high levels of lipase and proteases. The most common psychrotrophs found in raw milk during cold storage belong to the genus *Pseudomonas spp.*, which secrete most of the heat-resistant peptidases during milk cold storage (Ercolini *et al.*, 2009; Baur *et al.*, 2015).

During the late exponential or early stationary growth phase, *Pseudomonas* bacteria produce thermoresistant alkaline metalloproteases (AprX), generally at a bacterial count of 10^{7} to 10^{8} cfu/ mL (Stoeckel *et al.*, 2016). These metalloproteases preferably hydrolyse κ-casein (κ-CN), then β-casein (β-CN) and then α_{S1}-casein (α_{S1}-CN), and their activities are optimal at a pH between 7 and 9, in a temperature range between 30 and 45 ˚C (Decimo *et al.*, 2014; Martins *et al.*, 2015). Zhang *et al.* (2018) reported that increasing concentrations of AprX resulted in increasing hydrolysis, mainly of κ-CN, and gelation in UHT milk. Bagliniere *et al.* (2013) inoculated 0.2 mg/ L of purified AprX into milk and

observed increased proteolysis of caseins over storage and destabilisation of the produced UHT milk after 8 days of storage. In the case of cheese manufacture, the activity of bacterial proteases can affect the casein content, which may result in low yield, off-flavour (i.e., bitterness) and texture problems (Cousin, 1982; Fox *et al.*, 2017). Mankai *et al.* (2012) observed a low dry matter content in Gouda cheeses produced from milk stored at 4 ˚C for 48, 72 and 96 h, and related this to the activity of psychrotrophic proteases leading to losses of protein in whey. Boulares *et al.* (2011) also associated decreased cheese yield with enzymes produced by gram-negative psychrotrophs. High levels of proteolysis can negatively affect cheese manufacture and sensory characteristics of the final product; however, in contrast, certain levels of proteolysis are necessary during cheese ripening. Proteolysis is essential in most cheese varieties, as it is responsible for textural changes (i.e., hardness, elasticity, cohesiveness) and development of flavour during cheese ripening (Fox *et al.*, 2017).

Some studies have correlated psychrotrophic bacterial counts with proteolysis levels in milk, highlighting the negative impact levels higher than 10^6 cfu/ mL can have on cheese-making properties of milk (clotting time, curd yield) and cheese sensory characteristics (Leitner *et al.*, 2008; Ricciardi *et al.*, 2015). However, psychrotrophs can produce a range of thermo-resistant enzymes, which can be active in different conditions (e.g., temperature, pH). Therefore, in addition to psychrotrophic counts, further studies are necessary to understand the activity and impact of different levels of proteases produced by psychrotrophs on the quality of milk used for cheese production. Those investigations could aid in elucidating to what extent those proteases could have a positive or negative impact on cheese manufacture. Therefore, the objective of this study was to

investigate the effect of different activity levels of a thermo-resistant protease, produced by a *Pseudomonas* strain, on the cheese-making properties of milk and proteolysis.

6.2. Materials and methods

6.2.1. Experimental design

The experimental design of this study is shown in Figure 6.1. Fresh raw milk was collected during milking from the milk line, inoculated with different levels of protease and stored at 4 °C for 72 h. After 0, 48 and 72 h, rennet coagulation properties and proteolysis levels in the samples were assessed. The experiment was repeated in triplicate over a 3-week period.

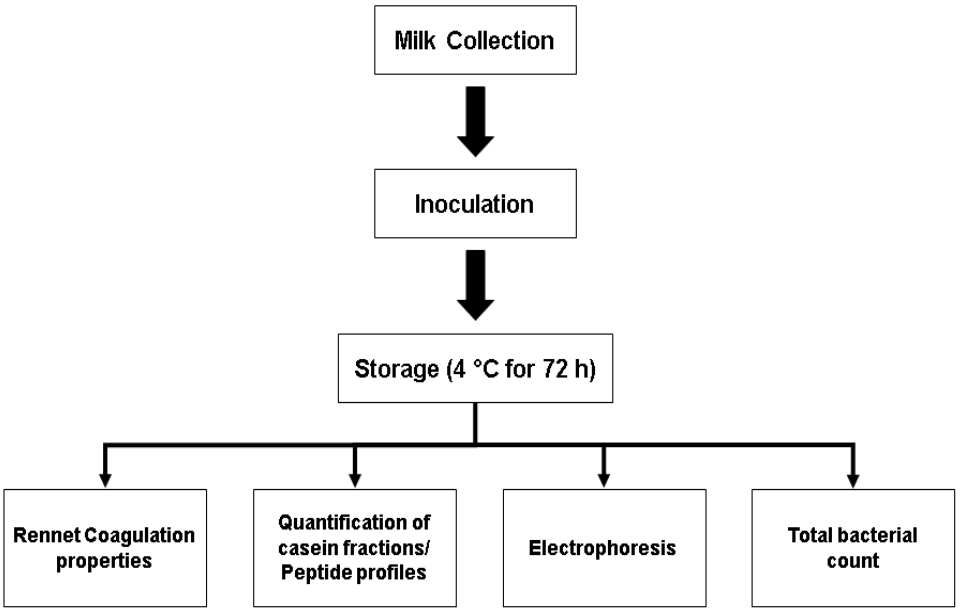


Figure 6.1. Experimental design.

6.2.2. Culture of *Pseudomonas fluorescens* and protease extraction

Pseudomonas fluorescens (ATCC 17556) was grown in nutrient broth (meat extract 3 g/ L, meat peptone 5 g/ L; Sigma Aldrich, Dublin, Ireland) for 48 h at 26 ˚C with stirring at 90 rpm. After, 1 mL of the inoculated nutrient broth was spread-plated on blood agar plates (horse blood agar, 7% concentration, base No. 2; Oxoid, Basingstoke, UK) and plates were incubated for 24 h at 26 ˚C. Bacterial colonies were transferred to 100 mL of sterilised reconstituted skim milk powder (RSMP; 0.1% w/v). The inoculated RSMP bottles were incubated for 120 h at 10 ˚C with stirring at 90 rpm. Afterwards, RSMP was centrifuged at 20,000 g at ambient temperature for 30 min and the supernatant containing protease was collected.

6.2.3. Azocasein assay

The production of protease by the strain was verified using azocasein (Sigma Aldrich, Dublin, Ireland) as substrate, as described by Andreani *et al.* (2016). The results were used to determine the activity of the protease. One unit of protease activity was defined as the required amount of enzyme able to hydrolyse azocasein resulting in an increase of 1 unit of absorbance per mL of sample (supernatant) per minute (Leighton *et al.*, 1973). The following equation was used for calculations:

$$Proteolytic\ activity\ (U.mL^{-1}.min^{-1}) = \frac{(ABS_{sample} - ABS_{neg} - ABS_{blank}) * 10 * F_d}{t}$$

Where,

ABS_{sample}, ABS_{neg}, ABS_{blank} = absorbance of the sample, negative control and blank, respectively

F_d = factor of dilution, which is equal to 1.00 as samples were not diluted

t = time of reaction

In addition, samples of the same supernatant were separated in two groups to determine if the protease would still be active after pasteurisation: one group of three samples was heated to 72 ˚C for 15 s, while other three samples were not pasteurised. Afterwards, those samples were analysed using the azocasein assay for comparison.

6.2.4. Milk collection and inoculation

At the Teagasc Animal and Grassland Research and Innovation Centre (Moorepark, Cork, Ireland), spring-calving cows were milked in a 30-unit side-by-side milking parlor, with milking commencing at 07:00 h. The milking equipment used, udder preparation and sanitation of equipment were as described by Paludetti *et al.* (2018). Over 3 weeks, raw milk was collected once weekly and transferred directly from the milking machine milk line to 5 x 500 mL sterilised bottles. Milk was transported to the Milk Quality Laboratory (Teagasc, Moorepark, Cork, Ireland) in cooling boxes.

Milk samples were inoculated based on the protease activity determined using the azocasein assay (0.030 ± 0.006 U/ mL.min). Samples were inoculated

with 5, 20 and 50 mL of supernatant containing protease per L of milk, corresponding to 0.15, 0.60 and 1.50 U/ L of milk (treatments P1, P4 and P10, respectively). One milk sample was not inoculated to be used as control (C), while one sample of milk was inoculated with 50 mL of supernatant from RSMP that was not inoculated with *P. fluorescens* and was used as the negative control sample (P10 neg). All milk samples were stored at 4 ˚C for up to 72 h.

6.2.5. Total bacterial count

After 0, 48 and 72 h, milk samples from each treatment were tested in duplicate for total bacterial count (TBC). The analysis was performed according to the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004). The TBC was estimated using Petrifilm aerobic count plates, a ready-to-use medium (3M, Technopath, Tipperary, Ireland). Samples were incubated for 48 h at 32 ˚C (Laird *et al.*, 2004).

6.2.6. Rennet coagulation properties

The rennet coagulation properties of the inoculated milk were assessed using a Formagraph instrument (Model 11700, Foss Electric, HillerØd, Denmark) after 0, 48 and 72 h of storage. Prior to analysis, milk samples were pasteurised at 72 ˚C for 15 s in a water bath. In order to mimic Cheddar cheese manufacture, the pH was measured when samples were cooled to 32 ˚C and, when necessary, adjusted to 6.55 with 4% (v/v) lactic acid solution. The milk volumes were renneted with chymosin (1:20 v/v dilution of double strength Chy-max; Pfizer Inc., Milwaukee, WI, USA) at a rate of 36 µL/ 10 mL, incubated at 32 ˚C and the coagulation properties monitored over a 30 min period. The properties measured

were rennet coagulation time (RCT, min), curd firmness at 30 min (a30, mm) and curd-firming time (k20, min).

6.2.7. Quantification of casein fractions

The caseins fractions (κ-CN, α_{S1}-CN; α_{S2}-casein, α_{S2}-CN; β-casein, β-CN; α-lactalbumin, α-lac; β-lactoglobulin A, β-Lg A, β-Lactoglobulin B, β-Lg B; and total casein) were identified and quantified (in duplicate) in the inoculated milk samples by high-performance liquid chromatography (HPLC). Milk samples (200 µL) were diluted in 3,780 µL of dissociating buffer (7 M urea and 20 mM Bis-Tris propane, pH 7.5). Twenty µL of mercaptoethanol were added to the diluted samples, after which they were filtered through 0.22-µm filters. The equipment used was an Agilent 1200s system (Agilent Technologies, Santa Clara, CA, USA) with a quaternary pump and a multiwavelength detector. The casein fractions were separated in the reversed-phase mode using an Agilent Poroshell 300SB C18 column (2.1 mm x 7.5 mm; Agilent Technologies). The gradient elution and peak detection were performed according to the method of Mounsey and O'Kennedy (2009).

6.2.8. Peptide profiles

The peptide profiles of milk samples collected after 0, 48 and 72 h were obtained by HPLC. The non-protein fractions of the milk samples were extracted using trichloroacetic acid, according to the procedure described in IDF method 20-4 (IDF, 2001). The extracts were filtered using 0.45-µm syringe cellulose filters (25 mm diameter, Chromafil Xtra RC-45/25, Macherey-Nagel, Dublin, Ireland) and 50 µL were injected onto the column to obtain the profiles. The

separation of milk peptides was performed in the reversed-phase mode using an Agilent Zorbax 300SB C8 column (4.6 mm ID x 150 mm; Agilent Technologies). The gradient elution and peak detection methodology was an adaption of the methodology of Rohm *et al.* (1996). The HPLC equipment used was an Agilent 1200s system (Agilent Technologies, Santa Clara, CA, USA) with a quaternary pump and a multiwavelength detector.

6.2.9. Polyacrylamide gel electrophoresis (SDS-PAGE)

The individual proteins of the milk samples collected after 0, 48 and 72 h of storage were identified using pre-cast sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Novex Technologies, ThermoFischer Scientific) under reducing and non-reducing conditions. The inoculated milk samples were diluted in Milli-Q water to a protein concentration of approximately 5.7 µg/ µL, and then further diluted in SDS sample buffer [NuPAGE LDS Sample Buffer (4x; ThermoFisher Scientific, Waltham, MA)]. For reducing SDS-PAGE conditions, dithiothreitol [NuPAGE Sample Reducing Agent (10x); concentration = 500 mmol/ L, (ThermoFisher Scientific)] was added to the samples at a level of 10% (v/v) of the sample total volume. Samples were heated to 70 °C for 10 min, cooled, and loaded on 12% Bis-Tris SDS-PAGE gels (10 µg/ well). A low range protein ladder was also loaded on the gels (10 µg/ well; Spectra Multicolor Low Range Protein Ladder, ThermoFisher Scientific). The gels were run in SDS running buffer [NuPAGE MOPS SDS Running Buffer (1x)] at 200V for 50 min. After, the gels were stained for 24 h with Instant Blue Coomassie (Expedeon, UK).

6.2.10. Statistical analysis

The results of the azocasein test for the pasteurised and non-pasteurised supernatants were statistically compared using the MIXED procedure in SAS 9.3 (SAS Institute Inc., Cary, NC).

Least square means for the main effects of storage time and added protease activity, as well as the interaction between storage time and added protease activity, were calculated using the GLIMMIX procedure also in SAS 9.3. The fixed effects included in each model were storage time (0, 48 and 72 h) and added protease activity (0, P10 neg, P1, P4, P10). The inoculated milk volumes within trial week were considered the experimental unit. The response variables were: pH; RCT, k20 and a30; κ-CN, α_{S1}-CN, α_{S2}-CN, β-CN, α-lac, β-Lg A, β-Lg B and total casein. Residual checks were made to ensure that the assumptions of the analysis were met. The Tukey test (at 5% error probability) was used to compare the means for all variables.

6.3. Results

6.3.1. Total bacterial count

The TBC of milk samples from different treatments and stored for up to 72 h are shown in Table 6.1. The TBC of those samples significantly increased with level of protease activity ($P<0.0001$) in the milk samples and over storage time ($P<0.0001$) (Table 6.1). The interaction between storage time and added protease activity did not affect TBC ($P>0.05$).

6.3.2. Measurements of pH in milk

The pH measurements were not affected by added protease activity (P = 0.52). The mean (± S.E.) pH measured in samples C, P10 neg, P1, P4 and P10 were 6.51, 6.53, 6.53, 6.54, 6.53 ± 0.01, respectively. The pH was also significantly affected by storage time (P<0.0001) and interaction between added protease activity and storage time (P = 0.01). No decreases in pH were observed over storage time, except in samples P10. At 0, 48 and 72 h the pH measured in samples P10 were 6.58, 6.55 and 6.47 at 0, 48 and 72 h, respectively (P = 0.001).

In addition, the mean (± S.E.) fat (3.44 ± 0.30%) and protein (3.58 ± 0.06%) contents did not differ significantly between treatments and during storage of milk (data not shown).

6.3.3. Azocasein assay and protease activity

The mean (± S.E.) result obtained for the supernatant samples that were not pasteurised was 0.5469 ± 0.1093 nm. Based on this results, the mean (± S.E.) protease activity in the supernatant was 0.030 ± 0.006 U/ mL.min. Also, the result obtained for non-pasteurised samples was not statistically different from the results obtained for the samples that were pasteurised (0.5352 ± 0.1143 nm, P>0.05).

Table 6.1. Mean (± S.E.) total bacterial count (log_{10} cfu/ mL) of control (C) and negative control (P10 neg) milk samples, as well as samples inoculated with 0.15, 0.60 and 1.50 U/ L (treatments P1, P4, P10, respectively), stored for up to 72 h.

TBC (log_{10} cfu/ mL)	**Protease activity**					**S.E.**
	C	**P10 neg**	**P1**	**P4**	**P10**	
0 h	3.11^{A}	3.22^{A}	4.14^{A}	4.46^{A}	4.77^{A}	0.24
48 h	3.96^{AB}	4.26^{A}	5.45^{A}	6.00^{B}	6.00^{B}	0.24
72 h	5.33^{B}	5.30^{B}	8.00^{B}	8.00^{C}	8.00^{C}	0.24

$^{A-B}$Values within a column not sharing common superscripts differ significantly ($P < 0.05$).

6.3.4. Proteolysis

6.3.4.1. Casein fractions

The significance of the main effects of storage time, added protease activity and interaction between added protease activity and storage time on the concentration of caseins and major whey proteins of milk are shown in Table 6.2. The concentrations of those proteins measured in the milk samples from the different treatments stored for up to 72 h are also shown in Table 6.2.

The concentrations of α_{S2}-CN and β-CN were affected by storage time. The concentrations of all caseins were significantly affected by added protease activity and interaction between protease activity and storage time.

The mean concentrations of α-lac were affected by storage time, β-Lg A was affected by added protease activity and β-Lg B was affected by the interaction between those factors.

The casein chromatograms of the samples C, P10 neg, P1, P4 and P10, stored for up to 72 h, are shown in Figure 6.2. The chromatograms of samples C and P10 neg are similar and indicated low levels of proteolysis over storage. In relation to the inoculated samples, the levels of proteolysis increased with the activity of protease over storage time. The chromatograms indicated that κ-CN and β-CN were the main proteins hydrolysed by the protease over time. Small decreases were also observed in α_{S1}-CN peaks with the increase in added protease activity. In samples P10, decreases in the concentrations of α_{S1}-CN and α_{S2}-CN after 48 h and decreases in the concentrations of β-Lg A and B after 72 h were greater compared to other samples (Table 6.2).

6.3.4.2. Peptide profiles

The peptide profiles of samples C, P10 neg, P1, P4 and P10, stored for up to 72 h at 4 °C, are shown in Figure 6.3. The peptide profiles of the samples C and P10 neg were similar and low levels of proteolysis were observed. Over storage time, the hydrolysis of proteins in milk increased with added protease activity.

Table 6.2. Contents of casein fractions (κ-CN, α_{s1}-CN, α_{s2}-CN, β-CN, α-lac, β-Lg A, β-Lg B and total casein contents) in control (C) and negative control (P10 neg) milk samples and samples inoculated with 0.15, 0.60 and 1.50 U/ L (treatments P1, P4, P10, respectively) stored for up to 72 h, and significance of the main effects of protease activity, storage time and the interaction between protease activity and storage time on those contents.

Protease activity	Time (h)	κ-CN (mg/ mL)	α_{s1} – CN (mg/ mL)	α_{s2} – CN (mg/ mL)	β- CN (mg/ mL)	α-lac (mg/ mL)	β-Lg A (mg/ mL)	β-Lg B (mg/ mL)	Total casein (mg/ mL)
C	**0**	5.30	13.53	3.01	12.71	1.13	1.96	2.62	39.75
	48	5.66	12.44	3.25	11.51	1.13	1.95	2.54	38.48
	72	6.32	13.36	3.54	12.37	1.18	2.10	2.60	41.46
P10 neg	**0**	6.20	14.42	3.93	13.18	1.26	2.20	2.67	43.88
	48	6.35	13.65	3.97	12.43	1.21	2.21	2.51	42.33
	72	5.95	13.60	3.73	12.38	1.21	2.03	2.42	41.33
P1	**0**	4.13	12.60	2.94	11.56	1.05	1.80	1.96	36.54
	48	4.96	14.79	3.72	11.25	1.23	2.11	2.74	40.83
	72	5.16	14.35	4.07	10.53	1.26	2.07	2.74	40.18
P4	**0**	4.04	13.14	3.47	11.63	1.11	1.96	2.69	38.05
	48	3.69	11.78	3.28	8.28	1.12	1.81	2.53	32.51
	72	4.13	13.54	3.85	8.64	1.27	1.76	2.85	36.02
P10	**0**	4.25	13.39	3.79	13.18	1.04	1.87	2.64	37.77
	48	3.70	8.05	2.89	7.72	1.04	1.87	2.40	27.68
	72	3.32	8.13	2.86	6.60	1.19	1.17	2.02	26.30
S.E.		0.25	0.31	0.14	0.28	0.05	0.08	0.15	1.05
Significance									
Storage time		0.40	**<0.0001**	0.09	**<0.0001**	**0.02**	0.14	0.47	**0.01**
Protease activity		**0.002**	**<0.0001**	**0.01**	**<0.0001**	0.22	**0.02**	0.06	**0.0004**
Protease activity*storage time		**0.02**	**<0.0001**	**0.001**	**0.001**	0.20	0.18	**0.04**	**0.001**

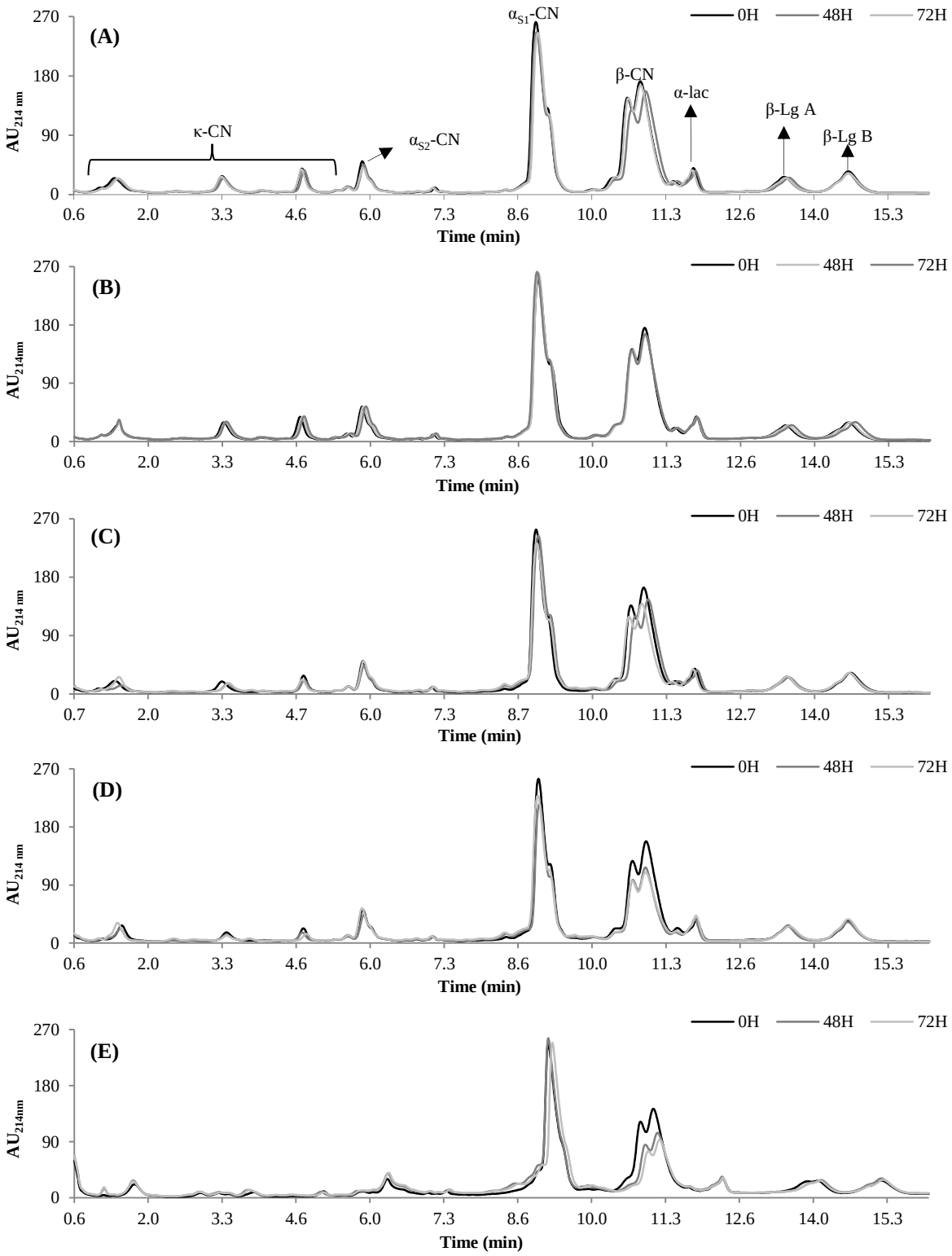


Figure 6.2. Separation of bovine milk caseins by reversed-phase HPLC (measured in absorbance units, AU). Chromatograms of **(A)** control and **(B)** negative control samples and samples inoculated with **(C)** 0.15 (P1), **(D)** 0.60 (P4) and **(E)** 1.50 (P10) U/ L stored for up to 72 h are shown.

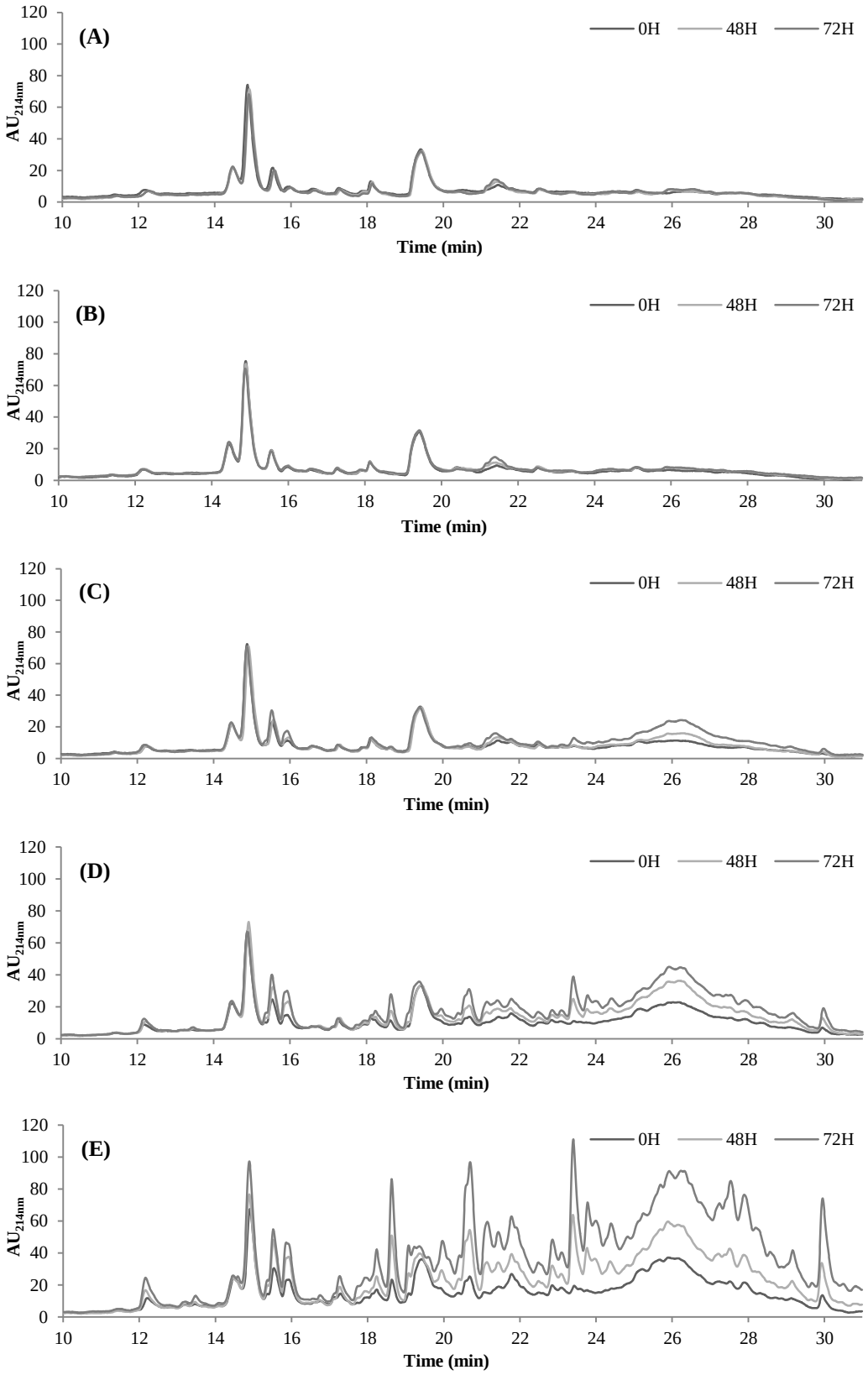


Figure 6.3. Separation of bovine milk peptides by reversed-phase HPLC (measured in absorbance units, AU). Chromatograms of **(A)** control and **(B)** negative control (P10 neg) samples and samples inoculated with **(C)** 0.15 (P1), **(D)** 0.60 (P4) and **(E)** 1.50 (P10) U/ L stored for up to 72 h are shown.

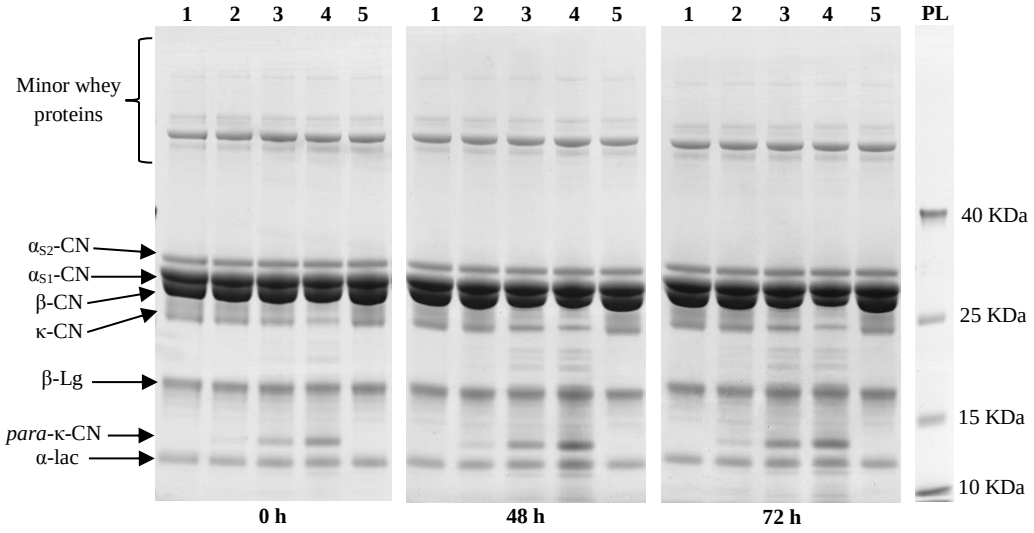
6.3.4.3. Electrophoresis (SDS-PAGE)

Non-reducing and reducing SDS-PAGE electrophoretograms of all milk samples stored for up to 72 h are shown in Figures 6.4.A and B, respectively.

The electrophoretograms of the samples C (lane 1) and P10 neg (lane 5) were similar and no apparent indication of proteolysis was observed. In contrast, the electrophoretograms of samples P1, P4 and P10 indicated that the protease was active mainly on κ-CN and β-CN, bands for which became of a less intense colour and decreased in size over time. In addition, a small decrease in bands corresponding to α_{S1}-CN was noted with increasing level of protease activity. The decrease was more noticeable in samples P10.

The intensity of the peptide bands from 10 kDa to 25 kDa, which are products of the hydrolysis of caseins, increased with level of protease activity. Those bands were observed in samples tested after inoculation (0 h of storage), indicating that the protease is highly active in milk. Also, a peptide band around 14 kDa (above ala) was observed in lanes corresponding to samples P1, P4 and P10 (lanes 2, 3 and 4, respectively) and its intensity increased with level of protease activity and storage time.

(A) Non-reducing



(B) Reducing

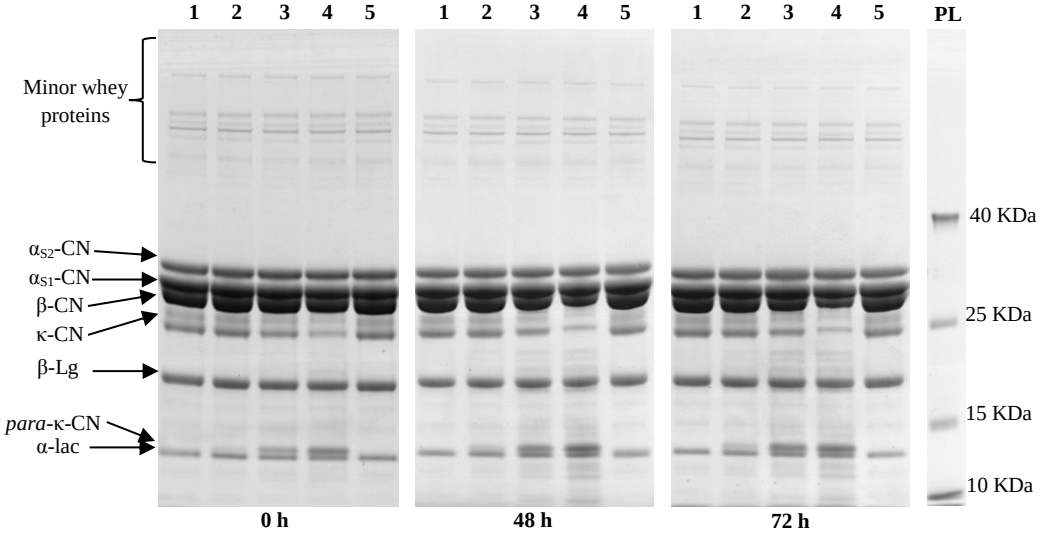


Figure 6.4. SDS-PAGE electrophoretogram of milk samples stored for up to 72 h under **(A)** non-reducing and **(B)** reducing conditions. Lane 1 corresponds to the control samples, while lanes 2 to 5 correspond to samples inoculated with 0.15 (P1), 0.60 (P4), 1.50 (P10) U/ L and negative control (P10 neg), respectively. Lane PL corresponds to a low range protein ladder.

6.3.5. Rennet coagulation properties

The significance of the main effects of storage time, added protease activity and interaction between added protease activity and storage time on the rennet coagulation properties of milk are shown in Table 6.3. The parameters RCT, k20 and a30 obtained for the milk samples from the different treatments, stored for up to 72 h, are shown in Table 6.4. It was not possible to assess the rennet coagulation parameters of the milk samples P10 after 48 h, as those samples did not form a coagulum after rennet addition.

Rennet coagulation time decreased significantly with increasing level of protease activity (C, P10 neg, P1 and P4: 21.54, 19.63, 16.64 and 16.81± 1.16 min, respectively; $P<0.05$). Over 72 h, a decrease in RCT was observed when testing samples P1. The results also indicated that RCT may tend to increase over time for samples P4.

The parameter a30 (mm) was affected by the interaction between level of protease activity and storage time ($P = 0.002$). Over 72 h, the parameter a30 did not vary significantly for samples C and P10 neg (Table 6.4). The variations in a30 measured in samples P1 were also not significant over 72 h; however, an increase in that parameter was observed after 48 h of storage. In relation to samples P4, a30 decreased significantly after 48 h ($P = 0.001$; Table 6.4).

It was not possible to measure the parameter k20 in samples P4 and P10 after 72 h and 48 h, respectively, as those samples did not reach a curd firmness of 20 mm. Consequently, the data set obtained at 0 and 48 h was separated from the data obtained after 72 h for statistical analysis. After 48 h, the k20 measured for samples C, P10 neg, P1 and P4 were not significantly affected by storage time ($P = 0.56$), added protease activity ($P = 0.32$) and interaction between those effects

(P = 0.09). Samples C, P10 neg and P1 reached a curd firmness of 20 mm after 72 h, and the parameter k20 for those samples did not differ statistically (P = 0.84).

Table 6.3. The significance of the main effects of protease activity, storage time and the interaction between protease activity and storage time on the rennet coagulation time (RCT) and curd firmness at 30 min (a30).

Rennet Coagulation Properties	Significance		
	Storage time	**Protease activity**	**Protease activity*storage time**
RCT	0.06	**0.02**	0.21
a30	0.06	0.08	**0.002**

*The significance levels obtained for k20 measurements were not included in this table as those measurements were separated in two groups (group 1: data obtained at 0 and 48 h; group 2: data obtained only at 72 h) for the statistical analysis, differently than for other parameters.

Table 6.4. Mean (± SD) rennet coagulation time (RCT), curd firmness at 30 min (a30) and curd-firming time (k20) of control (C) and negative control (P10 neg) milk samples and samples inoculated with 0.15 (P1), 0.60 (P4) and 1.50 (P10) U/ L, stored for up 72 h.

