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Evaluation of the Manufacture of Cheese from Micellar Casein Concentrate or using Novel Coagulants

Thesis presented by

Bozhao Li, B.Sc.

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University College Cork

School of Food and Nutritional Sciences

Head of School/Department: Prof. Mairéad Kiely

Supervisor(s): Prof. Paul L. H. McSweeney and Prof. Alan L. Kelly

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Declaration

I hereby declare that the work described in this thesis is entirely my own and has not been submitted to any other university of higher education instituted, or for any other academic award in this university.

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Bozhao Li

September 2022

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Summary

Novel materials and coagulants for cheese manufacture are currently of interest since the development of membrane filtration technology and gene recombination technology may offer opportunities for innovation in cheese manufacture. A novel dairy material – micellar casein concentrate (MCC) – is the co-product of whey protein recovery. As the main protein source in cheese is casein, MCC has the potential to be a starting material for cheese manufacture. The objective of the work presented in the first part of this thesis was to evaluate the feasibility of the manufacture of Cheddar and Quarg cheeses from micellar casein concentrate. In addition, camel chymosin has been reported to cause less proteolysis as a coagulant for cheese manufacture compared to bovine chymosin. The suitability of manufacture of Cheddar cheese using a novel camel chymosin with structural changes was also investigated.

The rennet and acid coagulation properties of micellar casein concentrate were evaluated. MCC had a higher casein in total protein content compared to low heat skim milk powder (LHSMP), and shorter rennet coagulation time and higher gel strength were found in MCC compared to that of LHSMP. A gelation pH value greater than 5 was found in MCC. MCC produced by cold microfiltration (MF) formed acid-induced gels with high strength at pH 4.6, while the gel strength of acid-induced gels formed by warm MF MCC reached the highest at a pH value of around 5 and decreased below this value due to rearrangements of the casein network. The suitability of the manufacture of Cheddar cheese from MCC was subsequently investigated; standard control milk, skim milk with cream, reconstituted MCC with cream and reconstituted LHSMP with cream were used

for comparison. The use of MCC led to increased proteolysis compared to the other treatments, linked to higher plasmin and chymosin activities in the cheese. Increased springiness, cohesiveness and meltability were found in Cheddar cheese manufactured from MCC. For the manufacture of Quarg cheese, lower moisture and higher protein contents were found in cheese made from MCC compared to that made from LHSMP. Cheese made from hot MF MCC showed the highest hardness compared to that made from LHSMP or cold MF MCC. Higher glycomacropeptide (GMP) content was found in cheese whey made from MCC.

The suitability of manufacture of Cheddar cheese using a modified fermentation-produced camel chymosin (mCC) was investigated; fermentation-produced bovine chymosin (BC) and camel chymosin (CC) were used for comparison. The use of mCC led to reduced proteolysis compared with BC or CC, and higher instrumental and sensory hardness and lower meltability were found in cheeses made using CC or mCC compared to BC. Descriptive sensory analysis indicated less sulphur and barny flavour in cheese made with CC and mCC, while cheese made using mCC showed the lowest brothy flavour and bitter taste. Finally, the proteolytic specificity of the three generations of chymosin on NaCN at pH 5.2 with 5% NaCl and 6.5 and in proteolysis of Cheddar cheese made using these coagulants were investigated. Many peptides were identified through liquid chromatography-mass spectrometry (LC-MS) in both NaCN digests and Cheddar cheese made using each chymosin. Other than the majority of peptides produced by BC and CC reported in the literature, some new peptides were identified in this study as well. The proteolytic activity of mCC was relatively lower than that of BC and CC.

Overall, the results presented in this thesis will support the innovation and

application of new materials for the manufacture of cheese and other dairy products and add to the understanding of the properties of three generations of chymosin when used in cheese manufacture.

Introduction and research objectives

About 8,000 years ago, cheese was first produced and, nowadays, numerous types of cheese are well-established. Applications of novel streams and novel coagulants are still, however, of continued attention and may help advance cheese production or modify cheese production and that of other dairy products that are linked to cheese manufacture, e.g., whey protein ingredients and glycomacropeptides (GMP).

Microfiltration (MF) fractionates milk proteins according to their molecular weight and the production of cheese using membrane-separated material is a growing area. MF retentate (micellar casein concentrate, MCC), a novel dairy material, can be utilised in the functional and nutritional areas and has a potential to be the starting material of cheese manufacture (Crowley et al., 2018; Li et al., 2020; Xia et al., 2022). The operating temperature of MF may change the properties of resultant MCC since a portion of the β-CN and colloidal calcium phosphate (CCP) in milk will fractionate from casein micelle into the serum phase at low temperatures (≤ 5 ℃) (Zulewska et al., 2018). With the differences mentioned above, warm MF (normally 45-55 ℃) and cold MF may generate retentates with different coagulating and cheese-making properties.

Based on the coagulation method (acid-coagulation or rennet-coagulation) or coagulant used, cheese can be roughly divided into two major groups. The coagulation properties of milk or novel cheese-making materials under both conditions are essential to understand in assessing their potential for cheese manufacture (Fox et al., 2015).

Rennet-induced coagulation involves two phases. Chymosin, which is the principal enzyme in most coagulants, cleaves the Phe_{105}-Met_{106} bond of κ-CN to initiate coagulation in the first phase (Morr, 1975), and reduces the steric and electrostatic stabilisation of *para*-casein micelles, which triggers the aggregation of *para*-casein micelles to form a network in the second phase (Cheryan et al., 1975). Bovine chymosin is the most commonly used enzyme for cheese manufacture, but chymosin preparations extracted from lambs and camels are also commercially available (Scott, Robinson & Wilbey, 1998).

Acid-induced coagulation coagulates casein micelles by decreasing the pH of the milk to 4.6, i.e., the isoelectric point of casein, which neutralises the negative charge of casein micelles, dissolves some of the colloidal calcium phosphate crosslinks and reduces the steric and electrostatic stabilisation between micelles. Without those forms of stabilisation, casein micelles aggregate to form a gel network (De Kruif, 1998; Horne, 2002; Lucey & Singh, 1997).

The proteolytic specificity and general proteolytic activity of a chymosin are important factors for cheese manufacturers to consider (Bansal et al., 2009). Camel chymosin has been reported to be a suitable substitute of bovine chymosin as it has higher milk clotting activity and lower proteolytic activity compared to bovine chymosin (Kappeler et al., 2006; Langholm Jensen et al., 2013). A modified form of camel chymosin (CHY-MAX ® Supreme; Chr. Hansen, Hoersholm, Denmark) has been produced through a structure-based technique and was found to have increased specific milk-clotting activity and more limited proteolytic specificity compared to the original camel chymosin (Jäckel et al., 2017; Jäckel et al., 2019).

The processes of cheese manufacture and changes occurred during cheese ripening were described and reviewed in Chapter 1. Based on the review of literature, there are many gaps in the general knowledge of cheese manufacturing properties of MF retentates. Novel aspects of cheese manufacture were investigated in this study which might be of interest in fields involving cheese and other dairy products in the future including

- The rennet- and acid-coagulation properties of warm and cold MF retentates were analysed in Chapter 2 to evaluate the cheese-making properties of the novel dairy material.
- In Chapter 3, a study is presented on the manufacture of Cheddar cheese from reconstituted MCC and cream, with standardised cheese milk, skim milk and cream and reconstituted low heat skim milk powder and cream for comparison in the composition, proteolysis, and functionalities of cheese.
- For acid cheese, the lab-scale and pilot-scale manufacture of Quarg cheese from warm and cold MF retentates was evaluated based on the composition, proteolysis, structure, and texture of cheese as reported in Chapter 4.

Besides novel ingredients for cheese manufacture, the application of novel coagulants on cheese manufacture was also investigated. Coagulants mainly influence the gel strength and primary proteolysis of cheese, which are essential for the functionalities of cheese (Bansal et al., 2009). The proteolytic specificity and cheese manufacture possibility of a commercial fermentation-produced modified camel chymosin were characterised in Chapter 5 and 6.

- The suitability of Cheddar cheese manufactured using three generations of commercial fermentation-produced chymosins (bovine chymosin, camel

chymosin, and modified camel chymosin) were also assessed, focusing on their rennet coagulation properties, and composition, proteolysis, peptide profiles, texture and flavour of the resulting cheese (Chapter 5).

- The action of these chymosins in casein hydrolysates and in cheese were analysed in Chapter 6, using the Cheddar cheeses manufactured in Chapter 5 and sodium-caseinate-chymosin digested samples as substrates to compare the peptide profiles and the corresponding cleavage sites of the cheese with the *in vitro* hydrolysates.

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CHAPTER 1

Cheese manufacture and ripening

Bozhao Li, Alan L. Kelly and Paul L. H. McSweeney

School of Food and Nutritional Sciences, University College Cork, Cork, Ireland.

Declaration

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Chapter 1

Abstract

Principles of the manufacture and ripening of rennet and acid-coagulated cheese are discussed in this chapter. Rennet or acid coagulation is essential for the manufacture of cheese, and various methods can be applied to study the coagulation properties of milk. In cheese manufacture, many processes are then used to concentrate the casein and fat content of milk in the form of cheese. Membrane filtration processes are a relatively novel technology that alters the cheesemaking properties and composition of milk; cheese manufacture with membrane filtration processes has the potential to make cheese with tailored functionality.

Chapter 1

1.1 Introduction

Cheese is a common and convenient dairy product in markets all over the world; it can be served as a dessert, snack, food ingredient or sandwich filler. All types of cheese are manufactured by the coagulation of milk.

In the bovine milk system, casein, which accounts for 80% of milk protein, exists in the form of casein micelles. Casein micelle in milk consists of four types of casein: κ-casein, α_{S1}-casein, α_{S2}-casein, and β-casein, typically found with a ratio of 1.5: 4: 1: 3.5 (Dalgleish & Corredig, 2012). Casein micelles are distributed evenly in the milk system and are prevented from aggregating through steric stabilisation and electrostatic repulsion. For most types of cheese, casein micelles undergo rennet coagulation or acid coagulation that eliminates the electrostatic repulsion and removes steric stabilisation, leading to destabilisation of the micelles and the formation of a gel structure.

The essential process of cheese manufacture is transforming milk to curd, which involves about a 10-fold concentration of casein and fat, while the whey proteins, soluble salts and lactose are separated in the cheese whey. Normally, milk for cheese manufacture is standardised and pasteurised. Cheese manufacture involves steps including gelation, acidification, cutting, heating, salting, pressing, and ripening, and occasionally other processes depending on the cheese type. The family of cheese and cheese-related products are complex and can be classified according to their coagulation method (see Fig. 1.1).

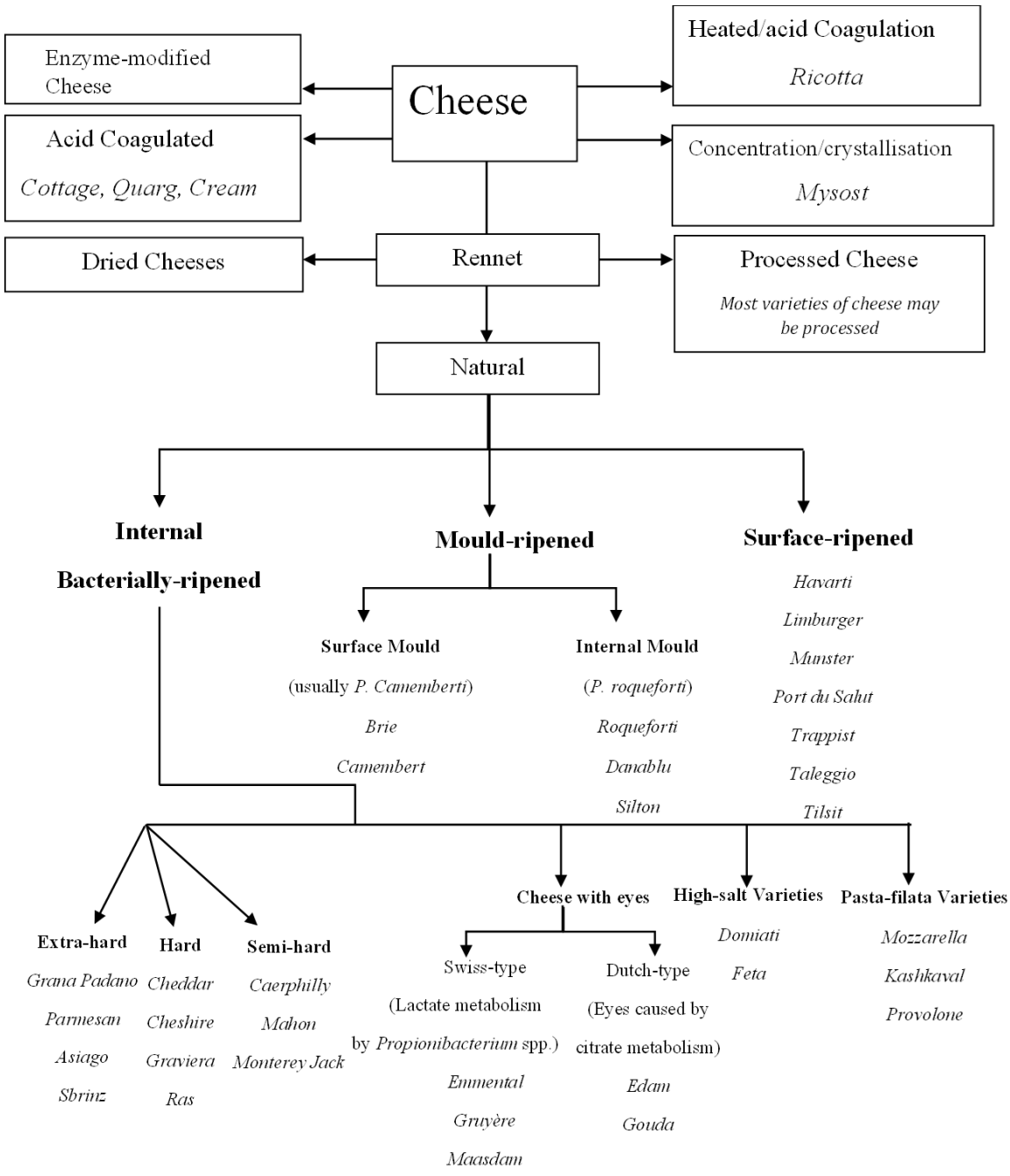

Fig. 1.1. Cheese family scheme based on the coagulation method and ripening conditions (McSweeney et al., 2004).

Chapter 1

Rennet, an enzyme preparation consisting of one or more proteinases, initiates coagulation through cleavage of κ-casein on the surface of casein micelles, producing *para*-κ-casein and glycomacropeptide (GMP; Morr, 1975) in the first phase of rennet coagulation. In the second phase, without the negative charges on κ-casein attached to casein micelles, at a suitable temperature (>~20 ℃), casein micelles aggregate with each other to form a gel network (Cheryan, Van Wyk, Olson, & Richardson, 1975). Many types of rennet, including those from bovine, camel (Bansal et al., 2009), plant (Scott, Robinson, & Wilbey, 1998) and microbial (Preetha & Boopathy, 1997) sources, can be used for rennet coagulation. This enzymatic coagulation can be used for the manufacture of many types of rennet-coagulated cheeses e.g., Cheddar, Gouda, Mozzarella.

Different methods are used to characterise the rennet coagulation properties of milk such as rennet coagulation time (Kübarsepp, Henno, Kärt, & Tupasela, 2005), and rheometry (Moynihan et al., 2014). The coagulation properties of milk are influenced by temperature (Espinoza-Molina, Acosta-Muñiz, Sepulveda, Zamudio-Flores, & Rios-Velasco, 2016; Ulusu, Şentürk, Kuduğ, & Gökçe, 2016), pH (Nájera, De Renobales, & Barron, 2003), Ca^{2+} (Balcones, Olano, & Calvo, 1996; Patel & Reuter, 1986), pre-heat treatment (Karoui & Kamal, 2017), enzymatic concentration, levels of non-micellar casein (Gaygadzhiev, Massel, Alexander, & Corredig, 2012; Lin, Kelly, O'Mahony, & Guinee, 2017), the presence of whey proteins (Gamlath, Leong, Ashokkumar, & Martin, 2018), and fat globules (Guinee et al., 1997). Syneresis of the coagulum is a related phenomenon following rennet coagulation and is essential for cheese manufacture (Hansen et al., 2010).

The principle of acid coagulation is that when the pH decreases to about 4.6 – the isoelectric point of the caseins – negative charges on the surface of casein micelle are neutralised, which initiates the aggregation of casein micelles. Acidification usually occurs through fermentation of lactose to lactic acid by lactic acid bacteria (LAB), but on occasion, it can be achieved by the introduction of mineral acids, the addition of an acidogen such as glucono-delta-lactone (GDL), or by using carbon dioxide (Lucey, 2016). Acid-coagulated cheeses such as Cottage, Quarg and cream cheeses are manufactured by fermentation of skim milk, milk and cream, respectively. Preheating of milk (Lucey, Teo, Munro, & Singh, 1997), temperature (Ahmed, Rashida, & Najma, 2006), and buffering capacity of milk affect acid coagulation properties of milk (Lucey, Hauth, Gorry, & Fox, 1993). Acid coagulated cheeses can be consumed fresh while most rennet-coagulated cheeses undergo a ripening period of between 4 weeks and 2 years to develop the desired texture, flavour, aroma of the cheese (Fox, 1989). A small amount of rennet may be added during the manufacture of acid-coagulated cheese such as Quarg to make the gel firmer (Kelly & O'Donnell, 1998).

As a relatively recent development in milk pretreatment for cheese-making, membrane filtration technology can be used to fractionate milk contents according to their molecular sizes. Membrane filtration technologies include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO). These four kinds of membrane filtration are classified according to the membrane pore size and the pressure that is applied (Ayral, Julbe, Rouessac, Roualdes, & Durand, 2008). Membrane filtration has been used for the standardisation of cheese milk and concentration of milk for cheese

manufacture (Mistry & Maubois, 2017). Through membrane filtration, the buffering capacity, rennet coagulation properties and rheological properties of milk can be affected; different consequences of cheese manufacture from membrane-filtered milk are reported (Green & Grandison, 1993; Mistry & Kosikowski, 1985). Membrane filtration can be applied in cheese manufacture for standardising cheese milk (Brandsma & Rizvi, 1999), concentrating cheese milk (Govindasamy-Lucey, Jaeggi, Martinelli, Johnson, & Lucey, 2011), and using membrane filtration retentates for cheese manufacture (Li et al., 2020; Xia et al., 2020).

This chapter will discuss the principles and protocols of rennet and acid-coagulated cheese manufacture and the applications of membrane filtration for cheese manufacture.

1.2 Casein System

In the bovine milk system, the casein micelles are polydisperse and include four kinds of casein, i.e., κ-casein, α_{S1}-casein, α_{S2}-casein, and β-casein, occluded water and calcium phosphate nanoclusters. Casein micelles provide and transfer the energy, calcium, phosphate, and protein that are required during the growth of mammals (Li & Zhao, 2019; McMahon & Oommen, 2013). The dual-binding model hypothesises that caseins within the micelle are bound through both hydrophobic and electrostatic interactions (Horne, 1998). The hydrophobic interactions are from the phosphate groups on the casein molecules. In all models of the casein micelle, including the recent and widely accepted Holt/Dalgleish model, κ-casein is located on the surface of casein micelle; in the interior of casein micelles, calcium phosphate nanoclusters are attached to the

caseins. β-Casein is bound through hydrophobic interactions and may dissociate from casein micelles at low temperatures, as water channels exist inside the micelle (Dalgleish, 2011). The κ-casein, as a protruding molecule from the casein micelle, reaches out to the surrounding environment (McMahon & Oommen, 2008). Through the effect of the negative charge on κ-casein and the "hairy layer" of this protein on the surface of casein micelle, casein micelles are prevented from aggregation with each other through electrostatic repulsion and steric stabilisation.