Protease activity	RCT (min)			a30 (mm)			k20 (min)		
	0 h	**48 h**	**72 h**	**0 h**	**48 h**	**72 h**	**0 h**	**48 h**	**72 h**
C	23.0 ± 1.6^{a}	19.6 ± 0.9^{a}	22.0 ± 3.5^{a}	$17.36 \pm 2.3^{A,ab}$	$31.42 \pm 4.0^{A,a}$	$21.67 \pm 10.4^{A,a}$	*	5.39 ± 0.5^{a}	7.00 ± 0.8^{a}
P10 neg	17.7 ± 3.9^{ab}	19.5 ± 0.4^{a}	21.7 ± 2.5^{a}	$28.14 \pm 5.7^{A,a}$	$27.22 \pm 5.0^{A,ab}$	$25.10 \pm 10.1^{A,a}$	6.58 ± 1.3^{a}	6.89 ± 1.8^{a}	6.39 ± 1.7^{a}
P1	20.3 ± 2.2^{a}	15.2 ± 1.1^{a}	14.4 ± 1.2^{a}	$23.27 \pm 6.1^{A,ab}$	$34.08 \pm 5.4^{A,a}$	$32.60 \pm 6.7^{A,a}$	7.86 ± 3.0^{a}	6.28 ± 1.3^{a}	7.28 ± 2.3^{a}
P4	14.4 ± 6.5^{ab}	16.7 ± 3.9^{a}	19.3 ± 1.2^{a}	$32.78 \pm 9.0^{A,a}$	$17.93 \pm 6.5^{AB,b}$	$5.61 \pm 1.6^{B,b}$	6.03 ± 2.2^{a}	9.31 ± 1.6^{a}	*
P10	7.8 ± 4.0^{b}	**	**	27.98 ± 14.2^{a}	**	**	6.08 ± 0.6^{a}	**	**

$^{A-B}$Values within a row not sharing common superscripts differ significantly ($P < 0.05$).

$^{a-b}$Values within a column not sharing common superscripts differ significantly ($P<0.05$).

*Samples did not reach a curd firmness of 20 mm.

**Data missing due to the no coagulation of those samples.

6.4. Discussion

According to the azocasein test, the strain of *Pseudomonas fluorescens* (ATCC 17556) produced active proteases when incubated at 10 ˚C. This result is in accordance with the study from Marchand *et al.* (2009), in which the same strain presented proteolytic activity at 7, 22 and 30 ˚C. In relation to the level of protease activity, Liao and McCallus (1998) cultured *P. fluorescens CY091*, centrifuged the media and obtained a protease activity varying from 5 to 8 U/ mL in the supernatant. Those results differ from the activity obtained in this study, indicating that other strains may produce proteases with higher levels of activity. The production of protease could depend on several factors, such as bacterial strain, culture media, time and temperature of incubation (McKellar, 1982).

The protease produced was thermo-resistant, as its activity was maintained after pasteurisation and therefore it would remain active during cheese manufacture. In addition, it is now widely shown that multiple *Pseudomonas* strains produce heat-resistant proteases (Baglinière *et al.*, 2013; Machado *et al.*, 2017; Marchand *et al.*, 2017).

The difference in TBC and bacterial growth between non-inoculated samples (C and P10 neg) and inoculated samples (P1, P4 and P10) over storage time at 4 °C could be due to the presence of *P. fluorescens* cells in the supernatant used for inoculation. As the amount of supernatant added increased according to the aimed protease activity, the levels of TBC and bacterial growth also increased. The strains of *P. fluorescens* probably contributed to the increase in TBC, because this strain can grow at temperatures up to 30 ˚C, which is close to the incubation temperature applied to measure mesophilic bacterial numbers (32 ˚C) (Marchand *et al.*, 2009).

After 72 h, the pH of samples P10 decreased significantly, which could be due to the high levels of bacteria in those samples. The high bacterial activity could have increased the acidity of milk with the conversion of lactose into lactic acid. The decrease in pH could have potentially resulted in the coagulation of milk samples P10 after 48 h, as the acidity could have influenced the net charge of the casein micelles (Fox *et al.*, 2017).

The similarity between the results obtained for samples C and P10 neg, as well as how they differed from the results obtained for the inoculated samples, indicated that the method of production and extraction of proteases were effective and that protease was active in milk.

The casein concentrations (Table 6.2), as well as the results from the chromatograms (Figures 6.2 and 6.3) and electrophoretograms (Figure 6.4), indicated an increase in proteolysis levels with added protease activity over storage time. The protease tested preferably hydrolysed κ-CN and β-CN; the rate of hydrolysis of α_S-CNs increased with level of protease activity. The breakdown of α_S-CNs and major whey proteins were more noticeable when the level of protease activity was increased to 1.50 U/ L (P10, Figures 6.2 and 6.4; Table 6.2). Similar results were observed by Zhang *et al.* (2015) when investigating the degradation on milk proteins by a thermo-resistant protease produced by *Pseudomonas fluorescens BJ-10*.

Statistical analysis indicated that ala and β-Lg B levels were affected by storage time and interaction between added protease activity and storage time, respectively. However, no trends over time were observed for the concentrations on those proteins and variations observed were minimal (Table 6.2). Small variations in the concentrations of whey proteins were also observed in the

chromatograms (Figure 6.2). The only trend observed was the decrease in the whey protein peaks when the protease activity was 1.50 U/ L (samples P10).

The hydrolysis of the caseins affected the rennet coagulation properties of the milk from different treatments. Overall, the increase in the level of protease activity resulted in a significant decrease in RCT. At 0 h, that trend was observed (Table 6.4) and could be linked to the hydrolysis of κ-CN, which is important for the coagulation of milk during cheese-making. During coagulation, the Phe_{105}-Met_{106} bond of κ-CN is hydrolysed and the N-terminal that remains bound in the casein network is called *para*-κ-casein (*para*-κ-CN). Due to changes in charges, the *para*-casein micelles are then able to aggregate in the presence of calcium ions (Fox *et al.*, 2017). In the electrophoretograms (Figure 6.4), a peptide band around 14 kDa (above α-lac) was observed in lanes corresponding to samples P1, P4 and P10 (2, 3 and 4, respectively) and its intensity increased with level of added protease activity and storage time. A similar result was obtained by Timotijevic *et al.*, (2006) when testing different concentrations of rennet and the authors identified that band as corresponding to *para*-κ-CN. According to Jackman *et al.* (1985), psychrotrophic bacterial proteases include endopeptidases capable of hydrolysing the phenylalanine-methionine bond of κ-CN in a similar manner to rennet. Therefore, the increase in added protease activity could have resulted in increasing hydrolysis rates of κ-CN into *para*-κ-CN, and consequently the decrease of RCT in inoculated milk samples.

After 48 h, the RCT of samples P1 continued to decrease over storage time. In contrast, the RCT of samples P4 tended to increase over time, while samples P10 did not coagulate after 48 h (Table 6.4). In addition, the firmness (a30) of the coagulum formed when testing samples P1 increased over time, while

the firmness of the coagulum of samples P4 significantly decreased over time (P = 0.01). The increase in RCT is potentially due to the enzymatic degradation of caseins. The α_S-CN and β-CN constitute the basic microstructure of the curd, and reduced levels of those caseins results in slow curd formation and a soft coagulum as observed. Protease activity can affect the caseins to such an extent that coagulation of milk does not occur (St-Gelais and Hache, 2005; Amenu and Deeth, 2007), as observed in samples P10. The proteolysis of caseins could result in curd shattering, high losses of fat in the whey and reduced cheese yields (Fox *et al.*, 2017). In relation to samples P1, the rate of hydrolysis of α_S-CN and β-CN was lower and therefore the structure of the casein micelles was less affected (Lu *et al.*, 2017). Consequently, the coagulum produced was firmer compared to other inoculated samples. The firmness measured in those samples was also higher than the firmness of control samples, possibly due to the higher rate at which κ-CN was hydrolysed into *para*-κ-CN.

Finally, the inoculated samples had a similar TBC after 48 and 72 h of storage and still had different results in relation to proteolysis levels. Therefore, those results were mainly due to the protease activity added and not the bacterial count.

6.5. Conclusion

This study demonstrated that the protease produced by the psychrotrophic bacteria tested was thermo-resistant and can affect the cheese-making properties of milk. The rennet coagulation time and curd firmness were affected over storage time, as the protease was mainly active on essential caseins for the formation and structure of the coagulum (κ-CN and β-CN, respectively). The results indicated

that, to a certain extent, protease activity can be favourable to accelerate the coagulation of milk and produce a firmer coagulum. However, increased levels of protease can result in the hydrolysis of α_S-CNs and major whey proteins, and consequently a softer coagulum is produced or the coagulation of milk is not possible. Therefore, this study highlighted the importance of controlling initial levels of psychrotrophs in milk, as the activity of their protease could affect the processability of milk over storage time. Further studies are necessary to determine the effect of those proteases on the manufacture and quality of cheese in an industrial scale. Additionally, as milk has a complex microbiota, it would be relevant to characterise other psychrotrophic bacterial proteases to determine their resistance to heat treatment and activity in milk.

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Chapter 7

Effect of *Pseudomonas fluorescens* proteases on the quality of Cheddar cheese

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Declaration: Analysis, including measurement of nitrogen fractions, casein fractions through HPLC, bacterial numbers in milk, pH in cheese, composition of Cheddar cheese and whey, as well as proteolysis levels in cheese (pH 4.6 SN/ TN, quantification of free aminoacids and Urea-PAGE electrophoresis) were all conducted by Lizandra Paludetti, at Teagasc, Moorepark Food Research Centre. The extraction of protease, inoculation of milk and manufacture of Cheddar cheese was also carried out by Lizandra Paludetti. Dr. Tom O'Callaghan aided with the collection, standardisation and pasteurisation of raw milk. Dr. Tom O'Callaghan and Dr. Jeremiah Sheehan aided on planning the experimental design and gave guidance regarding the preparation of milk and manufacture of Cheddar cheese. All experimental data were analysed and the chapter written by Lizandra Paludetti. Dr. David Gleeson and Prof. Alan L. Kelly provided overall supervision of the project and support of experimental design and data interpretation.

Abstract

The objective of this study was to investigate the effect of different activity levels of a thermo-resistant protease, produced by a *Pseudomonas fluorescens* strain, on the manufacture, proteolysis and quality of Cheddar cheese. Fresh raw milk was collected, standardised and pasteurised at 72 ˚C for 15s. Milk was inoculated to obtain a protease activity of 0.15 and 0.60 U/ L (treatments P1 and P4, respectively), while one sample was not inoculated to be the control (C). The milk containers were stored at 4 °C for 48 h and Cheddar cheese was manufactured after 0 and 48 h of storage. Results indicated that the protease was active in milk during 48 h of storage; however, its effect on the milk composition was minimal. β-CN was preferentially hydrolysed by the protease over storage time, followed by κ-CN. The mean cheese yield and recovery of fat and protein obtained for all cheeses were not affected by added protease activity (P>0.05). These results also indicated that the protease presented low activity during cheese manufacture, possibly due to the processing conditions (e.g., pH). Consequently, the composition, pH, patterns of proteolysis and hardness of all cheeses produced were similar and in accordance with parameters expected for that type of cheese, independently of the added protease activity level. However, slight increases in proteolysis were observed in cheeses with higher levels of protease and that were produced using milk stored for 48 h. Both cheeses had higher concentrations of FAA compared to control, while Urea-PAGE electrophoretograms indicated a higher breakdown of caseins in those P4 samples. This can be related to possible increases in proteolytic bacteria numbers in milk during storage. Therefore, to a certain extent, psychrotrophic bacterial proteases may not affect the manufacture and quality of Cheddar cheese during ripening. Finally, the control of initial levels

of proteolytic bacteria in raw milk is essential, as proteolysis influences on the development of flavour and texture in cheese.

Keywords: *Pseudomonas fluorescens*, psychrotrophic bacteria, milk storage, thermoresistant protease, Cheddar cheese.

7.1. Introduction

The world production of cheese is approximately 19 x 10^6 tonnes per year, and has increased at an average annual rate of approximately 4% over the past 30 years. The increase in cheese consumption could be due to the positive dietary image of the product, convenience and flexibility in use, as well as the great diversity of flavours and textures. Cheddar cheese is one of the most important cheese varieties worldwide and is one of the main dairy products exported by Ireland and the United Kingdom (Fox *et al.*, 2017). The quality of milk supplied for cheese manufacture is one of the main factors that can affect the quality of the final product.

The microbiota of raw milk during cold storage is mainly composed of Gram-negative and Gram-positive psychrotrophic bacteria. The most common psychrotrophs identified in raw milk belong to the genera *Pseudomonas spp.* (Ercolini *et al.*, 2009; Ribeiro Junior, 2017). Most of the lipases and proteases produced by this bacterial species are thermo-resistant, and can withstand heating to 100 ˚C for 30 min. Those enzymes are produced when psychrotrophic bacterial counts (PBCs) are higher than 6.0 $\log_{10}$ cfu/ mL and are not eliminated after heat treatment, affecting the quality of cheese products (Fox, 1989; Fox *et al.*, 2017). According to Sørhaug and Stepaniak (1997), PBCs between 6.5 to 7.5 $\log_{10}$ cfu/ mL can cause rancidity in hard cheeses, while counts between 7.5 to 8.3 $\log_{10}$ cfu/ mL can cause off-flavours (i.e., rancidity and soapy taste) and reduced cheese yield. Most psychrotrophic proteases are metalloproteases, which preferentially hydrolyse κ-casein (κ-CN), then β-casein (β-CN) and then α_{s1}-casein (α_{s1}-CN), potentially causing reductions in cheese yield (Decimo *et al.*, 2014). Boulares *et al.* (2011) and Mankai *et al.* (2012) observed decreases in cheese yield which

were associated with the loss of protein into whey due to the activity of proteases produced by psychrotrophic bacteria. Even though certain microbial proteolysis can be an undesirable by-product in raw milk during storage and cheese manufacture, specific starter cultures are added into milk to promote proteolysis during ripening for the development of texture and flavour in different cheese varieties.

Several studies have investigated the effects of thermo-resistant psychrotrophic bacterial proteases on UHT milk, identified and characterised the proteases produced by *Pseudomonas*, and assessed their proteolytic activity (Marchand *et al.*, 2009; Mateos *et al.*, 2015; Caldera *et al.*, 2016). However, the dynamics and activity of those proteases during the manufacturing process of a range of other dairy products, their impact on raw milk and consequent implications for the quality of the final product still require further investigations. Cheese products, for example, can vary greatly in relation to their processing parameters, pH and microbiota, which can be determinant parameters of the types and activity of proteases in those products. Therefore, the aim of this study was to investigate the effect of different activity levels of a thermo-resistant protease, produced by a *Pseudomonas fluorencens* strain, on the manufacture, proteolysis and quality of Cheddar cheese.

7.2. Materials and methods

7.2.1. Culture of *Pseudomonas fluorescens* and protease extraction

Pseudomonas fluorescens (ATCC 17556) was grown in nutrient broth (meat extract 3 g/ L, meat peptone 5 g/ L; Sigma Aldrich, Dublin, Ireland) for 72 h at 26 ˚C with stirring at 90 rpm. After this, 1 mL of the inoculated nutrient broth

was spread plated on blood agar plates (horse blood agar, 7% concentration, base No. 2; Oxoid, Basingstoke, UK) and plates were incubated for 24 h at 26 ˚C. Bacterial colonies were transferred to 100 mL of sterilised reconstituted skim milk powder (RSMP, 10% w/v). The inoculated RSMP bottles were incubated for 120 h at 10 ˚C with stirring at 90 rpm. Afterwards, RSMP was centrifuged at 20,000 g at 25 ˚C for 30 min and the supernatant containing protease was collected. In a previous trial, the authors determined that the protease was thermoresistant, as it still was active after being heated at 72 ° C (temperature applied during milk pasteurisation) (Chapter 6).

7.2.2. Milk collection

At the Teagasc Animal and Grassland Research and Innovation Centre (Moorepark, Cork, Ireland), spring-calving cows were milked in a 30-unit side-by-side milking parlor, with milking commencing at 07:00 h. The milking equipment used, udder preparation and sanitation were as described by Paludetti *et al.* (2018). This experiment was repeated in triplicate over a 3-week period. During that period fresh raw milk was collected once per week and directly transferred from the milking machine line to three sanitised containers. The containers were transported to the Biofunctional Food Engineering facility, at the Teagasc Food Research Centre (Moorepark, Cork, Ireland), where milk was standardised, pasteurised and used for the production of Cheddar cheese.

7.2.3. Milk standardisation, pasteurisation and inoculation

Raw milk was skimmed using a tabletop cream separator (Milky FJ 130 ERR, Janschitz-gmbh, Germany), and, the fat and protein contents in the

separated cream and skim milk were measured by using infrared absorption spectroscopy (MilkoScan FT6000, Foss Ireland Ltd, Dublin, Ireland). Pearson's square calculations were used to determine the amount of skim milk and cream that should be mixed to achieve a protein-to-fat ratio of 0.95:1.00. The standardised milk was pasteurised at 72 ˚C at a flow rate of 2 L/ min with a holding time of 15 s using a Microthermics Lab heat exchanger (MicroThermics, NC, USA).

The pasteurised milk was divided into 3 sanitised containers (40 L/ container). In a previous study, the activity of the protease extract was determined (0.030 U/ mL.min, Chapter 6) and, based on that value, the milk containers had different amounts of supernatant added to obtain different activity levels in the milk samples. One milk container was not inoculated to be used as control (C), while 5 and 20 mL of supernatant per L of milk were added to the other two containers, to obtain activity levels of 0.15 and 0.60 U/ L, respectively (treatments P1 and P4, respectively). Those concentrations were selected based on a previous study carried out by the authors, in which curd formation was observed after adding rennet to milk samples containing those levels of protease activity. All milk containers were stored at 4 ˚C for 48 h and Cheddar cheese was produced after 0 and 48 h of inoculation. The experimental design of this study is shown in Figure 7.1.

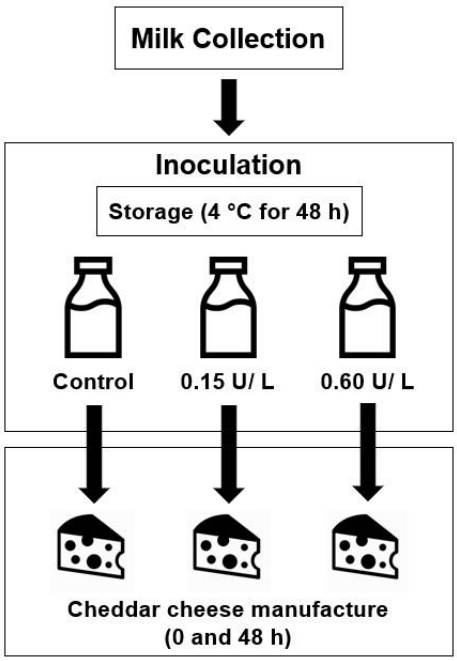


Figure 7.1. Experimental design.

7.2.4. Milk composition

After 0 and 48 h of storage, the composition of each milk was assessed. The nitrogen (%N), non-casein nitrogen (%NCN) and non-protein nitrogen (%NPN) contents of the milk samples were determined using the Kjeldahl method, as described in the methods IDF 20-3 (IDF, 2004a), 29-1 (IDF, 2004b) and 20-4 (IDF, 2001), respectively, using a Tecator Digestor Auto and Kjeltec 8400 distiller (Foss Electric, Hillerød, Denmark). The non-casein protein (%NCP) was calculated by multiplying %NCN by 6.38. The pH of milk was also measured after 0 and 48 h of storage, prior to cheese manufacture.

The casein fractions (κ-CN; α_{S2}-casein, α_{S2}-CN; α_{S1}-CN; β-CN; α-lactalbumin, α-lac; β-lactoglobulin A and B, β-Lg A and B; and total casein) were identified and quantified (in triplicate) in the milk samples by high-performance liquid chromatography (HPLC). Milk samples (200 µL) were diluted in 3.78 mL of dissociating buffer (7 M urea and 20 mM Bis-Tris propane, pH 7.5). Twenty

µL of mercaptoethanol were added to the diluted samples, and after they were filtered through 0.22-µm filters. The equipment used was an Agilent 1200s system (Agilent Technologies, Santa Clara, CA, USA) with a quaternary pump and a multiwavelength detector. The casein fractions were separated in the reversed-phase mode using an Agilent Poroshell 300SB C18 column (2.1 mm x 7.5 mm; Agilent Technologies). The gradient elution and peak detection were performed according to the method of Mounsey and O'Kennedy (2009).

7.2.5. Microbiological count in milk samples

The total bacterial count (TBC) and psychrotrophic bacterial count (PBC) were measured in all milk samples at 0 and 48 h, after pasteurisation and inoculation. Both bacterial counts were estimated using Petrifilm aerobic count plates, a ready-to-use medium (3M, Technopath, Tipperary, Ireland). Samples were incubated at 32 °C for 48 h to determine TBC, while samples were incubated at 7 °C for 10 days to determine PBC (Laird *et al.*, 2004). Those tests were performed according to the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004).

7.2.6. Cheddar cheese production

After 0 and 48 h of inoculation and storage, milk containing different protease activity levels were used to manufacture Cheddar cheese. Therefore, during a 3-week period, two cheesemaking trials were undertaken per week. During each period, 10 L of milk were transferred into jacketed, stainless steel cheese vats, which contained automated variable speed cutting and stirring equipment. The milk from different treatments were inoculated at 32 ˚C with

strains of lactic acid bacteria (R-604, *Lactococcus lactis sub. cremoris* and *Lactococcus lactis sub. lactis*, Chr. Hansen Ltd., Cork, Ireland). After 60 min, rennet (Chy-Max Plus, Chr. Hansen Ltd.), diluted in Milli Q water (approximately 0.1% v/v), was added to each milk vat at a level of 2 mL/ L.

Small-amplitude oscillatory rheometry (AR 2000ex, TA Instruments, New Castle, DE) was used to determine curd firmness (storage or elastic modulus, G'), by using a concentric-cylinder measuring geometry (cylindrical bob and cup). The dynamic changes in rheology during the coagulation process were monitored using a dynamic time sweep analysis, as described by Mateo *et al.* (2010) and Lamichhane *et al.* (2018), but at 32 ˚C. The gels were cut at a curd firmness of 35 Pa.

The cut programme of 3 min consisted of alternating between cutting (40 s) and healing (20 s). The curd/ whey mixture was stirred continuously for 15 min and then cooked. The temperature was increased at a rate of approximately 0.2 ˚C/ min from 32 to 38 ˚C. Once the pH reached 6.15, the whey was drained and the curds were retained in the vat to promote further syneresis. The curds were inverted every 15 min and pH was monitored over that period (cheddaring). Once the pH reached 5.30, the curds were milled and salted (2.7% w salt /w cheese). The curds from each vat were weighed and molded in 2 x 500 g moulds, pressed at 150 kPa overnight, weighed again and vacuum packed. The cheeses were stored at 4 ˚C for 14 days and ripened at 8 ˚C for 180 days.

The cheese yield (Y%) was calculated as the ratio between the weight of cheese produced (g) to the weight of milk used (g) multiplied by 100. Moisture adjusted yield was not calculated as the moisture contents of the cheeses was not significantly different. The fat and protein recovery (%REC_{FAT} and %REC_{PROT},

respectively) were calculated as the ratio of the weight (g) of the curd components (fat and protein, respectively) to the same component of milk (g) multiplied by 100.

7.2.7. Cheese and whey composition

At 7 days post-manufacture, the moisture (IDF, 1982), protein (IDF, 1993), salt (IDF, 1988) and fat of all cheeses were determined. The fat content was measured by nuclear magnetic resonance, using a CEM Smart Trac System method (Cartwright *et al.*, 2005). Based on the content of those parameters, the content of total solids (TS), salt-in-moisture (S/M), moisture in the nonfat substance (MNFS) and fat in dry matter (FDM) were calculated. The pH of the cheeses was monitored after 7, 14, 90 and 180 days of ripening, by mixing 20 g of cheese with 12 g of deionised water (British Standards Institution, 1976).

The fat and protein contents in whey were determined according to the IDF standard methods 22 (IDF, 2008) and 20-3 (IDF, 2004a), respectively.

7.2.8. Assessment of proteolysis

7.2.8.1. pH 4.6-soluble nitrogen and free amino acids

The pH 4.6-soluble fractions of the cheeses ripened for 14, 90 and 180 days (expressed as % of total nitrogen) were obtained as described by Fenelon and Guinee (2000a). The nitrogen content of those fractions (pH 4.6-SN/TN) were determined in duplicate by the macro-Kjeldahl method (IDF, 1986). The levels of free amino acids (FAA) were measured as described by Sheehan *et al.* (2007). The following amino acids were quantified: aspartic acid (asp), threonine (thre),

serine (ser), glutamic acid (glu), glycine (gly), alanine (ala), cysteine (cys), valine (val), methionine (met), isoleucine (ile), leucine (leu), tyrosine (tyr), phenylalanine (phe), histidine (his), lysine (lys), tryptophan (tryp), arginine (arg) and proline (pro).

7.2.8.2. Reversed-phase high-performance liquid chromatography

The hydrolysates peptide profiles of pH 4.6-soluble fractions were obtained by reversed-phase HPLC using an ultra-performance liquid chromatographer (UPLC). The pH 4.6-soluble fractions were filtered through 0.22 µm cellulose filters. An aliquot (10 µL) of each filtered extract was injected on the column (Acquity UPLC Peptide BEH C18, 130 Å, 1.7 m, 2.1 x 100 mm) and the flow rate was 0.20 mL/ min. The equipment consisted of a Waters Acquity UPLC H-Class Core System with an Acuity UPLC TUV Detector (dual wavelength) and Acquity Column Heater 30-A. The system was interfaced with Empower 3 software (Water Corp., Milford, MA, USA). The core system includes an Acquity UPLC H-Class quaternary solvent manager, an H-Class Samples Manager-FTN and a CH-A column heater. Elution was monitored at 214 nm and a mobile phase of two solvents was used: 0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water (A) and 0.1% (v/v) TFA in acetonitrile (B). In the elution gradient program used, the percentage of solvent B increased at a constant rate from 3% to 40% over 45 min, after which the % B increased to 85% in 2 min, was held at 85% B for 2 min, and then returned to 3% B over 8 min before injection of the next sample.

7.2.8.3. Urea polyacrylamide electrophoresis

Urea polyacrylamide electrophoresis (Urea-PAGE) was carried out on all cheeses after 14, 90 and 180 days of ripening. The analysis was performed using a PROTEAN II xi cell vertical slab gel unit (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, UK), using a separating and stacking gel system. The method used was an adaption of the methodology used by Andrews (1983) and Shalabi and Fox (1987). Cheese was dissolved in 1 mL of sample buffer [0.75 g Tris (hydroxymethyl)-methylamine, 49 g urea, 0.7 mL mercaptoethanol and 0.15 g bromophenol blue] on a protein basis (4.75 mg protein). Samples were incubated at 55 °C for 15 min and after filtered through glass wool to remove fat deposits. Sodium caseinate powder was used as a non-hydrolysed casein control and was dissolved to give an equivalent concentration of protein. The voltages applied when samples ran through the stacking and separating gels were 280 and 300 V, respectively. Gels were stained for 24 h with Instant Blue Coomassie (Expedeon, UK) and after scanned using a dual lens Epson Perfection V700 Photo Model J221A with Epson Scan software (Epson Deutschland GmbH, Meerbusch, Germany). The bands were identified as described by McSweeney *et al.* (1994) and Mooney *et al.* (1998).

7.2.9. Texture analysis

The hardness of each cheese were measured after 14, 90 and 180 days of ripening. All cheeses were cut into six cube-shaped samples (25 mm^3) using a Cheese Blocker (Bos Kaasgreedschap, Bodengraven, the Netherlands), wrapped in tin foil and stored at 4 ˚C for 1 h prior to analysis. The texture profile was assessed using a TAHDi analyser (Stable Micro Systems, Goldalming, Surrey,

UK), equipped with a 75-mm (diameter) compression plate and a 50-kg load cell. Each cube was taken from the refrigerator and immediately compressed, in 2 successive bites, to approximately 40% of their original height at a rate of 1.00 mm/ sec (Henneberry *et al.*, 2015). Hardness was calculated as described by Chevanan *et al.* (2006).

7.2.10. Statistical analysis

The main effects of storage time and added protease activity, as well as the interaction between storage time and added protease activity, on the parameters measured in the inoculated milk were investigated. The least square means of those effects were calculated using the GAUSSIAN procedure in SAS 9.3 (SAS Institute Inc., Cary, NC). The fixed effects included in each model were storage time (0 and 48 h) and added protease activity (C, P1 and P4). The containers in which inoculated milk was stored within week were considered the experimental unit. The response variables were: N%, NCN%, NPN%, and NCP%; κ-CN, α_{S1}-CN, α_{S2}-CN, β-CN, α-lac, β-Lg A and B, total casein contents and pH; TBC and PBC.

The influence of those same effects was also investigated on parameters measured in the cheeses and whey produced. When the cheeses were considered the experimental unit, the response variables were: Y%, $\%REC_{FAT}$, $\%REC_{PROT}$ and composition after 7 days (fat, protein, moisture, salt, TS, S/M, MNFS, FDM and pH). When the whey produced was considered the experimental unit, the response variables were the fat and protein contents.

Finally, the influence of those same effects and ripening time (14, 90 and 180 days) on the parameters measured during the ripening of the cheeses

produced (experimental unit) was determined. The least square means of those effects were also calculated using the GAUSSIAN procedure in SAS 9.3 (SAS Institute Inc., Cary, NC). The response variables were: pH, pH 4.6-SN/TN, concentrations of amino acids (asp, thre, ser, glu, gly, ala, cys, val, met, ile, leu, tyr, phe, his, lys, tryp, arg and pro) and hardness. In the case of the pH, the analysis also included the data measured at 7 days of ripening.

Residual checks were made to ensure that the assumptions of the analyses were met. The Tukey test (at 5% error probability) was used to compare the means for all variables.

7.3. Results

7.3.1. Milk composition

The mean (± S.E.) composition parameters and pH measured in milk from the different treatments are shown in Table 7.1, as well as the effects of storage time, added protease activity and interaction between them.

The mean (± S.E.) %N significantly decreased with increasing level of protease activity (C: 4.10, P1: 3.94 and P4: 3.91 ± 0.05%) (Table 7.1). The %NCN and %NCP of milk samples were also affected by added protease activity. However, the mean %NCN varied minimally, while the mean %NCP increased significantly with level of protease activity (Table 7.1).

In relation to the casein fractions, none of the factors significantly affected the level of κ-CN; however, decreases in κ-CN levels with increasing level of protease added were observed (Table 7.1). The mean contents of β-CN, α-lac, β-Lg A and B decreased significantly over storage time (Table 7.1). The significant

differences in α-lac and β-Lg A and B concentrations throughout storage time were considered minimal.

The casein chromatograms of all milk samples, stored for up to 48 h, are shown in Figure 7.2. β-CN content decreased with increasing level of protease added.

The pH of the milk samples was affected by storage time; however, the decreases observed over time were minimal.

7.3.2. Microbiological counts in milk

The mean TBC and PBC measured at 0 and 48 h in all milk samples, as well as the effects of storage time, added protease activity and their interaction, are shown in Table 7.2. The mean TBC increased significantly with added protease activity and storage time, while PBC increased significantly with storage time; it is noted that the enzyme preparation used was not pasteurised, and so may have contained live cells.

Table 7.1. Contents of nitrogen fractions, caseins and major whey proteins, and pH, in control (C) milk samples and milk samples inoculated with 0.15 (P1) and 0.60 (P4) U/ L stored for up to 48 h; significance of the main effects of storage time, protease activity and the interaction are indicated.

Components	Protease activity						S.E.	Significance		
	C		P1		P4			Storage time	Protease activity	Storage time*Protease activity
	0 h	48 h	0 h	48 h	0 h	48 h				
%N	4.17	4.20	4.01	3.87	3.90	3.93	0.09	0.80	**0.04**	0.68
%NCN	0.14	0.14	0.14	0.14	0.15	0.15	0.004	0.39	**0.04**	0.89
%NPN	0.04	0.03	0.03	0.04	0.04	0.04	0.001	1.00	0.07	0.30
%NCP	0.87	0.88	0.89	0.92	0.97	1.00	0.03	0.45	**0.01**	0.95
κ-CN (µg/ µL)	7.68	6.97	6.75	6.35	5.97	5.82	0.34	0.09	0.06	0.56
α_{S2}-CN (µg/ µL)	5.15	4.72	4.61	4.50	4.82	4.63	0.20	0.30	0.42	0.12
α_{S1}-CN (µg/ µL)	15.03	14.10	14.02	13.92	15.22	14.33	0.38	0.83	0.32	0.05
β-CN (µg/ µL)	15.12	14.15	14.05	12.74	14.10	11.63	0.38	**0.0002**	0.05	0.05
α-lac (µg/ µL)	1.24	1.06	1.08	1.04	1.11	1.07	0.06	**0.03**	0.52	0.20
β-Lg A (µg/ µL)	2.76	2.37	2.37	2.36	2.47	2.37	0.11	**0.03**	0.42	0.08
β-Lg B (µg/ µL)	3.12	2.67	2.75	2.63	2.90	2.74	0.11	**0.01**	0.41	0.12
Total casein (µg/ µL)	50.10	46.05	45.64	43.56	45.52	43.67	1.50	**0.01**	0.22	0.49
pH	6.60	6.58	6.59	6.58	6.61	6.59	0.01	**0.005**	0.24	0.61

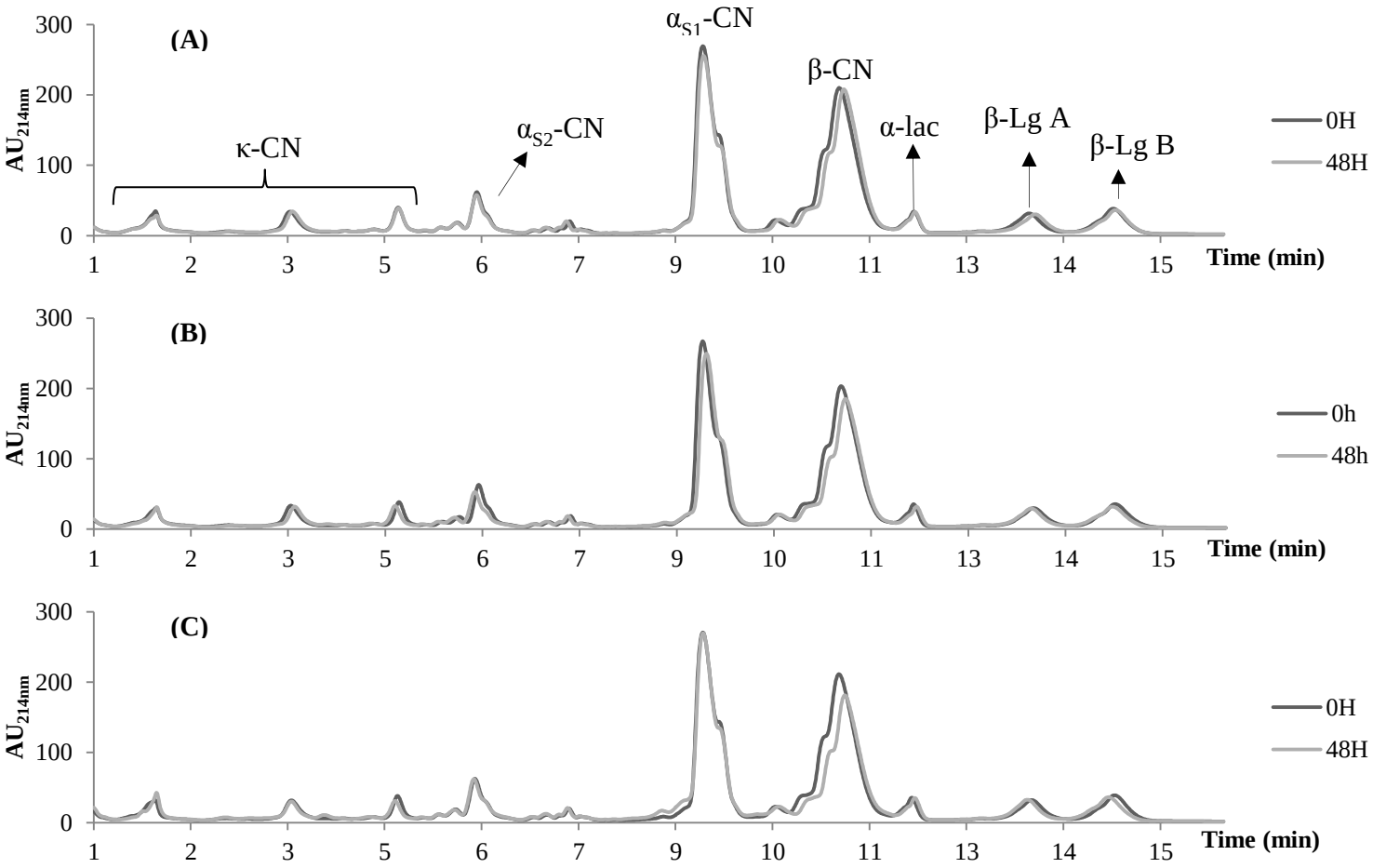


Figure 7.2. Separation of bovine milk caseins by reversed-phase HPLC (measured in absorbance units, AU). Chromatograms of **(A)** control milk samples and samples inoculated with **(B)** 0.15 and **(C)** 0.60 U/ L stored for up to 48 h are shown.