1.3 Pre-treatment of Cheese Milk

Cheese manufacture is a process that transforms the original material, milk, into the final product, cheese, by concentrating the fat and casein in milk about 10-fold.

Bovine milk is the most common starting material for cheese manufacture. Low heat skim milk powder, micellar casein concentrate, whey-protein-depleted cheese milk, and other dairy ingredients are used as raw materials for cheese manufacture on occasion (Freeman, Bucy, & Hassan, 1970; Li et al., 2020; Xia et al., 2020).

The composition of the milk is frequently standardised to a specific protein-to-fat ratio (PFR) or casein-to-fat ratio to achieve the desired target cheese composition. Milk can be separated into skim milk (0.04-0.07% fat) and cream by separation, and, after determining the PFR of milk, either cream or skim milk should be used to adjust the PFR of milk to the desired value. Standardisation can be facilitated using rapid compositional analysis through spectroscopic techniques such as Fourier-transform infrared (FTIR) methods;

for example, the MilkoScan system can measure protein, fat and lactose contents of milk simultaneously in a short time based on the infrared light-scattering profile of milk samples and is commonly used in dairy laboratories and the dairy industry today. The casein-to-fat ratio has a significant effect on the composition, component recoveries and yield (Guinee, Mulholland, Kelly, & Callaghan, 2007). The typical casein to fat ratio used for Cheddar cheese manufacture is 0.7:1.0. Increase in PFR of cheese milk gives higher levels of moisture, protein, Ca, and P and the lower moisture-in-non-fat-substances, fat-in-dry-matter and salt-in-moisture in the resultant cheese. In some cases, skim milk or skim milk membrane filtration permeate may be used to increase the protein content to reach the target PFR value.

Raw milk may carry pathogens like *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium tuberculosis* or *Staphylococcus aureus*, which could cause disease to humans, and spoilage organisms like *Pseudomonas fluorescens* that can produce heat-stable proteinases and lipases that affect milk quality. Therefore, pasteurisation, an essential process in milk and dairy product manufacture, is usually practised. Commonly, two kinds of pasteurisation methods are used: continuous pasteurisation and batch pasteurisation. Continuous pasteurisation involves heating the milk at 72 ℃ for 15 seconds, in what is called the high temperature short time (HTST) process, using a plate heat exchanger, while batch pasteurisation involves heating milk to 63-65℃ for 30 minutes and is typically only found today in small-scale (e.g., artisanal, farmhouse) cheese plants. Although pasteurisation inactivates vegetative cells, bacterial endospores with higher thermal resistance will not be killed and may germinate and be active again after pasteurisation. Also, not all spoilage

organisms can be destroyed during pasteurisation; therefore, it is necessary to keep the pasteurised milk at 4 ℃ until the beginning of cheese manufacture if it is not used immediately.

1.4 Rennets and coagulants

Rennet coagulated cheeses constitute perhaps ~75% of the world's entire cheese manufacture, and most rennet-coagulated cheese varieties are ripened (McSweeney, Ottogalli, & Fox, 2004). The manufacture of rennet-induced cheeses includes various steps including standardisation, pasteurisation, acidifying, gelling, cutting, heating, pressing, ripening, and other processes depending on the type of cheese to be manufactured. The protocols for some common rennet-coagulated cheeses are summarised in Fig. 1.2 a-d (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015).

Rennet is the common name for preparations from the abomasa of ruminant mammals used to coagulate milk that contain enzymes including chymosin, pepsin and, on occasion, active lipase. It is the main enzyme source that is used to make cheese by coagulating casein micelles (Singh, Singh, & Sachan, 2019). The cleavage site of rennet on κ-casein normally is the peptide bond between Phe_{105}-Met_{106}; this hydrolysis produces *para*-κ-casein, caseinomacropeptide (CMP) and glycomacropeptide (GMP) normally containing *N*-acetyl neuraminic acid and other carbohydrate moieties (Morr, 1975), which initiates the coagulation of milk (Sandra, Alexander, & Dalgleish, 2007). On reducing the negative charge on κ-casein and removing the physical barrier of steric stabilisation, casein micelles coagulate and associate to form the gel network (Gaygadzhiev et al., 2012).

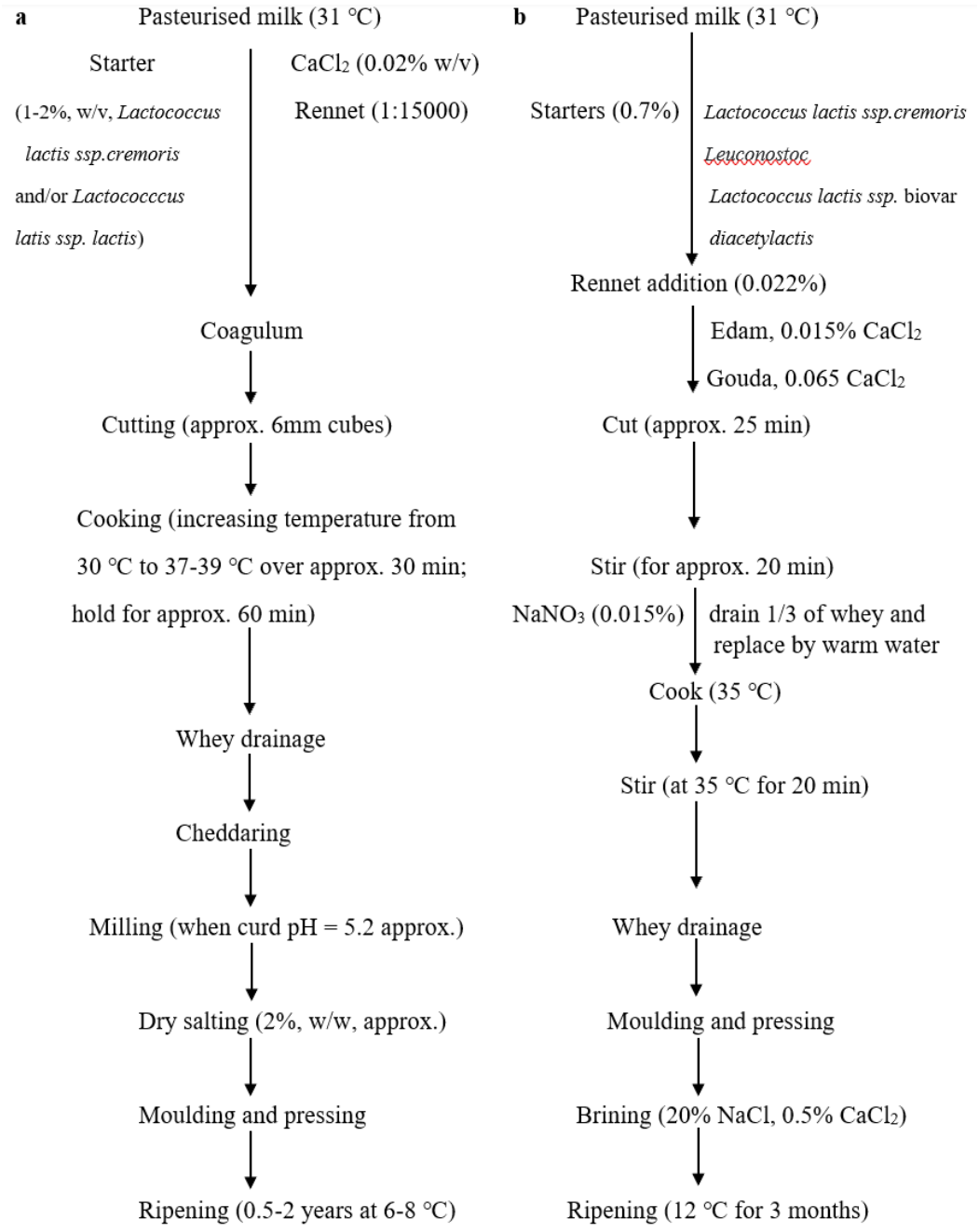

Fig. 1.2. Methods for manufacture of (a) Cheddar, (b) Gouda, (c) Emmental and (d) Parmigiano-Reggiano (Fox et al., 2015).