Table 7.2. Mean (± S.E.) total (TBC) and psychrotrophic (PBC) bacterial counts measured in control (C) milk samples and samples inoculated with 0.15 (P1) and 0.60 (P4) U/ L stored for up to 48 h, and significance of the main effects of storage time, protease activity and the interaction between them.

Bacterial count (log_{10} cfu/ mL)	**Protease activity**						**S.E.**	**Significance**		
	C		**P1**		**P4**			**Storage time**	**Protease activity**	**Storage time*Protease activity**
	0 h	**48 h**	**0 h**	**48 h**	**0 h**	**48 h**				
TBC	2.21	3.27	2.41	4.32	3.41	5.00	0.34	**<0.0001**	**0.04**	0.13
PBC	2.13	3.32	3.25	4.51	4.02	4.78	0.54	**0.007**	0.17	0.73

7.3.3. Cheese yield

The mean Y%, $\%REC_{FAT}$ and $\%REC_{PROT}$ obtained for all cheeses manufacture after 0 and 48 h of milk storage are shown in Table 7.3. The $\%REC_{FAT}$ was affected by storage time (P = 0.03), while none of the factors affected the mean Y% or $\%REC_{PROT}$.

7.3.4. Cheese and whey composition

The effects of storage time, added protease activity and interaction between those factors on cheese and whey composition are shown in Table 7.4. Only the FDM content was affected by storage time. None of the factors influenced the fat and protein contents in whey.

The mean (± S.E.) pH measured in the cheese samples from the different treatments and stored for 7, 14, 90 and 180 days are shown in Table 7.5. The pH of cheese was affected by ripening time (7 d: 5.13, 14 d: 5.16, 90 d: 5.21, 180 d: 5.26 ± 0.03; P = 0.01) and treatment (C: 5.17, 5: 5.10, 20: 5.20 ± 0.02; P<0.001). The pH was not affected by storage time (P = 0.05), ripening time and storage time (P = 0.96), ripening time and treatment (P = 0.99), storage time and treatment (P = 0.10) and interaction between ripening time, storage time and treatment (P = 0.99).

Table 7.3. Mean (± S.E.) cheese yield (Y%), fat recovery (%REC_{FAT}) and protein recovery (%REC_{PROT}) measured in control (C) milk samples and samples inoculated with 0.15 (P1) and 0.60 (P4) U/ L stored for up to 48 h, and significance of the main effects of storage time, protease activity and the interaction between them.

Parameter	Protease activity						S.E.	Significance		
	C		P1		P4			Storage time	Protease activity	Storage time*Protease activity
	0 h	48 h	0 h	48 h	0 h	48 h				
Y%	11.40	11.94	11.56	11.48	11.38	11.17	0.1	0.66	0.13	0.27
%REC_{FAT}	86.73	85.91	86.93	85.24	86.85	85.15	0.5	**0.03**	0.87	0.72
%REC_{PROT}	72.23	70.16	76.54	75.76	70.84	70.76	1.1	0.54	0.68	0.37

Table 7.4. Contents of fat, protein, moisture, salt, total solids (TS), salt-in-moisture (SM), moisture in the nonfat substance (MNFS), fat in dry matter (FDM) of the cheeses produced using control (C) milk and milk inoculated with 0.15 (P1) and 0.60 (P4) U/ L, and fat and protein contents in whey obtained during manufacture of Cheddar cheese; and significance of the main effects of storage time, protease activity and the interaction between them.

	Protease activity							**Significance**		
	C		**P1**		**P4**		**S.E.**	**Storage time**	**Protease activity**	**Storage time* Protease activity**
Cheese	**0 h**	**48 h**	**0 h**	**48 h**	**0 h**	**48 h**				
Fat%	32.08	31.58	31.29	31.46	31.49	31.85	0.30	0.97	0.34	0.50
Protein%	26.38	25.66	23.81	25.57	24.37	24.99	0.71	0.47	0.16	0.43
Moisture%	34.76	35.82	36.00	36.21	35.74	35.69	0.51	0.39	0.41	0.62
Salt%	1.39	1.45	1.28	1.29	1.42	1.31	0.06	0.83	0.21	0.50
TS%	65.24	64.18	64.00	63.79	64.26	64.31	0.51	0.39	0.41	0.62
SM%	4.03	4.06	3.55	3.57	3.99	3.69	0.23	0.69	0.22	0.75
MNFS%	51.16	52.33	52.39	52.83	52.16	52.36	0.53	0.22	0.43	0.68
FDM%	49.18	49.30	48.89	49.32	49.01	49.54	0.12	**0.04**	0.10	0.47
Whey										
Fat%	0.32	0.35	0.32	0.32	0.31	0.35	0.02	0.31	0.82	0.67
Protein%	0.92	1.06	0.96	1.18	0.92	1.22	0.09	0.09	0.47	0.84

Table 7.5. Mean (± S.E.) pH measured throughout the ripening (7, 14, 90 and 180 days) of Cheddar cheeses produced using control (C) milk and milk inoculated with 0.15 (P1) and 0.60 (P4) U/ L and stored for up to 48 h.

Protease activity	Time (h)	Ripening time (d)			
		7	14	90	180
C	0	5.19 ± 0.30	5.20 ± 0.29	5.25 ± 0.26	5.29 ± 0.30
	48	5.05 ± 0.01	5.08 ± 0.00	5.11 ± 0.00	5.15 ± 0.00
P1	0	4.95 ± 0.01	5.00 ± 0.05	5.06 ± 0.05	5.11 ± 0.05
	48	5.10 ± 0.27	5.13 ± 0.27	5.18 ± 0.28	5.23 ± 0.28
P4	0	5.17 ± 0.19	5.18 ± 0.20	5.20 ± 0.22	5.25 ± 0.21
	48	5.17 ± 0.26	5.19 ± 0.30	5.20 ± 0.28	5.26 ± 0.26

7.3.5. Proteolysis during cheese ripening

The mean %pH 4.6 SN/ TN obtained for cheeses C, P1 and P4, throughout ripening time, are shown in Table 7.6. The mean nitrogen content quantified in those fractions was affected by ripening time ($P<0.0001$); however, level of added protease ($P = 0.17$), storage time ($P = 0.71$) or interactive parameters evaluated did not affect that parameter.

The concentrations of all FAAs increased significantly over ripening time, with the exception of lys (Table 7.7). The mean concentrations of the FAA measured in all cheeses after 180 days of ripening are shown in Figure 7.3. Leucine and glutamic acid were the most abundant FAA quantified in all cheeses produced using milk stored for 0 and 48 h, with approximately 250 and 235 mg/ kg at 180 days, respectively, followed by phe, val, lys, hys and pro (approximately 190, 110, 93, 90 and 74 mg/ kg of cheese, respectively) (Figure 7.3).

The peptide peaks obtained for the pH 4.6-soluble fractions of the cheeses at 180 days of ripening were broadly similar (Figure 7.4). Differences were

observed in relation to the height of those peaks between samples, indicating possible quantitative but not qualitative differences in the levels of proteolysis in terms of relative concentrations of those peptides.

Electrophoresis indicated a progressive increase in the breakdown of α_{S1}-CN and β-CN throughout ripening time (Figure 7.5). The pattern of proteolysis over ripening was similar between cheeses manufactured using non-inoculated and inoculated milk after 0 h of storage. In contrast, an increase in the breakdown of β-CN with increasing levels of protease activity was observed in cheeses manufactured using milk stored with enzyme added for 48 h. The increase in the intensity of a band denoted by X, which is possibly a product of protein breakdown, also indicated increased proteolysis in cheese samples from treatment P4 manufactured after 48 h of milk storage.

The hardness of the cheeses was affected by ripening time (14 d: 265.9, 90 d: 225.6, 180 d: 127.7 ± 10.3 N; $P<0.0001$). Hardness was not affected by storage time ($P = 0.54$), added protease activity ($P = 0.13$), interaction between ripening time and treatment ($P = 0.73$), interaction between storage time and treatment ($P = 0.61$) and interaction between ripening time, storage time and treatment ($P = 0.98$).

Table 7.6. Mean (± S.E.) levels of %pH 4.6 SN/TN (g/ 100 g) measured during ripening (14, 90 and 180 days) of cheeses produced with control (C) milk and milk inoculated with 0.15 (P1) and 0.60 (P4) U/ L and stored for up to 48 h.

Protease activity	**Time (h)**	**Ripening time (d)**		
		14	**90**	**180**
C	0	4.68 ± 1.33	14.74 ± 1.74	20.55 ± 0.56
	48	6.31 ± 0.23	17.45 ± 0.32	22.53 ± 0.56
P1	0	5.35 ± 1.92	16.55 ± 3.68	22.58 ± 4.07
	48	5.58 ± 0.97	15.97 ± 1.04	21.35 ± 0.71
P4	0	5.60 ± 1.24	16.74 ± 1.70	22.71 ± 0.66
	48	5.92 ± 1.71	15.99 ± 2.10	22.05 ± 1.13

Table 7.7. Significance of the main effects of storage time, protease activity, ripening time, interaction between storage time and protease activity, interaction between ripening time and protease activity and interaction between storage time, protease activity and ripening time on the content of free amino acids (asp, thre, ser, glu, gly, ala, cys, val, met, ile, leu, tyr, phe, his, lys, tryp, arg and pro) contents (mg/ kg of cheese) measured in the pH 4.6 soluble fractions of control (C) cheese samples and cheeses manufactured with milk containing 0.15 (P1) and 0.60 (P4) U/ L of protease activity.

Amino acid (mg/ kg of cheese)	**Significance**					
	Storage time	**Protease activity**	**Ripening time**	**Storage time* protease activity**	**Ripening time* protease activity**	**Storage time*protease activity*ripening time**
asp	0.21	0.52	**0.0007**	0.26	0.60	0.27
thre	0.60	0.49	**0.002**	0.37	0.24	0.13
ser	0.54	0.67	**0.01**	0.39	0.67	0.59
glu	0.85	0.84	**0.03**	0.28	0.92	0.34
gly	0.91	0.83	**0.02**	0.37	0.59	0.21
ala	0.77	0.81	**0.006**	0.36	0.33	0.11
cys	0.11	0.18	**<0.0001**	0.95	0.74	0.89
val	0.96	0.86	**0.008**	0.34	0.58	0.19
met	0.98	0.82	**0.0002**	0.32	0.65	0.18
ile	0.60	0.89	**0.007**	0.11	0.81	0.64
leu	0.64	0.94	**<0.0001**	0.09	0.80	0.47
tyr	0.20	0.20	**<0.0001**	0.50	0.45	0.85
phe	0.60	0.47	**<0.0001**	0.55	0.40	0.77

Table 7.7. Continuation.

Amino acid (mg/ kg of cheese)	Significance					
	Storage time	Protease activity	Ripening time	Storage time* protease activity	Ripening time* protease activity	Storage time*protease activity*ripening time
his	0.41	0.54	**<0.0001**	0.25	0.59	0.70
lys	0.80	0.95	0.07	0.45	0.67	0.18
tryp	0.30	0.73	**<0.0001**	0.19	0.71	0.69
arg	0.31	0.32	**<0.0001**	0.51	0.50	0.83
pro	0.21	0.52	**0.0007**	0.26	0.60	0.27

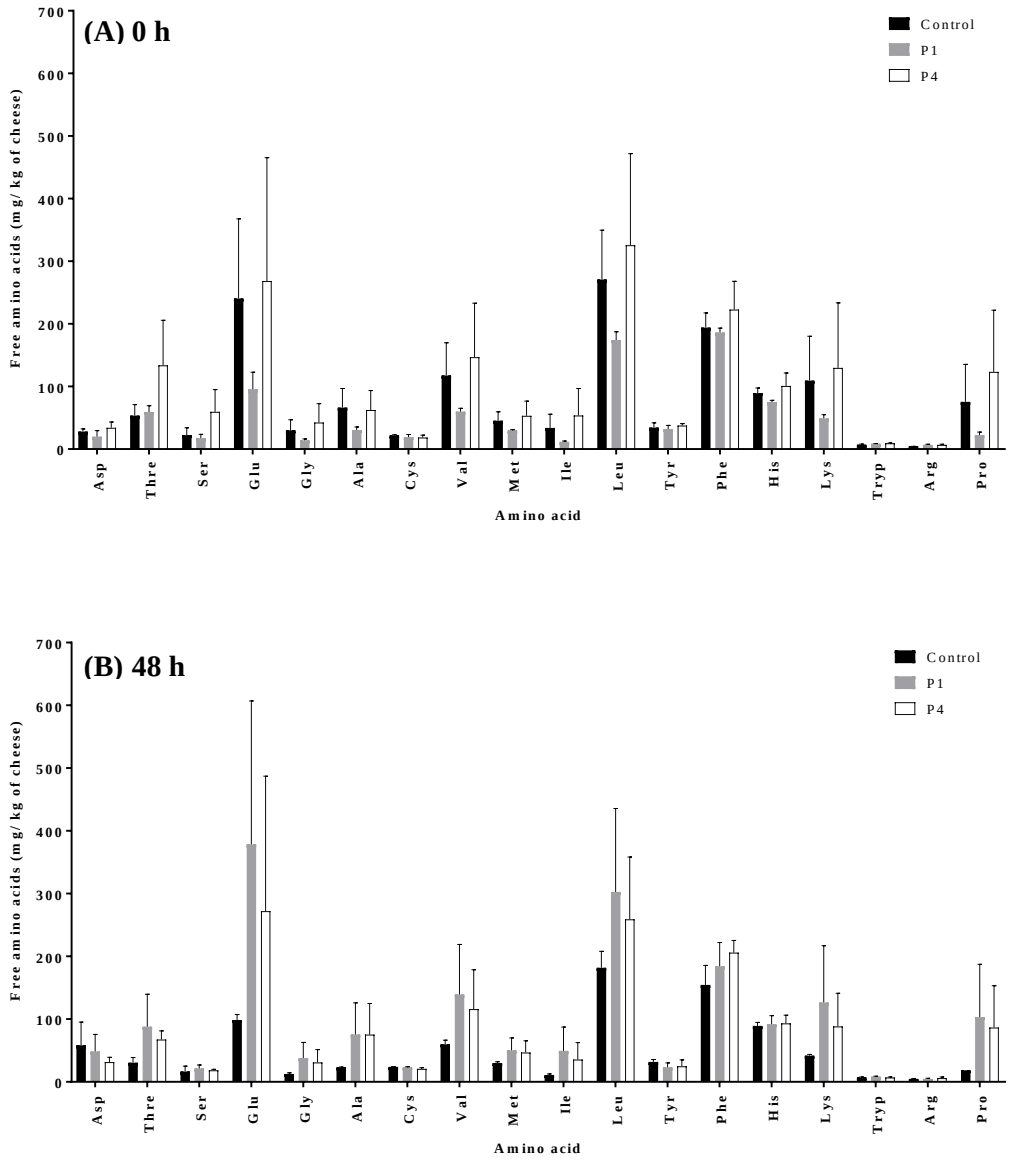


Figure 7.3. The effect of milk treatments on the mean levels of individual FAA in pH 4.6-soluble nitrogen extracts from Cheddar cheeses at 180 days of ripening. The charts correspond to cheeses produced using milk after (A) 0 h and (B) 48 h of storage. Data presented are means of data from 3 replicate trials. Error bars show the SEM from 3 replicate trials.

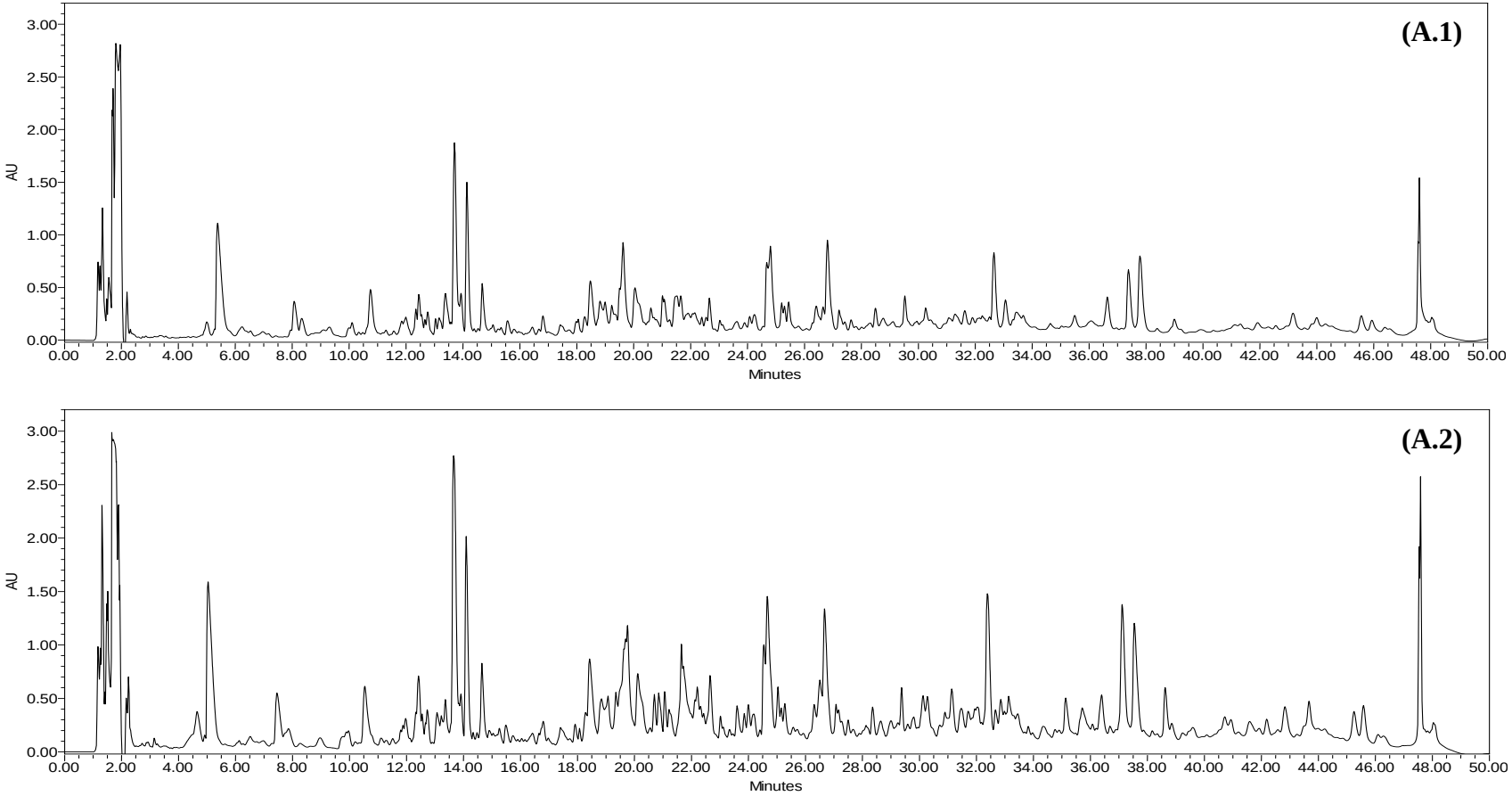


Figure 7.4. Reversed-phase UPLC profiles of peptides in the pH 4.6 soluble fraction of Cheddar cheeses manufactured using milk with added protease activity levels of **(A)** 0 (control), **(B)** 0.15 and (**C)** 0.60 U/ L, after **(1)** 0 and **(2)** 48 h of storage, at 180 days of ripening.

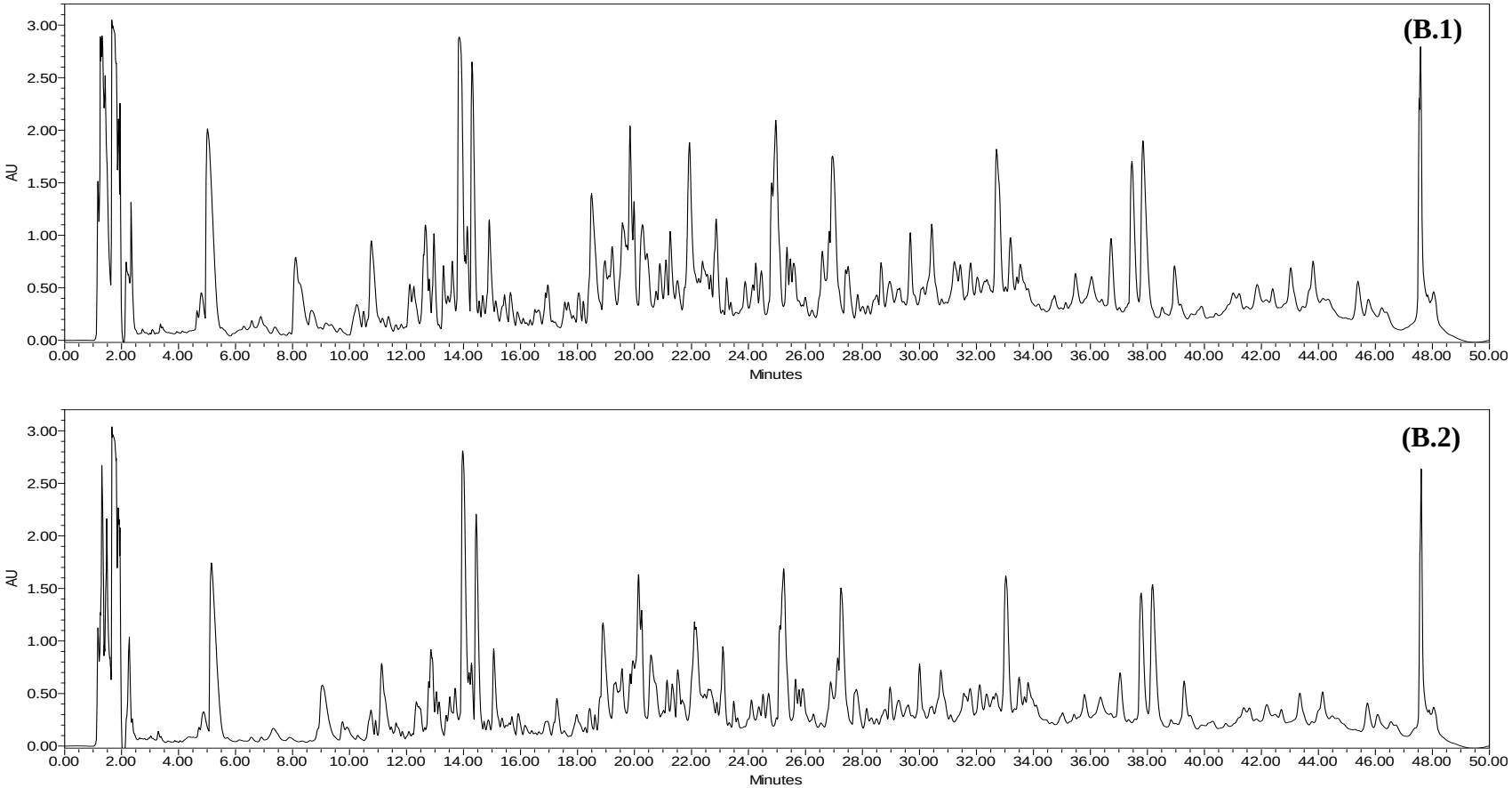
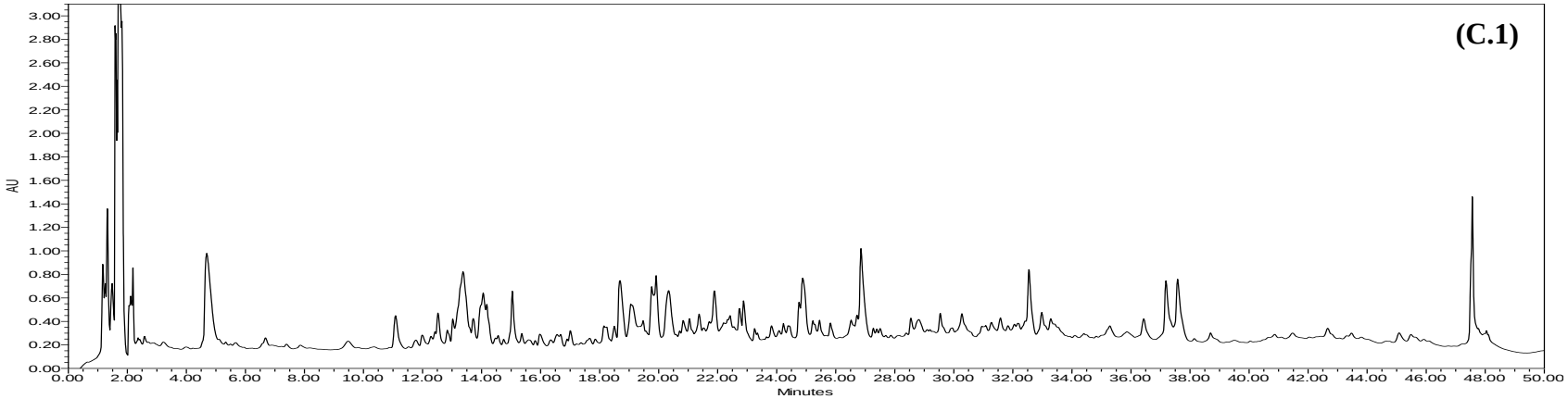


Figure 7.4. Continuation



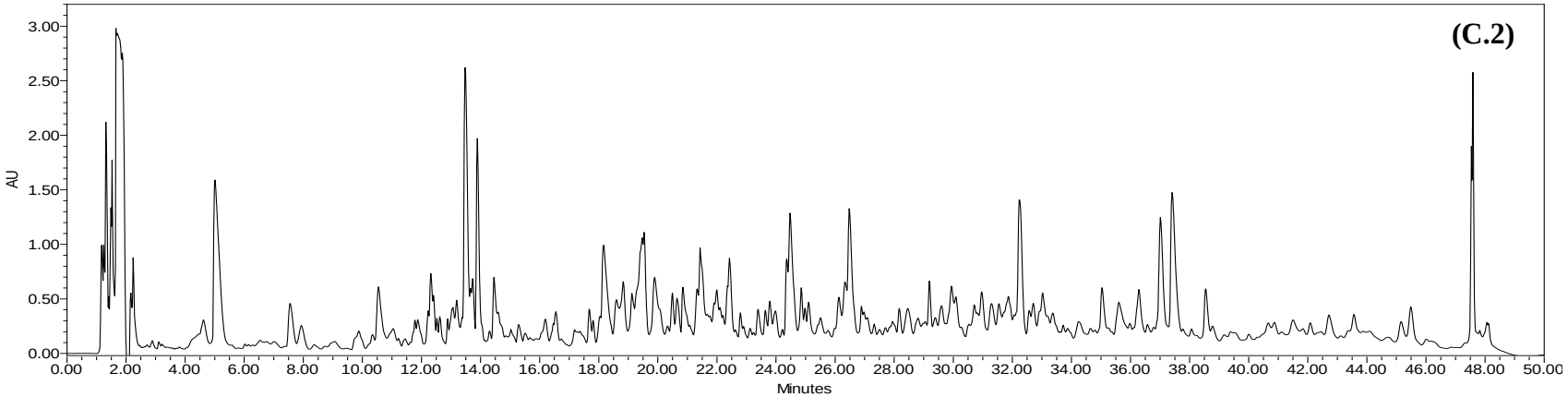


Figure 7.4. Continuation

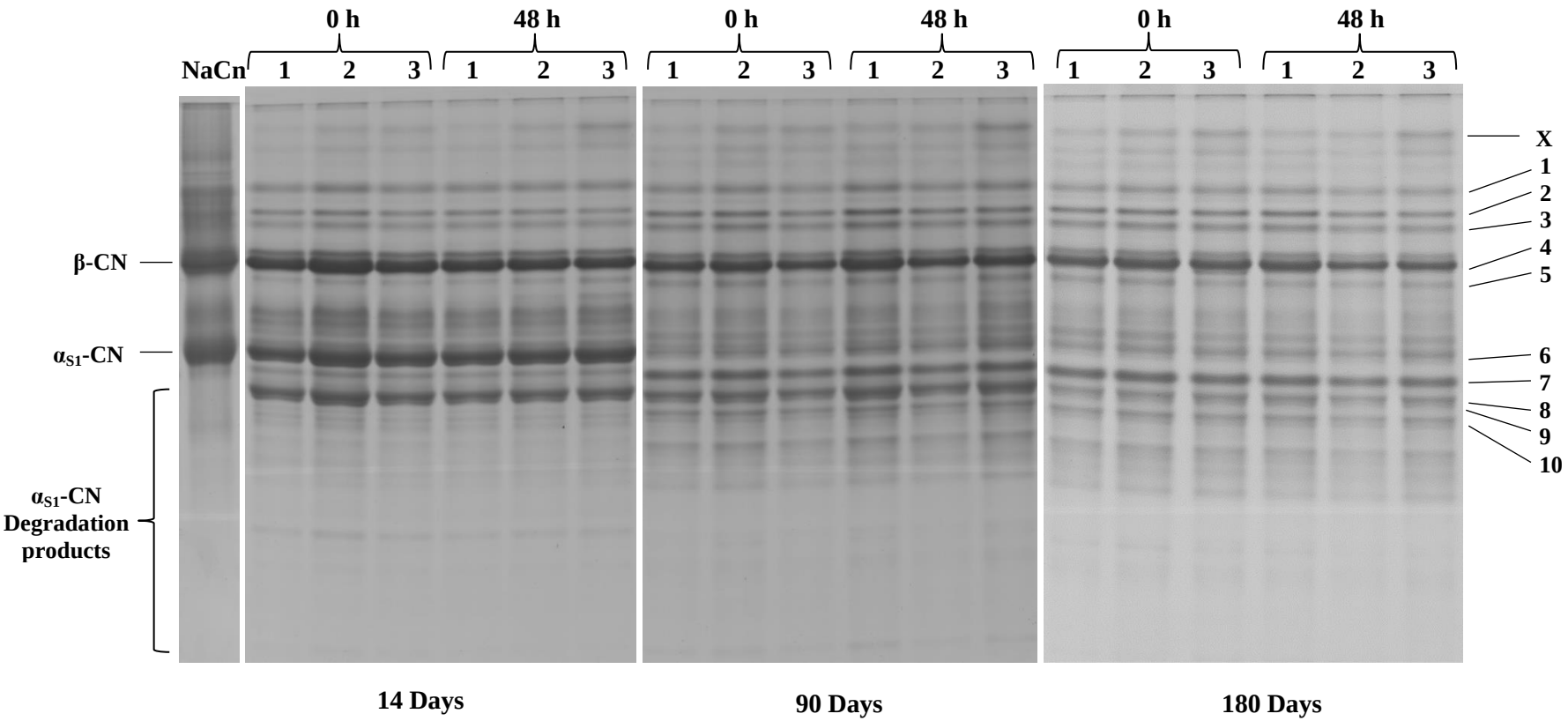


Figure 7.5. Urea-polyacrylamide gel electrophoretograms of Cheddar cheeses stored for 14, 90 and 180 days. Those cheeses were produced using control (C) milk and milk inoculated with 0.15 (P1) and 0.60 (P4) U/ L (lanes 1, 2 and 3, respectively) after 0 and 48 h of storage. Sodium caseinate (lane NaCn), loaded at an equivalent weight of protein (4.75 mg per lane) was included as an unhydrolysed casein control. Protein bands were identified according to Mooney *et al.* (1998) and McSweeney *et al.* (1994): **1** – β-CN(f106-209) (γ2); **2** – β-CN(f29-209) (γ1); **3** – β-CN(f108-209) (γ3); **4** - β-CN; **5** – β-CN(f1-192); **6** – α_{S1}-CN; **7** - α_{S1}-CN(f102-199); **8** - α_{S1}-CN(f24 – 199); **9** - α_{S1}-CN(f121-199); **10** - α_{S1}-CN(f33-*).The band that increased intensity with protease concentration is denoted by **X**.

Table 7.8. Mean (± S.E.) hardness (N) measured during ripening (14, 90 and 180 days) of Cheddar cheeses produced with control (C) milk and milk inoculated with 0.15 (P1) and 0.60 (P4) U/ L stored for up to 48 h.

Protease activity	Time (h)	Ripening time (d)		
		14	90	180
C	0 h	258.7 ± 51.7	226.5 ± 88.6	121.2 ± 28.0
	48 h	225.1 ± 51.8	211.0 ± 16.0	108.1 ± 13.6
P1	0 h	248.5 ± 76.4	210.4 ± 48.7	122.6 ± 40.4
	48 h	242.0 ± 90.1	221.6 ± 33.0	119.1 ± 13.4
P4	0 h	281.9 ± 97.5	225.1 ± 43.4	127.6 ± 19.7
	48 h	314.8 ± 125.0	244.9 ± 43.1	134.7 ± 36.0

7.4. Discussion

The slight decreases in %N and increases in %NCP with added protease activity level are an indication that the protease was active in the milk during storage. The increases in %NCP could be due to the amount of supernatant that were added to the milk, aiming to achieve a range of levels of protease activity. During the protease extraction, some of the NCP fraction of the RSMP could have also been separated in the supernatant.

In relation to caseins, the addition of protease seemed to have mainly affected the mean β-CN and κ-CN contents over storage time (Table 7.1 and Figure 7.2). A difference of approximately 1.5 and 2.0 µg/ µL in the mean κ-CN and β-CN concentrations, respectively, were observed between samples C and P4. Similarly, Baglinière *et al.* (2013) reported that a thermo-resistant protease (AprX) produced by a *Pseudomonas* strain preferably hydrolysed β-CN, and Matéos *et al.* (2015) observed rapid hydrolysis of κ-CN and β-CN in similar proportions by AprX. However, other studies (Zhang *et al.*, 2015; Zhang *et al.*, 2018) reported that thermo-resistant proteases produced by *Pseudomonas* strains

preferably hydrolyse κ-CN followed by β-CN. Those differences in results could be due to the methodology used to investigate hydrolysis or differences in the specificity of the protease isolated. β-CN, along with α_S-CN, constitutes the basic microstructure of cheese and decreases in its concentration in milk could affect the rennet clotting of milk and curd formation (St-Gelais and Hache, 2004).

In addition, the increase in the breakdown of caseins and whey proteins in milk with increasing level of protease activity over storage time could have resulted in the loss of amino acids or peptides into whey, which could have resulted in the increase in %N in whey samples over time as shown in Table 7.4. Decreases in $\%REC_{PROT}$ with increasing level of protease added were also observed (Table 7.3) and could be due to the breakdown of proteins in milk during storage.

The increases in TBC and PBC with added protease activity and over storage could be due to *P. fluorescens* cells in the supernatant used for inoculation; the initial TBC and PBC increased as the amount of supernatant added was increased to achieve experimental levels of protease activity in the milk samples.

While the level of protease activity and storage time had little effect on the composition of milk and cheese, the statistical differences observed in the mean Y%, $\%REC_{FAT}$, $\%REC_{PROT}$ and whey composition were also minimal (Tables 7.3 and 7.4). Those results could also indicate that the protease had a low activity when subjected to the processing conditions (e.g., variations in pH). In addition, the composition of the cheeses produced were similar to the one obtained by Auldist *et al.* (2016) (moisture: 37.5%, fat: 31%, protein: 25.5%, salt: 1.70%, SM:

4.60%, FDM: 49.5%) and reported by Fox *et al.* (2017) (moisture: 37.2%, protein: 25.4%, fat: 33.1%, salt: 1.80%, SM: 4.8%).

The variations in the mean pH between treatments were considered small. After 7 days of ripening, the mean pH of the cheeses from each treatment varied from 4.95 to 5.15, which was within the ranges specified for *Premium* quality Cheddar as defined by Fox (1975) and Pearce and Gilles (1979). Furthermore, the pH of the cheeses increased approximately 0.1 unit after 180 d of ripening, which is as reported by Fox *et al.* (2004). The increase in pH during ripening is due to the formation of alkaline nitrogenous compounds and/ or catabolism of lactic acid (Fox *et al.*, 2004).

The pattern of proteolysis investigated in each experimental cheese (quantification of pH 4.6 SN/ TN, concentration of FAAs, peptide profiles, Urea-PAGE electrophoresis) indicated that the protease added had minimal impact on proteolysis during ripening.

The quantification of the pH 4.6 SN/TN is a measurement of proteolysis in cheeses, as this fraction contains the peptides resulting from casein breakdown (Hou *et al.*, 2014). The magnitude of the increase in pH 4.6 SN/TN over ripening (from approximately 5.0% at 14 d to 22.0% at 180 d; Table 7.6) was similar between all cheeses and is in agreement with previous reports for Cheddar cheese (Fenelon *et al.*, 2000, Wang *et al.*, 2011, Hou *et al.*, 2014). The progressive breakdown of the casein matrix during ripening also increases concentrations of FAAs (Fox, 1996), as was observed in all cheese samples in this study (Figure 7.3). Fenelon & Guinee (2000b) and McCarthy *et al.* (2017) also observed high levels of leu, glu, phe, val, lys, hys and pro in Cheddar cheeses.