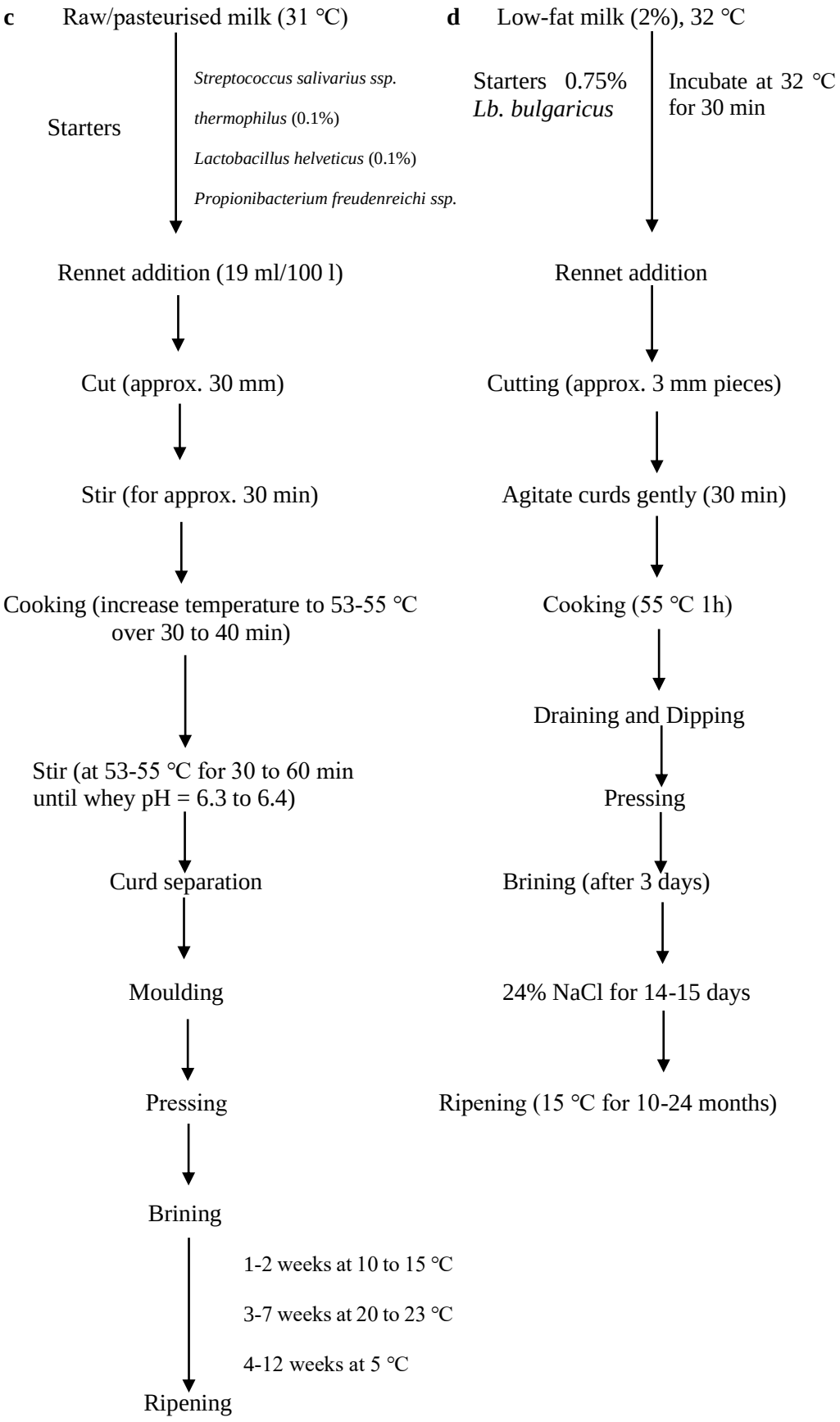

Fig. 1.2. Continued.

Chapter 1

There are many sources of coagulant, including animal, vegetable and microbial rennets. Animal rennet originates from the fourth stomach (abomasum) of young ruminants. Depending on the age of the animal, the chymosin content in rennet as a percentage of coagulating enzyme activity varies from 50%-95%; the remainder is mainly pepsin (Addis, Piredda, & Pirisi, 2008). Other than the most widely used bovine chymosin, camel chymosin and lamb chymosins are also commercially available. Fermentation-produced chymosin (FPC), which is bovine or camel chymosin secreted by genetically modified microorganisms, is also used for cheese manufacture. With the development of genetic engineering, animal enzymes like chymosin can be produced by recombinant strains of *Aspergillus niger* or *Kluyveromyces lactis* to obtain better and more consistent quality rather than extracting it from the abomasum of the animal (Kappeler et al., 2006). Camel chymosin shares 85% of its amino acid sequence with calf chymosin and has 70% higher rennet coagulation activity than calf chymosin and 25% of the proteolytic activity of calf chymosin (Dalgleish, 2011; Sandra et al., 2007). Unlike calf chymosin, camel chymosin has two additional positively charged residues that attract the negative charge in κ-casein, which therefore makes camel chymosin bind more readily to casein micelles; this higher electrostatic interaction of camel chymosin makes the milk coagulation activity of camel chymosin higher than that of bovine chymosin (Kappeler et al., 2006; Langholm Jensen et al., 2013). The proteolytic specificity of camel chymosin, which has been studied in the context of Cheddar and Mozzarella cheese manufacture (Bansal et al., 2009; Moynihan et al., 2014), shows the same cleavage site on κ-CN, i.e., the Phe_{105}-Met_{106} bond (Møller, Rattray, & Ardö, 2012).

Enzymes naturally produced by microorganisms (e.g., *Rhizomucor miehei*) are also sometimes used for cheese manufacture (Preetha & Boopathy, 1997). Coagulants from plants such as *Cynara cardunculus, Ficus carica, Arctium minus* and *Solanum dobium* are very occasionally used for traditional cheese manufacture.(Scott et al., 1998) The proteolytic activity of plant rennet is usually much higher than that of rennet extracted from animals, and the preference of plant rennets could be for other bonds on κ-casein rather than Phe_{105}-Met_{106}, leading to a reduction in cheese yield or flavour problems (Lo Piero, Puglisi, & Petrone, 2002).

1.4.1 Rennet Coagulation

The enzymatic coagulation of milk for the manufacture of many varieties (Cheddar, Gouda, Mozzarella etc.) is also called rennet coagulation. Rennet coagulation is a biochemical reaction that includes two stages. In the first stage, due to the action of rennet on κ-casein bond Phe_{105}-Met_{106}, the κ-casein is cleaved into *para*-κ-casein and GMP, which is the peptide that is cleaved off from the casein micelle. GMP is a promising ingredient in the food area, due to its biological activity properties to human health and ease of its recovery from whey, as GMP mainly partitions into the whey during cheese manufacture (Jauregui-Rincón, Salinas-Miralles, Chávez-Vela, & Jiménez-Vargas, 2018). The casein with GMP remoed is called *para*-casein.

Para-casein aggregates in the second stage of rennet coagulation. During this second stage, when around 85% of the total κ-casein is hydrolysed, the stability of the casein micelle is reduced. This is due to the reduced negative charge on casein micelle (the zeta potential of casein micelle is reduced from -

20 mV to -10 mV), and removal of the steric stabilising layer, both of which promote aggregation of casein micelles through hydrophobic interactions. The calcium ions in serum also help the aggregation of casein micelles by crosslinking casein micelles. The second stage of rennet coagulation can only happen in the presence of calcium ions when the temperature is greater than 20 ℃, while the first stage takes place the temperatures > 0 ℃ (Cheryan et al., 1975). Through aggregation, casein micelles form a gel network (Fig. 1.3).

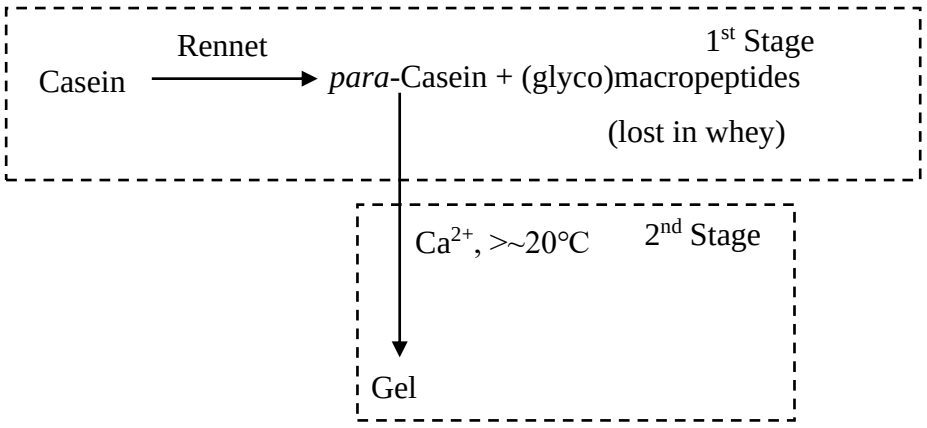

Fig. 1.3. The first and second stages of enzymatic coagulation of milk (Fox et al., 2015).

1.4.2 Factors that Influence Milk Coagulation Properties

Several common factors influence milk coagulation properties: temperature, pH, enzymatic concentration, preheating and concentrations of $Ca^{2+}$and casein. As pH decreases (e.g., from 6.8 to 6.0), the first stage of rennet coagulation will be promoted and RCT decreases; this is because the optimum pH of rennet for cleavage of the Phe-Met bond is around 5 and the second stage is also helped somewhat as electrostatic repulsion between micelles is reduced at low pH (Lopez, Lomholt, & Qvist, 1998; Nájera et al., 2003). The optimum temperature for chymosin is around 40 ℃ and, therefore, RCT is lowest when

the coagulation temperature is close to 40 ℃ (Ulusu et al., 2016); Espinoza-Molina et al. (2016) reported that the optimum temperature of bovine chymosin is between 35-40 ℃. However, the most common coagulation temperature for cheese manufacture is about 30 ℃, as this favours mesophilic starter bacteria. The RCT is inversely correlated with enzyme concentration. Preheating (< 70 ℃) of milk slightly decreases RCT because heating causes precipitation of colloidal calcium phosphate (CCP), which influences the stability of casein micelle and decreases pH. Preheating at over 70 ℃ causes denaturation of the whey protein β-lactoglobulin (β-Lg), which interacts with κ-casein through disulphide bonds, therefore inhibiting rennet coagulation (Karoui & Kamal, 2017). The concentration of Ca^{2+} (in the range < 10 mM) has a positive effect, mainly on the second stage of rennet coagulation, and influences gel firmness (Balcones et al., 1996; Patel & Reuter, 1986).

Other particles, including non-micellar casein, whey proteins and fat globules, can also influence rennet-induced coagulation and should also be considered during cheese manufacture. The addition of non-micellar casein, e.g., sodium caseinate, inhibits the rennet coagulation of casein micelles by attaching to rennet-hydrolysed casein micelles, which delays their aggregation through steric repulsion forces, while the rennet coagulation properties can be restored through adding Ca^{2+}, although some inhibition by sodium caseinate still occurs (Gaygadzhiev et al., 2012; Lin et al., 2017).

Whey protein denaturation by severe heating process suppresses rennet coagulation of casein micelle, but native whey proteins also inhibit casein micelle aggregation; the increase of the whey protein to casein ratio in milk thus

inhibits rennet-induced coagulation of milk, as whey proteins create a physical barrier that prevents *para*-casein aggregation (Gamlath et al., 2018).

The size of fat globules in milk influences rennet-induced coagulation (Luo et al., 2017). With smaller size of fat globules, milk coagulates gels with more compact structure within shorter time compared to that with larger size of fat globules. This is attributed to the weakening of gel structure caused by the coalescence and creaming of fat globules (Luo et al., 2017).

1.4.3 Measurement of Milk Coagulation Properties

The international milk chymosin unit (IMCU) is a defined measure of chymosin or rennet activity. One IMCU mL^{-1} is the activity of chymosin required to coagulate 10 mL of bovine milk, at pH 6.5, within 100 s at 30 °C (McSweeney, Fox, & Olson, 1995). The concentration of commercial chymosins is about 200 - 600 IMCU mL^{-1} which is quite concentrated for rennet coagulation. The common dosage of bovine chymosin for Cheddar cheese manufacture is around 60 IMCU L^{-1} milk.

Different methods may be used to describe or quantify milk coagulation properties (MCP). The Berridge method is used to measure the rennet coagulation time (RCT), which is the time for milk to start to coagulate after the addition of rennet. IDF Standard 157: 2007 is the updated standard method for measuring RCT. In the Berridge method, a milk sample is placed in a rotating bottle, the temperature of which is controlled through a water bath. When rennet is added to the sample, a timer is set and the sample in the rotating bottle should be carefully observed. The RCT is determined when white flecks can be observed visually. The Berridge method is subjective as it relies on the

experience and judgement of the operator to identify the time when flecks appear; different operators may observe different RCT from the same sample.

An optical method that measures the infrared light backscatter at 880 nm of milk during rennet coagulation has been developed, which is objective and as reliable as the Berridge method (Tabayehnejad, Castillo, & Payne, 2012). The use of Fourier-transform mid-infrared (FT-MIR) spectroscopy for the prediction of MCP of ovine, buffalo, and bovine milk has been studied. Although the prediction accuracy of the FT-MIR spectroscopy method is low, it can be used as a tool to approximately distinguish the coagulation stages of milk (early-stage (<10 min), mid-stage (10-20 min), and late-stage (20-30 min)) to increase the efficiency of cheese manufacture (Manuelian et al., 2019, 2017).

Curd firmness after milk coagulation is also an important aspect of MCP. A rheometer uses tiny strain rheological tests like dynamic low amplitude oscillatory shear rheology which will not damage a sample (Foegeding & Drake, 2007; Gunasekaran & Ak, 2003; Tunick, 2011). Rheometers can precisely and continuously measure the storage modulus (G', the energy stored during each oscillatory shear cycle), loss modulus (G", the energy transformed to heat during each oscillatory shear cycle) and loss tangent (tan δ, the ratio of the loss modulus to storage modulus) of a sample by applying sinusoidal oscillatory shear to the sample (Guinee, Feeney, Auty, & Fox, 2002). As rennet coagulation proceeds, the storage modulus, which represents gel firmness, increases. Researchers have used the gel firmness to indicate when to cut the curd (Bansal et al., 2009; Moynihan et al., 2014); this method can also be used to indicate the gelation time, which is often defined as the time when G' is >1 Pa (Moynihan et al., 2014). The following parameters relating to cheese manufacture are used to describe

MCP as well: maximum curd-firming rate, A_{40} and tan δ 40 (the G and tan δ value after 40 min of rennet addition, respectively), K_{35} and K_{70} (the time required for the sample to reach 35 and 70 Pa, respectively) and cutting window (calculated from K_{35} and K_{70}) (Panthi et al., 2019).

There are several in-vat methods to determine the curd strength during the manufacture, e.g., knife test, hot wire sensor and near infra-red (NIR) reflectance. The knife test uses a sharp, flat, and clean tool to cut the curd at an angle of 45° to around 5 cm deep and then turn the blade through 90 degrees to lift the gap with the knife. If there is a clean break of the curd with whey drainage, the curd is ready to be cut, and otherwise, the coagulation requires more time to complete. The hot wire sensor uses a wire to pass a current through the milk sample which generates heat that dissipates easily by convection currents; when the milk coagulates, the heat is less easily dissipates, increasing the temperature and conductivity of the wire. NIR reflectance sensors determine the cutting time of the gel by measuring light reflectance through milk coagulation (O'Callaghan, Mulholland, Duffy, O'Donnell, & Payne, 2001).

The microstructure of gels, which consists of a coarse molecular network of casein formed by chains, strips and brunches of aggregated casein micelles, can be observed through confocal laser scanning microscopy (CLSM) or electron microscopy (EM; Kalab, Allan-Wojtas, & Phipps-Todd, 1983). CLSM is an optical technology that uses spatial pinholes to block the defocused light during imaging, thereby improving the optical resolution and contrast of photomicrographs. This technique requires minimal preparation for the sample and users can dye protein and fat with different coloured probes to distinguish them. Unlike CLSM, samples for EM should be carefully dehydrated, fixed,

embedded, sectioned, and stained before observation. Several studies have used EM to observe the microstructure of acid and rennet gels (Benyahia-Krid, Attia, & Zidoune, 2010; Davies, Shankar, Brooker, & Hobbs, 1978; Mellema, Heesakkers, Van Opheusden, & Van Vliet, 2000; Mottar, Bassier, Joniau, & Baert, 1989; Parnell-Clunies, Kakuda, & Smith, 1987).

1.4.4 Acidification

The pH plays an important role in all types of cheese manufacture. The initial pH of cheese milk is around 6.6. For rennet cheese manufacture, pH decreases to about 5 within 6 to 24 hours. The rate of pH decrease depends on the buffering capacity of milk (Lucey et al., 1993); buffering capacity is the ability of a system to resist change in pH upon the addition of an acid or an alkali (Allaby & Park, 2013). In milk, buffering capacity is contributed by salts (like inorganic phosphate, citrate), organic acids and milk proteins (caseins and whey proteins) and is affected by physical or chemical treatments during manufacture, such as heat treatment, high-pressure treatment, salt addition and membrane filtration.

The enzyme activity and proteolysis are related to pH. The chymosin activity increases when the pH of milk decreases from 6.9 to 5.0 (Espinoza-Molina et al., 2016). Milk pH also affects the strength of rennet coagulated gels. When pH gets lower (6.0), the coagulation time becomes shorter and the rate of gel formation increases, regardless of whether chymosin or plant coagulants are used (Esteves, Lucey, Wang, & Pires, 2003). A lower pH gives better syneresis which relates to the moisture content of cheese and whey drainage (Patel, Lund, & Olson, 1972). Microbial growth is also affected by pH as environmental pH

relates to the energy yield, redox reactions of microorganisms, therefore affecting microbial respiration (Jin & Kirk, 2018). The change of pH influences the calcium equilibrium in the system; when the pH is below 5.6, CCP is dissolved by hydrogen ions to calcium ions and hydrogen phosphate, and Ca^{2+} is then released from the casein micelle to the milk serum phase (Lucey & Fox, 1993). At the whey drainage stage, the released Ca^{2+} is lost in whey.

1.4.5 Syneresis

Syneresis is a phenomenon that happens after the coagulation of milk. Local stresses that occur in the gel structure causes local rearrangements of the protein matrix, leading to the expulsion of whey, and resulting in syneresis (Hansen et al., 2010). Syneresis is related to MCP and traditionally is measured through physical separation or tracer methods. Physical separation methods measure the weight of whey and gel to indicate the extent of syneresis, which requires cautious handling to minimize the expulsion of whey during measurement (Hansen et al., 2010). Tracer methods submerge the curd into dyed ultrafiltration milk permeate and measure syneresis through the dilution of tracers determined by absorbance (Unger Grundelius, Lodaite, Östergren, Paulsson, & Dejmek, 2000). Low-field nuclear magnetic resonance (LF-NMR) is a spectroscopic technique that quantifies and monitors the state or location of water in the food system, with the advantage that it is not destructive nor invasive to sample and requires no or very little sample preparation. LF-NMR can detect the release of protons from water and, therefore, is also used to continuously measure endogenous syneresis (syneresis that happens due to the

pressure from gel network formation or mechanical factors) (Hansen et al., 2010).

1.4.6 Other Steps

After cutting, the curds tend to sink to the bottom of the cheese vat and fuse into a large curd when the whey is drained. “Cheddaring” is the unique process for Cheddar cheese that traditionally involves cutting the drained curd into large chunks and stacking the chunks one over another to press the curd under its own weight, which also keeps the curd pieces warm, thus promoting uniform acidification. During this process, physicochemical changes happen to the curd. Additional lactose is transformed to lactic acid, causing a pH decrease from 6.15 to 5.2. The decrease in pH leads to the solubilisation of CCP, increasing the composition of Ca^{2+} and decreasing the casein-bound Ca, which, therefore, increases *para*-casein hydration. The pressure of the weight and the *para*-casein hydration results in a limited flow of the *para*-casein network (Guinee, 2016).

Besides cheddaring, various steps like cooking, stirring, whey drainage, and pressing can be applied to the curd to promote dehydration and acidification during the cheese manufacture, depending on the type of cheese. In the production of Dutch cheese varieties such as Edam and Gouda, the curd is cooked by washing with warm water to drain the whey, reduce the lactose content and control the pH of cheese.