Storage time did not statistically affect the concentrations of each FAA in cheese; however, some trends in relation to ripening and added protease activity were observed. Cheeses P1 and P4, produced using milk stored for 48 h, had higher concentrations of glu, gly, ala, val, met, ile, leu, lys and pro than control cheeses. These increases in proteolysis in those cheeses could be related to the increase in bacterial counts with added protease activity that was observed in milk samples. Possibly, bacteria cells that remained in the milk after pasteurisation or *Pseudomonas* cells that were in the supernatant could have contributed to an increased concentration of enzymes in milk during storage, consequently increasing the proteolytic activity in the cheese produced with that milk. Concentrations of FAA are associated with the development of flavour in cheese (Fox *et al.*, 2017) and the prolonged storage of milk containing high levels of proteolytic bacteria could affect the sensory quality of the product.

Urea-PAGE electrophoretograms also showed a slight increase in proteolysis in cheese samples P4, manufactured after 48 h of milk storage, the increase in intensity of band X, above β-CN, was greater compared to other samples (Figure 7.5). Furthermore, the proteolysis between all cheese samples were similar independently of level of protease activity. During ripening, α_S-CN is degraded more extensively than β-CN into smaller fragments by proteolytic enzymes (St-Gelais and Hache, 2004), as was observed in the electrophoretograms.

The electrophoretograms obtained in Chapter 6 indicated that the protease potentially have a specificity, in relation to casein hydrolysis, similar to that of chymosin. This could explain the similarity between the peptide peaks obtained for control cheese samples and samples with added protease at 180 days of

ripening (Figure 7.4). In addition, the decrease in some of the peptide peaks could indicate that those could have possibly been further hydrolysed by the protease during ripening.

Proteolysis is a determinant factor of the final texture and flavour in cheeses (Fox *et al.*, 2017). As the levels of proteolysis were similar between samples, it is perhaps not surprising that the mean hardness values obtained for the cheeses from each treatment were also similar. The decrease in cheese hardness with ripening was expected. The softening of cheese texture during ripening is a result of the hydrolysis of the casein matrix, solubilisation of calcium in the early stages of ripening, and a decrease in the water activity of the curd (McSweeney, 2004; Fox *et al.*, 2017). The latter occurs as a consequence of changes in water-binding by the carboxylic acid and amino groups formed on hydrolysis. The chemical composition of cheese (e.g., fat and moisture content) can also impact on the hardness of cheese during ripening. In addition, as reported by Lamichhane *et al.* (2019), hydrolysis of α_s- and β-CNs influences the fracture stress and strain of cheeses, respectively. The decreases in hardness between 14 and 180 d of ripening observed in this study were similar to decreases reported by McCarthy et al. (2016) and Hickey et al. (2017) for Cheddar-style cheeses (approximately 100 N).

Finally, the results suggested that the protease was not extensively active in the cheese matrix. An azocasein test was carried out to investigate if the protease was transferred into the whey during manufacture of each cheese. The results of the test were not significantly different (0 h; C: 0.0653 ± 0.0645 nm, P5: 0.0748 ± 0.0665 nm, P20: 0.0917 ± 0.0951 nm; 48 h, C: 0.0953 ± 0.1193 nm, P5: 0.1739 ± 0.0454 nm, P20: 0.1468 ± 0.2049 nm; $P>0.05$), indicating that the

protease remained in the cheese matrix. The activity of thermo-resistant proteases, produced by *Pseudomonas* bacteria, is optimal at a pH value between 7 and 9 and at a temperature range between 30 and 45 ˚C (Martins *et al*, 2015). Therefore, the low pH and storage temperature of the cheeses could have affected the protease activity. Other cheese types are characterized by higher ripening temperatures and pH, such as Swiss cheese (stored at approximately 22 °C for 4 to 6 weeks), Mozzarella cheese (heated to 57 °C during stretching) and smear ripened cheeses (e.g., Camembert, which pH is approximately 7.0) (Fox *et al.*, 2017), and may be more significantly affected if the protease is active in the matrix.

7.5. Conclusion

The levels of activity of the *Pseudomonas* protease tested had a minimal impact on the proteolysis levels in milk and Cheddar cheese produced. Processing and storage conditions of milk and cheese could have influenced the activity of the protease and, consequently, the manufacture and quality of the Cheddar cheeses produced were not affected. Independently of the added protease activity level tested, the composition and levels of proteolysis during ripening in all cheeses were similar and consistent with parameters expected for that type of product. The levels of protease activity tested seemed to have also not affected the quality of whey, a valuable by-product obtained in cheese manufacture. Therefore, the production of psychrotrophic bacterial proteases in milk during storage might not contribute to increase proteolysis in cheese to a certain extent. The prolonged storage of milk containing high levels of proteolytic enzymes indicated that higher levels of protease than tested could potentially affect proteolysis in cheese during

ripening. Further studies may be necessary to determine the effects of those higher concentrations on cheese and whey.

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Chapter 8

General discussion

Declaration: This chapter was written by Lizandra F. Paludetti, with corrections and comments from Prof. Alan L. Kelly from University College Cork, and Dr. David Gleeson from Teagasc Moorepark.

8.1. General summary

Milk production in Ireland has been expanding as a result of the removal of milk quotas in 2015. In 2014, the reported milk production was 5 billion litres and it was projected to increase to 7.5 billion litres by 2020; however, this production had already been reached in 2018 (Central Statistics Office, 2019). This scenario is an opportunity for Irish dairy processors to expand its market worldwide; however, complying with international quality standards is critical to holding market share.

The microbiological and compositional quality of raw milk are the main determinants of its processability and safety, and consequently have a major influence on the quality of dairy products. As a result, Irish dairy processors have been applying measures to improve the control of factors that can affect the quality of raw milk, such as penalising dairy suppliers when bacterial levels are higher than acceptable, monitoring farm management practices, and monitoring concentrations of residues originating from sanitation products.

In the context of improving the quality of raw milk and dairy products, this thesis has generated knowledge in relation to milk production on-farm (storage and cooling conditions), processability of milk, as well as the impact of microbiological and compositional quality of raw milk on the quality of Irish dairy products of high commercial value (milk powder and Cheddar cheese).

The enumeration of bacteria in raw milk samples from bulk tanks after the first milking was an approach that allowed an observation of the effect of initial microbiological count and storage temperature on the quality of raw milk during storage (Chapter 2). Milk obtained from only one milking was used as this milk is stored for longer compared to the milk added in bulk tanks from subsequent

milkings. The addition of fresh milk to bulk tanks everyday may also mask the effects of bacterial growth and enzymatic activity on raw milk (O'Connel *et al.*, 2016). Even though the study conducted was laboratory-based and did not represent a real farm scenario, it still showed an opportunity for improvement of the quality of Irish milk.

Temperature is a parameter that has impact on the bacterial growth and consequently on the composition of milk during storage (Chapter 2). Cooling milk to 2 ˚C can allow the extension of storage with minimal impact in bacterial counts and breakdown of caseins and other proteins, while a small increase in the temperature to 4 ˚C can promote a considerable increase in those levels. Also, the maintenance of low temperatures throughout storage is more effective on preserving milk quality than pre-cooling milk (Chapter 3).

Nevertheless, independently of temperature, the initial microbiological count in milk is the main limiting factor of storage time in bulk tanks and, consequently, is the main determinant of its quality (Chapter 2). High initial levels of total (TBC) and psychrotrophic (PBC) bacterial counts in raw milk (> 3.70 $\log_{10}$ cfu/ mL) can reduce the effectiveness of storage temperature on reducing bacterial growth. Therefore, farm management practices are still the most important factor to determine microbiological quality of raw milk. The sanitation of milking equipment and parlor, hygiene during milking and cow management should be appropriately carried out (O'Brien, 2009; O'Connell, 2015; Vithanage *et al.*, 2017).

In Ireland, the effect of seasonality on farm production conditions also challenges dairy suppliers to consistently achieve low bacterial levels in milk throughout the year (Doyle *et al.*, 2017). Appropriate equipment sanitation and

cow management are especially critical during late-lactation, which coincides with the period when herds are housed indoors due to winter conditions. Thermoduric and thermophilic bacterial counts can potentially be higher in milk produced during those months due to housing and poorer hygienic conditions (O'Connell, 2015; Doyle *et al.*, 2017). Consequently, variations in the microbiological quality of milk can directly impact dairy products manufactured during different seasons. High bacterial counts in late-lactation milk, especially of microorganisms that survive high temperatures, can affect the effectiveness of thermal treatments in reducing those levels (Chapter 4). This could have implications in the safety of dairy products, particularly products for infants, as some thermoduric and thermophilic microorganisms can be pathogenic (Gleeson et al., 2013). Some examples are bacteria that belong to the *Bacillus cereus* group and sulphite-reducing *Clostridia*. Monitoring the microbiological load of milk throughout a dairy manufacturing process can be useful to identify the effect that different suppliers have on the overall quality of raw milk delivered to the factory, as well as, contamination points throughout the production line.

The microbiological quality of raw milk is also of major importance to processors as thermo-resistant proteases can be produced by certain microorganisms, especially *Pseudomonas fluorescens* strains (Lopez-Fandino *et al.*, 1993). Bacteria from the species *P. fluorescens* mainly comprise the population of psychrotrophs in milk, which are the main bacteria type to increase levels during storage and are possibly the main contributors to increases in proteolysis in milk (Chapter 2). The effects of the activity of their thermo-resistant proteases are well known in products subjected to high temperatures, such as gelation in UHT milk (Bagliniere *et al.*, 2013; Zhang *et al.*, 2018), however, they

can also affect the cheese making properties of milk (Chapter 6). Increasing levels of added protease activity increased the hydrolysis of β- and α_S-caseins in milk over storage time. As a result, a softer coagulum was produced or the coagulation of milk was not possible, as β- and α_S-caseins are related to the structure formation of the coagulum (Amenu and Deeth, 2007).

In contrast, the levels of protease activity used during the pilot-plant experiment (0.15 and 0.60 U/ mL, Chapter 7) affected minimally the quality of milk and Cheddar cheese, as well as the manufacture of such product. Those results could be positive from the perspective of a cheese manufacturer; however, it is not known if the activity levels tested are representative of the levels that could be measured in raw milk supplied to factories. In Chapter 7, results indicated that further increases in the bacterial proteolytic activity during storage could have potentially affected proteolysis in Cheddar cheese during ripening. Therefore, considering those results and the potential effect that thermo-resistant proteases could have on cheese-making properties of milk (Chapter 6), it would be relevant to correlate PBCs in milk with levels of protease activity. Psychrotrophic bacterial counts could also be adopted as another quality indicator of raw milk supplied for processing.

Variations in pH during Cheddar cheese manufacture, as well as the low storage temperatures, seemed to have influenced the activity of the protease (Chapter 7). Thus, investigations regarding the effect of other processing conditions on the protease activity would be relevant, considering that Irish cheese manufacturers have been exploring opportunities to diversify its products. Those studies could focus on cheeses that are stored at higher temperatures, such as Swiss cheese, which is stored at approximately 22 ˚C for 4 to 6 weeks, or cheeses

that have a high final pH, such as smear ripened cheeses (e.g., the pH of Camembert cheese is 7.0) (Fox *et al.*, 2017; Hartmann *et al.*, 2017). It would also be important to determine if higher levels of protease would have an effect on the composition of whey obtained during cheese manufacture. This information would be useful to processors, as whey became a high-value by-product in the international market, which is used for the production of a wide range of nutritional products.

International markets are also setting stringent specifications regarding the concentrations of chlorine related residues (chlorate, CHLO; perchlorate, PCHLO and trichloromethane, TCM) in dairy products. Consequently, it became highly important to Irish processors to control those concentrations in raw milk, which has led to investigating sanitation practices that contribute to their increase. Monitoring residue concentrations throughout the manufacturing process of SMP, from individual suppliers until the final product, showed that production season also has an effect on those concentrations (Chapter 5). The concentrations of CHLO and TCM tended to be higher in late-lactation milk and, consequently, in the SMP and cream produced, respectively. Those differences could be due to changes in the sanitation practices adopted during summer and winter months. Therefore, the consistent use of appropriate sanitation practices during winter months is critical to control residue levels in raw milk. Changes in practices could have also affected the microbiological quality of milk supplied during winter (Chapter 4). New studies have been testing alternatives to reduce residue levels during processing, such as the use of microfiltration; however, it is still more effective from an economical point of view to control levels at the farm (Wang *et al.*, 2013; McCarthy *et al.*, 2018).

In addition, by monitoring the concentrations of CHLO throughout the manufacture of SMP, it was shown that some operations may cause a large increase in CHLO in dairy products (Chapter 5). The CHLO concentrations can increase approximately 50 times in SMP after evaporation and spray-drying. This information could be useful for processors to determine what should be the concentration of CHLO in the raw milk supplied to the factory. For example, in the case of the SMP manufacturing process, results indicated that CHLO levels should be lower than 0.0025 mg/ kg in raw milk to produce SMP with lower levels than the specification allow (0.100 mg/ kg). Those results also indicate that the use of chorate-based products and chlorinated water should be reviewed for the sanitation of processing equipment. Moreover, the prediction of residue concentrations in milk prior to processing, based on the volumes and concentrations in each milk volume supplied, could also be a useful tool to determine the quality of milk prior to processing.

Levels of residual iodine in raw milk are another concern for the Irish dairy industry, as the consumption of excessive amounts can lead to dysfunctions in the thyroid gland (Leung & Braverman, 2014). The initial iodine concentrations in raw milk are the main determinants of the concentration in the final product, and therefore actions to control levels in milk should be focused on Irish farms (Chapter 5). The main factor to be monitored is the level of iodine supplemented to dairy herds, especially during early and late-lactation and during winter milk production (O'Brien *et al.*, 2013). In addition, in the case of SMP manufacture, even though the levels in raw milk were lower than the required by the manufacturer (150 µg/ L), the levels in the SMP produced were higher than the required. Therefore, the limit iodine concentration in raw milk destined for milk

powder production should be lower than the current specification, which could possibly be decreased to 100 µg/ L.

The continuous growth of Irish milk production and changes in electricity pricing systems have been also challenging dairy suppliers to sustainably increase the production and to produce high quality milk. The prolonged storage of milk on-farm may impact on production costs related to cooling, requiring alternatives to decrease energy usage (Upton *et al.*, 2013). The operation of highest energy consumption on Irish dairy farms is cooling using bulk tanks and, therefore, the use of pre-cooling systems would be effective in substantially reducing the costs related to that operation (Chapter 3). Single-stage plate coolers are the systems which mostly decrease the energy usage compared to double-stage plate coolers. Energy requirements can be high when using a double-stage plate cooler as it requires an ice bank; however, this system could be useful on farms that already use an ice bank for cooling milk within bulk tanks. As electricity is one of the main contributors to production costs, alternatives for optimising energy usage on-farm may contribute to improvement of the cost competitiveness of Irish dairy products (Upton *et al.*, 2013).

In conclusion, the microbiological quality and composition of raw milk are still the main factors to pose challenges for dairy processors to ensure the consistent production of high quality and safe products. The present research explored opportunities for dairy suppliers to improve the quality of raw milk and increase production sustainably, as well as providing relevant information for processors on how the quality of raw milk can affect its processability and manufacture of milk powder and Cheddar cheese.

8.2. Proposals for further research

The following suggested studies would provide further understanding of the influence of microbiota and bacterial thermo-resistant proteases on the composition and processing properties of milk. In addition, further studies in relation to the use of different sanitation products could aid on ensuring the safety of dairy products, while studies using pre-cooling systems could aid on improving production costs on-farms.

- Screening of the microbiota of milk from different Irish dairy farms during storage. The present research indicated that bacterial growth in raw milk was possibly affected by its microbiota, therefore, the suggested study would aid in understanding the dynamics of growth of different microbial strains during storage. Microorganisms that mainly increase in numbers during storage could be identified and characterised in relation to their proteolytic activity, as well as their possible effects on the composition of milk;
- The present research indicated that the activity of the thermo-resistant protease tested was possibly affected by low storage temperatures or low pH obtained during Cheddar cheese manufacture. Therefore, it would be relevant to identify and characterise this protease regarding its activity when subjected to refrigeration temperatures and low pH ranges. This information could indicate what manufacturing processes and dairy products are more likely to be affected by the protease;
- The levels of protease activity tested had a minimal effect on the composition of milk and Cheddar cheese; however, it is not known if those are representative levels of the protease activity that could be in

raw milk supplied to factories. A study could be conducted to determine the potential concentrations and activity of thermo-resistant proteases in raw milk containing different psychrotrophic bacterial counts. The production and activity of those proteases could be monitored in farm bulk tanks over storage time, as well as their effects on milk composition. This study could mimic the milking routines, storage time and temperature usually used on farms, and the selection of PBC could be based on levels usually measured in raw milk supplied to processors. A correlation between protease activity and PBC could also be established based on the results. This information could be useful for processors to determined what levels of PBC could affect the processability of milk and quality of dairy products.

- In relation to CHLO and TCM residues, Irish processors are currently planning to ban the use of chlorine-based products on farms as a measure to avoid the formation of those residues in milk. However, chlorine-based products are widely used due to their strong bactericidal properties (Garcia-Villanova *et al.*, 2010) and, therefore, it would be necessary to determine if non-chlorine products are as effective on reducing bacterial numbers in raw milk, especially pathogenic microorganisms. Dairy farms that supply milk to different processors and that are already using non-chlorine cleaning products could participate in this study. Raw milk samples from those farms could be analysed for several microorganisms and questionnaires regarding their cleaning routines could be applied. The results could aid processors in

identifying suppliers having difficulties to adapt their cleaning routines, as well as, identify the most effective non-chlorine products.

- The present thesis indicates that pre-cooling systems would be efficient to decrease production costs on farms. However, those costs could also be affected if the monetisation of the public supply and waste water occurs in Ireland. Therefore, a study to identify opportunities for the reuse of water on farm would provide useful information for dairy farmers. A modelling of the influence of water reuse and pre-cooling systems on milk production costs could also be investigated. This would be relevant to demonstrate that the implementation of those alternatives to decrease energy and water usage can contribute to improving the cost competitiveness of Irish dairy products.

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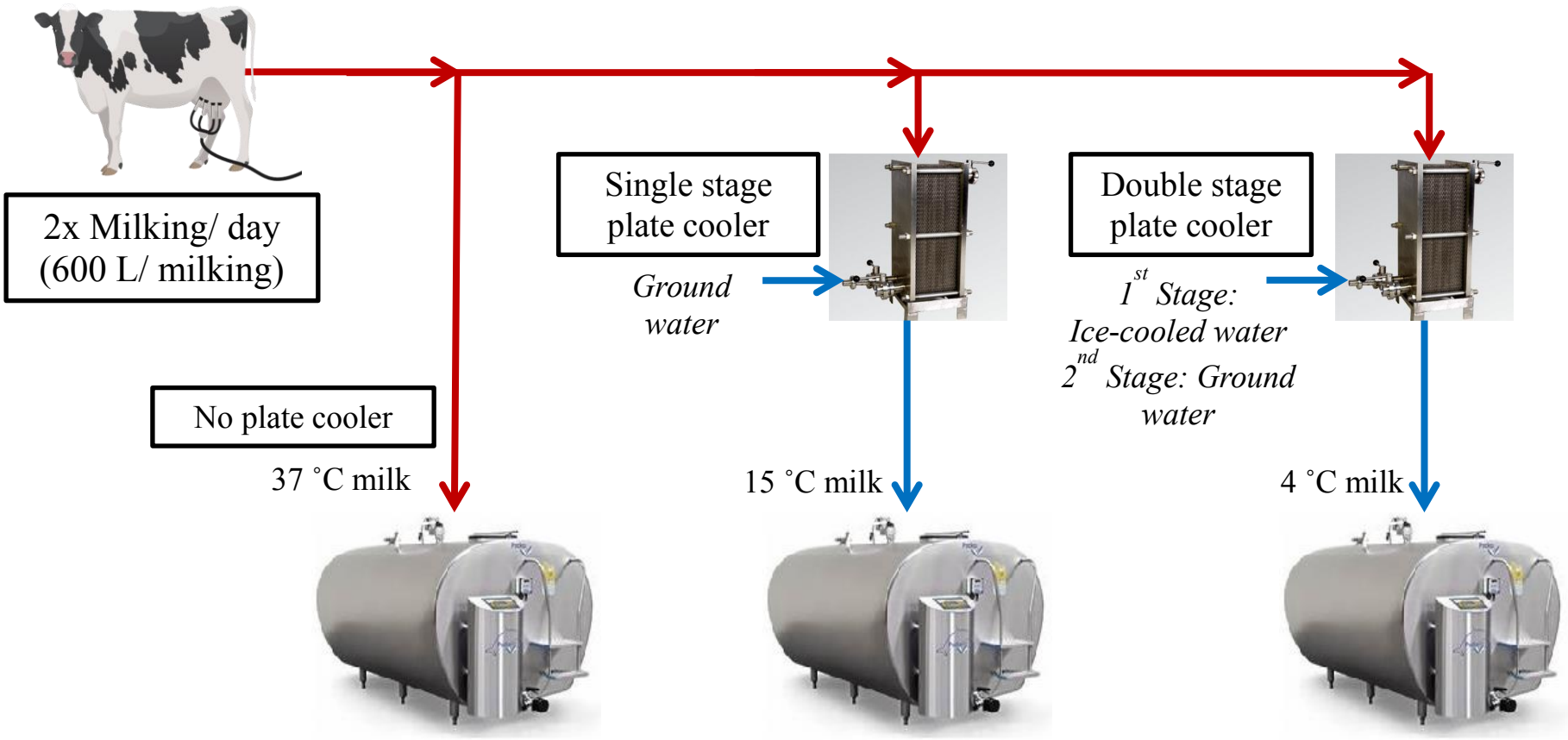
Appendix

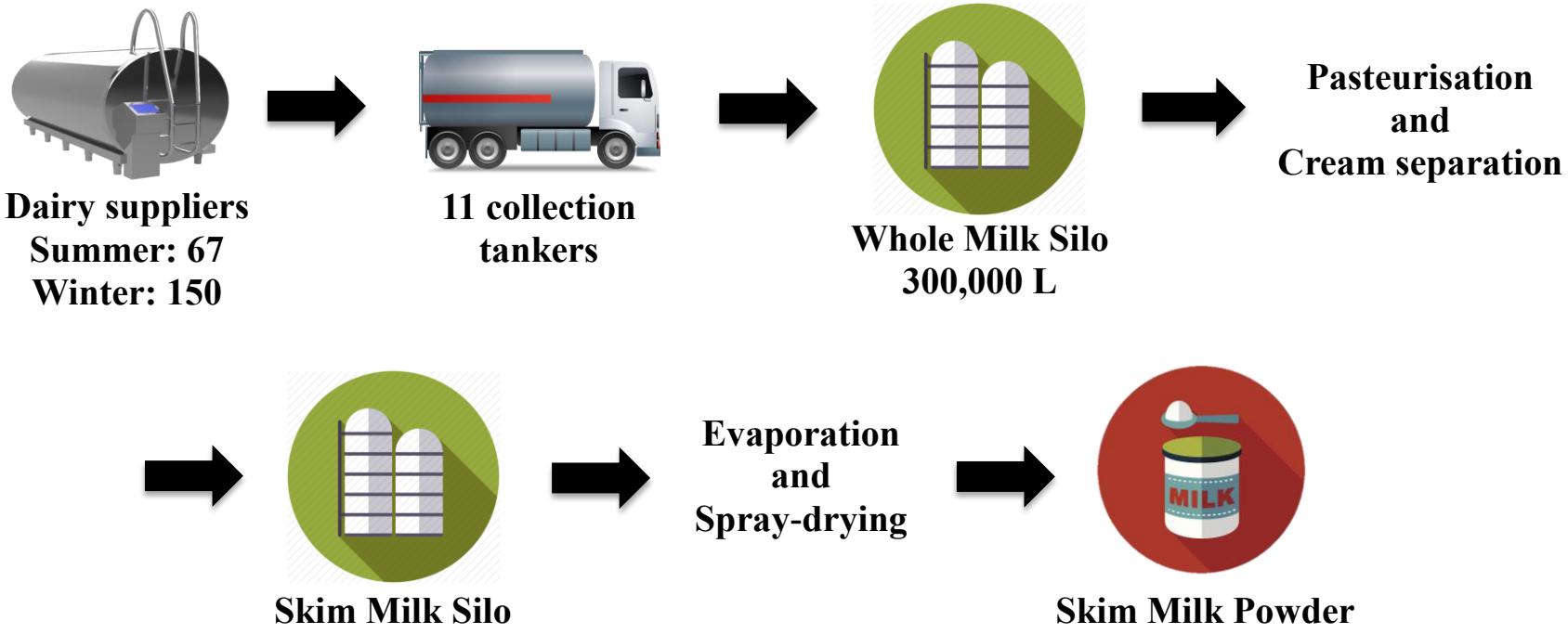
Experimental Designs

CHAPTER 3 - The effect of different pre-cooling rates and cold storage on milk microbiological quality and composition



CHAPTER 4 - Microbiological quality of milk from farms to milk powder manufacture: an industrial case study

CHAPTER 5 - Monitoring residue concentrations in milk from farm and throughout a milk powder manufacturing process



Conference contributions

Control of chlorate and trichloromethane residue levels in bulk tank milk

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Chlorate (CHLO) and trichloromethane (TCM) residues are formed when chlorine-based sanitation products are used inappropriately for milking equipment cleaning. Those residues are a concern in milk powder and butter manufacture, respectively. The aim of this study was to measure CHLO and TCM in 67 farm bulk milk tanks during mid- and late-lactation, and to investigate the main factors that influence these residue levels in milk. The CHLO and TCM levels were assessed using gas chromatography and high performance liquid chromatography with tandem mass spectroscopy, respectively. Questionnaires regarding sanitation practices were completed on farms. Differences between lactation periods with regard to residue levels, and the effect of equipment cleaning practices on TCM and CHLO in milk, were calculated using the MIXED procedure in SAS 9.3. The median TCM levels in milk during mid- and late-lactation were significantly different (0.0005 mg/kg [CI: 0.0004 - 0.0006 mg/kg] and 0.0011 mg/kg [CI: 0.0009 – 0.0014 mg/kg], respectively; P<0.0001), and were lower than the limit applied by butter manufacturers (0.0015 mg/kg). However, 6 and 21 farms in mid- and late-lactation, respectively, had TCM levels greater than that limit. In mid-lactation, less milk samples had CHLO detected (14 samples [range: 0.0010 – 0.0070 mg/kg]) compared to late-lactation (32 samples [range: 0.0010 – 0.6500 mg/kg]). In mid-lactation, all of the samples had CHLO levels lower than the European default limit for milk (0.010 mg/kg); however, in late-lactation 5 samples had CHLO levels greater than that limit. The higher levels of TCM and CHLO in late-lactation were associated with incorrect sanitation practices on-farm: insufficient rinse water used after milking (P=0.03) and after the detergent wash (P=0.01) and detergent type used (P=0.010). Generally, less milk is stored in bulk tanks during late-lactation, due to the advancing stage of lactation; consequently, residue concentrations may be higher in late-lactation milk. This study highlighted production conditions that could be targeted to minimize TCM and CHLO levels in farm milk.

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Microbial quality of milk from grass based production system and impact on milk powder quality

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Abstract

The microbial quality of bovine milk, produced on a pasture based system on 67 dairy farms, and its influence on milk powder quality were evaluated. The diet of the cows consisted mainly of perennial ryegrass with an additional 2.0 kg DM / cow / day of concentrates. Milk was sampled from farm bulk tanks, as well as from the 11 collection tankers, whole milk (WMS) and skim milk (SMS) silos. Skim milk powder (SMP) was also sampled. The average total bacterial count (TBC) for the farm milk samples was 3.78 ± 0.08 $\log_{10}$ cfu mL^{-1}, which was similar to that observed in the tankers (3.90 ± 0.41 $\log_{10}$ cfu mL^{-1}) and increased in the WMS (5.89 ± 0.02 $\log_{10}$ cfu mL^{-1}). Pasteurisation decreased TBC (SMS: 2.61 ± 0.20 $\log_{10}$ cfu mL^{-1}), while evaporation and drying resulted in further reductions of TBC in SMP (2.36 ± 0.09 $\log_{10}$ cfu g^{-1}). Thermal processes were not efficient in reducing thermoduric bacterial counts (LPC), which did not vary greatly from farm (0.90 ± 0.11 $\log_{10}$ cfu mL^{-1}) to SMS (1.85 ± 0.10 $\log_{10}$ cfu mL^{-1}). In conclusion, milk of good microbial quality can be produced from grass-based systems, resulting in high quality milk powder.

Keywords: milk quality, milk powder, pasture-based systems, microbiological quality

Introduction

Ireland is the 10th largest dairy exporter in the world, supplying dairy products to 130 countries. The Irish industrial milk production is currently expanding, due to the end of the milk quotas and to the increase in demand for dairy products worldwide. The maintenance of high milk quality is essential to hold market share and produce dairy products in accordance with specific quality parameters. On Irish dairy farms, pasture-based seasonal calving is the main milk production system, coordinating the peak of the lactation period and milk production with grass growth peak. Farming systems that are pasture-based can contribute to the production of milk with high fat content (due to a diet rich in fibre) and high protein content. Such milk is rich in fatty acids, vitamins and volatile compounds (flavours, terpenes) favourable to human nutrition and health. In this study, the microbiological quality and composition of milk produced from

pasture-based systems on 67 commercial dairy farms were evaluated and monitored throughout the milk powder manufacturing process, and the effect on the final product quality was evaluated.

Materials and methods

To undertake the manufacturing process within the factory, a minimum quantity of milk was required (296,003 L), which was supplied by a total of 67 dairy farms during the mid-lactation period (May 2016). The dairy farms that participated in this study were located in the Kilkenny and Waterford regions of Ireland. The diet of the cows on the farms (Holstein-Friesian, 120 DIM) consisted mainly of perennial ryegrass with on average an additional 2.0 kg DM / cow / day of concentrates. The average milk volume collected from each farm was 4,418 L, which was stored in bulk tanks for an average of 48 h, prior to tanker collection. Collection tankers (11) transported the milk from the farms (approximately 6 farms / tanker) to a commercial skim milk powder factory and the milk collected was stored in the whole milk silo (WMS) for approximately 5.5 h at 4.6 ˚C. Milk was then pasteurised (high temperature/ short time treatment), cream was separated and skim milk was stored in the skim milk silo (SMS). The skim milk underwent evaporation and a spray-drying process to produce skim milk powder (SMP) (21,940 kg). Milk samples were collected from the top inlet of the 67 farm bulk tanks, collection tankers (11) and the WMS and SMS. During the start, middle and final stages of the spray dryer run, 9 x 25 kg bags of powder were collected. A representative sample was collected from each bag (300 g) and reconstituted using deionised water (1:10 dilutions). All samples were tested for total bacterial count (TBC), psychrotrophic (PBC), proteolytic (PROT), thermoduric (Laboratory pasteurisation count – LPC), thermophilic (THERM), presumptive *Bacillus cereus* (BAC) and sulphite-reducing *Clostridia* (SRC) bacterial counts, as well as fat, protein and lactose contents were also measured in the samples. Somatic cell count (SCC) was measured in the all samples, except in the SMP samples.

Results and discussion

The TBC and PBC levels in the farm bulk tank milk samples varied from 2.48 to 4.97 $\log_{10}$ cfu mL^{-1} and from 2.84 to 4.67 $\log_{10}$ cfu mL^{-1}, respectively. The TBC levels were below the European limit (TBC: 5.00 $\log_{10}$ cfu mL^{-1}, EC no 853/2004) and below the typical limit applied by some Irish milk processors (4.70 $\log_{10}$ cfu mL^{-1}). Twelve farms had PBC levels higher than the European limit (PBC: 4.22 $\log_{10}$ cfu mL^{-1}), possibly due to the milk storage temperature within the bulk tanks, as low temperatures are favourable for the growth of psychrotrophs. The average PROT was below the limit suggested by Vyletelova *et al.* (2000) (4.65 $\log_{10}$ cfu mL^{-1}), at which proteolytic bacteria starts to produce high levels of heat-resistant enzymes. The LPC levels were below typical Irish

milk processor specifications and varied from 2.70 to 3.00 $\log_{10}$ cfu mL^{-1}. Milk of good microbiological quality was produced on-farm, given that TBC, PBC, PROT and LPC are below the limits cited. The European legislation or dairy processors have no specifications for thermophilic bacteria in milk. The farm milk samples also had a low level of contamination with BAC (non-detected to 2.00 $\log_{10}$ cfu mL^{-1}) and SRC (non-detected to 1.00 $\log_{10}$ cfu mL^{-1}). The TBC, PBC, PROT and LPC levels in the collection tankers samples were also below the limits cited. The TBC, PBC and PROT levels were higher in the WMS than in the collection tanker samples, which could be due to the silo or transference equipment (pipes, pumps, filters) cleaning practices. The TBC was above the limit specified in legislation for milk prior to processing (5.48 $\log_{10}$ cfu mL^{-1}; EC no 853/2004). The temperature applied during pasteurisation (75 ˚C, 25 s) was effective in significantly reducing the TBC, PBC and PROT levels, as observed in the SMS samples. The subsequent processing, where high temperatures were applied, also contributed to further reductions in those bacterial counts, as observed in the SMP samples (Table 1). The LPC and THERM levels were similar in the WMS and SMS samples, indicating that pasteurisation was not efficient in reducing those bacteria numbers, possibly due to the high temperatures applied, which are favourable for the growth of thermoduric and thermophilic bacteria; also, spores can survive high temperatures. The highest bacterial counts in the SMP samples were LPC and THERM; however, THERM counts were below the industrial limit applied in the USA for milk powder (4.00 $\log_{10}$ cfu g^{-1}). The composition of the milk transported by the tankers was similar to the composition of the raw milk collected from the corresponding farms, and no differences were noticed in the WMS, as expected (Table 2). After cream separation, the fat content in the SMS samples decreased, while the protein and lactose contents remained the same (Table 2). The average SCC in the farm milk samples was 135 ± 73 x 10^3 cells mL^{-1} (range: 36 to 342 x 10^3 cells mL^{-1}) and was below the European threshold limit (400 x 10^3 cells mL^{-1}). The average SCC in the tanker milk samples and WMS were similar: 139 ± 42 x 10^3 cells mL^{-1} and 126 ± 3 x 10^3 cells mL^{-1}, respectively. The SCC decreased in the SMS samples (98 ± 8 x 10^3 cells mL^{-1}), possibly due to the separation of somatic cells with the cream.

Table 1. Average (± SD) total (TBC), psychrotrophic (PBC), proteolytic (PROT), thermoduric (LPC) and thermophilic (THERM) bacterial counts of milk samples from the farm bulk tanks, collection tankers, whole milk silo (WMS) and skim milk silo (SMS), and of the skim milk powder (SMP) samples.

Samples	Bacterial counts ($\log_{10}$ cfu mL^{-1})				
	TBC	PBC	PROT	LPC	THERM
Farm bulk tanks	3.78 ± 0.08	3.74 ± 0.09	3.54 ± 0.17	1.26 ± 0.11	0.90 ± 0.11
Collection tankers	3.90 ± 0.41	3.70 ± 0.55	3.64 ± 0.35	1.38 ± 0.44	1.43 ± 0.68
WMS	5.89 ± 0.02	6.00	5.45 ± 0.62	1.55 ± 0.17	2.00 ± 0.14
SMS	2.61 ± 0.20	2.00	2.00	1.69 ± 0.07	1.85 ± 0.10
SMP[1]	2.36 ± 0.09	0.99 ± 0.48	1.24 ± 0.53	2.45 ± 0.09	3.63 ± 0.12

[1]Results given in $\log_{10}$ cfu g^{-1}.

Table 2. Average (± SD) fat, protein and lactose contents of the milk samples from the farm bulk tanks, collection tankers, whole milk silo (WMS) and skim milk silo (SMS), and of the skim milk powder (SMP) samples.

Samples	Fat %	Protein %	Lactose %
Farm bulk tanks	3.76 ± 0.05	3.44 ± 0.02	4.89 ± 0.01
Collection tankers	3.76 ± 0.12	3.44 ± 0.06	4.90 ± 0.04
WMS	3.85 ± 0.01	3.42 ± 0.01	4.88 ± 0.03
SMS	0.08 ± 0.05	3.54 ± 0.01	5.08 ± 0.01
SMP (reconstituted)	0.09 ± 0.03	3.45 ± 0.05	4.88 ± 0.08

Conclusion

Milk of good microbiological and compositional quality was produced from grass-based systems, contributing to the production of good quality milk powder. The differences in bacterial counts between production stages are indications of the growth potential of the bacteria in the milk, or even an indication of possible contamination sources. Also, the reduction of bacterial counts is an evidence of the effectiveness of pasteurisation. This study can aid industry in the development of new sanitation procedures, process controls or optimization of processes parameters and practices to control bacterial numbers, in order to ensure the consistent production of safe high-quality dairy products.