Salting of cheese contributes to cheese flavour and functionality and initiates preservative action. With the effect of salt, the osmotic pressure of food is increased, and the water activity is depressed, therefore, the growth of the bacteria is terminated or inhibited. Besides, salting affects the flavour of cheese

directly and through influencing the metabolic and enzymatic activities that relate to cheese pH, flavour compounds, and degradation of casein and fats (Guinee & Fox, 2017). Different amounts of salt (from 0.4% for Emmental to 5% for blue cheeses to greater than 10% for Feta) is added to cheese usually through brine or as dry salt mixed with the milled curds (Bisig, 2014; Hammam, Kapoor, Salunke, & Metzger, 2022).

Most cheeses are pressed in moulds of characteristic size and shape and may be vacuum packed for ripening. Some hard cheeses are waxed to protect the cheese from airborne bacteria and moulds and to prevent moisture evaporation during ripening.

1.4.7 Cheese Ripening

Freshly made rennet-coagulated cheeses are usually flavourless and have a rubbery texture. The ripening period of rennet-coagulated cheeses, required to develop the desired flavour and texture, varies from 2 weeks to 2 years (Fox, 1989), although some types of cheese, e.g., acid-coagulated varieties, can be consumed without ripening.

During the ripening period, chemical, biological, and biochemical reactions occur that produce flavour compounds and modify the texture of cheese. During the ripening of cheese, three interlinked series of primary biochemical changes happen in the cheese: (i) metabolism of citrate and residual lactose and catabolism of lactate; (ii) lipolysis and fatty acid metabolism; and (iii) proteolysis and catabolism of amino acids. Proteolysis during cheese ripening, along with the changes in casein-bond calcium are responsible for the texture development (from hard and tough to soft and elastic) of cheese.

The ripening of cheeses is influenced by agents from four to six sources: cheese milk, coagulant, starter LAB, non-starter lactic acid bacteria (NSLAB), secondary and adjunct cultures, and other exogenous enzymes. Cheese milk contains about 60 indigenous enzymes, many of which are associated with the casein micelles or fat globules and retained in cheese curd; only heat-stable enzymes can survive HTST pasteurisation and contribute to cheese ripening (Kelly & McSweeney, 2003).

1.4.7.1 Proteolysis in Cheese

Proteolysis can be classified into three stages: proteolysis in milk before cheese manufacture, proteolysis during cheese manufacture, and proteolysis in cheese during ripening (Fox, 1989). The effects of each of these stages of proteolysis impact the manufacture and quality of cheese.

Proteolysis in milk before cheese manufacture can be divided into proteolysis from extracellular proteinases that are secreted from psychrotrophic bacteria and leucocytes and proteolysis from the indigenous milk proteinase (plasmin). Psychrotrophs are bacteria that can grow and reproduce at low temperatures, while their optimal growth temperature is above 15-20 ℃ (Moyer & Morita, 2007). Raw milk provides a favourable environment for many bacterial species, including psychrotrophs, that can contaminate or impair the quality of milk during refrigerated storage at the farm, or during transportation and processing (McPhee, Griffiths, & John, 2011; Pinto, Martins, & Vanetti, 2006; Sørhaug & Stepaniak, 1997). Although pasteurisation kills a considerable number of microorganisms, the quality of dairy products can still be affected by the microbial activities of raw milk (Nörnberg, Friedrich, Weiss, Tondo, &

Brandelli, 2010). When the population of psycrhrotrophs is greater than 10^6 cfu/ml, impaired recovery of milk solids, increased moisture, pasty texture, and off-flavour may result in cheese (Fox, 1989).

Other than proteinases produced by psychrotrophs, indigenous milk proteinases, principally plasmin, affect cheese quality (Ismail & Nielsen, 2010). The plasmin system is a complex proteinase-proteinase inhibitor system in milk (Fig. 1.4; Ismail & Nielsen, 2010). Most of the potential plasmin activity exists as the inactive zymogen plasminogen in milk. Plasminogen can be activated to plasmin by plasminogen activators (of which there are tissue-type and urokinase-type) (Bastian & Brown, 1996; Grufferty & Fox, 1988). In the plasmin system, there are also plasminogen activators inhibitors and plasmin inhibitors that can influence plasminogen activators and plasmin, depending on the manufacturing conditions (Bastian & Brown, 1996; Grufferty & Fox, 1988).

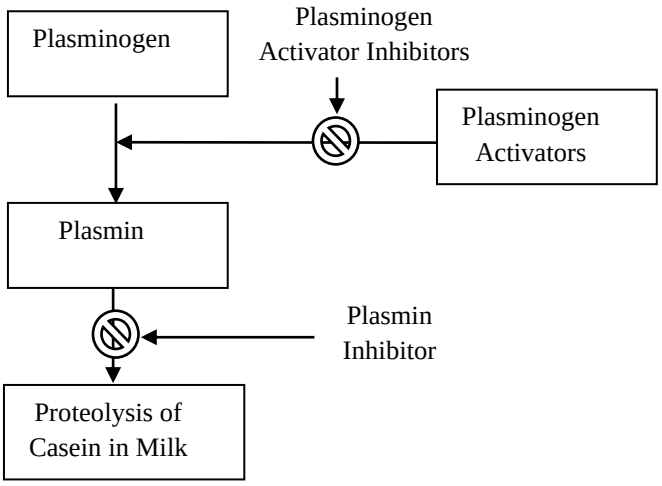

Fig. 1.4. Plasmin system of bovine milk (Ismail & Nielsen, 2010).

Plasmin prefers to cleave peptide bonds on the carboxyl side of L-Lys and L-Arg residues (Kelly & McSweeney, 2003). Plasmin mainly hydrolyses β-CN to β-CN (f29-209), β-CN (f106-209), and β-CN (f108-209) (Eigel et al., 1984; Fox, 1989) as there are three principal plasmin-sensitive bonds in β-CN (Ismail & Nielsen, 2010). Plasmin also hydrolyses α_{S1}-, α_{S2}-, and κ-CN to lesser or greater extents (Grufferty & Fox, 1988). Plasminogen, plasmin, and plasminogen activators bind with casein micelles through lysine-binding and electrostatic forces (Baer, Ryba, & Collin, 1994). The factors that affect plasmin association with casein micelles include storage temperature of milk, pH, ionic strength, hydrolysis of casein by plasmin, and microbial proteolysis (Ismail & Nielsen, 2010).

During cheese manufacture, a small part of the coagulant is retained in cheese curd and plays an essential role in cheese ripening. The level of residual coagulant varies from 15% of activity in Gouda cheese to 50% of activity for high moisture cheese like Camembert (Garnot, Molle, & Piot, 1987; Stadhouders, 1975).

The maximum total number of LAB is achieved at the end of cheese manufacture. Due to their numbers decreasing by about two log cycles within 1 month, their intracellular enzymes are released and can hydrolyse protein and thereby contribute to cheese ripening (Ganesan, Dobrowolski, & Weimer, 2006; Stuart, Chou, & Weimer, 1999). Proteinases or peptidases of NSLAB and secondary or adjunct cultures (microorganisms that are not added as the primary starter) are mostly like that of starter LAB (McSweeney, 2007). *Penicillium camemberti* and *Penicillium roqueforti* produce aspartyl proteinases, metalloproteinases, and a carboxypeptidase (Gripon, 1993). Some types of

cheese, like smear-ripened cheese, requires yeasts and bacteria to grow at the surface of the cheese after cheese manufacture. The growth of fungi (e.g., *Debaryomyces. hansenii* and *Geotrichum candidum*) and bacteria (e.g., *Staphylococcus saprophyticus*) increases the pH to a value over 6.0, allowing a Gram-positive microbiota to develop which generates a sticky surface with a red-orange colour (Bockelmann, 2011).

Exogenous enzymes (e.g., pre-gastric lipase for Romano or Provolone cheeses) may be used to modify or accelerate cheese ripening (Fox, 1988) or for the production of enzyme-modified cheeses (Kilcawley, Wilkinson, & Fox, 1998). Exogenous enzymes may cause an increase in the bitterness of cheese; cheese manufactured from milk with high levels of added peptidase was found to have higher bitterness (Hicks, O'Leary, & Bucy, 1988).

The production of small peptides and free amino acids contributes to cheese flavour. Free amino acids are also involved in a series of reactions catalysed by aminotransferases that convert amino acids to α-keto acids, which can be degraded to many flavour compounds. Too much proteolysis may create excessive levels of hydrophobic peptides that contribute to the bitterness of cheeses, while an optimal level of hydrophobic peptides is advantageous to cheese flavour (Gomez, Garde, Gaya, Medina, & Nuñez, 1997; Visser, 1993).

The level of proteolysis during cheese ripening varies depending on the ripening time of cheese. Proteolysis in unripened cheese, e.g., Mozzarella is limited (IDF, 2007); the proteolysis of cheeses such as Gouda and Cheddar is medium (Li et al., 2020; Wemmenhove, Wells-Bennik, Stara, Van Hooijdonk, & Zwietering, 2016); and some cheeses such as Blue cheeses undergo extensive proteolysis during ripening (Mane, Ciocia, Beck, Lillevang, & McSweeney,

2019). The coagulant is the major proteolytic agent in most types of cheese, while the activity of coagulants can be reduced due to coagulation denaturation at high cooking temperatures for varieties like *pasta filata*, Emmental, and Parmesan.

The measurement of pH 4.6-SN/TN (soluble nitrogen/total nitrogen) through Kjeldahl may be used to quantify the overall levels of proteolysis in cheese (Kuchroo & Fox, 1982; IDF, 1986), while more precise and deeper information on proteolysis can be acquired through methods such as electrophoresis, reversed-phase HPLC or UPLC. Urea-polyacrylamide gel electrophoresis (urea-PAGE) separates proteins and polypeptides according to their size/charge ratio and mainly characterises the primary proteolysis of cheese (Li et al., 2020; Mane et al., 2019). Reversed-phase HPLC or UPLC can be used for mapping the profiles of small peptides in cheese according to their hydrophobicity and is commonly used in cheese research (Bansal et al., 2009; Mane et al., 2019). More than 3000 peptides have been detected from different types of cheeses using liquid chromatography mass-spectrometry (Mane et al., 2019; Taivosalo et al., 2018). Free amino acids that reflect the levels of secondary proteolysis can be measured by a number of methods, e.g., in pH 4.6-soluble fractions of cheese fractionated using trichloroacetic acid (TCA) following the method of McDermott et al. (2016) and separation of amino acids through HPLC followed by fluorescent detection (Kabelová, Dvořáková, Čížková, Dostálek, & Melzoch, 2009).

Urea-PAGE analysis indicates that α_{S1}-CN is fully hydrolysed in Cheddar cheese after 3-4 months of ripening (Mooney, Fox, Healy, & Leaver, 1998). Primary proteolysis of Cheddar cheese is mainly caused by chymosin and

plasmin (Farkye & Fox, 1992; Mooney et al., 1998). Peptide bond Phe_{23}-Phe_{24} of α_{S1}-CN is the most susceptible to chymosin during ripening; peptide bond Leu_{101}-Lys_{102} is hydrolysed by chymosin later, followed by Phe_{32}-Gly_{33}, Leu_{98}-Lys_{99} and Leu_{109}-Glu_{110} (Mooney et al., 1998). β-CN is slowly hydrolysed by chymosin at the ionic strength of the aqueous phase in cheese during ripening, but about half of β-CN can be hydrolysed by plasmin at peptide bonds Lys_{28}-Lys_{29}, Lys_{105}-His/Gln_{106} and Lys_{107}-Glu_{108}, generating γ^1, γ^2 and γ^3-CNs (β-CN (f28-209), (105-209) and 107-209), respectively) (Mooney et al., 1998).

As for the proteinases of starter bacteria, *in vitro*, β-CN is susceptible to the cell wall-associated proteinase of the *Lactococcus* starters, while in cheese the proteinase tends to be active on peptides released from α_{S1}-CN through hydrolysis by chymosin or from β-CN by plasmin (Boutrou, Mollé, & Leonil, 2001; Visser, Robben, & Slangen, 1991).

The number of viable starter cells decreases after the pressing of cheese curd at the end of cheese manufacture. Intracellular endopeptidases (PepO, PepF), aminopeptidases (including PepN, PepA, PepC, PepX), tripeptidases and dipeptidases are released through lysis from the dead cells and hydrolyse peptides to free amino acids (Rattray & Fox, 1999; Upadhyay, McSweeney, Magboul, & Fox, 2004). The proteinases of NSLAB cause little proteolysis in Cheddar cheese but can produce free amino acids (Fox & McSweeney, 1996).

The degree of proteolysis affects the functionality and flavour of the cheese (Fox, Law, McSweeney, & Wallace, 1993). The level of soluble N at pH 4.6 as a percentage of total N (pH 4.6-SN/TN) is correlated with the hardness and meltability of Cheddar cheese (Wang et al., 2011). As levels of secondary proteolysis of cheese increase, free water can be associated with the charged

groups released from peptides, therefore increasing the cohesiveness and springiness of cheese (O'Mahony, Lucey, & McSweeney, 2005). The flavour compounds of cheese are mainly found in the water-soluble fraction (Aston, 1986) which is related to proteolysis. Compounds with carbonyl groups have been found to react with the amino acids released from proteolysis, and therefore contribute to the development of cheese flavour (Griffith & Hammond, 1989; Visser, 1993).

As mentioned before, camel chymosin exhibits higher clotting activity while being less proteolytic than bovine chymosin. Due to lower levels of primary proteolysis, Cheddar cheese manufactured using camel chymosin has been shown to exhibit higher hardness and less bitterness than that made using calf chymosin (Bansal et al., 2009; Møller, Rattray, Sørensen, & Ardö, 2012). Therefore, camel chymosin might be an option for manufacturing cheese with modified functionalities.

1.4.7.2 Metabolism of Lactate and Residual Lactose and Citrate in Cheese

The fermentation of lactose to lactic acid is a fundamental biochemical reaction by lactic acid bacteria in all types of cheese. About 98% of the lactose in cheese milk is lost in whey during cheese manufacture (Fox, Lucey, & Cogan, 1990). The metabolism of residual lactose in cheese occurs at the beginning of ripening. LAB normally metabolise lactose to L-lactic acid. Lactic acid is modified primarily by NSLAB. In most types of cheese, L-lactate is converted to D-lactate (Thomas & Crow, 1983). In Swiss-type cheese, L-lactate is converted to CO_2, acetate, and propionate, of which CO_2 is the reason for eye formation and add to the characteristic flavour (Turner, Morris, & Martley,

1983). Lactate is metabolized to CO_2 and H_2O by surface mould and probably by surface bacteria in smear-ripened cheese, which contributes to the increase in pH characteristic of such cheeses and is responsible for textural changes. Some lactate may be oxidized to acetate by *Pediococcus* in Cheddar and Dutch-type cheeses (Fox et al., 1990).

The level of citrate in cheese is low, and it can be converted to diacetyl, acetoin and 2,3-butanediol by Cit^+ strains of *Lactococcus* or by *Leuconostoc*. Diacetyl contributes to the flavour, and CO_2 produced by citrate metabolism is responsible for the minimal eye formation found in Dutch-type cheeses (Fox et al., 1990).

1.4.7.3 Lipolysis and Metabolism of Fatty Acids in Cheese

Lipid oxidation is not a reaction that commonly occurs in cheese, which is attributed to the low redox potential of cheese (McSweeney & Sousa, 2000), while the breakdown of triglycerides to free fatty acids (FFAs) by enzymatic hydrolysis (lipolysis) is critical for cheese flavour formation. In most circumstances, the level of lipolysis is low and is mostly induced by the limited lipolytic activity of LAB and NSLAB, or indigenous milk lipase, especially in raw milk cheese (Thierry et al., 2017). Lipids are good carriers and precursors of flavour compounds due to their hydrophobic nature. Certain FFAs can be perceived and given sensory characteristics by humans through trigeminal nerve and taste, which makes FFAs essential for the flavour and palatability of cheese and the preferences of customers for cheese (Poette et al., 2014).

Reduced-fat cheese lacks FFAs and typical flavour compared to full-fat cheese (Foda, Hammond, Reinbold, & Hotchkiss, 1974; Wijesundera, Drury,

Muthuku-Marappan, Gunasekaran, & Everett, 1998). Full-fat Cheddar cheese manufactured using mineral or vegetable oil presented an atypical flavour (Foda et al., 1974; Wijesundera & Watkins, 2000). Cheddar cheese manufactured from skim milk and milk fat extracted from butter showed a lower Cheddar flavour score than that of cheese made from whole milk (Foda et al., 1974). No significant differences in Cheddar cheese flavour were found between cheese made from whole milk or skim milk with cream (Wijesundera & Drury, 1999). These results indicate that the effect of milk FFAs on cheese flavour may be irreplaceable.

Severe lipolysis of cheese happens in certain Italian cheeses and Blue cheeses, the flavour of which is contributed by fatty acids or its degradation products (Fox et al., 2015). For other cheeses like Cheddar and Gouda, the level of lipolysis is low and the contribution of lipolysis to cheese quality and flavour has received comparatively little attention (Thierry et al., 2017).

FFAs play an important role in cheese flavour, and different chemical reactions transform FFAs into other flavour compounds e.g., methyl ketones, secondary alcohols, esters, and lactones (Thierry et al., 2017).

Methyl ketones are important flavour compounds in Blue cheese (Gallois & Langlois, 1990). FFAs undergo the β-oxidation to alkan-2-ones and alkan-2ol through the catabolic activity of *Penicillium* spp. (McSweeney & Sousa, 2000). Esters are transformed from FFAs in many kinds of cheese (Thierry et al., 2017) through esterification and alcoholysis (Liu, Holland, & Crow, 2004). Secondary alcohols, which may be responsible for the off-flavours in cheese, are formed through enzymatic reduction of methyl ketones (Engels, Dekker, De Jong, Neeter, & Visser, 1997; Fox et al., 2015). Lactones are compounds with a ring structure

formed through intramolecular esterification of hydroxy fatty acids (Christie, 1983; Molimard & Spinnler, 1996). Lactone levels in Cheddar cheese increase most quickly at the beginning of ripening and are present at concentrations considerably over their thresholds for flavour perception (Jolly & Kosikowski, 1975).

1.5 Acid-Coagulated Cheeses

Acid-coagulated cheeses are manufactured through the acidification of milk, skim milk or cream. The principle of the manufacture of acid-coagulated cheeses is different to that of rennet coagulated cheeses. Unlike induction of coagulation by rennet at about pH 6.4-6.6, acid coagulation happens when pH is close to the isoelectric point of casein, 4.6 (Fox, O'Connor, McSweeney, Guinee, & O'Brien, 1996).

Acid coagulation, following a decrease in milk pH, is critical to the manufacture of certain cheeses, yoghurt, and other cultured dairy products. Due to the different composition and different amino acid sequences, each protein has zero net charge at a unique pH, called the isoelectric point. The isoelectric point of the caseins is at pH 4.6. As mentioned in the rennet coagulation section, the casein micelle consists of four different casein proteins and calcium phosphate nanoclusters held together through both hydrophobic and electrostatic interactions (Bogenrief & Olson, 1995; Dalgleish, 2011).