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Iodine levels in bulk tank milk produced by dairy cows during the mid- and late-lactation periods

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Application

The consumption of excess iodine by humans is related to thyroid dysfunctions. Bovine milk is one of the main iodine sources for humans and therefore it is relevant to monitor on-farm factors that could contribute to high iodine levels in milk.

Introduction

Two major factors influencing milk iodine levels are (i) ingestion of iodine through animal feeds and (ii) application of teat disinfectants containing iodine to cows. Incorrect feeding management, such as utilisation of rations with higher levels of iodine than required or unnecessary supplementation, can result in high concentrations of iodine in milk. Borucki Castro et al. (2012) reported a linear relationship between dietary iodine content and milk iodine concentration. With regard to the use of iodine teat disinfectants, the incomplete removal of those products from the teat surface prior to cluster attachment can increase the risk of direct transfer of iodine to milk. Iodine may also be absorbed through the teat skin, with consequent effects on milk iodine levels. The British Agricultural Research Council, the German Society of Nutrition & Physiology and the US National Research Council recommend that the daily iodine consumption should be 10 to 12 mg/cow/day. The aim of this study was to investigate the concentration of milk iodine levels on Irish dairy farms during mid- and late-lactation periods and identify the possible sources of this iodine in the milk.

Material and methods

This study was undertaken on 67 spring-calving dairy farms (herd size: 21 to 469 cows), located in the Kilkenny and Waterford region of Ireland, during the mid- (June) and late- (October) lactation periods. Milk samples were collected from the top inlet of each of 67 bulk tanks using sterilised sample dippers, after appropriate agitation. Iodine was quantified through inductively coupled plasma mass spectroscopy, using an Agilent ICPMS 7700x system. Questionnaires were

completed on-farm, in which information on iodine content in animal feed and use of teat disinfectants containing iodine was captured.

Results

Average milk iodine levels on the 67 dairy farms were similar during the mid- and late-lactation periods: at 116 ppb (range: 10–561 ppb) and 109 ppb (range: 3–1121 ppb), respectively. During the mid- and late-lactation periods, 13 and 12 farms, respectively, produced milk with iodine levels higher than 150 ppb, which is the target level for the production of milk powder. Among those farms, 5 farms had high levels of iodine in both lactation periods. Milk iodine levels ranged between 178 and 561 ppb and between 154 and 1121 ppb on the farms with iodine levels higher than 150 ppb in the mid-lactation and late-lactation periods, respectively. The daily iodine intake from the dairy rations on a per cow basis on the farms that were higher than the 150 ppb limit varied from 31.8 to 100 mg of iodine/cow/day (mid-lactation) and 75.0 to 87.5 mg iodine/cow/day (late-lactation). It was not possible to observe a linear relationship between dietary iodine content and milk iodine concentration in the present study, given that the questionnaires did not cover other potential factors that could contribute to iodine levels in milk (e.g., boluses, addition of iodine to drinking water, mineral licks) and the contribution of grass. Regarding teat disinfection, in the mid- and late-lactation period, 4 of the 13 farms and 2 of the 12 farms, respectively, were using iodine teat disinfectants. Those products were also used on other farms among the 67 (mid-lactation: 3 farms; late-lactation: 3 farms); however, the iodine levels in their milk samples was lower than 150 ppb.

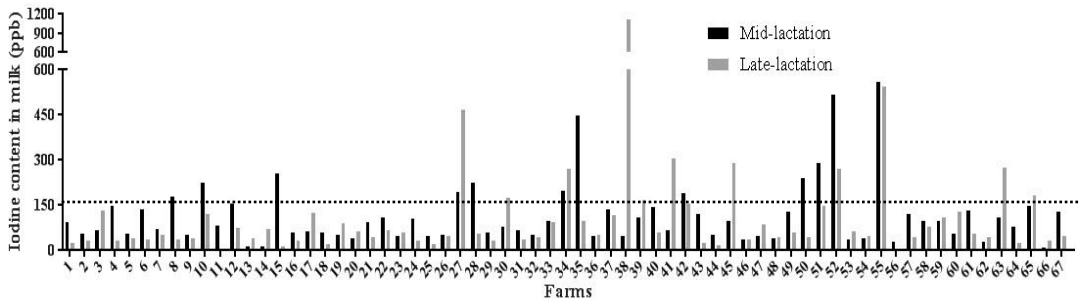

Figure 1. Iodine levels in milk samples collected during the mid- and late-lactation period on 67 dairy farms.

Conclusion

The concentration of iodine in Irish dairy rations is higher than necessary and combined with the misuse of iodine-based teat disinfectants will result in higher levels of iodine than required in milk destined for infant formula manufacture.

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The effect of milk cooling rate on milk microbiological quality and composition

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Application

Pre-cooling milk prior to it entering the bulk tank is effective in rapidly reducing milk temperature and could be useful in reducing bacterial growth during storage, thereby preserving milk quality.

Introduction

Milk leaves the cow's udder at approximately 35 ˚C, which is a favourable temperature for bacterial growth (Walstra et al., 2006). Therefore, milk cooling and refrigerated storage are necessary after milking, in order to reduce bacterial growth rates. An increase in bacterial counts in milk could result in hydrolysis of protein, fat and lactose, thus affecting milk processing characteristics and nutritional value. The pre-cooling of milk using plate heat exchangers (prior to entering the bulk tank) rapidly reduces milk temperature and also could rapidly reduce bacterial growth rates. The aim of this study was to investigate the effect of pre-cooling milk at different rates on the microbiological quality and composition of milk.

Material and methods

Spring-calving dairy cows (n=210) were milked in a 30-unit side-by-side milking parlour, twice daily over two three-week periods. The volume of milk collected during each milking was distributed equally into three identical bulk tanks (morning: 800 L/ tank; afternoon: 500 L/ tank), using shut-off valves to control the flow rate. Prior to the milk entering the bulk tanks, three pre-cooling treatments were applied: no plate cooler (NP), single-stage (SP) and double-stage (DP) plate cooler; which pre-cooled milk to 32.0 ± 1.4 ˚C, 17.0 ± 2.8 ˚C and 6.0 ± 1.1 ˚C, respectively. In the SP treatment, milk exchanged heat with ground water (at approximately 15 ˚C). In the DP treatment, milk was pre-cooled in two stages: ground and ice-water (at approximately 0 ˚C) were used in the first and second stage, respectively. Milk was added to the bulk tanks twice daily for 72 h and stored at 3 ˚C. Milk line samples were collected to access the microbiological

quality of milk entering the tanks. After the initial morning milking, duplicate milk samples were collected from each bulk tank once the milk temperature reached 3 ˚C, corresponding to 0 h samples (one milking). The subsequent samples (24, 48 and 72 h) were collected prior to the addition of milk from the morning milkings of the following days, when the bulk tanks contained milk from 2, 4 and 6 milkings, respectively. The samples were analysed for a range of bacteria, as well as composition and somatic cell count (SCC). The data were analysed using the MIXED procedure in SAS 9.3, with period (1 and 2), week (1, 2 and 3), pre-cooling system (NP, SP and DP) and storage time (0, 24, 48, and 72 h) as fixed effects.

Results

The average total bacterial count (TBC) for the milk line samples was 3.35 ± 0.29 $\log_{10}$ cfu/ mL, indicating that milk of good microbiological quality was produced. The TBC means at 0 h for NP, SP and DP were 3.55, 3.57 and 3.50 ± 0.09 $\log_{10}$ cfu/ mL, respectively; while the psychrotrophic bacterial count (PBC) means were 3.11, 3.04 and 3.07 ± 0.11 $\log_{10}$ cfu/ mL, respectively. The bulk tanks that received the NP, SP and DP treatments cooled milk to 3 ˚C in 2 h, 1 h and 20 min, respectively. The differences in cooling times were expected to affect the initial bacterial counts within each tank; however, there was no significant difference between treatments (P>0.05). The different pre-cooling treatments also did not affect any of the bacterial counts over the storage period up to 72 h (P>0.05). This could be due to the reduction of the blend temperature within each bulk tank as the volume of milk at 3 ˚C increased after each milking; consequently, the milk was cooled faster. However, after 72 h, numerical differences were observed between the bacterial counts in the milk volumes subjected and not subjected to pre-cooling. The TBC means at 72 h for NP, SP and DP were 3.90, 3.77 and 3.71 ± 0.09 $\log_{10}$ cfu/ mL, respectively; the mean PBC were 3.38, 3.28 and 3.25 ± 0.11 $\log_{10}$ cfu/ mL, respectively. The low bacterial growth rates also indicate that the storage temperature of 3 ˚C was effective in preventing an increase in bacterial numbers over the storage period. The pre-cooling treatments and storage time had no impact on milk composition (P>0.05). The average fat, protein, lactose and total solids were: 4.42 ± 0.08 %, 3.58 ± 0.07 %, 4.78 ± 0.07 % and 13.38 ± 0.13 %, respectively.

Conclusion

The bacterial count and composition of milk are minimally impacted when the milk was stored at 3 ˚C for 72 h, whether the milk is pre-cooled or not; however, milk entering the tank should have good initial microbiological quality. Considering the numerical differences in bacterial counts between treatments at 72 h, the use of the SP or DP pre-cooling systems is recommended to maintain low levels of bacteria and possibly reduce cooling energy costs.

Acknowledgements

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Monitoring residues in milk from farm to milk powder manufacture

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Introduction

Trichloromethane (TCM) and chlorate residues are formed in milk and dairy products when sanitation products containing chlorine are inappropriately used for cleaning milking and processing equipment.

Aim

To monitor TCM and chlorate concentrations in farm milk bulk tanks, and through the manufacturing stages to final product.

Methods

TCM and chlorate concentrations were measured in milk from 67 farm bulk tanks, 11 collection tankers that transported that milk to a factory (approximately 6 farms/tanker), whole milk silo (WMS) that stored milk from the tankers (300,000 L) and in a skim milk silo (SMS) after pasteurization and cream separation. Residues were also measured in powder collected at the start, middle and end of a spray dryer run. TCM levels were assessed using gas chromatography, while chlorate was quantified using High Performance Liquid Chromatography and Tandem Mass Spectroscopy. Milk powder samples were reconstituted in deionized water (1:10) to quantify TCM. Questionnaires were also completed on farms regarding sanitation practices.

Results

TCM and chlorate concentrations were above the detection limit suggested for raw milk in 6 (>0.0015 mg/kg) and 8 farms (>0.0010 mg/kg), respectively; suggesting incorrect sanitation practices. The average TCM and chlorate concentrations in farm milk were 0.0007 and 0.0006 mg/kg, respectively. The TCM level remained the same in tankers and WMS and decreased in the SMS (0.0001 mg/kg), indicating that TCM was separated with cream. Chlorate concentrations slightly increased in tankers (0.0008 mg/kg) and WMS (0.0010 mg/kg) and remained the same in the SMS; however these averages are below the detection limit. Powder samples contained TCM and chlorate levels of 0.0001 and

0.057 mg/kg, respectively; the latter being lower than the suggested limit for infant formula (0.100 mg/kg).

Conclusion

This study highlighted production conditions that could affect residue concentrations in milk from farm level through the processing stages to powder manufacture.

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Microbiological quality of milk on farms, during transport, processing stages and in milk powder

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The microflora population in milk is an important determinant of milk processability and functional properties, as well as organoleptic qualities, shelf life and safety of dairy products. The objective of this study was to monitor the microbiological quality of milk from individual dairy farms, during transport, in factory silos and in milk powder subsequently manufactured. Milk was collected from 67 dairy farms by 11 tankers, which transported this blended milk to a factory, where milk was stored in a whole milk silo, pasteurised and skimmed, and used to produce milk powder. Milk samples were collected at each stage from farms to final product. Among the 12 microbiological analysis performed, samples were tested for: total bacterial count (TBC), psychrotrophic (PBC), thermoduric (LPC), thermophilic (THERM) bacterial counts and presumptive *Bacillus cereus* (BAC). Questionnaires were completed on farms in order to correlate hygiene and sanitation practices with the microbiological levels in milk. The average TBC and PBC for farm milks were 9,723 cfu/mL (range:450-80,000 cfu/mL) and 31,368 cfu/mL (range:500-10^6 cfu/mL), respectively, and those levels increased from farm to whole milk silo. The average TBC in tankers and whole milk silo samples were $1.2\pm1.2\times10^4$ and $7.75\pm0.4\times10^5$ cfu/mL, while PBCs were $1.3\pm2.7\times10^4$ and 1.0×10^6 cfu/mL respectively. After pasteurisation, TBC and PBC decreased to 450±238 cfu/mL and 50±58 cfu/mL, respectively. Evaporation and the drying processing stage contributed to the lower bacterial levels observed in milk powder (TBC: 233±49 cfu/g; PBC: 14±9 cfu/g). After pasteurisation, no presumptive BAC colonies were identified; however the process was not efficient in reducing LPC and THERM, which did not vary greatly from farm to skim milk silo (LPC: 38±10 cfu/mL; THERM: 69±30 cfu/mL). Farms presented milks of a high microbiological quality and where inferior quality milk was observed this was related to inadequate cow and equipment cleaning protocols. This study can aid in monitoring sanitation practices and process controls to ensure manufacture of safe and high-quality dairy products.

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Changes required in milking equipment cleaning practices to ensure low chlorine residues in milk

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Trichloromethane (TCM) is a residue in milk caused by the interaction of chlorine (hypochlorite) and milk. On the majority of farms in Ireland, the products used for cleaning milking equipment contain sodium hydroxide and sodium hypochlorite (detergent/steriliser). It is thought that incorrect use of these products and/or inadequate rinsing after washing may increase milk TCM levels. A detailed knowledge of the daily milking equipment cleaning practices on dairy farms is required in order to give effective advice to farmers on this issue. A survey of milking equipment cleaning procedures was conducted on 112 farms, previously identified as having milk containing TCM residue. The size of the water trough in the dairy was inadequate on 55% of farms and this is a key factor accounting for the insufficient water used for rinsing milking equipment after washing on 65% of farms. A minimum of 14 litres per milking unit is advised. Meanwhile inadequate rinsing of the bulk milk tank (after washing) was also identified on 30% of farms. Higher than the required volume of cleaning product was used for cleaning the milking machine and bulk milk tank on 18% and 26% of farms, respectively. Products with a high chlorine concentration (>3.5%) were used for cleaning bulk milk tanks on 16% of farms. Using cleaning products with >3.5% chlorine increase the likelihood of residues, particularly when rinse water volumes are inadequate. Even though an adequate chlorine concentration was present in all products used, additional chlorine was added to the wash solution on 15% of farms resulting in very high working solutions of chlorine. Reusing the cleaning solution more than once, using chlorine in a pre-milking rinse to sterilize equipment, and dipping clusters in a chlorine solution between individual cow milking's all represent additional individual causes of high milk TCM. These practices were observed on 19%, 5% and 4% of farms, respectively. Overall, there were 14 incorrect cleaning procedures identified on the farms visited with the number of faults per farm ranging from one to seven. All of these incorrect practices must be addressed to ensure minimum residue levels in milk leaving the farm.

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Influence of storage time on the microbiological load of raw milk destined for UHT processing

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In Ireland, dairy companies are interested in increasing the storage time of milk intended for UHT processing, which is currently stored on-farm for 24 h before collection. However, investigations are required regarding the effects of the extended storage time on the raw milk quality. This study assessed the effects of storing 24 h milk (two milkings) for a further three days on the microbiological load. Raw milk samples were collected from bulk tanks on six dairy farms and stored at 4 ˚C for 96 h; total bacterial (TBC), psychrotrophic bacteria (PBC) and thermoduric (TC) counts were measured at 24 h intervals. The initial TBC and PBC were $1.0 \times 10^4 \pm 0.3 \times 10^4$ cfu/mL and $8.3 \times 10^3 \pm 3.1 \times 10^3$ cfu/mL, respectively. The TBC and PBC increased significantly ($2.3 \times 10^6 \pm 1.9 \times 10^6$ cfu/mL, $P<0.05$ and $2.7 \times 10^6 \pm 1.9 \times 10^6$ cfu/mL, $P<0.01$, respectively), after 72 h. TC did not change up to and including 96 h ($P>0.05$); however, counts higher than acceptable levels were observed for four farms (200-900 cfu/mL) over the storage period. Milk was collected during the housing period, in which contamination by spore-forming bacteria is more likely. Good farm management practices are required to ensure minimal contamination of milk with TC. The TBC and PBC levels after 72 h were within the specification required for raw milk destined for UHT (50,000 and 300,000 cfu/mL, respectively); indicating that the storage time of milk could be extended beyond 24 h.

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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,

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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

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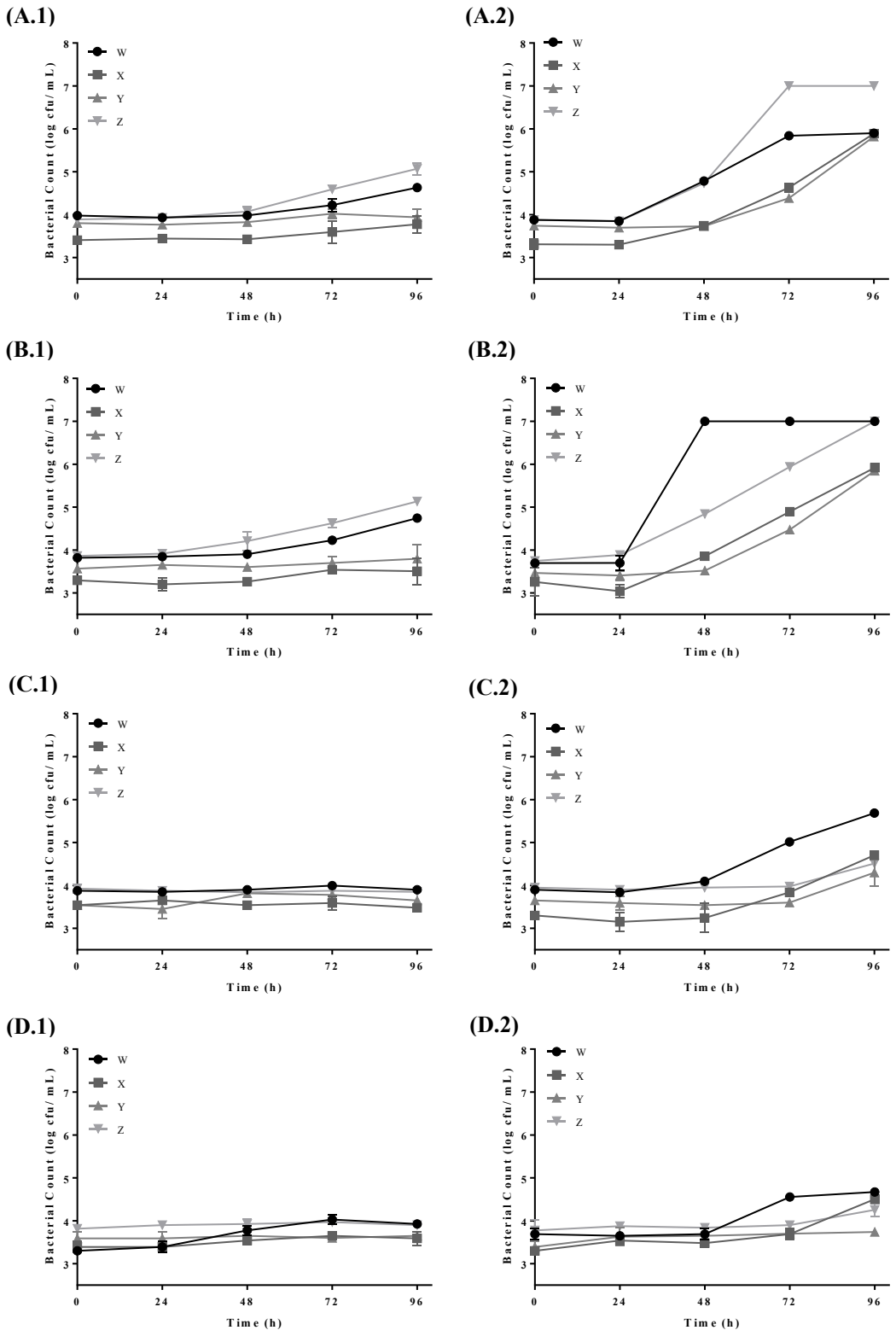

Figure 1. (A) Total bacterial count (TBC), **(B)** psychrotrophic bacterial count (PBC), **(C)** proteolytic (PROT) bacterial count and **(D)** lipolytic (LIP) bacterial count over 96 h for milk samples W, X, Y and Z stored at **(1)** 2°C or **(2)** 4°C.

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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

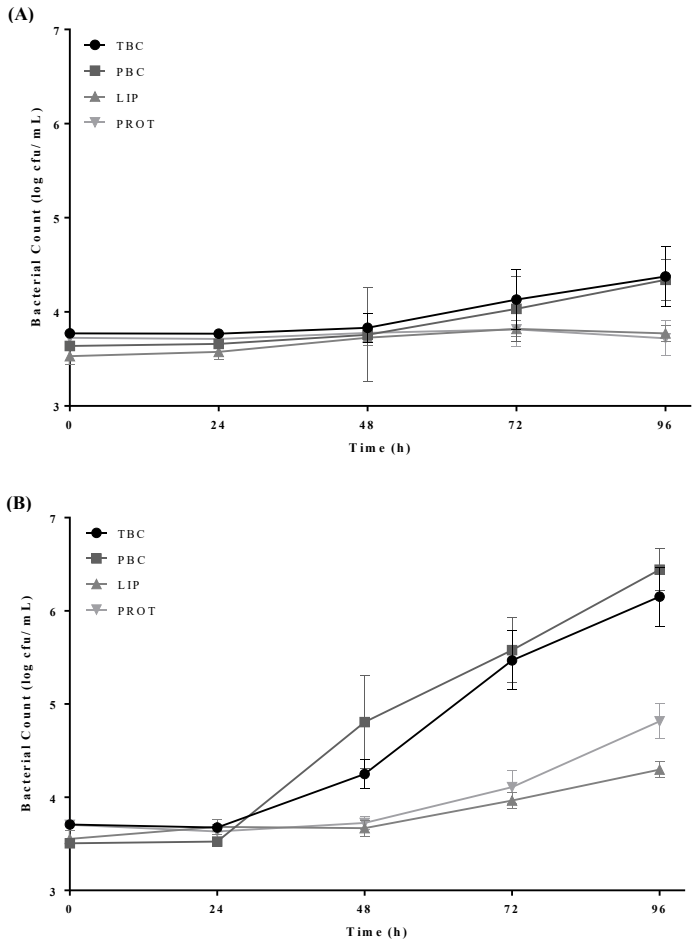

Figure 2. Average of the total bacterial count (TBC), psychrotrophic bacterial count (PBC), lipolytic (LIP) bacterial count and proteolytic (PROT) bacterial count over 96 h for milk samples from four dairy farms (W, X, Y and Z) stored at **(A)** 2°C and **(B)** 4°C.

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(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.

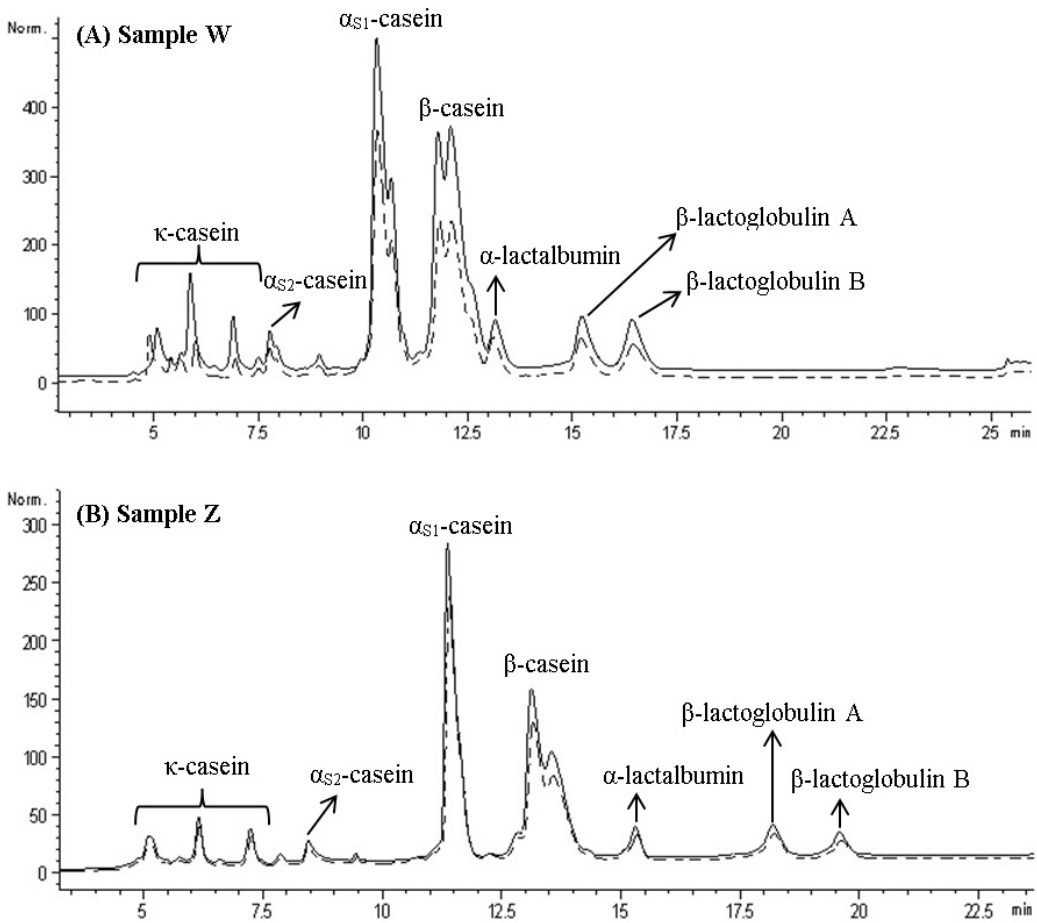

Figure 3. Separation of bovine milk proteins by reversed-phase high-performance liquid chromatography (HPLC). Chromatograms of samples **(A)** W and **(B)** Z stored at 4°C are shown. Full line (-) shows the 0 h sample; dashed line (– –) shows the 96 h sample.

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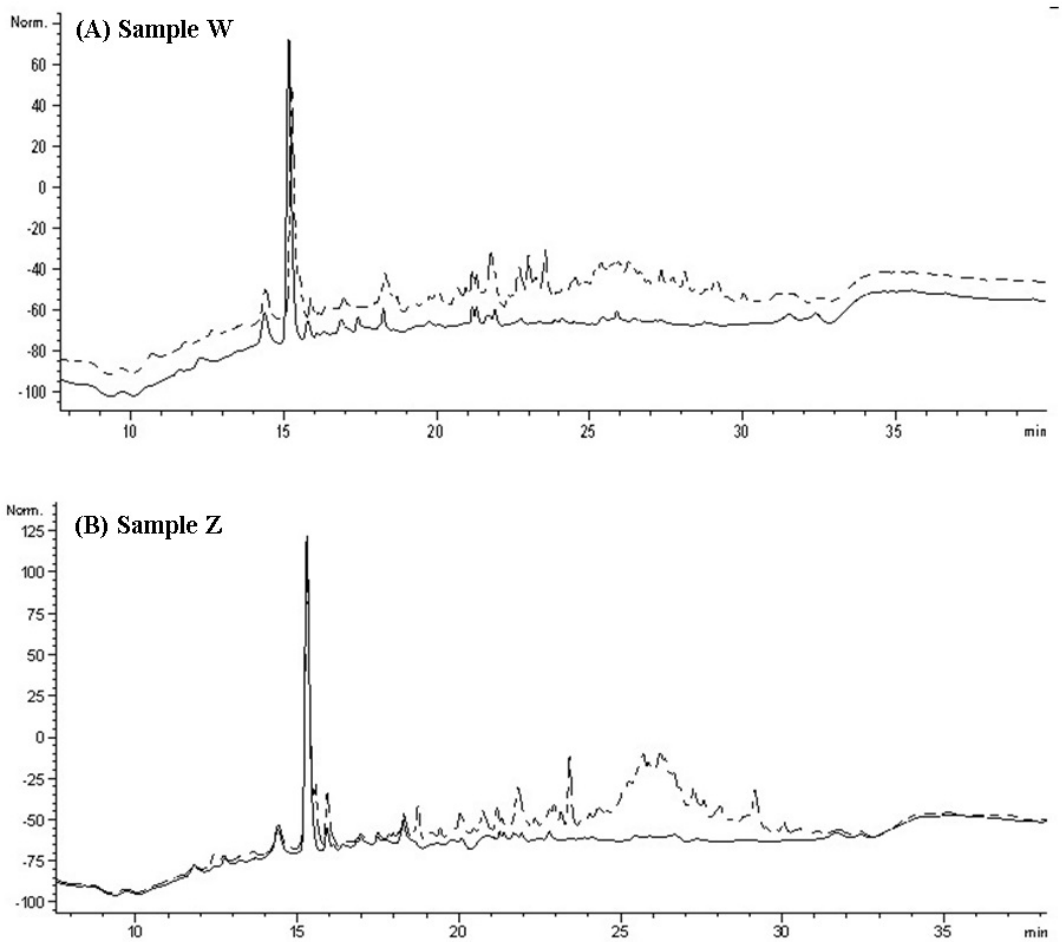

Figure 4. Separation of bovine milk peptides by reversed-phase high-performance liquid chromatography (HPLC). Chromatograms of samples **(A)** W and **(B)** Z stored at 4°C are shown. Full line (-) shows the 0 h sample; dashed line (– –) shows the 96 h sample.

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

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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

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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 ($P > 0.05$).

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

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The effect of different precooling rates and cold storage on milk microbiological quality and composition

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ABSTRACT

The objective of this study was to measure the effect of different milk cooling rates, before entering the bulk tank, on the microbiological load and composition of the milk, as well as on energy usage. Three milk precooling treatments were applied before milk entered 3 identical bulk milk tanks: no plate cooler (NP), single-stage plate cooler (SP), and double-stage plate cooler (DP). These precooling treatments cooled the milk to 32.0 ± 1.4°C, 17.0 ± 2.8°C, and 6.0 ± 1.1°C, respectively. Milk was added to the bulk tank twice daily for 72 h, and the tank refrigeration temperature was set at 3°C. The blend temperature within each bulk tank was reduced after each milking event as the volume of milk at 3°C increased simultaneously. The bacterial counts of the milk volumes precooled at different rates did not differ significantly at 0 h of storage or at 24-h intervals thereafter. After 72 h of storage, the total bacterial count of the NP milk was 3.90 ± 0.09 $\log_{10}$ cfu/mL, whereas that of the precooled milk volumes were 3.77 ± 0.09 (SP) and 3.71 ± 0.09 (DP) $\log_{10}$ cfu/mL. The constant storage temperature (3°C) over 72 h helped to reduce bacterial growth rates in milk; consequently, milk composition was not affected and minimal, if any, proteolysis occurred. The DP treatment had the highest energy consumption (17.6 ± 0.5 Wh/L), followed by the NP (16.8 ± 2.7 Wh/L) and SP (10.6 ± 1.3 Wh/L) treatments. This study suggests that bacterial count and composition of milk are minimally affected when milk is stored at 3°C for 72 h, regardless of whether the milk is precooled; however, milk entering the tank should have good initial microbiological quality. Considering the numerical differences between bacterial counts, however, the use of the SP or DP precooling systems is recommended to maintain low levels of bacterial counts and reduce energy consumption.

Key words: milk precooling, milk microbiological quality, energy, milk storage

INTRODUCTION

Milk cooling and refrigerated storage are necessary after milking to reduce bacterial growth rates. Milk leaves the udder at approximately 35°C, which is a favorable temperature for bacterial growth (Walstra et al., 2006). Thus, the microbial load could increase rapidly if milk is maintained at that temperature. According to Holm et al. (2004), cooling milk rapidly (below 6°C) is necessary to avoid the multiplication of microorganisms, especially psychrotrophs, which can grow at refrigeration temperatures but have optimal and maximal growth temperatures at >15 and 20°C, respectively (Moyer and Morita, 2007). Thus, the precooling of milk (before it enters the bulk tank) could further reduce the bacterial growth rate. A further possible benefit of precooling milk is the reduction of energy costs on-farm (Murphy et al., 2013).

The equipment used to precool milk consists of plate heat exchangers incorporating stainless steel plates in a sandwich arrangement, in which milk and cooling water flow in opposite directions through the spaces between alternate plates (Wang et al., 2007). This system may have 1 or 2 cooling stages, in which well water and well and chilled waters are used in the first and second stages, respectively. O'Connell et al. (2016) observed only a minimal increase in milk bacterial count over time when fresh milk from each milking event was precooled using a single-stage plate cooler before being added to the bulk milk tanks twice daily.

Total bacterial count (**TBC**) is the main test used by milk processors to assess milk microbiological quality and it quantifies aerobic mesophilic bacteria in milk. In conjunction with TBC, the psychrotrophic bacterial count (**PBC**) is used to assess the hygiene quality of milk and is an indicator of hygiene conditions on-farm (Harding, 1995; Robinson, 2002). Milk cooling reduces the growth rate of mesophilic and psychrotrophic bacteria, the optimum growth temperatures of which are

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between 20 and 45°C and <7°C, respectively (Frank and Yousef, 2004; Willey et al., 2008). Thermoduric and thermophilic bacteria are the other relevant groups of bacteria that are measured in milk. These bacteria are important because they can survive thermal treatments such as those frequently applied in dairy processing to reduce bacterial numbers (e.g., pasteurization; Murphy et al., 1999; Robinson, 2002). The main sources of those bacteria are in the cows' environment, because their vegetative cells and spores can be present in feed, forage, bedding material, dust, feces and soil (Scheldeman et al., 2005; Gleeson et al., 2013). *Clostridium perfringens* and *Clostridium botulinum* are the pathogenic thermoduric bacteria of most relevance to the dairy industry because of their heat-resistant spores and toxins (Wrigley, 1994; Fernandes, 2009).

Some mesophilic, psychrotrophic, thermoduric, and thermophilic bacterial strains have the ability to produce lipases and proteases. These enzymes hydrolyze fat and protein, resulting in sensorial defects and altering the physico-chemical properties and processability of milk (Chen et al., 2003; Deeth, 2006). Lipolytic activity produces flavors described as rancid and bitter (Deeth, 2006) and could, for example, result in loss of foaming and creaming ability during butter manufacture (Shelley et al., 1987). Celestino et al. (1997) reported that reconstituted UHT milk powder manufactured using 4-d-old raw milk had rancid and bitter flavors compared with UHT milk powder produced using fresh raw milk, probably due to bacterial protease and lipase activity. Therefore, the control of bacterial numbers in milk helps to preserve milk functionality, allowing the production of a range of dairy products in accordance with specific quality parameters.

The aim of this study was to investigate the effect of precooling milk at different rates on the microbiological quality and composition of milk, as well as on energy usage. This study was conducted in a manner that mimicked on-farm milk production conditions: morning and evening milkings, similar milk storage conditions, and use or not of precooling systems.

MATERIALS AND METHODS

Experimental Design

This experiment was carried out in the dairy parlor at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Cork, Ireland. Spring-calving dairy cows (n = 210) were milked in a 30-unit side-by-side milking parlor, twice daily over two 3-wk periods, with milking commencing at 0700 and 1430 h. Period 1 extended from June 13 to July 2, 2016, and period 2 extended from July 25 to August 13, 2016. Before milking, cows' teats were washed and disinfected with chlorhexidine foam teat cleaner (Deosan Teatfoam Advance AG104, Sealed Air, Johnson Diversey Ltd., Dublin, Ireland) and dried using individual paper towels. The milk was transferred from clusters through 16-mm (internal diameter) milk tubes to a mid-level milk line (72 mm, internal diameter), with a milk lift of 1.5 m. The milk was collected in a receiver jar and pumped through a 48-mm stainless steel pipe, using a variable speed milk pump, to the bulk milk tanks (Figure 1). Once the milk flow rate dropped to 0.2 kg/min, clusters were automatically removed, with a delay time of 20 s. A system to individually wash and disinfect each cluster between each individual cow milking (Cluster Cleanse, Dairymaster, Causeway, Kerry, Ireland) was used. After each milking, the milking equipment was rinsed with water (14 L per milking unit), followed by a hot (75°C) liquid detergent sterilizer wash (Liquid Gold, Dairymaster) circulated for 8 to 10 min in the milk line. Following this, the milking equipment was rinsed twice, and the final rinse contained peracetic acid (0.3–0.5% concentration). An acid-descale (Extra-strong descaler, Dairymaster) was incorporated into the wash regime before the detergent cycle once a week.

The volume of milk collected during each milking was distributed equally into 3 identical bulk milk tanks. The milk line for each bulk tank was fitted with shut-off valves, which were used to control the milk flow rate and guarantee an equal distribution of milk to the tanks. Each bulk tank had capacity of 4,000 L (Swiftcool, Dairymaster) and was fitted with a 5.5-Hp condensing unit. A screen on the front of each tank displayed the milk temperature, time, and milk volume. The milk was cooled to 3°C within the tanks and stored for up to 72 h from once the first milking entered the tank. Approximately 800 and 500 L of milk were added to each bulk milk tank during the morning and afternoon milkings, respectively. At the end of each 72-h storage period, the milk was collected and the bulk milk tanks were washed using a hot detergent/sterilizer wash (50°C). This was followed by a cold-water rinse and an additional rinse containing peracetic acid. An acid-descale wash product was used at every third wash.

Before entering the bulk tanks, the milk underwent 1 of 3 precooling treatments: no precooling (**NP**), single-stage (**SP**), or double-stage (**DP**) plate cooling (Figure 1). In the NP treatment, the ground water line was closed; therefore, no precooling was undertaken in that treatment. In the SP treatment (37 plates), the milk exchanged heat with ground water at approximately 15°C. In the DP treatment (45 plates), the milk was cooled in 2 stages; in the first stage, ground water was used (at approximately 15°C) and in the second stage,

ice water (at approximately 0°C) was used. Ice water was produced in an ice bank, a system with external melting ice on a coil thermal storage unit with an in-line coil array. In the NP, SP, and DP treatments, the milk entered the bulk tanks at average temperatures of 32 ± 1.4°C, 17 ± 2.8°C, and 6 ± 1.1°C, respectively. The temperature and volume of milk in each tank was recorded by an integrated system (Swiftcool, Dairymaster) and transmitted via Global System for Mobile communications (GSM) technology to a computer over the two 3-wk trial periods.

Milk Sampling

During each milking, a milk sample was collected from the milk line using a sterile Durham flask surrounded by ice to assess the quality of milk entering the tanks. After the initial morning milking, duplicate milk samples (30 mL) were collected from each bulk tank once the milk temperature within each tank reached 3°C, corresponding to 0-h samples (one milking). The subsequent samples (24, 48, and 72 h) were collected before the addition of each subsequent morning milk on subsequent days, when the bulk tanks contained milk from 2, 4, and 6 milkings, respectively. Before sample collection, the milk was agitated at 24 rpm for 1 min, and samples were collected from the top viewing inlet using sterilized sample dippers. Samples were transported to the laboratory in ice boxes, delivered within 30 min of collection, and analyzed. One sample from each tank was used for microbiological analysis and the other for compositional analysis and SCC.

Microbiological Analysis

Immediately on delivery to the laboratory, raw milk samples collected every 24 h were tested in duplicate for a range of bacteria. All the microbiological analyses were performed according to the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004). The TBC, PBC, thermoduric (laboratory pasteurization count, **LPC**), and thermophilic (**THERM**) bacterial counts were estimated using Petrifilm aerobic count plates, a ready-to-use medium (3M, Technopath, Tipperary, Ireland). Samples tested for LPC were pasteurized at 63°C for 30 min, with an additional 5 min that allowed time for the samples to reach the required temperature (Frank and Yousef, 2004); after

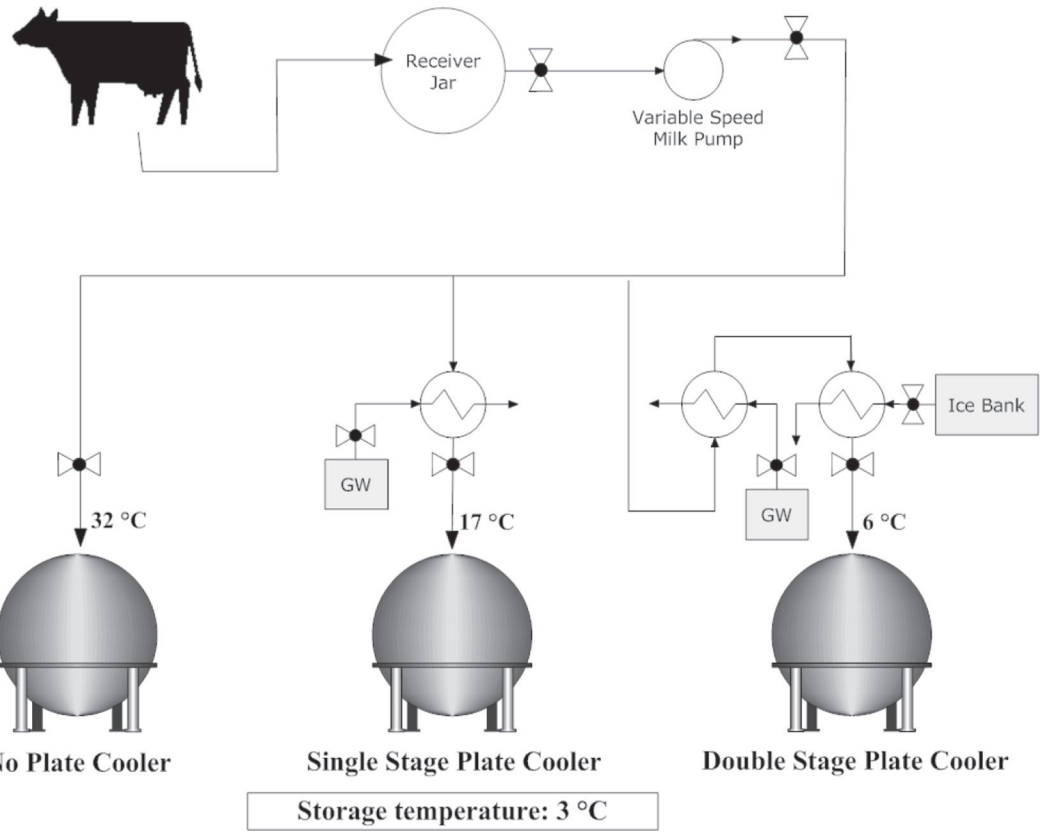

Figure 1. Experimental setup using 3 precooling systems: no plate cooler, single-stage plate cooler, and double-stage plate cooler. GW = ground water.

heating, the samples were cooled to 10°C in iced water before testing. Samples tested for TBC and LPC were incubated for 48 h at 32°C (Laird et al., 2004; Pantoja et al., 2009; O'Connell et al., 2016), whereas samples tested for PBC and THERM were incubated at 7 ± 1°C for 10 d and at 55°C for 48 h, respectively (Frank and Yousef, 2004). We are aware that using Petrifilm plates at 7°C or 55°C is outside the validated range. However, a pretrial experiment for THERM indicated that, at the same dilution, plate count agar plates were uncountable due to bacterial colonies spreading over the surface of agar plates, whereas Petrifilm plates were countable (data not shown). In other studies, Petrifilm plates have been used for PBC at 7°C (Ramsahoi et al., 2011). The number of bacterial colonies was assessed using a Petrifilm Plate Reader (3M, Technopath). The lipolytic bacterial count (**LIP**) was performed by spread-plating 100 μL of the appropriate dilutions on tributyrin agar with added glyceryl tributyrate (0.01 mL/mL of agar prepared; Sigma Aldrich, Dublin, Ireland). The proteolytic bacterial count (**PROT**) was estimated by spread-plating 100 μL of the diluted sample on calcium caseinate agar with added skim milk powder (2.5 mg/mL of agar; Merck, Darmstadt, Germany). For both methods, samples tested were incubated at 37°C for 48 h. Lipolytic bacteria colonies were identified as colonies surrounded by a clear zone in a turbid medium, whereas proteolytic bacteria colonies were identified as colonies surrounded by a clear zone in an opaque medium.

The sulfite-reducing *Clostridia* count (**SRC**) was assessed by pour-plating 1 mL of diluted sample in iron sulfite agar and incubating plates under anaerobic conditions for 72 h at 37°C, in accordance with ISO standard 15213 (ISO, 2003). Presumptive SRC colonies were identified as black colonies.

Composition and SCC

Raw milk samples collected every 24 h had their composition (fat, protein, lactose, and TS contents) and SCC determined using a Fossomatic FC (Foss Electric, Hillerød, Denmark) within 24 h after arrival in the laboratory.

Peptide Profiles

Milk samples were collected from the bulk milk tanks at 0 and 72 h to obtain the peptide profiles. Trichloroacetic acid (TCA) was used to extract the nonprotein fraction of the milk samples, according to the extraction procedure described in IDF method 20-4 (IDF, 2001). The extracts were not diluted but were filtered using 0.45-μm syringe cellulose filters (25 mm diameter, Chromafil Xtra RC-45/25, Macherey-Nagel, Dublin, Ireland). The HPLC equipment used was an Agilent 1200s system (Agilent Technologies, Santa Clara, CA), with quaternary pump and multi-wavelength detector. A Zorbax 300SB column (4.6 mm internal diameter × 150 mm; Agilent Technologies) was used to perform the separation of milk peptides. The gradient elution and peak detection methodology was an adaptation of the methodology of Rohm et al. (1996). Samples were injected onto the column (50 μL) in duplicate and the flow rate was 0.50 mL/min.

Assessment of Electricity Consumption

The energy consumption of each treatment, expressed in Watt-hours (Wh), was measured as the energy usage of each bulk milk tank when each of the precooling treatments was applied. For the DP treatment, the energy usage of the ice bank was also considered. Energy usage was assessed using energy analyzers (EM24 DIN) and Digi Connect wireless WAN cellular routers (Carlo Gavazzi Automation SpA, Lainate, Italy), which measured and transmitted the energy data, respectively. The cumulative energy usage was recorded every 1 min using the software program Powersoft (Carlo Gavazzi Automation SpA).

Statistical Analysis

This study was carried out following a Latin square design with repeated measures, in which samples were collected every 24 h, and each bulk tank (n = 3) received a different precooling treatment (NP, SP, DP) in each week (n = 3). Each Latin square was conducted over two 3-wk periods.

Least squares means for the main effects of period, week, storage time, and precooling system, as well as the interaction between storage time and precooling system, were calculated using the MIXED procedure in SAS 9.3 (SAS Institute Inc., Cary, NC). The fixed effects included in each model were period (1 and 2), week (1, 2, and 3), precooling system (NP, SP, and DP), and storage time (0, 24, 48, and 72 h). Repeated-measures models were used to account for correlations between time points. Tank within week was considered the experimental unit. The response variables were TBC, PBC, LPC, THERM, PROT, LIP, SRC, SCC, and fat, protein, lactose, and TS contents. 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 test (at 5% error probability) was used to compare the means for all variables.

RESULTS AND DISCUSSION

Microbiological Analysis

During the first milking occasion on the first day of each trial week, a milk sample was collected from the milk line before distribution of the milk to each bulk tank. The average (± SD) TBC of those milk samples was 3.35 ± 0.29 $\log_{10}$ cfu/mL, indicating that milk of good microbiological quality was produced. The TBC least squares means of milk samples from each bulk tank collected at 0 h (after first milking) was 3.54 ± 0.05 $\log_{10}$ cfu/mL. The similarity between the 2 TBC levels for those samples indicated that the precooling treatments did not affect the microbiological load and that milk of good microbiological quality entered each tank. The average (±SD) TBC of the milk line over 72 h of storage and the 2 trial periods was 3.55 ± 0.26 log cfu/mL. Good hygiene practices (e.g., teat preparation, individual cluster cleaning between milkings, and equipment wash routines) contributed to the high quality of the milk entering the bulk milk tanks.

The TBC levels at 0 h for NP, SP, and DP were 3.55, 3.57, and 3.50 ± 0.09 $\log_{10}$ cfu/mL, respectively; the PBC least squares means were 3.11, 3.04, and 3.07 ± 0.11 $\log_{10}$ cfu/mL; the LIP least squares means were 3.24, 3.26, and 3.28 ± 0.10 $\log_{10}$ cfu/mL; and the PROT least squares means were 3.20, 3.14, and 3.24 ± 0.07 $\log_{10}$ cfu/mL, respectively (Figure 2). The differences in the time required to cool the milk to 3°C (within the bulk tanks) were expected to affect those initial bacterial counts of the milk volumes; however, the bacterial counts were not significantly different ($P > 0.05$). The NP, SP, and DP treatments precooled the milk to average (±SD) temperatures of 32.0 ± 1.4°C, 17.0 ± 2.8°C, and 6.0 ± 1.1°C, respectively; and the average time taken to cool milk to 3°C within the bulk milk tanks on the first morning milking on each week was approximately 2 h, 1 h, and 20 min, respectively. Given the low initial bacterial counts, the difference in these bulk tank-cooling times was not sufficient to result in different bacterial levels at 0 h.

The different precooling treatments also did not affect any of the bacterial counts over the storage time up to 72 h ($P > 0.05$, Table 1). The volume of milk stored at 3°C increased in each bulk tank after each milking, resulting in a decrease in the blend temperature within the tanks over time. After the first 2 milking occasions, the milk volume produced at subsequent milkings blended with a higher volume of milk previously cooled to 3°C; consequently, the milk was cooled faster than that from the first 2 milking events. Therefore, the maintenance of low temperatures within the bulk tanks did not allow for significant increases in bacterial num-

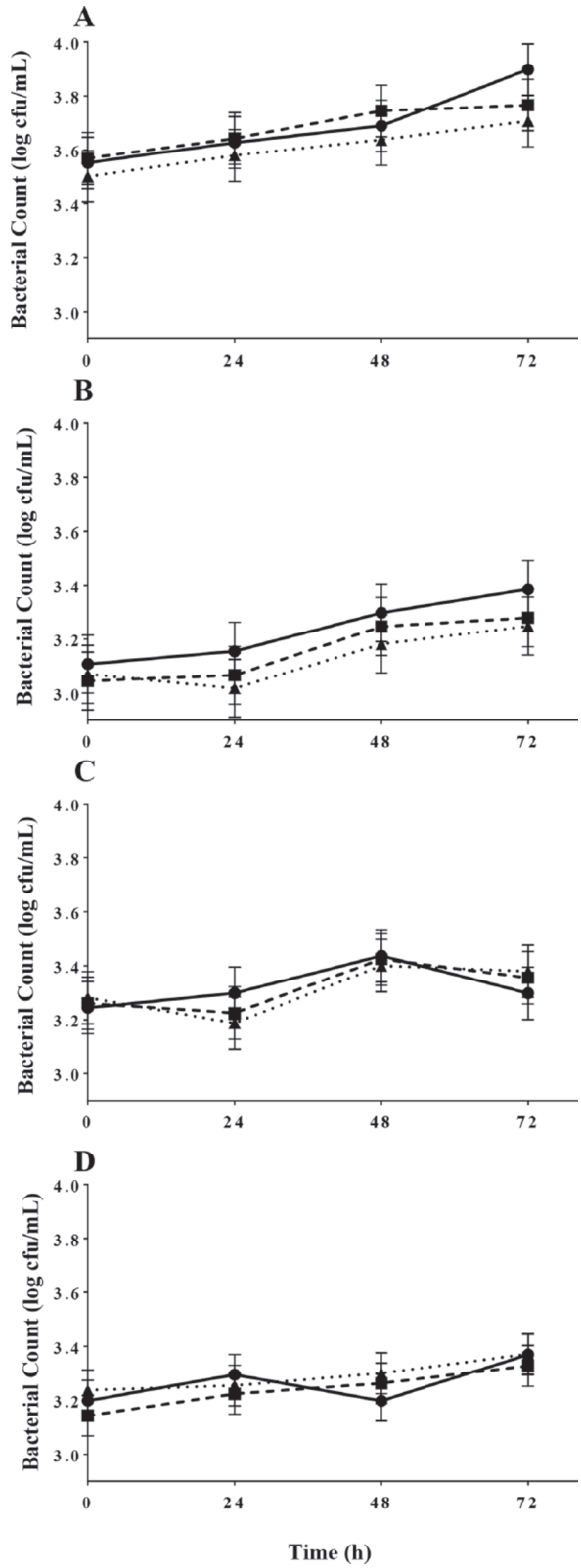

Figure 2. Effect of storage time and different precooling systems (● no plate cooler; ■ single-stage plate cooler; and ▲ double-stage plate cooler) on (A) total, (B) psychrotrophic, (C) lipolytic, and (D) proteolytic bacterial counts (±SD) in milk stored for 72 h.

Table 1. Significance of the main effects of period (2 × 3 wk), week (6 wk), storage time (72 h), and precooling systems (no plate cooler, single-stage plate cooler, and double-stage plate cooler), as well as the interaction between storage time and precooling system on bacterial counts of milk samples

	P-value				
Bacterial count[1]	Period	Week	Storage time	Precooling system	Precooling system × Storage time
TBC	0.23	0.02	0.004	0.61	0.93
PBC	0.002	0.18	<0.001	0.68	0.99
LIP	0.08	0.005	0.05	1.00	0.96
PROT	0.30	0.05	0.03	0.77	0.92
LPC	0.82	0.42	0.20	0.71	0.38
THERM	0.79	0.08	0.70	0.12	0.69

[1]TBC = total bacterial count, PBC = psychrotrophic bacterial count, LIP = lipolytic bacterial count, PROT = proteolytic bacterial count, LPC = thermoduric (laboratory pasteurization) bacterial count, and THERM = thermophilic bacterial count.

bers in the milk; consequently, the precooling system had no significant effect on bacterial counts over time. However, after 72 h, we observed a numerical difference between the bacterial counts in milks subjected to different precooling treatments (Figure 2). After 72 h, TBC and PBC (least squares means) in milk that was not precooled were 3.90 ± 0.09 and 3.38 ± 0.11 $\log_{10}$ cfu/mL, respectively. The SP milk precooled had TBC and PBC (least squares means) of 3.77 ± 0.09 and 3.28 ± 0.11 $\log_{10}$ cfu/mL, and that precooled using DP had similar TBC and PBC: 3.71 ± 0.09 and 3.25 ± 0.11 $\log_{10}$ cfu/mL, respectively.

Storage time affected TBC, PBC, and PROT (P = 0.004, $P < 0.001$, and $P = 0.03$, respectively, Table 1), which were 3.54 ± 0.05, 3.07 ± 0.06, and 3.19 ± 0.04 $\log_{10}$ cfu/mL at 0 h; and 3.79 ± 0.05, 3.30 ± 0.06, and 3.36 ± 0.04 $\log_{10}$ cfu/mL after 72 h, respectively (least squares means across precooling treatments). The increases in TBC and PBC were not considered biologically relevant because both were well below the European thresholds determined in document EC no 853/2004 [European Commission, 2004; TBC: 5.00 $\log_{10}$ cfu/mL (1.00×10^5 cfu/mL); PBC: 4.22 $\log_{10}$ cfu/mL (16,666 cfu/mL)] and typical TBC limits applied by some milk processors [e.g., 4.70 $\log_{10}$ cfu/mL (5.00×10^4 cfu/mL)]. The least squares means of PROT and LIP levels at 72 h (3.36 ± 0.04 $\log_{10}$ cfu/mL and 3.34 ± 0.06 $\log_{10}$ cfu/mL, respectively) were also not considered relevant as both were well below the limit suggested by Vyletelova et al. [2000; 4.65 $\log_{10}$ cfu/mL (44,668 cfu/mL), for each]; those authors suggested that LIP and PROT should be below this level to avoid the production of heat-resistant hydrolytic enzymes when milk is destined for dairy product manufacture, because such enzyme activities could result in loss of milk functional properties and sensory defects.

The PBC was different between the 2 trial periods (1 and 2; $P = 0.002$, Table 1), whereas TBC and LIP varied between weeks ($P = 0.02$ and $P = 0.005$, respectively, Table 1). Variations in bacterial population in milk at different periods could be related to the cows' health status (e.g., mastitis) or different bacteria strains present in the cows' environment (e.g., feed; Lafarge et al., 2004).

The LPC and THERM counts did not differ between periods and weeks, and storage time and precooling systems did not affect their levels ($P > 0.05$, Table 1). At 0 and 72 h, the least squares means of LPC were 0.80 and 0.83 ± 0.11 $\log_{10}$ cfu/mL, whereas THERM counts were 0.85 and 0.64 ± 0.13 $\log_{10}$ cfu/mL, respectively. A typical industry LPC specification can range from 2.70 to 3.00 $\log_{10}$ cfu/mL (500 to 1,000 cfu/mL), although there are no European legislation thresholds or dairy processor specifications for thermophilic bacteria in milk. According to Byrne and Bishop (1991), some species of *Micrococcus* do not grow well on Petrifilm, although those authors concluded that Petrifilm aerobic count plates are a suitable alternative to agar plates for determination of LPC. The SRC levels varied between 0 and 1 $\log_{10}$ cfu/mL (10 cfu/mL), indicating a low level of contamination with those organisms. Because of the low incidence, we could not determine the influence of storage and production conditions on these bacteria.

The milk volumes stored in the 3 tanks had low bacterial growth rates, indicating that the storage temperature was effective in preventing an increase in bacterial numbers in the milk over the storage period. O'Connell et al. (2016) stored milk in bulk milk tanks for over 96 h at 2 or 4°C and observed similar results to this study. In that study, milk stored at 2 or 4°C for over 72 h had average TBC, PBC, PROT, and LIP of 3.58, 3.11, 2.94, and 2.91 $\log_{10}$ cfu/mL, respectively. As well as the storage temperature, the initial microbial load of the milk will influence the microbial load over storage (Guinot-Thomas et al., 1995). Therefore, milk entering the tank has to be of high microbiological quality to obtain bacterial counts similar to those obtained after the storage period in the present study. Thus, to minimize

bacterial growth in milk during storage, it is important that appropriate cleaning practices (for milking equipment and cows) be carried out during milking.

Composition and SCC

The average (±SD) fat, protein, lactose, and TS contents of the sample collected from the milk line after the first milk occasion (first day of each trial week) were 4.52 ± 0.26, 3.58 ± 0.09, 4.76 ± 0.18, and 13.36 ± 1.93%, respectively. After the first morning milking, the milk samples (0 h) precooled at different rates had average (±SD) fat, protein, lactose, and TS contents similar to those in the milk line sample: 3.49 ± 0.09, 3.63 ± 0.06, 4.81 ± 0.06, and 12.53 ± 0.10% (NP treatment); 3.48 ± 0.16, 3.62 ± 0.05, 4.82 ± 0.06, and 12.52 ± 0.17% (SP treatment); and 4.14 ± 0.22, 3.59 ± 0.07, 4.76 ± 0.08, and 13.12 ± 0.21% (DP treatment), respectively. These results, compared with the milk line results, indicate that the precooling treatments did not affect the milk composition as would have been expected. The differences in the fat contents noted could be due to fat distribution when sampling.

The precooling treatments had no effect on milk composition ($P > 0.05$), and storage time did not affect fat, protein, or TS content ($P > 0.05$). After the 2 milking occasions on the first day, the contents of fat, protein, lactose, and TS (least squares means) were 4.41 ± 0.06, 4.59 ± 0.08, 5.78 ± 0.05, and 13.35 ± 0.07%, and after 72 h (6 milkings) were 4.44 ± 0.06, 4.58 ± 0.08, 5.79 ± 0.05, and 13.41 ± 0.07%, respectively. The protein contents (least squares means) were different in the 2 periods (period 1: 3.55 ± 0.002%; period 2: 3.63 ± 0.002%, $P = 0.0001$) and between weeks ($P = 0.02$), ranging from 3.54 to 3.72%. The lactose content was also different between periods (period 1: 4.82 ± 0.002%; period 2: 4.74 ± 0.002%, $P = 0.007$). As fresh milk was transferred to the tanks every day, the composition of milk stored within the bulk tanks may have varied according to the content of components in the fresh milk added to the tank on each milking occasion. Those variations in milk composition could be related to cows' physiology or days in milking (Linn, 1988). Also, the interval between milkings can affect milk composition, influencing the TS content of milk collected during the morning and afternoon (Ayadi et al., 2004).

The SCC between periods were statistically different ($P = 0.003$); however, there was a marginal difference of 36.6×10^3 cells/mL between periods 1 and 2, which is probably not biologically relevant. Furthermore, SCC in both periods (period 1: 115.9×10^3 cells/mL; period 2: 152.5×10^3 cells/mL) were below the European Union legislation threshold (400×10^3 cells/mL).

Peptide Profiles

High-performance liquid chromatography was performed to determine whether precooling treatments would result in different peptide profiles after 72 h of storage, thus indicating proteolysis. The chromatograms presented in Figure 3A, B, and C are an average of the chromatograms obtained for all milk samples precooled using the NP, SP, and DP systems, respectively, over the 2 periods. The chromatograms indicated no difference between the initial peptide concentrations in milk volumes precooled at different rates (0 h), no increase in concentrations over time, and no appearance of peaks that characterize proteolysis. We also noted in those chromatograms the absence of peaks after 20 min, indicating the lack of plasmin action, which hydrolyses β-, α_{S1}-, and α_{S2}-caseins into peptides and proteose-peptones (Crudden et al., 2005). Therefore, the application of different precooling treatments did not affect proteolysis levels in the milk.

The peptide peak at 15 min (unknown) is the only peptide whose concentration varied over 72 h and we observed only small differences between treatments for that peak. However, the variation in the concentration of this peptide could be caused by the addition of fresh milk to the tanks, the composition of which could vary, as previously mentioned. The low levels of proteolysis observed might be due to the low levels of proteolytic bacteria in the milk, which did not reach a level sufficient for significant production of proteolytic enzymes (4.65 $\log_{10}$ cfu/mL; Vyletelova et al., 2000). The low storage temperature applied over the storage period could have been effective in reducing the growth rate of proteolytic bacteria and is far from the optimum temperature for most enzymes (Kelly and Fox, 2006).

Energy Consumption

When the NP and SP treatments were used, the average energy usage (±SD) of the bulk tanks was 16.8 ± 2.7 and 10.6 ± 1.3 Wh/L of milk, respectively. The energy usage for the NP treatment was higher than that for the SP treatment because milk entered the bulk tank at a higher temperature (32.0 ± 1.4°C) and the compressor running time was longer to achieve the required storage temperature. For the DP treatment, the average energy usage (±SD) of the bulk milk tank and ice bank was 4.0 ± 0.5 and 13.6 ± 0.2 Wh/L of milk, respectively (total energy usage was 17.6 ± 0.5 Wh/L of milk).

Considering the similar bacterial counts between the precooling treatments and the energy usage of each treatment over the 72-h storage period, the SP system would achieve low levels of bacterial counts over storage

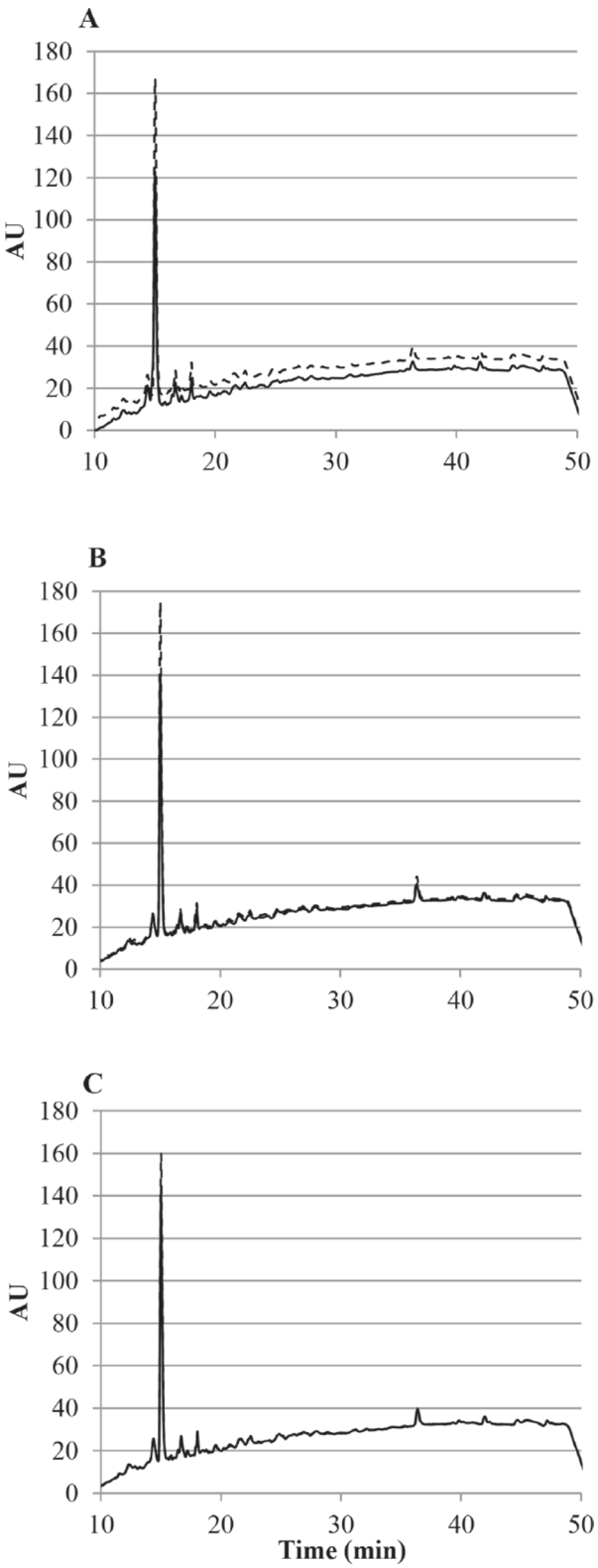

Figure 3. Separation of bovine milk peptides by reversed-phase HPLC (measured in absorbance units, AU). Chromatograms of samples precooled using (A) no plate cooler, (B) single-stage plate cooler, and (C) double-stage plate cooler and stored at 3°C are shown. Solid line = 0 h; dashed line = 72 h.

time and lower energy usage rates compared with the DP treatment. Energy usage was higher for the DP system than for the SP system because of the energy requirements to produce ice. However, this system could be recommended for farms in which an ice bank system is already being used for cooling milk within the bulk tank.

The energy consumption of each treatment is within the ranges reported by Shine et al. (2018), who surveyed 58 Irish commercial dairy farms regarding energy consumption at milking. In that study, the average (±SD) energy usages reported were 12.68 ± 5.20, 10.54 ± 2.55, and 14.94 ± 5.45 Wh/L for NP, SP, and DP systems, respectively. The variation in results between studies could be due to the age of the bulk tanks, the size of the tanks, and how they were installed. The ice bank energy usage in the present study was similar to average usage reported in a survey of 25 Irish commercial dairy farms (13.0 Wh/L; Murphy et al., 2013) and similar to the average value reported by Upton et al. (2013; 19.2 Wh/L, range: 16.0–21.8 Wh/L).

CONCLUSIONS

The microbiological load of milk precooled at different rates did not differ statistically at 0 h or over the 72 h of storage, indicating no significant difference between the precooling treatments. No technologically relevant variations were observed in milk composition, and no considerable enzymatic activity was observed, possibly because of the good microbiological quality of the milk. This study suggests that the bacterial count and composition of milk are minimally affected when milk is stored at 3°C for 72 h whether the milk is precooled or not; however, milk entering the tank should have good initial microbiological quality. Regarding energy usage, the SP treatment required less energy than the other treatments to maintain an equivalent microbiological load in milk. Considering that the milk volumes undergoing the SP and DP treatments had the lowest bacterial counts over 72 h of storage, it may be beneficial and economical to incorporate the DP system on farms that already use an ice bank bulk milk tank and SP system on other farms. Precooling good quality milk with an SP or DP system and subsequent storage at 3°C for 72 h can maintain good microbiological and compositional quality of milk with reduced energy consumption.

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Microbiological quality of milk from farms to milk powder manufacture: an industrial case study

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Abstract

The experiments reported in this research paper aimed to track the microbiological load of milk throughout a low-heat skim milk powder (SMP) manufacturing process, from farm bulk tanks to final powder, during mid- and late-lactation (spring and winter, respectively). In the milk powder processing plant studied, low-heat SMP was produced using only the milk supplied by the farms involved in this study. Samples of milk were collected from farm bulk tanks (mid-lactation: 67 farms; late-lactation: 150 farms), collection tankers (CTs), whole milk silo (WMS), skim milk silo (SMS), cream silo (CS) and final SMP. During mid-lactation, the raw milk produced on-farm and transported by the CTs had better microbiological quality than the late-lactation raw milk (e.g., total bacterial count (TBC): 3.60 ± 0.55 and 4.37 ± 0.62 log 10 cfu/ml, respectively). After pasteurisation, reductions in TBC, psychrotrophic (PBC) and proteolytic (PROT) bacterial counts were of lower magnitude in late-lactation than in mid-lactation milk, while thermoduric (LPC—laboratory pasteurisation count) and thermophilic (THERM) bacterial counts were not reduced in both periods. The microbiological quality of the SMP produced was better when using mid-lactation than late-lactation milk (e.g., TBC: 2.36 ± 0.09 and 3.55 ± 0.13 cfu/g, respectively), as mid-lactation raw milk had better quality than late-lactation milk. The bacterial counts of some CTs and of the WMS samples were higher than the upper confidence limit predicted using the bacterial counts measured in the farm milk samples, indicating that the transport conditions or cleaning protocols could have influenced the microbiological load. Therefore, during the different production seasons, appropriate cow management and hygiene practices (on-farm and within the factory) are necessary to control the numbers of different bacterial groups in milk, as those can influence the effectiveness of thermal treatments and consequently affect final product quality.

Bovine milk is used to produce a wide range of dairy products and nutritional ingredients. Each dairy product has to conform with specific quality parameters determined by regulatory authorities and international markets, which could be related to safety, nutritional value, physical and sensory characteristics. Bacterial numbers in milk are one of the main factors that can impact those parameters, and their control throughout processing is essential to achieve dairy products of high quality (Kable *et al.*, 2016). The first stage of the milk supply chain is the farm, where factors such as cow management, stage of lactation and equipment cleaning protocols can affect bacterial numbers in milk (O'Connell *et al.*, 2015). A variety of microorganisms could grow in milk, including: mesophilic, psychrotrophic, lipolytic, proteolytic, thermoduric and thermophilic bacteria, as well as pathogenic bacteria. Huck *et al.* (2008) observed that some spore-forming bacteria (*Bacillus*, *Paenibacillus* and *Sporosarcina*) were identified throughout the processing stages of fluid milk production, from the farm to the packaged product, suggesting that multiple potential entry points for those bacteria into milk are at the farm. Therefore, the production of raw milk under appropriate hygienic conditions is critical to control bacterial numbers, as thermal treatments during dairy processing cannot always completely reduce the bacterial load.

Several studies have focused on quantifying and identifying bacterial types in raw milk on-farm and their effect on dairy products (Barbano *et al.*, 2006; Quigley *et al.*, 2013*a*; Murphy *et al.*, 2016). However, the combined influence of farm practices, storage conditions, transport and processing conditions on the microbiological quality of final product is not well understood and further investigations are necessary. Kable *et al.* (2016) reported that the microbiota in collection tankers (CTs) can be highly diverse and differ according to season. This diversity may be attributed to contributing on-farm factors, such as cattle skin, bedding, feed, human handling, milking equipment, and on-site bulk tanks used for storage. Thus, each individual supplier could impact differently on the levels of different bacterial groups in the

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milk within CTs that collect milk from multiple farms. When milk is collected from farm bulk tanks, it is still prone to further increases in bacterial populations, which can arise due to inappropriate equipment sanitation and storage conditions or processing parameters that are favourable for rapid bacterial multiplication (Teh *et al.*, 2011; Cherif-Antar *et al.*, 2016). Therefore, dairy processors have to adopt good manufacturing practices and monitor several critical control points throughout the manufacturing processes to guarantee food safety and conformity with legislation or specifications. For example, one of the challenges regarding equipment sanitation concerns heat-resistant spore-forming bacteria. These bacteria can develop cleaning-resistant biofilms on the interior surfaces of pipelines or equipment, enabling cross-contamination of finished products (Jindal *et al.*, 2016). Processing parameters could also have an impact on bacterial load, especially thermal treatments. For example, the temperature programme and holding time during pasteurisation should be appropriate to reduce the microbial load and the number of viable pathogens in milk (Tucker, 2015).

The objective of this study was to monitor the microbiological quality of milk throughout the processing of low-heat skim milk powder (SMP), from individual farm bulk tanks to the final powder produced, during mid- and late-lactation periods, addressing the hypothesis that stage of lactation and/or environmental factors related to time of year will influence microbiological quality. This study will aid in determining the association between the quality of milk and subsequent SMP produced, as well as the impact of processing parameters on milk and SMP quality. To our knowledge, this is the first such study that tracked milk quality from individual farms to final product.

Materials and methods

Milk collection and skim milk powder manufacture

This study was conducted on commercial dairy farms and in a milk powder processing plant, which produced SMP only using the milk supplied by the farms involved in this study. This experiment was carried out during the mid- and late-lactation periods (May 2016 and December 2016, respectively), which corresponded to spring and winter in Ireland. During those periods, cows were grazing outdoors and housed indoors, respectively. The dairy farms involved in this study were located in the Kilkenny and Waterford regions of Ireland. During mid-lactation, 67 Irish dairy farms supplied sufficient milk to the factory to undertake the manufacturing process; during late-lactation, 150 dairy farms were necessary, due to the lower milk yield per cow during that period. During mid- and late-lactation, the average (±SD) milk volume collected from each farm was 4418 ± 3,066 l and 1786 ± 1,905 l, respectively. Collection tankers ($n = 11$) transported a total of 296 003 l and 267 932 l of milk to a commercial SMP factory during mid- and late-lactation, respectively. Those volumes were stored in a whole milk silo (WMS) within the factory. Subsequently, the milk was pasteurised by applying a high temperature/short time (HTST) treatment (75 °C, 25 s). After pasteurisation, the cream was separated and stored in the cream silo (CS), while the skim milk was stored in the skim milk silo (SMS). The skim milk was evaporated in a triple-effect evaporator and afterwards underwent spray-drying process. Approximately 22 000 kg of low-heat SMP were produced during both lactation periods that this study was carried out. Further details regarding the processing parameters are described in the supplementary material.

Sampling procedure

During mid- and late-lactation, samples were collected from the top inlet of the 67 and 150 farm bulk tanks, respectively, using sterilised sample dippers. On arrival at the processing plant, samples were collected from the top inlet of each CT ($n = 11$) using sterilised dippers. Samples were also collected from the top and bottom sampling ports of both WMS and SMS using industrial syringes. Additionally, in late-lactation, cream samples were collected from the top and bottom of the CS using industrial syringes, as that cream was produced only using the milk supplied by the 150 farms. All silo samples were collected after the whole milk, skim milk or cream was completely transferred to the respective silos. Additionally, three 25-kg SMP bags were collected within the factory at the start, middle and final stages of the spray-dryer run, giving a total of 9 bags. Powder samples were reconstituted using deionised water (1:10 dilution).

All samples collected in mid-lactation and samples from the factory collected during late-lactation (CT, WMS, CS, SMS and SMP samples) were analysed in the milk quality laboratory in Teagasc Moorepark (Fermoy, Co. Cork, Ireland). Due to the high number of farm milk samples collected in late-lactation, those samples were analysed at the laboratory in the factory. A schematic drawing of the SMP manufacturing process is shown in supplementary Fig. S1, as well as the sampling points.

Microbiological analysis

All samples collected during mid-lactation and the CT, WMS, CS, SMS and SMP samples collected during late-lactation were tested in duplicate for a range of bacterial species. All the microbiological analyses were performed according to the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004). Total (TBC), psychrotrophic (PBC), thermoduric (Laboratory Pasteurisation Count—LPC) and thermophilic (THERM) bacterial counts were measured using Petrifilm aerobic count plates (ready to use media; 1 ml of diluted sample on each plate) (3M, Technopath, Tipperary, Ireland), in accordance with the procedures described by Laird *et al.* (2004). The LPC test consisted of pasteurising the milk samples at 63 °C for 35 min, including time to allow samples to reach the required temperature (Frank and Yousef, 2004); afterwards, the samples were cooled to 10 °C using iced water before testing. Samples tested for TBC and LPC were incubated for 48 h at 32 °C, while samples tested for THERM were incubated for 48 h at 55 °C. The Petrifilms corresponding to the PBC test were incubated for 10 d at 7 ± 1 °C (Frank and Yousef, 2004). The authors are aware that using Petrifilm at 7 or 55 °C is outside the validated temperature range for that media. However, a pre-trial experiment for THERM indicated that, at the same dilution, plate count agar plates were uncountable due to bacterial colonies spreading over the surface of agar plates, whereas Petrifilm plates were countable (data not shown). Regarding PBC, other studies have been using Petrifilm for that test at 7 °C (Ramsahoi *et al.*, 2011). A Petrifilm Plate Reader (3M, Technopath, Tipperary, Ireland) was used to assess the number of bacterial colonies.

The proteolytic bacterial count (PROT) test consisted of spread plating the diluted sample (100 µl) on calcium caseinate agar with added skim milk powder (Merck, Darmstadt, Germany). Plates were incubated at 37 °C for 48 h. Proteolytic bacterial colonies were identified as colonies surrounded by a clear zone in an opaque medium.

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The TBC of the 150 farm milk samples collected during late-lactation were analysed within the factory using a MilkoScan FT2 system (Foss Electric, Hillerød, Denmark).

Statistical analysis

The statistical analyses were performed using the software SAS 9.3 (SAS Institute, 2016). The bacterial counts means (TBC, PBC, PROT, LPC and THERM) of each CT were predicted using the volume and bacterial count measured in the milk of all farms that supplied each CT. The same bacterial counts were predicted for the WMS using the volume and bacterial counts measured in the milk of all CTs that supplied that silo. Those predictions were calculated as volume weighted means with estimated confidence interval. The actual bacterial counts measured in each CT and WMS samples were compared to the respective confidence interval for those predicted means of the bacterial counts. Agreement plots were also used to check for bias in the relationship between actual and predicted bacterial count means. There were insufficient numbers of samples from the factory (WMS, SMS and SMP samples) to determine the statistical differences between the bacterial counts measured in those samples. Therefore, only numerical differences between those samples were reported in this research paper to indicate the possible variations in bacterial load throughout the process. This study was performed once during each mid- and late-lactation periods.

Results

Mid-lactation study

The mean bacterial counts (TBC, PBC, PROT, LPC and THERM) of the samples from the farm bulk tanks, CTs, WMS, SMS and samples of SMP, which were collected during the mid-lactation period, are shown in Table 1. Small increases were observed when comparing all mean bacterial counts of the farm bulk tanks and CTs (Table 1). Pronounced increases in the TBC, PBC and PROT were observed in the WMS samples when compared to the CT samples (Table 1). The mean TBC, PBC and PROT were lower in the SMS samples compared to the WMS samples; however, the LPC and THERM levels were not different from each other (Table 1).

The comparisons between the actual bacterial counts of each CT sample with the respective confidence interval for the predicted means, which were calculated considering the volume and bacterial count of each farm's milk supplied to each CT, are shown in supplementary Table S1. The TBC, PBC, PROT, LPC and THERM of two, three, one, two and four CT samples, respectively, were not within the respective confidence intervals. The comparisons between the actual bacterial counts of the WMS samples and the respective confidence interval for the predicted means, which were calculated considering the volume and bacterial count of each CT milk supplied to the silo, are shown in Supplementary Table S2. The mean TBC, PBC, PROT and THERM of the WMS samples were not within the respective confidence intervals.

Late-lactation study

The mean bacterial counts (TBC, PBC, PROT, LPC and THERM) of the samples from the farm bulk tanks, CTs, WMS, CS, SMS and samples of SMP, that were collected during late-lactation period, are shown in Table 1. The mean TBC of the CT samples was higher than the mean TBC of the farm milk samples. The mean TBC, PBC and PROT of the WMS samples were higher than the CT samples means. The mean TBC, PBC and PROT of the SMS samples were lower compared to the WMS samples, while their LPC and THERM levels were similar (Table 1).

The comparisons between the actual mean TBC measured in each CT sample with the respective confidence interval for the predicted means, which were calculated considering the volume and TBC of each farm milk supplied to each CT, are shown in the supplementary Table S3. The mean TBC of nine CT samples (1, 3, 5, 6, 7, 8, 9, 10 and 11) were not within the respective confidence intervals. The comparisons between the actual bacterial counts of the WMS samples with the respective confidence interval for the predicted means, which were calculated considering the volume and bacterial count of each CT milk supplied to the silo, are shown in Supplementary Table S2. The mean TBC, PBC and PROT of the late-lactation WMS samples were not within the respective confidence intervals.

Discussion

Production season or storage conditions can affect the bacterial counts of different types of microorganisms in milk, which can impact on the final quality of SMP. In mid-lactation, the mean TBC and PBC of the farm milk samples were below the European limits (EC no. 853/2004): 5.00 and 4.22 $\log_{10}$ cfu/ml, respectively. The TBC was also below the typical limit of 4.70 $\log_{10}$ cfu/ml applied by some Irish milk processors (Table 1). The mean PROT of the farm samples was below the limit suggested by Vyletelova *et al.* (2000) (4.65 $\log_{10}$ cfu/ml), at which proteolytic bacteria would produce high levels of heat-resistant proteases. The mean LPC of the mid-lactation farm milk samples was lower than the typical industry specifications, which can range from 2.70 to 3.00 $\log_{10}$ cfu/ml. Thermoduric and thermophilic bacterial colonies were not detected in 8 and 24 farm milk samples, respectively. In mid-lactation, some individual farm milk samples had TBC, PBC, PROT and LPC higher than the specified limits. However, considering that the milk volumes from all farms would be blended for processing, the comparisons between the weighted mean bacterial counts and the known specifications for raw milk indicated that good quality milk was delivered to the factory for processing in mid-lactation.

The mean TBC of late-lactation farm bulk tank milk samples was also lower than the European and industrial limits; however, 49 farm samples had TBC above those specifications. Statistical comparisons between the mean TBC of the farm samples collected during mid- and late-lactation were not possible, as the group of farms involved in the mid- and late-lactation studies were different and samples from those groups were analysed in different laboratories. However, the figures gave an indication that lower quality milk was produced in late-lactation. The variations in the counts of different bacterial types between lactation periods could be related to seasonal differences in bacterial strains in the environment, cow management, cows' health status (especially mastitis), on-farm hygiene practices, or milk storage conditions (Linn, 1988; Lafarge *et al.*, 2004).

In mid-lactation, the mean TBC, PBC, PROT and LPC of the CT milk samples were below the limits determined by the European legislation, industry and literature cited, while in late-lactation the mean TBC and PBC were higher than the European limits (Table 1). The TBC, PBC, PROT, LPC and

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Table 1. Mean (±SD) total bacterial count (TBC), psychrotrophic (PBC), proteolytic (PROT), thermoduric (LPC—Laboratory pasteurisation count) and thermophilic (THERM) bacterial counts of the samples collected from the farm bulk tanks, collection tankers (CTs), whole milk silo (WMS), cream silo (CS), skim milk silo (SMS) and samples of skim milk powder (SMP) from the mid- and late-lactation periods.

Mid-lactation bacterial counts (log_{10} cfu/ml)	Farm bulk tanks[a] (*n* = 67)	CT[a] (*n* = 11)	WMS (*n* = 2)	CS[b] (*n* = 2)	SMS (*n* = 2)	SMP [d] (*n* = 9)
TBC	3.60 ± 0.55 (2.65 to 4.90)	3.90 ± 0.40 (3.22 to 4.62)	5.89 ± 0.02		2.61 ± 0.20	2.36 ± 0.09 (2.26 to 2.50)
PBC	3.54 ± 0.65 (2.70 to 6.00)	3.70 ± 0.53 (2.74 to 5.97)	6.00 ± 0.00		2.00 ± 0.00	1.21 ± 0.15 (1.00 to 1.40)
PROT	3.50 ± 0.56 (3.00 to 5.10)	3.66 ± 0.29 (3.30 to 4.30)	5.72 ± 0.62		2.00 ± 0.00	1.36 ± 0.30 (1.00 to 1.70)
LPC	1.35 ± 0.33 (1.00 to 2.60) [e]	1.44 ± 0.28 (1.00 to 1.98)	1.58 ± 0.17		1.69 ± 0.07	2.45 ± 0.08 (2.30 to 2.51)
THERM	1.43 ± 0.47 (1.00 to 2.52) [e]	1.62 ± 0.35 (1.00 to 2.47)	2.02 ± 0.14		1.85 ± 0.10	3.63 ± 0.11 (3.50 to 3.79)
Late-lactation bacterial counts (log_{10} cfu/ml)	**Farm bulk tanks[a,c] (*n* = 150)**	**CT [a] (*n* = 11)**	**WMS (*n* = 2)**	**CS (*n* = 2)**	**SMS (*n* = 2)**	**SMP [d] (*n* = 9)**
TBC	4.37 ± 0.62 (3.60 to 7.16)	5.12 ± 0.53 (4.32 to 5.96)	5.84 ± 0.09	2.32 ± 0.09	5.00 ± 0.00	3.56 ± 0.08 (3.44 to 3.69)
PBC		5.25 ± 0.58 (4.15 to 5.97)	5.80 ± 0.04	1.15 ± 0.21	5.00 ± 0.00	2.07 ± 0.10 (1.90 to 2.19)
PROT		4.09 ± 0.72 (3.30 to 5.95)	4.68 ± 0.40	4.27 ± 0.27	2.52 ± 0.35	2.18 ± 0.26 (2.00 to 2.54)
LPC		2.60 ± 0.23 (2.35 to 2.99)	2.55 ± 0.03	2.33 ± 0.01	2.61 ± 0.17	3.51 ± 0.09 (3.33 to 3.62)
THERM		2.72 ± 0.19 (2.51 to 2.98)	2.74 ± 0.06	4.54 ± 0.01	2.63 ± 0.04	3.58 ± 0.09 (3.41 to 3.69)

[a]Weighted means calculated considering the volumes and bacterial counts of each farm or CT sample.
[b]Cream samples were not collected during mid-lactation.
[c]Only TBC was measured in the late-lactation farm milk samples.
[d]Bacterial counts in log_{10} cfu/g.
[e]Weighted means calculated not considering the samples in which those bacteria were not detected.
n = number of samples analysed in duplicate
Ranges are given between parentheses.

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THERM of the CTs milk were higher in late-lactation compared to mid-lactation, possibly due to the production of milk of inferior quality on-farm during that period. Also, the longer milk collection periods in late-lactation (approximately 8 h) could have contributed to the increased bacterial numbers in the CTs. The CT milk samples that had the bacterial counts higher than the upper confidence limit (mid-lactation: TBC, PBC, PROT, LPC and THERM; late-lactation: TBC; supplementary Tables S1 and S3) indicated that those bacterial numbers could have been influenced by the transport duration, CT cleaning protocol, temperature during transport or by the impact of individual farm suppliers (Kable *et al.*, 2016).

In both lactation periods, some of the bacterial counts measured in the WMS samples were higher than the respective upper confidence limits (mid-lactation: TBC, PBC, PROT and THERM; late-lactation: TBC, PBC and PROT; supplementary Table S2). The increase in those bacterial counts could be due to the conditions of the equipment in the milk transfer line (from the CT to the silo) (e.g., pump system and filters), non-effective silo clean-in-place routine, storage time or favourable storage temperature for the growth of some bacterial strains, or could be a result of blending raw milk from different origins and levels of contamination (Pinto *et al.*, 2006).

In mid- and late-lactation, the mean TBC of the WMS samples was higher than the limit determined for raw milk prior to processing (5.48 $\log_{10}$ cfu/ml; EC no. 853/2004). However, the temperature-time binomial applied during pasteurisation (75 °C, 25 s) reduced the TBC, PBC and PROT, as observed in the SMS samples (Table 1). In both lactation periods, pasteurisation was not efficient in reducing the LPC and THERM, when comparing the figures obtained for the WMS and SMS samples (Table 1), as those bacterial types are capable of surviving the temperatures applied in thermal treatments (Delgado *et al.*, 2013; Quigley *et al.*, 2013*b*). Thermoduric bacteria are able to survive pasteurisation temperatures (above 63 °C), while thermophilic bacteria are able to survive and grow at 55 °C or above (Frank and Yousef, 2004). The decreases in TBC and PBC after pasteurisation were of lower magnitude in late-lactation than in mid-lactation (Table 1), indicating that milk may contain higher numbers of heat-resistant bacteria strains during winter. Furthermore, in late-lactation, the THERM levels were higher in the CS samples compared to the WMS and SMS samples (Table 1). Given that cream separation occurred after pasteurisation, the relative abundance of thermophiles in pasteurised whole milk was possibly higher than prior to pasteurisation. Thermophilic bacteria could have migrated with the fat globules due to density (Graham, 2004) or the high levels could be related to the cleaning of the silos, as the persistence of thermophilic bacteria is related to the formation of biofilms (Burgess *et al.*, 2010).

Mid-lactation raw milk had better microbiological quality than late-lactation milk, consequently, the SMP produced using mid-lactation milk had lower bacterial counts than that made from late-lactation milk (Table 1). Laboratory-based studies indicated that when TBC in milk is higher than 5.00 $\log_{10}$ cfu/ml, the solubility index of SMP can increase, as well as the free fat acid content, while the heat stability decreases (Muir *et al.*, 1986; Celestino *et al.*, 1997). In relation to thermoduric and thermophilic bacteria, there are no European limits determined for milk powder. However, the SMP produced using mid- and late-lactation milk had THERM levels in accordance to the North American dairy industry requirements (less than 4.00 $\log_{10}$ cfu/g) (Wehr and Frank, 2004). Furthermore, it is likely that evaporation and spray-drying processes may have contributed to further reductions in TBC, PBC and PROT in the SMP in both periods.

This study highlights the importance of controlling bacterial levels in milk on-farm and during manufacturing, as processing parameters might not be able to reverse the negative effects of high bacterial levels, consequently compromising the quality of dairy products. For example, when in sufficient numbers, certain bacteria strains can produce lipases and proteases, which could not be eliminated in pasteurisation and could affect essential technological properties of milk for dairy products manufacture (Muir, 1996; Barbano *et al.*, 2006). Hygiene practices, cow management and processing parameters can affect the abundance of different bacterial types in milk; and therefore, those should be adequate to guarantee milk powder high quality and safety (Craven *et al.*, 2010; Watterson *et al.*, 2014).

In conclusion, this was the first study that monitored the quality of milk from farm bulk tank, through processing stages, to skim milk powder. We found evidence that stage of lactation and/or environmental factors related to time of year did influence microbiological quality, but the experimental design did not allow us to statistically validate the hypothesis. The effects of milk quality parameters on the quality of low-heat skim milk powder were observed, as well as how those parameters were affected throughout the manufacturing process. The good microbiological quality of the mid-lactation farm milk resulted in the production of milk powder with lower bacterial counts in contrast to the powder produced during late-lactation with milk of inferior quality. The season and/or stage of milk production had an influence on the abundance of different bacterial types in milk, which could impact the effectiveness of thermal treatments and consequently affect final product quality. Also, the differences in bacterial counts between production stages are indications of the growth potential of the bacteria in the milk, or even an indication of possible contamination sources in the specific production stage in which changes were observed. The results observed can aid industry in targeting sources of contamination throughout processing stages and practices to control bacterial numbers, in order to ensure the consistent production of safe high-quality dairy products throughout the year.

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Microbiological quality of milk from farms to milk powder manufacture: an industrial case study

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Short title: **Microbiological quality from farm to milk powder**

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SUPPLEMENTARY FILE

Materials & Methods

Milk collection and skim milk powder manufacture

The raw milk harvested during mid- and late-lactation were stored within the bulk tanks for an average (± SD) of 44 ± 11 h (range: 2 - 52 h) and 70 ± 19 h (range: 24 – 217 h) prior to tanker collection, at 3.1 ± 0.7 ˚C (range: 0.9 to 4.5 ˚C) and 3.3 ± 1.2 ˚C (range: 0.5 to 9.5 ˚C), respectively. During mid- and late-lactation, the milk volume collected from each farm ranged from 298 to 21,572 L and from 114 to 10,525 L, respectively. Each collection tanker (CT) collected milk from approximately 6 and 14 farms in mid- and late-lactation, respectively; and the temperature in the CTs ranged from 3.7 to 4.2 ˚C. The milk stored in the whole milk silo (WMS) was stored approximately 5.5 h (time between the transference of the first CT milk and the eleventh CT milk to the silo), at an average (± SD) temperature of 4.6 ± 0.2 ˚C, and agitated for 1 min every 29 min. The whole milk was pasteurised by applying a high temperature/ short time (HTST) treatment, during which the milk was heated to 75 ˚C for 25 s. After cream separation, the cream content in the skim milk was 0.075%. In the triple-effect evaporator the skim milk was concentrated from 9% w/w to 52% w/w of total solids content and the final moisture content was 48% w/w. The average moisture content of the skim milk powder (SMP) produced was 3.2 ± 0.2% w/w. The commercial processing plant in which this experiment was carried out detains further details regarding the processing parameters.

Sampling procedure

After agitation, 300-mL milk samples were collected from each farm bulk tanks, CTs, WMS, cream silo (CS) and SMS. All milk samples collected in mid-lactation and samples from the factory collected during late-lactation (CT, WMS, CS and SMS samples) were transported to the milk quality laboratory in Teagasc Moorepark in cooling boxes (<4 ˚C) within 6 h. After delivery, samples were sub-divided into 30-mL sterile bottles for microbiological analysis and analysed within 2 h. The milk samples were manually agitated to avoid unequal fat distribution.

In relation to the low-heat SMP samples, 100 g were taken from the top, middle and bottom of each bag; these were mixed to obtain a representative 300-g sample from each bag. These powder samples were reconstituted using deionised water (1:10 dilutions) and sub-divided into 30-mL sterile bottles for microbiological analysis.

Table S1. Comparison of mean total (TBC), psychrotrophic (PBC), proteolytic (PROT), thermoduric (laboratory pasteurisation count – LPC) and thermophilic (THERM) bacterial counts measured in each collection tanker (CT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) during mid-lactation and those predicted (± standard error; S.E.) from the combined farm samples in each CT.

Bacterial counts	CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean CT bacterial count (log_{10} cfu/ mL)	Predicted bacterial count (weighted means; S.E.)† (log_{10} cfu/ mL)	95% CI‡		Mean CT bacterial counts covered by predicted C.I.
							LCL	UCL	
TBC									
	1	4	23771	5,943 ± 1,271	3.99	3.93 ± 0.09	3.64	4.23	Yes
	2	5	26503	5,301 ± 2,385	4.38	3.7 ± 0.27	2.95	4.45	Yes
	3	6	29122	4,854 ± 1,763	3.90	3.82 ± 0.32	2.98	4.65	Yes
	4	6	23780	3,963 ± 2,683	4.18	3.64 ± 0.23	3.06	4.22	Yes
	5	8	27585	3,448 ± 2,214	3.88	3.51 ± 0.19	3.05	3.97	Yes
	6	7	28628	4,090 ± 1,208	4.15	3.57 ± 0.2	3.08	4.06	No
	7	7	27188	3,884 ± 2,064	4.62	3.87 ± 0.33	3.06	4.67	Yes
	8	7	28470	4,067 ± 2,437	3.64	3.9 ± 0.08	3.71	4.09	No
	9	2	27147	13,574 ± 11,312	3.22	3.03 ± 0.07	2.2	3.86	Yes
	10	5	25248	5,050 ± 3,877	3.45	3.27 ± 0.13	2.93	3.62	Yes
	11	10	28561	2,856 ± 1,764	3.54	3.35 ± 0.12	3.08	3.62	Yes
PBC									
	1	4	23771	5,943 ± 1,271	3.99	3.61 ± 0.28	2.71	4.51	Yes
	2	5	26503	5,301 ± 2,385	3.52	3.36 ± 0.18	2.86	3.87	Yes
	3	6	29122	4,854 ± 1,763	4.04	3.83 ± 0.33	2.97	4.68	Yes

4	6	23780	3,963 ± 2,683	3.56	3.51 ± 0.11	3.22	3.8	Yes
5	8	27585	3,448 ± 2,214	3.74	3.36 ± 0.25	2.76	3.95	Yes
6	7	28628	4,090 ± 1,208	3.80	3.45 ± 0.1	3.21	3.69	No
7	7	27188	3,884 ± 2,064	5.97	4.11 ± 0.54	2.78	5.45	No
8	7	28470	4,067 ± 2,437	3.60	3.97 ± 0.12	3.67	4.28	No
9	2	27147	13,574 ± 11,312	2.74	3.04 ± 0.04	2.48	3.6	Yes
10	5	25248	5,050 ± 3,877	3.23	3.35 ± 0.17	2.48	3.6	Yes
11	10	28561	2,856 ± 1,764	3.51	3.29 ± 0.11	3.04	3.55	Yes
PROT								
1	4	23771	5,943 ± 1,271	3.70	3.71 ± 0.15	3.24	4.17	Yes
2	5	26503	5,301 ± 2,385	3.70	3.61 ± 0.41	2.48	4.73	Yes
3	6	29122	4,854 ± 1,763	3.65	3.68 ± 0.27	2.98	4.38	Yes
4	6	23780	3,963 ± 2,683	3.98	3.61 ± 0.28	2.9	4.33	Yes
5	8	27585	3,448 ± 2,214	3.74	3.41 ± 0.15	3.05	3.76	Yes
6	7	28628	4,090 ± 1,208	3.30	3.67 ± 0.24	3.08	4.26	Yes
7	7	27188	3,884 ± 2,064	4.30	4.03 ± 0.26	3.39	4.67	Yes
8	7	28470	4,067 ± 2,437	3.40	3.33 ± 0.09	3.1	3.56	Yes
9	2	27147	13,574 ± 11,312	3.84	3.06 ± 0.12	1.52	4.61	Yes
10	5	25248	5,050 ± 3,877	3.30	3.05 ± 0.05	2.9	3.2	No
11	10	28561	2,856 ± 1,764	3.40	3.37 ± 0.1	3.14	3.6	Yes
LPC								

	1	4	23771	5,943 ± 1,271	1.54	1.21 ± 0.06	1.01	1.42	No
	2	5	26503	5,301 ± 2,385	1.18	1.35 ± 0.13	0.99	1.71	Yes
	3	6	29122	4,854 ± 1,763	1.00	1.07 ± 0.3	0.3	1.84	Yes
	4	6	23780	3,963 ± 2,683	1.48	1.34 ± 0.07	1.16	1.52	Yes
	5	8	27585	3,448 ± 2,214	1.98	0.79 ± 0.25	0.21	1.38	No
	6	7	28628	4,090 ± 1,208	1.30	1.24 ± 0.32	0.45	2.02	Yes
	7	7	27188	3,884 ± 2,064	1.60	1.12 ± 0.20	0.62	1.62	Yes
	8	7	28470	4,067 ± 2,437	1.18	0.96 ± 0.18	0.51	1.41	Yes
	9	2	27147	13,574 ± 11,312	1.70	0.48 ± 0.95	0	12.56	Yes
	10	5	25248	5,050 ± 3,877	1.70	1.44 ± 0.1	1.17	1.71	Yes
	11	10	28561	2,856 ± 1,764	1.30	1.26 ± 0.08	1.09	1.44	Yes
THERM									
	1	4	23771	5,943 ± 1,271	1.30	0.65 ± 0.34	0	1.73	Yes
	2	5	26503	5,301 ± 2,385	1.00	1.41 ± 0.19	0.88	1.94	Yes
	3	6	29122	4,854 ± 1,763	1.74	0.87 ± 0.32	0.03	1.7	No
	4	6	23780	3,963 ± 2,683	1.00	1.08 ± 0.35	0.17	1.99	Yes
	5	8	27585	3,448 ± 2,214	1.00	0.19 ± 0.15	0	0.56	No
	6	7	28628	4,090 ± 1,208	1.84	1.55 ± 0.33	0.73	2.37	Yes
	7	7	27188	3,884 ± 2,064	1.70	0.7 ± 0.3	0	1.44	No
	8	7	28470	4,067 ± 2,437	1.40	1.4 ± 0.12	1.12	1.69	Yes
	9	2	27147	13,574 ± 11,312	2.47	0.51 ± 1.0	0	13.15	Yes

10	5	25248	5,050 ± 3,877	1.95	0.73 ± 0.25	0.05	1.42	No
11	10	28561	2,856 ± 1,764	1.48	0.92 ± 0.28	0.28	1.55	Yes

†Weighted means were calculated considering the volume of milk supplied by each farm.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table S2. Comparison of mean total (TBC), psychrotrophic (PBC), thermoduric (laboratory pasteurisation count – LPC) and thermophilic (THERM) bacterial counts measured in the whole milk silo (WMS) during mid- and late-lactation and those predicted (± standard error; S.E.) from the combined collection tanker (CT) samples.

Stage of lactation	Bacterial count (log_{10} cfu/ mL)	Mean (± SD) bacterial count (WMS)	Predicted bacterial count (weighted means; S.E.)†	95% CI‡		Mean CT bacterial counts covered by predicted C.I.
				LCL	UCL	
Mid-lactation						
	TBC	5.89 ± 0.02	3.9 ± 0.13	3.62	4.18	No
	PBC	6.00 ± 0.00	3.7 ± 0.17	3.33	4.08	No
	PROT	5.72 ± 0.62	3.66 ± 0.09	3.45	3.87	No
	LPC	1.58 ± 0.17	1.46 ± 0.09	1.27	1.65	Yes
	THERM	2.02 ± 0.14	1.64 ± 0.11	1.39	1.88	No
Late-lactation						
	TBC	5.84 ± 0.09	5.1 ± 0.17	4.73	5.47	No
	PBC	5.80 ± 0.04	5.25 ± 0.18	4.84	5.66	No
	PROT	4.68 ± 0.40	4.09 ± 0.23	3.58	4.6	No
	LPC	2.55 ± 0.03	2.61 ± 0.07	2.44	2.77	Yes
	THERM	2.74 ± 0.06	2.73 ± 0.06	2.59	2.86	Yes

Mean (± SD) volume of milk measured per tanker in mid- and late-lactation were 26,909 ± 1,902 L and 24,357 ± 3,768 L, respectively.
†Weighted means were calculated considering the volume of milk supplied by each tanker.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table S3. Comparison of mean total bacterial counts (TBC) measured in each collection tanker (CT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) during late-lactation and those predicted (± standard error; S.E.) from the combined farm samples in each CT.

CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean TBC of each CT (log_{10} cfu/ mL)	Predicted TBC (weighted means; S.E.)† (log_{10} cfu/ mL)	95% CI‡		Mean TBC of each CT covered by predicted C.I.
						LCL	UCL	
1	15	25,743	1,716 ± 2,135	5.64	4.38 ± 0.16	3.95	4.66	No
2	7	19,853	2,836 ± 3,542	5.33	5.12 ± 0.32	4.35	5.89	Yes
3	8	23,460	2,933 ± 2,381	5.96	4.8 ± 0.34	4.0	5.6	No
4	13	24,221	1,863 ± 1,401	4.32	4.14 ± 0.08	3.96	4.33	Yes
5	10	24,274	2,427 ± 2,558	4.64	4.34 ± 0.12	4.06	4.61	No
6	14	24,729	1,766 ± 2,489	5.90	4.24 ± 0.25	3.71	4.77	No
7	19	28,583	1,504 ± 1,168	4.86	4.4 ± 0.08	4.23	4.56	No
8	27	28,322	1,049 ± 881	4.81	4.24 ± 0.08	4.08	4.4	No
9	18	27,606	1,534 ± 1,794	4.84	4.17 ± 0.11	3.93	4.4	No
10	8	15,774	1,972 ± 1,002	5.40	4.27 ± 0.13	3.95	4.59	No
11	13	25,367	2,306 ± 2,221	4.66	4.15 ± 0.06	4.02	4.29	No

†Weighted means were calculated considering the volume of milk supplied by each farm.
‡Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

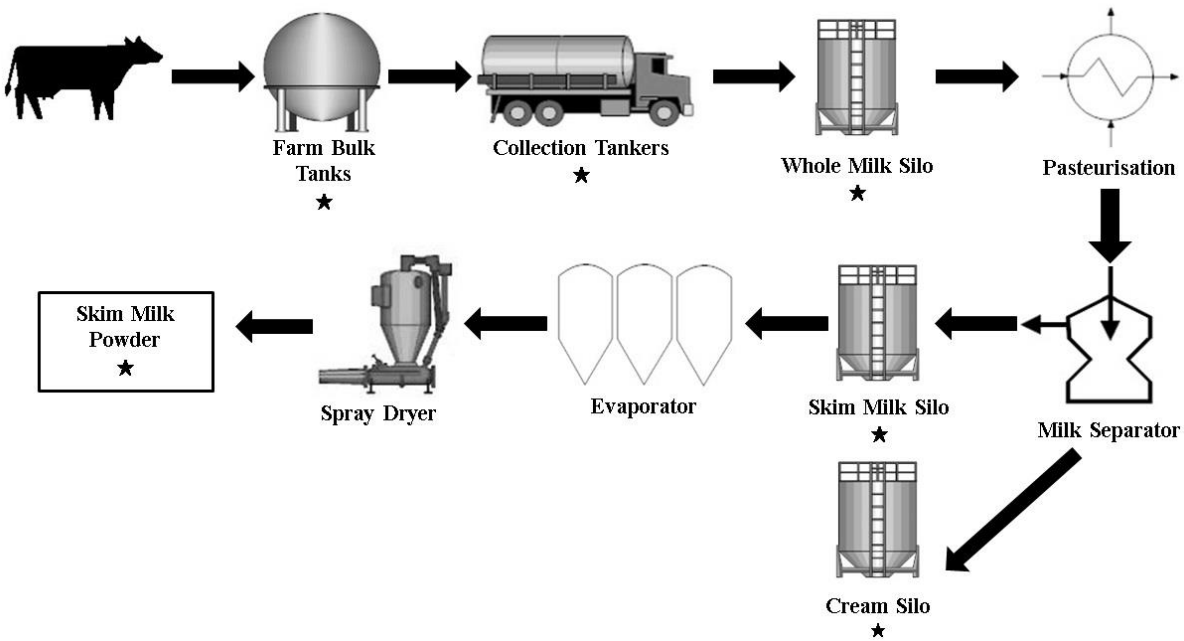

Figure S1. Milk supply chain and manufacturing process for conversion to low-heat skim milk powder, conducted in the mid- and late-lactation periods. The sampling points are indicated with a ★.

Journal of Dairy Research

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Monitoring residue concentrations in milk from farm and throughout a milk powder manufacturing process

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Research Article

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Abstract

The experiments reported in this research paper aimed to investigate differences in the levels of chlorate (CHLO), perchlorate (PCHLO), trichloromethane (TCM) and iodine residues in bulk tank (BT) milk produced at different milk production periods, and to monitor those levels throughout a skim milk powder (SMP) production chain (BTs, collection tankers [CTs], whole milk silo [WMS] and skim milk silo [SMS]). Chlorate, PCHLO and iodine were measured in SMP, while TCM was measured in the milk cream. The CHLO, TCM and iodine levels in the mid-lactation milk stored in the WMS were lower than legislative and industrial specifications (0.0100 mg/kg, 0.0015 mg/kg and 150 µg/l, respectively). However, in late-lactation, these levels were numerically higher than the mid-lactation levels and specifications. Trichloromethane accumulated in the cream portion after separation. Perchlorate was not detected in any of the samples. Regarding iodine, the levels in mid-lactation reconstituted SMP were higher than that required by manufacturers (100 µg/l), indicating that the levels in milk should be lower than 142 µg/l. The higher residue levels observed in late-lactation could be related to the low milk volume produced during that period and changes in sanitation practices, while changes in feed management could have affected iodine levels. This study could assist in controlling and setting limits for CHLO, TCM and iodine levels in milk, ensuring premium quality dairy products.

International markets are setting high specifications for milk and dairy product quality, including stringent guidelines on concentrations of residues that could occur in milk. Potential milk contaminants of most concern include chlorate (CHLO, ClO_3^-), perchlorate (PCHLO, ClO_4^-) and trichloromethane (TCM, $CHCl_3$), which arise as a consequence of sanitation with chlorine products.

Chlorate and PCHLO were reported to result in thyroid dysfunctions (EFSA, 2015), while TCM could possibly be carcinogenic to humans (ICAR, 1999). There are a few studies available that have discussed contributing factors on-farm (Gleeson *et al.*, 2013; Ryan *et al.*, 2013) but the dynamics of residue concentrations when subjected to different milk processing conditions are not fully understood. Sodium hypochlorite, chlorine gas or dioxide may be used for the sanitation of water, while chlorine-based detergents are used for the sanitation of milking or processing equipment. Chlorine products generally have good bactericidal properties and are widely used because of their effectiveness and low cost (Garcia-Villanova *et al.*, 2010). The decomposition of chlorine compounds results in the production of oxyhalide species (ClO^- and ClO_2^-), which react and form CHLO. Further reactions of CHLO with those oxyhalides result in the formation of PCHLO (Gordon and Tachiyashiki, 1991). Residual chlorine, CHLO or PCHLO on the surfaces of processing equipment can contaminate milk (Asami *et al.*, 2013). The contamination of infant formula with CHLO is a major concern due to the risk of intoxication in infants, which have lower tolerance than adults. The contact of chlorine with milk could also result in the formation of TCM (Tiefel and Guthy, 1997). Chlorinated hydrocarbons accumulate in fat-rich fractions, so products such as butter and cream could contain high concentrations of TCM if milk is contaminated with high levels (Hubbert *et al.*, 1996).

Excessive levels of residual iodine in raw milk are another concern in the Irish dairy industry, especially in the manufacture of infant formula. Iodine is an essential micronutrient for the synthesis of hormones by the thyroid gland (Leung and Braverman, 2014). Even though iodine is a nutrient of extreme importance to the human organism, the daily consumption of iodine at higher levels than recommended could result in dysfunctions of the thyroid gland. Bovine milk is one of the main sources of iodine for humans and its content depends on the daily iodine intake by dairy cows (Flachowsky *et al.*, 2014). The US National Research Council (2001) recommends that the daily iodine intake per cow should be 10 mg, which is the reference value applied in Ireland. The utilisation of rations with higher levels of iodine than

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required or overfeeding cows can result in excessive iodine concentrations secreted into milk. Over supplementation of Irish herds is of most concern during early and late-lactation and during winter milk production (O'Brien *et al.* 2013). O'Brien *et al.* (1999) recorded an average of 227 μg/l iodine in Irish milk, while concentrations of 510 and 180 μg/l were recorded for December and June, respectively. Those levels were not a food safety concern at the time. However, processors are currently requiring lower levels of iodine in raw milk destined for the production of infant formula, in order to meet requirements of the international market. Some Irish dairy processors require that raw milk should contain less than 150 μg/l of iodine. Other iodine sources in milk include mineral-added water, boluses, mineral licks and grass (Magowan *et al.* 2010). The use of iodine-based teat disinfectants could also contribute to iodine content in milk, as these products are absorbed through the teat skin if not completely removed prior to milking (Flachowsky *et al.*, 2014).

The first objective of this study was to investigate changes in the CHLO, PCHLO, TCM and iodine levels throughout the milk production chain, from farm to dairy product, in two different milk production periods (mid- and late-lactation). Chlorate, PCHLO and iodine were measured throughout the production stages of skim milk powder (SMP), while TCM was measured throughout the production stages of milk and cream, which were destined for butter manufacture. The second objective was to investigate differences in residue levels in bulk tank (BT) milk produced during mid- and late-lactation. The milk used in this study was produced on commercial dairy farms and processed in a commercial SMP processing plant.

Materials and methods

Sampling procedure at the farms and throughout a skim milk powder manufacturing process

In Ireland a seasonal spring-calving production system is practiced, with all cows calving within a 10-week period approximately (February to April). This experiment was performed on one occasion during each of mid- (May; 80 DIM) and late-lactation (December; 290 DIM). The farms that supplied milk to the factory (mid-lactation: 67 farms; late-lactation: 150 farms), milk storage conditions on-farm, amount of milk produced, milk collection and the skim milk powder manufacturing process was the same as described by Paludetti *et al.* (2019). A schematic drawing of the SMP manufacturing process is shown in supplementary Figure S1.

In mid-lactation, samples were collected at various points of the manufacturing process between the farm BTs and the SMP [BTs, collection tankers (CTs), whole milk silo (WMS), skim milk silo (SMS) and final SMP] and were tested for CHLO, PCHLO and iodine. In late-lactation, samples were collected at various points between the CTs and the SMP [CTs, WMS, cream silo (CS), SMS and final SMP] and were tested for CHLO, PCHLO and iodine. In both lactation periods, TCM was quantified in all samples, with exception of the SMP samples (supplementary Figure S1). Due to the high number of farms (150) necessary to supply sufficient milk volume to undertake the manufacturing process in late-lactation (December), it was not possible to undertake collection and analysis of all individual BT samples. The collection of samples and preparation of SMP samples for analysis were performed as described by Paludetti *et al.* (2019).

Comparison between the residue levels in the same 67 farm bulk tanks in mid- and late-lactation (May and November)

The concentrations of CHLO, PCHLO, TCM and iodine residues were measured in raw milk produced on the same 67 dairy farms sampled in mid-lactation (May, 80 DIM) and in late-lactation period (November; 260 DIM), to investigate the effect of milk production period on residue levels. Milk samples were collected as described by Paludetti *et al.* (2019).

Quantification of chlorate and perchlorate

The quantification of CHLO and PCHLO was performed by high-performance liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS) with ESI electrospray ionisation in negative mode (−ESI). The mid-lactation milk and SMP samples, as well as the 67 late-lactation farm BT samples, were analysed in the laboratory of Labor Friedle GmbH group (Labor Friedle GmbH, Von-Heyden-Straβe 11, D-93105, Tegernheim, Germany), while the late-lactation samples from the factory (CT, WMS, SMS and SMP samples) were analysed in Teagasc Ashtown (Dublin, Ireland). The methodologies used are based on the procedures described in the European Quick Polar Pesticides method (QuPPe) (EURL-SRM, 2015). In the present study, some of the milk samples were analysed by both laboratories and the results were statistically similar ($P > 0.05$). The detection limit of CHLO and PCHLO in milk was 0.0010 mg/kg and in SMP was 0.010 mg/kg.

Quantification of trichoromethane

Trichloromethane was quantified in the milk using static head-space gas chromatography (HS-GC) with electron capture detector (ECD), fitted with a low thermal mass system (LTM) (Agilent 7890A, Agilent Technologies, Santa Clara, California, USA). The trichloromethane detection limit in this analysis was 0.0001 mg/kg. The methodology applied was an adaption of the procedure of Resch and Guthy (1999). This analysis was performed in the Milk Quality laboratory in Teagasc Moorepark (Fermoy, Co. Cork, Ireland).

Quantification of iodine

Iodine was quantified in milk and reconstituted SMP samples using inductively coupled plasma mass spectrometry (ICP-MS), using an Agilent ICP-MS 7700x (Agilent Technologies, Santa Clara, California, USA). The methodology used was based on the procedures described in the standard method for the determination of iodine compounds in foodstuffs (BS EN 15111:2007, 2007). Standard solutions of Tellurium and 1% TMAH were used to obtain a calibration curve. The limit of detection was 1.31 μg/l. The mid-lactation milk and SMP samples were analysed in Teagasc Moorepark (Fermoy, Co. Cork, Ireland), while the late-lactation milk and SMP samples were analysed in FBA laboratories (Capoquinn, Co. Waterford, Ireland). Those laboratories used the same methodology and samples analysed by both laboratories had statistically similar results ($P > 0.05$).

Statistical analysis

Influence of individual farm milk volumes on the residue concentration in each CT and influence of CT milk on the residue concentration in WMS

The statistical analyses were performed using the software SAS 9.3 (SAS Institute, 2016). In mid-lactation, the iodine and TCM

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concentrations of each CT were predicted using the volume and iodine or TCM concentrations measured in the milk of all farms that supplied each respective CT. In mid- and late-lactation, the iodine and TCM concentrations in the WMS were also predicted using the volume and iodine or TCM concentrations in the milk of all CTs that supplied that silo. Those predictions were calculated as volume-weighted means with estimated confidence intervals. The actual iodine or TCM concentrations measured in each CT and WMS samples were compared to the respective confidence interval for those predicted means. Agreement plots were also used to check for bias in the relationship between actual and predicted means. It was not possible to perform the same analyses with the CHLO and PCHLO results, due to the low number of samples in which those residues were detected.

Comparison between the residue levels in the same 67 farm bulk tanks in mid- and late-lactation (May and November)

Differences between the adjusted least square means of the 67 mid- and late-lactation milk samples, collected in May and November, were calculated using the MIXED procedure in SAS 9.3 (SAS Institute, 2016). The fixed effects included in each model were lactation period (mid- and late-lactation) and farms (numbered from 1 to 67). Farms were considered the experimental unit and the response variable was iodine or TCM. Residual checks were made to ensure that the assumptions of the analysis were met.

It was not possible to statistically determine the differences between CHLO and PCHLO levels measured in mid- and late-lactation milk samples, due to insufficient number of samples in which those residues were detected. McNemar's test was applied to compare the number of BT milk samples in mid- and late-lactation that had CHLO and TCM concentrations ≥0.0010 and 0.0015 mg/kg, respectively. The GLM procedure was used to determine the regression relationship between CHLO and TCM concentrations.

Results

The mean CHLO, TCM and iodine concentrations of samples collected during mid- and late-lactation (May and December, respectively) throughout the milk powder production chain are shown in Table 1.

Chlorate and perchlorate

In mid-lactation (May), CHLO was detected in 14 of the 67 BT and 6 of the 11 CT samples. The weighted mean CHLO concentration was calculated at the basis of the milk volume supplied by those farms and CTs (Table 1). The volume-weighted mean CHLO concentrations of these farms and CTs were numerically similar. In late-lactation (December), CHLO was detected in 6 of the 11 CT samples also, but the volume-weighted mean of those samples was higher compared to mid-lactation (Table 1). In both mid- and late-lactation, the mean CHLO concentration in the WMS and SMS were numerically similar (Table 1).

The mean CHLO concentration of the SMP samples was higher in late-lactation (December) compared to mid-lactation (May) (Table 1). In both lactation periods, the CHLO concentration in powder increased approximately 50 times compared to the concentrations in SMS samples. In mid-lactation, the CHLO levels in the SMP samples decreased throughout the spray-dryer run. At the start, middle and end of the spray-drying process, the CHLO levels were: 0.0630 ± 0.0020, 0.0610 ± 0.0060 and 0.0470 ± 0.0020 mg/kg, respectively. In contrast, the CHLO concentration of the late-lactation SMP samples did not vary throughout the spray-dryer run (start: 0.124 ± 0.003 mg/kg; middle: 0.129 ± 0.011 mg/kg; end: 0.126 ± 0.006 mg/kg).

Perchlorate was not detected in any of the mid- and late-lactation samples collected throughout the manufacturing process.

Trichloromethane

Trichloromethane was detected in all BT and CT samples collected in mid-lactation (May) and in all CT samples collected in late-lactation (December). The volume-weighted mean TCM concentration of those samples was calculated considering the milk volume supplied by each BT or CT (Table 1). The volume-weighted mean TCM concentration of the CT milk samples was higher in late-lactation compared to mid-lactation. In mid-lactation, the volume-weighted mean TCM concentrations of the milk samples from the BTs and CTs were numerically similar. The mean TCM concentrations of the milk samples from the CTs and WMS were also numerically similar in both mid- and late-lactation.

The comparisons between the actual TCM concentration and the respective confidence interval for the predicted means for each mid-lactation CT sample, are shown in the Supplementary Table S1. The TCM concentrations in all of the mid-lactation CT samples were within their respective confidence intervals. A similar comparison for the mid- and late-lactation WMS samples is shown in the Supplementary Table S2. The TCM concentration in the WMS samples were also within their respective confidence interval in mid- and late-lactation.

In both lactation periods, the mean TCM concentration decreased in the SMS samples compared to the WMS samples, as expected (Table 1).

Iodine

In mid-lactation, the volume-weighted mean iodine concentration was numerically higher in the BT samples than in the CT samples. The volume-weighted mean iodine concentration of all of the CTs was numerically higher in late-lactation than in mid-lactation. In mid-lactation, the mean iodine concentrations in the CTs and WMS were similar, while in late-lactation, the mean concentration was numerically higher in the CTs compared to the WMS (Table 1). In both lactation periods, the iodine concentrations increased in SMS samples and, consequently, as levels were higher in late-lactation BT milk, the iodine concentration in SMP was higher in late-lactation than in mid-lactation.

The comparisons between the actual iodine concentrations of each mid-lactation CT sample with the respective confidence interval for the predicted means are shown in the Supplementary Table S3, while such comparison for the mid- and late-lactation WMS samples are shown in the Supplementary Table S4. All the iodine concentrations measured in each mid-lactation CT sample were within the respective confidence intervals, as well as the WMS samples collected in mid- and late-lactation.

Comparison between the residue levels in the same 67 farm bulk tanks in mid- and late-lactation (May and November)

The number of BT samples in which CHLO was detected was significantly higher in late-lactation (32 out of the 67 samples) than in mid-lactation (14 out of the 67 samples) ($P < 0.0001$). Also, in

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Table 1. Mean (± SD) chlorate (CHLO), trichloromethane (TCM) and iodine concentrations in samples collected from the farm bulk tanks (BTs), collection tankers (CTs), whole milk silo (WMS), skim milk silo (SMS), cream silo (CS) and samples of skim milk powder (SMP) from the mid- and late-lactation periods

	Mid-lactation		
	CHLO (mg/kg)	TCM (mg/kg)	Iodine (μg/l)
Farm BTs ($n = 67$)[a]	0.0021 ± 0.0019 (0.0010 to 0.0070)[b]	0.0009 ± 0.0008 (0.0002 to 0.0043)	142.2 ± 129.2 (10.4 to 561.2)
CT ($n = 11$)[a]	0.0020 ± 0.0010 (0.0010 to 0.0030)[c]	0.0009 ± 0.0003 (0.0006 to 0.0015)	134.2 ± 89.6 (58.3 to 390.8)
WMS ($n = 2$)	0.0010 ± 0.0000	0.0009 ± 0.0000	135.5 ± 7.6
SMS ($n = 2$)	0.0010 ± 0.0000	0.0002 ± 0.0000	142.1 ± 9.1
SMP ($n = 9$)	0.0570 ± 0.0090[d]		142.2 ± 10.0 (120.2 to 153.5)
	Late-lactation		
	CHLO (mg/kg)	TCM (mg/kg)	Iodine (μg/l)
CT ($n = 11$)[a]	0.0410 ± 0.0554 (0.0020 to 0.1550)[c]	0.0020 ± 0.0007 (0.0010 to 0.0033)	437.6 ± 155.2 (225 to 709)
WMS ($n = 2$)	0.0025 ± 0.0000	0.0018 ± 0.0000	419.0 ± 2.8
SMS ($n = 2$)	0.0025 ± 0.0000	0.0005 ± 0.0000	450.0 ± 7.1
CS ($n = 2$)		0.0190 ± 0.0000	
SMP ($n = 9$)	0.1263 ± 0.0071[d]		398.2 ± 22.8 (257 to 425)

[a]Weighted means and standard deviations calculated considering the volumes of milk and residues concentrations of each farm or CT sample.
[b]Weighted mean CHLO of the 14 bulk tank milk samples in which chlorate was detected.
[c]Weighted mean CHLO of the CT milk samples in which chlorate was detected (mid-lactation: 6 samples; late-lactation: 6 samples).
[d]Results for non-reconstituted skim milk powder n = number of samples; ranges are given between parentheses.

contrast to mid-lactation, 8 out of the 67 late-lactation BT samples contained 0.0010 mg/kg of PCHLO. The volume-weighted mean TCM concentration was significantly higher in late-lactation (0.0015 ± 0.0014 mg/kg; range: 0.0003 to 0.0074 mg/kg) than in mid-lactation (0.0009 ± 0.0008 mg/kg; range: 0.0002 to 0.0043 mg/kg) ($P < 0.0001$). The volume-weighted mean iodine concentrations of the BT samples in mid- and late-lactation (142.2 ± 129.2 and 119.7 ± 151.6 μg/l, respectively) were not statistically different ($P = 0.63$).

Discussion

Residues related to the use of chlorine

Concentrations of CHLO and PCHLO were monitored throughout the production chain of SMP in mid- and late-lactation (May and December, respectively). In Europe, a default threshold limit of 0.0100 mg/kg of CHLO and PCHLO is applied for milk (EU Regulation 396, 2005). In mid-lactation (May), the volume-weighted mean CHLO concentration in the 14 BTs and 6 CTs (in which CHLO was detected) were lower than that limit; however, in late-lactation (December), the mean CHLO concentration of the 6 CTs (in which CHLO was detected) was higher than the EC limit and higher than the volume-weighted mean concentration in mid-lactation.

In mid-lactation, the CHLO concentrations in each of the CTs could have been diluted as CHLO was not detected in 53 of the BTs. For example, CHLO was not detected in 4 CT milk samples, as only one of the BT milk volumes contributing to each of those CTs contained CHLO. Additionally, CHLO was not detected in most of the BT milk supplied to the 6 CTs in which CHLO was detected, indicating that the sanitation of those CTs could possibly have influenced the CHLO levels. In both mid- and late-lactation, as CHLO was not detected in most of the CT milk volumes, the CHLO concentrations could have also been diluted in the WMS; therefore, it is likely that the sanitation practices of the silos did not influence the CHLO levels. Consequently, the mean CHLO concentrations in the WMSs were lower than the EC limit of 0.0100 mg/kg. However, as the milk supplied to the factory during late-lactation contained higher levels of CHLO than the mid-lactation milk, the CHLO levels in the WMS in late-lactation were higher compared to mid-lactation; consequently, the CHLO levels in the SMP were higher in late-lactation than in mid-lactation.

In mid-lactation, the mean CHLO concentration of the SMP samples was lower than the limit applied by some Irish infant formula manufacturers (0.100 mg/kg). The difference of 0.0016 mg/kg between the mean CHLO concentration of the SMP samples collected at the end and start of the spray-drying run, indicated that the sanitation of the spray-dryer could have contributed to the CHLO levels in SMP. The interior surface of the spray-dryer could have contained residual CHLO, and the majority of that residue was transferred to the first batch of evaporated skim milk that entered the equipment. In late-lactation, the mean CHLO concentration of the SMP samples was higher than 0.100 mg/kg, indicating that the CHLO level in the bulk milk stored in the WMS should had been lower than 0.0025 mg/kg. Even though no variations in the CHLO concentration were observed in SMP samples collected throughout the spray-drying run in late-lactation, sanitation practices of that equipment could have also contributed to the increased CHLO levels in SMP. Additionally, the variations in CHLO concentrations throughout the spray-drying run that were observed in mid-lactation and not observed in late-lactation could be due to differences in the sanitation practices between production periods.

The concentrations of TCM were also monitored throughout the production chain of SMP in mid- and late-lactation (May and December, respectively). There are no European regulations that have defined a standard TCM limit for milk or dairy products; however, Irish dairy processors apply a limit of

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0.0015 mg/kg to milk destined for the production of lactic butter which should have less than 0.0300 mg/kg of TCM, as required by the export market (Ryan *et al*., 2013). In mid-lactation, the mean TCM concentrations of the BTs, CTs and WMS were all lower than that limit; while, in late-lactation, the mean TCM concentrations of the CTs and WMS were higher than that limit and higher than the concentrations in mid-lactation. The agreement between the TCM concentrations of each mid-lactation CT sample and the contributions of each BT milk volume supplied, as well as the agreement between the TCM concentrations of the WMS samples and the contributions of each CT in both lactation periods, indicated that the cleaning protocol of the CTs or WMS did not contribute to any increases in the TCM levels in milk (Supplementary Tables S1 and S2).

In both lactation periods, the decrease in the TCM concentrations in the SMS in relation to the WMS was expected, due to the accumulation of TCM in the cream during separation (Hubbert *et al*., 1996; Table 1). As the levels of TCM were higher in late-lactation milk, the TCM concentration in late-lactation cream was possibly higher than the levels expected in cream produced with mid-lactation milk.

The concentrations of CHLO and TCM were also monitored in the same 67 farm BTs in mid- and late-lactation (May and November, respectively) to investigate if those concentrations could differ in milk produced by the same farm during different production periods. None of the mid-lactation BT samples contained CHLO levels higher than 0.0100 mg/kg (EC limit), while 5 late-lactation BT samples contained levels higher than that limit. In relation to TCM, the number of BT samples that contained levels greater than 0.0015 mg/kg was significantly higher in late-lactation (21 BT samples; range: 0.0016 to 0.0074 mg/kg) than in mid-lactation (7 BT samples, range: 0.0017 to 0.0043 mg/kg) ($P = 0.002$). Those increases in the levels of those residues in late-lactation could be related to changes in the sanitation practices on each farm. Chlorine detergent sterilisers should contain a maximum of 3.5% of chlorine and should be prepared and applied according to the manufacturer's instructions (Gleeson, 2016). According to Ryan *et al*. (2013), 14 l of rinse water per milking unit are recommended in order to totally remove the detergent solution, and the solutions should be rinsed immediately after the wash cycle. Additionally, the lower volume of milk produced per farm during late-lactation (1,683 ± 1,031 l) could have also contributed to the increase in CHLO or TCM levels during that period, as those residues could have been more concentrated. The presence of CHLO and TCM in milk was not correlated; therefore, if milk contains CHLO it will not necessarily contain TCM and *vice versa*. The contamination of milk with CHLO or TCM might be related to a combination of specific sanitation practices and further studies are necessary to determine them. In addition, the higher number of farms in late-lactation that supplied milk containing higher levels of CHLO or TCM indicated that extra care is required during that period for the production of milk powder or butter.

Iodine

Variations in the iodine concentrations were investigated throughout the production chain of SMP in mid- and late-lactation (May and December, respectively). The EFSA (2005) reported that the average iodine concentration in BT milk samples from several European studies was predominately between 100 and 200 µg/l, which were suitable to meet the required iodine daily intake for children and adults. Some Irish dairy processors specify that the iodine levels in raw milk should be lower than 150 µg/l to produce infant formula. In mid-lactation, the mean iodine concentration of the BT, CT and WMS samples were all lower than that limit; while in late-lactation, the mean concentrations of the CT and WMS were higher than that limit.

Flachowsky *et al*. (2014) suggested that iodine could undergo sublimation throughout processing, as more than 90% of iodine in milk is in the inorganic form. Small decreases in the mean iodine concentration observed from the BTs to CTs (mid-lactation) and from the CTs to WMS (late-lactation) could be associated with the sublimation of iodine (Table 1). The actual iodine concentrations measured in each CT (Supplementary Table S3) and WMS (Supplementary Table S4) were in agreement with the contributions of each BT and CT, respectively. However, the actual concentrations of each CT and WMS were slightly lower than the predicted concentrations, indicating that possibly a small amount of iodine underwent sublimation during transport and storage, but not sufficient to be significant. Those small losses could have resulted in those decreases in the mean iodine concentrations shown in Table 1.

In mid-lactation, two CT samples had levels higher than 150 µg/l (390.8 and 202.9 µg/l). One of those CTs collected milk from two farms that supplied milk containing 289.1 and 516.0 µg/l of iodine. The other CT collected milk from 5 farms; however, most of the volume collected was from one farm that supplied milk containing 561.2 µg/l of iodine. Therefore, it is important that individual milk suppliers control the iodine intake of their herds and correctly apply iodine-based teat disinfectants (US National Research Council, 2001; O'Brien *et al*., 2013). In late-lactation, all of the CT samples had levels higher than 150 µg/l, indicating that the iodine levels in BT milk were possibly higher in late-lactation than in mid-lactation. Those higher levels could be due to the contribution of the increased number of farms (150) and also due to high levels of iodine in ration supplied to the cows when indoors.

In both lactation periods, the mean iodine concentrations increased in the SMS when compared to the WMS. Prior to pasteurisation and cream separation, milk permeate (details were not disclosure by the manufacturer) is added to standardise the protein and lactose content in milk. That permeate could have contributed to an increase in the iodine content in the SMS.

The International Council for Control of Iodine Deficiency Disorders (ICCIDD; Delange *et al*. 1993) specified that the iodine content in reconstituted SMP should be lower than 100 µg/l. The mean iodine concentrations of the SMP produced in mid- and late-lactation were higher than that limit (Table 1). Therefore, in the case of the conditions of this study, the iodine levels in the bulk milk supplied to the factory should be lower than 142 µg/l to produce SMP containing iodine levels within the specification. Also, as the iodine levels were higher in late-lactation compared to mid-lactation milk, the iodine content in reconstituted SMP was also higher in late-lactation than in mid-lactation.

In order to investigate variations in the levels iodine in BT milk during different production periods, the concentrations of such residue were also measured in the same 67 farm BTs in mid- and late-lactation (May and November, respectively). In mid- and late-lactation, 13 and 12 BT samples had iodine concentrations higher than 150 µg/l, respectively. Questionnaires were completed on some of those farms, capturing information regarding animal feed. It was established that the majority of those farms were using concentrates from one manufacturer, which contained

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at least 10 and a maximum of 43 mg of iodine/kg of ration. Therefore, the iodine intake from ration per cow on those farms was likely to be higher than that recommended (10 mg per cow per day), as the average ration intake on those farms was 2.5 kg per cow per day. Other factors that were not included in the questionnaires could have contributed to iodine levels in milk such as grass, boluses, mineral-supplemented water and mineral licks. Furthermore, according to the questionnaires, five and two farms that were using iodine-based teat disinfectants supplied milk with iodine levels higher than 150 µg/l, in mid- and late-lactation, respectively. O'Brien *et al.* (2013) also observed increases in the iodine levels in milk when applying those teat disinfectants post-milking. Those increases are associated with the absorption of iodine through the teat skin, particularly if pre-milking teat preparation is not being conducted.

In conclusion, incorrect sanitation practices on-farm can result in increases in the CHLO or TCM levels in milk throughout the year, while the production of lower volumes of milk is an additional contributing factor in late-lactation; therefore, extra care is necessary during that period. Consequently, increases in the CHLO or TCM levels in milk result in increased residue levels in SMP or cream, respectively. Therefore, it is important to control the initial residue levels in milk destined for processing, especially considering that those could concentrate greatly after evaporation and spray-drying processes or cream separation. Appropriate sanitation practices should also be carried out within the processing plant to avoid increases in the residue levels throughout the processing stages. In relation to iodine, this study indicated that some Irish dairy herds are over supplemented with iodine, while the use of iodine-based teat disinfectants also contributed to high levels in some BT samples. Also, the iodine content of the SMP produced in mid-lactation was not within the required specification, even though the WMS milk had lower iodine levels than specified, indicating that the levels in BT milk should be even lower. Finally, it is possible to calculate the expected residue levels in milk stored in the CTs or WMS based on the volumes and residue levels of milk supplied by each dairy farm, which could aid dairy processors to identify the stages that may have contributed to increases in those levels. This study highlights the importance of controlling the contributing factors on-farm and in the processing plant in order to maintain residues at safe and market-acceptable levels.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919000578

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Monitoring residue concentrations in milk from farm and throughout a milk powder manufacturing process

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Short title: **Milk quality from farm to milk powder**

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SUPPLEMENTARY FILE

Materials & Methods

Comparison between the residue levels in farm bulk tanks in mid- and late-lactation

In late-lactation (November 2016), the average (± SD) milk volume that was stored in each BT of the 67 farms during sampling was 1,683 ± 1,031 L (range: 125 to 4,519 L), which were stored for an average (± SD) of 34 ± 15 h, at 3.3 ± 1.2 ˚C.

Quantification of trichoromethane

In each sample vial, 2 mL of milk or reconstituted SMP were added with 5 µL of internal standard and 5 µL of of ethanol. The internal standard consisted of a solution prepared using 2-bromo-1-chloropropane and ethanol (0.2 mg/ mL). Samples were placed on an autosampler tray (CTC analytics Combi-pal; CTC Analytics AG Industriestrasse 20 CH-4222, Zwingen, Switzerland) and were incubated for 15 min at 80 ˚C and agitated at 750 rpm. Samples were injected (500 µL) into an Agilent 19095J-121LTM column (10 m x 0.53 mm x 2.65 µm; Agilent Technologies, Santa Clara, California, USA) with a heated gas-tight syringe (90 ˚C). Helium was used as the carrier gas, and the column temperature was kept at a constant temperature of 200 ˚C, which decreased to 70 ˚C in the end of analysis.

Quantification of total iodine

In each vial, 1:1 (w/v) solutions of sample and 5% tetramethyl-ammonium hydroxide (TMAH) extraction solution were added and gently swirled. Vials were placed in an oven at 90 ˚C for 3 h and afterwards they were allowed to cool. The standards used for the calibration consisted of solutions of iodine with 0.5 mL of a Tellurium solution (1,000 µg/ mL), which contained 1% TMAH.

Table S1. Comparison of mean trichloromethane (TCM) concentrations measured in each collection tanker (CT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) during mid-lactation and those predicted (± standard error; S.E.) from the combined farm samples in each CT.

CT number	Number of farms	Total volume per tanker (L)	Mean (± SD) volume measured per farm (L)	Mean TCM concentration of each CT (mg/ kg)	Predicted TCM concentration (weighted means ± S.E.)† (mg/ kg)	95% CI‡		Mean TCM concentration of each CT covered by predicted C.I.
						LCL	UCL	
1	4	23771	5,943 ± 1,271	0.0015	0.0014 ± 0.0009	0.0000	0.0043	Yes
2	5	26503	5,301 ± 2,385	0.0008	0.0005 ± 0.0002	0.0000	0.0011	Yes
3	6	29122	4,854 ± 1,763	0.0012	0.0009 ± 0.0003	0.0001	0.0016	Yes
4	6	23780	3,963 ± 2,683	0.0012	0.0009 ± 0.0003	0.0002	0.0016	Yes
5	8	27585	3,448 ± 2,214	0.0008	0.0004 ± 0.0001	0.0002	0.0005	Yes
6	7	28628	4,090 ± 1,208	0.0011	0.0008 ± 0.0004	0.0000	0.0018	Yes
7	7	27188	3,884 ± 2,064	0.0006	0.0004 ± 0.0001	0.0002	0.0006	Yes
8	7	28470	4,067 ± 2,437	0.0007	0.0004 ± 0.0002	0.0001	0.0008	Yes
9	2	27147	13,574 ± 11,312	0.0010	0.0007 ± 0.00004	0.0002	0.0012	Yes
10	5	25248	5,050 ± 3,877	0.0007	0.0003 ± 0.0001	0.0000	0.0006	Yes
11	10	28561	2,856 ± 1,764	0.0008	0.0005 ± 0.0001	0.0003	0.0008	Yes

† Weighted means were calculated considering the volume of milk supplied by each farm or by each CT.

‡ Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table S2. Comparison of mean trichloromethane (TCM) concentrations measured in the whole milk silo (WMS) during mid- and late-lactation and those predicted (± standard error; S.E.) from the combined collection tankers (CTs) samples.

	Mean TCM concentration of the WMS (mg/ kg)	**Mean (± SD) volume measured per CT (L)**	**Predicted TCM concentration (weighted means ± S.E.)† (mg/ kg)**	**95% CI‡**		**Mean TCM concentration of WMS covered by predicted C.I.**
				LCL	**UCL**	
Mid-lactation	0.0009	26,909 ± 1,902	0.0007 ± 0.00009	0.0005	0.0009	Yes
Late-lactation	0.0018	24,357 ± 3,768	0.0019 ± 0.0002	0.0014	0.0024	Yes

† Weighted means were calculated considering the volume of milk supplied by each CT.

‡ Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table S3. Comparison of mean iodine concentrations measured in each collection tanker (CT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) during mid-lactation and those predicted (± standard error; S.E.) from the combined farm samples in each CT.

CT	Number of farms	Mean (± SD) volume measured per farm (L)	Total volume per CT (L)	Iodine concentration measured in each CT sample (µg/ L)	Predicted iodine concentrations (weighted means ± SE) (µg/ L) †	95% CI ‡		Mean iodine concentration of each CT covered by predicted CI
						LCL	UCL	
1	4	5,943 ± 1,271	23,771	83.9	89.2 ± 21.8	19.8	158.6	Yes
2	5	5,301 ± 2,385	26,503	81.8	90.0 ± 23.8	23.9	156.2	Yes
3	6	4,854 ± 1,763	29,122	120.0	117.9 ± 45.6	0.6	235.3	Yes
4	6	3,963 ± 2,683	23,780	58.3	61.2 ± 8.5	39.3	83.7	Yes
5	8	3,448 ± 2,214	27,585	125.9	141.0 ± 27.8	75.4	206.7	Yes
6	7	4,090 ± 1,208	28628	138.4	144.1 ± 55.7	7.9	280.3	Yes
7	7	3,884 ± 2,064	27188	112.0	116.7 ± 15.7	78.4	155.1	Yes
8	7	4,067 ± 2,437	28470	76.3	82.9 ± 20.9	31.6	134.1	Yes
9	2	13,574 ± 11,312	27147	390.8	335.7 ± 91.6	0	1,500	Yes
10	5	5,050 ± 3,877	25248	202.9	282.7 ± 121.2	0	619.7	Yes
11	10	2,856 ± 1,764	28561	80.0	101.7 ± 12.1	74.3	129.1	Yes

† Weighted means were calculated considering the volume of milk supplied by each farm.

‡ Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

Table S4. Comparison of mean iodine concentrations measured in the whole milk silo (WMS) during the mid- and late-lactation periods and those predicted (± standard error; S.E.) from the combined collection tankers (CTs) samples.

	Mean (± SD) iodine concentration of the WMS (µg/ L)	Mean (± SD) volume measured per CT (L)	Predicted iodine concentration (weighted means ± SE) (µg/ L) †	95% CI ‡		Mean iodine concentration of the WMS covered by predicted CI
				LCL	UCL	
Mid-lactation	135.5 ± 7.6	26,909 ± 1,902	134.2 ± 28.3	71.0	197.3	Yes
Late-lactation	419.0 ± 2.8	24,357 ± 3,768	421.4 ± 50.5	308.8	534.0	Yes

† Weighted means were calculated considering the volume of milk supplied by each CT.

‡ Confidence interval (CI), lower (LCL) and upper (UCL) confidence limits.

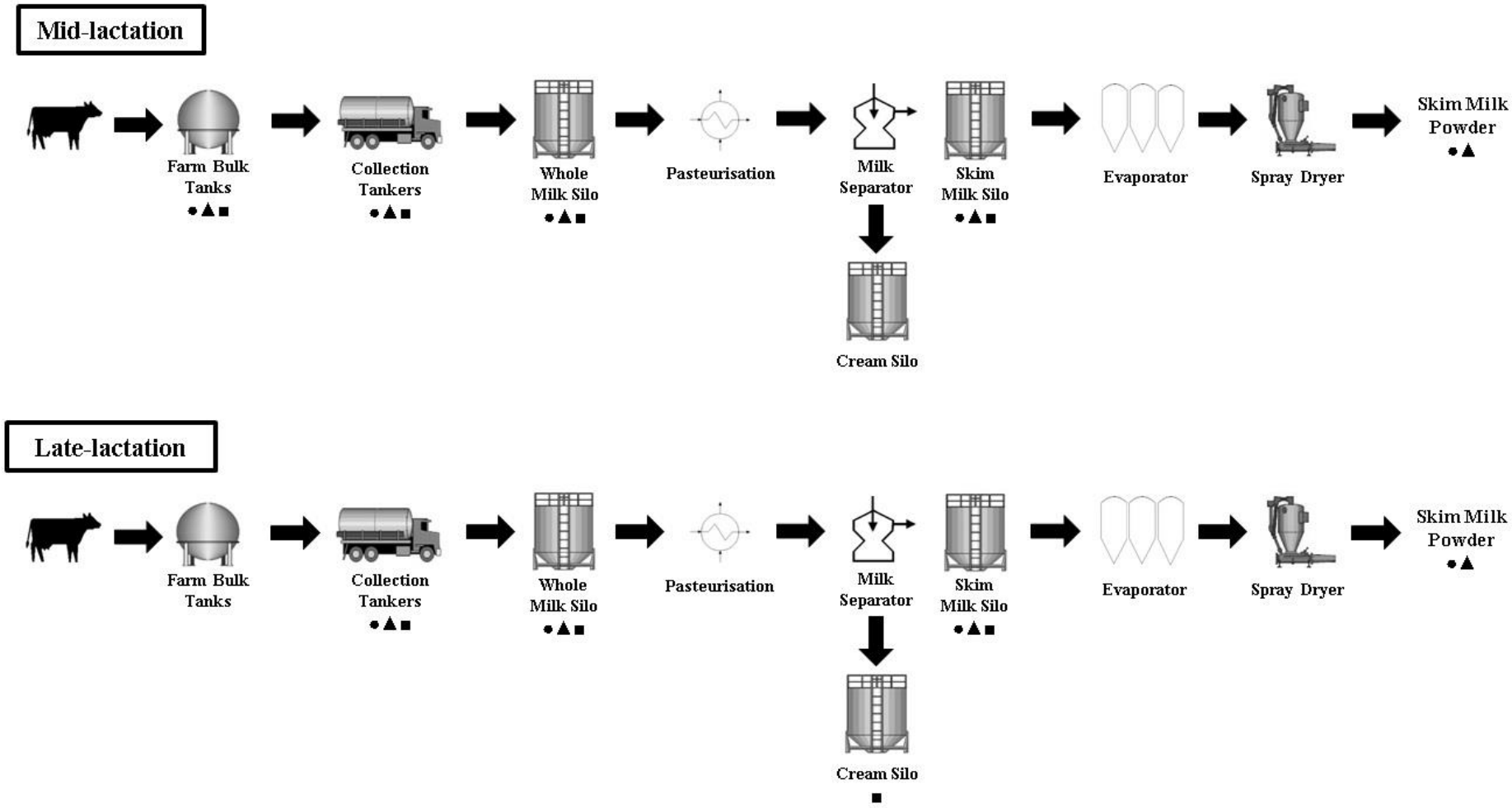

Figure S1. Milk supply chain and manufacturing process for conversion to low-heat skim milk powder, conducted in the mid- and late-lactation periods. The sampling points for chlorate (CHLO) and perchlorate (PCHLO), iodine and trichloromethane (TCM) are indicated with a ●, ▲and ■, respectively.