The principle of acid coagulation is to decrease the pH of the milk to 4.6, which initiates a series of interactions that coagulate the casein micelles. When the milk is acidified to a pH value ~5.1 at temperatures > 20 ℃, physicochemical interactions happen in the casein micelle; most of the CCP in the casein micelles is dissolved, Ca^{2+} is released to the milk serum, and the

charge on each casein is changed, while the ionic strength of milk increases. On acidification of milk to pH ~ 4.6, the negative charges of the casein micelles are neutralised and the repulsion reactions between micelles are reduced. These changes initiate the aggregation of casein micelles. The coagulation starts from the formation of chains and chains are linked together to form the 3D network structure (Mulvihill & Grufferty, 1995). Acid casein gels can be formed from milk or sodium caseinate, and the acid casein gels usually have similar properties, which indicates that CCP does not play an important role during acid coagulation since it is lost from the micelle before milk reaches pH 4.6 (Lucey, Van Vliet, Grolle, Geurts, & Walstra, 1997).

Acidification can be achieved in various ways: (i) the addition of mineral acids, (ii) the addition of LAB starters, (iii) the addition of GDL, (iv) injection of carbon dioxide, or a combination of these methods (Lucey, 2016). The addition of mineral acids such as hydrochloric or sulfuric acids to milk introduces hydrogen ions into the environment and immediately decreases the pH of milk, which is called fast acidification. Fast acidification typically reduces the pH of the milk to a range of 4.2-4.6 and the caseins precipitate to produce a coarse acid casein curd. After cooking and washing, whey proteins, mineral and lactose will be removed from acid curd. The acid casein curd could be further dried and milled to acid casein powder, which is used to produce caseinates (Carr & Golding, 2016; Mulvihill & Ennis, 2003).

Unlike fast acidification, the use of LAB or GDL gradually generates acid to decrease pH over a much longer time. Mesophilic LAB such as *Lactococcus. lactis* subsp. *lactis* and *Lactococcus. lactis* subsp. *cremoris* are widely used for acidification and flavour development in acid cheese manufacture. At the

optimum temperature, LAB transforms lactose into lactic acid to generate energy. As the total number of LAB increases, more lactic acid is produced and pH decreases slowly to the isoelectric point of casein, causing coagulation of casein and, therefore, forming an acid gel. For acid coagulation initiated by LAB, lactic acid is continuously produced to reach a low pH, which eventually actually inhibits the growth of LAB. The acidification by LAB becomes more efficient as the temperature is closer to the optimum temperature for the growth of the starter. The optimum growth temperature range for LAB is between 20 and 35 ℃ (Ahmed et al., 2006).

GDL is a pH-neutral acidogen that generates gluconic acid gradually when GDL is hydrolysed by water. Since the acid production by GDL is more consistent and controllable compared to that of LAB, it is often used to study acid coagulation of milk. For milk acidified with GDL, the higher the acidifying temperature, the faster acidification will be, as the hydrolysis of GDL is promoted.

The difference between the use of GDL and LAB is the pH decrease rate of the sample. At the same temperature, GDL initiates a faster drop in pH, while LAB requires a period to grow and multiply before decreasing pH. The final pH can be controlled by the level of GDL.

To describe the acid coagulation of milk, the pH of the milk sample could be measured as the acidifying time progresses. Acid coagulation properties can be monitored by dynamic small-amplitude oscillatory rheology. Parameters like storage modulus, loss modulus and loss tangent are recorded to reflect the hardness change and acid coagulation time. During acidification, the measurement of pH and rheology may be performed simultaneously to give

plots of G' versus pH over the course of time (Meletharayil, Patel, Metzger, & Huppertz, 2016).

Preheating of milk, the temperature of acidification and buffering capacity of milk are key factors that affect acid coagulation of milk. Unlike rennet coagulation, preheating of milk promotes acid coagulation, as whey protein will be denatured by heat treatment and associated with κ-casein. When the pH decreases, heated milk is easier to coagulate, as the electrostatic repulsion is reduced by denatured whey protein through hydrophobic interactions and disulphide bonds. The gel formed from unheated milk could undergo particle rearrangements during coagulation that makes the casein network hard to provide gel firmness and results in a low G' gel (Lucey et al., 1997). Gels coagulated from heated milk were found to be firmer and more stable due to the crosslinks from denatured whey proteins (Lucey, Tamehana, Singh, & Munro, 2000).

The manufacture of acid cheeses involves pre-treatment of milk, slow quiescent acidification, coagulation, whey drainage, and curd treatments (Fig. 1.5). In the manufacture of some cheeses like Quarg, Cottage, and Fromage frais, the use of low levels of rennet is common and thus rennet coagulation and acid coagulation of milk are combined to form firmer coagulum and reduce the loss of casein during whey separation (Fox et al., 1996; Kelly & O'Donnell, 1998). In the combined coagulation of milk, the dosage of rennet is substantially less compared rennet coagulation varieties. From a protein structure perspective, acidification dissolves CCP and alters the inner and outer casein micelle structure, and acidification promotes rennet coagulation as the rennet activity increases with the decreased pH (from pH 6.7 to ~5) (Li & Zhao, 2019). Rennet

coagulation facilitates the acid coagulation of milk through hydrolysing κ-casein and reducing the surface negative charge of casein micelles. The acidification of partially renneted casein micelles may result in a higher gelation pH and a higher final G' value (Gastaldi et al., 2003).

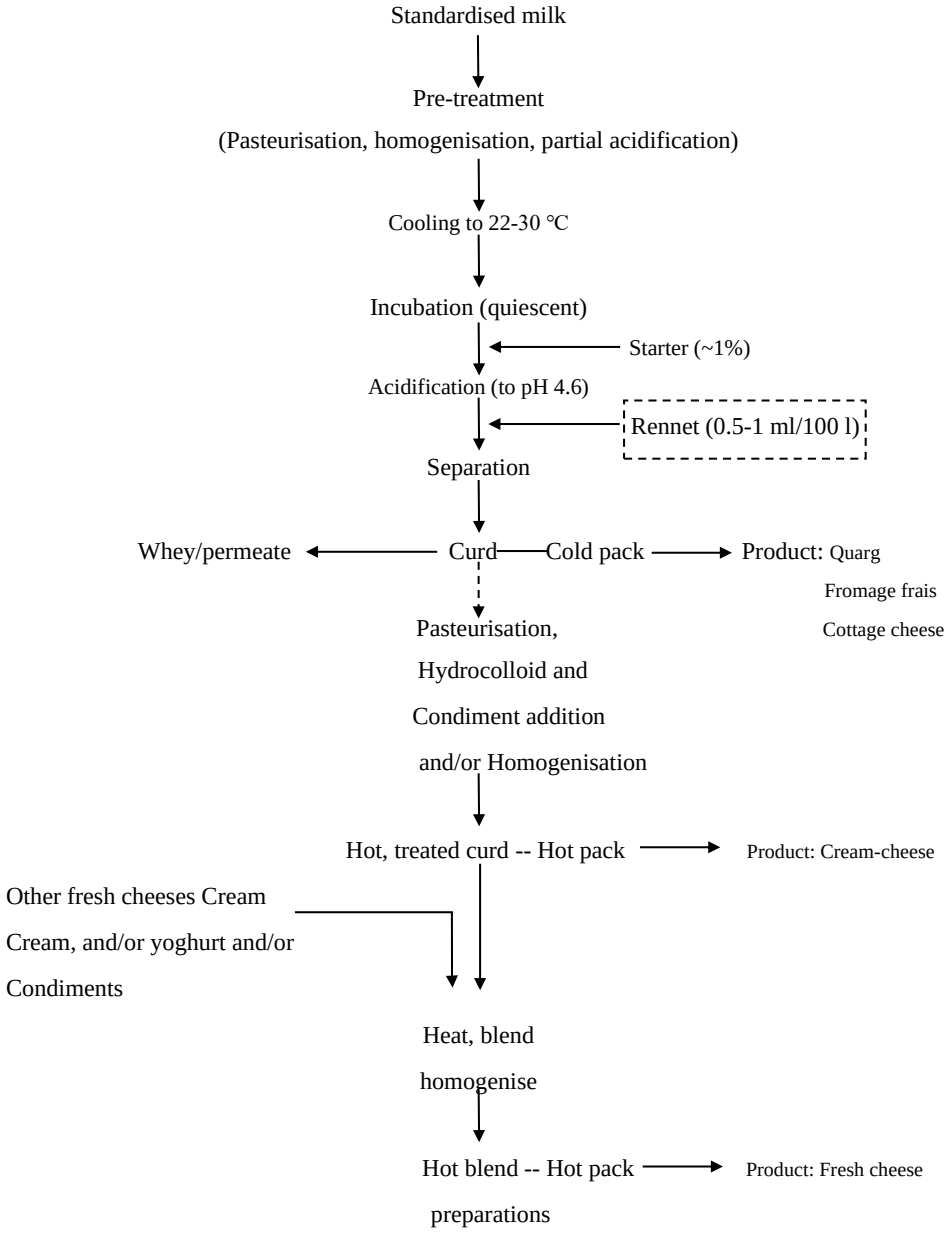

Fig. 1.5. Methods for acid-coagulated cheese manufacture (Fox et al., 1996).

1.6 Cheese Manufacture with Membrane Filtration

Membrane separation technology has been used in different aspects of dairy processing. With the application of membrane filtration, cheese plant efficiency and cheese yield can be increased, many plants worldwide use membrane filtration to manufacture various cheeses (Tamime, Robinson, & Kiers, 2006).

1.6.1 Membrane filtration

Membrane filtration processes including microfiltration (MF), ultrafiltration (UF) nanofiltration (NF) and reverse osmosis (RO) can be used to separate skim milk into different streams according to molecular size (Govindasamy-Lucey et al., 2011). Different membrane filtration processes are characterised by different pore sizes of the membrane and different pressures are applied to the membranes to fractionate streams (Fig. 1.6).

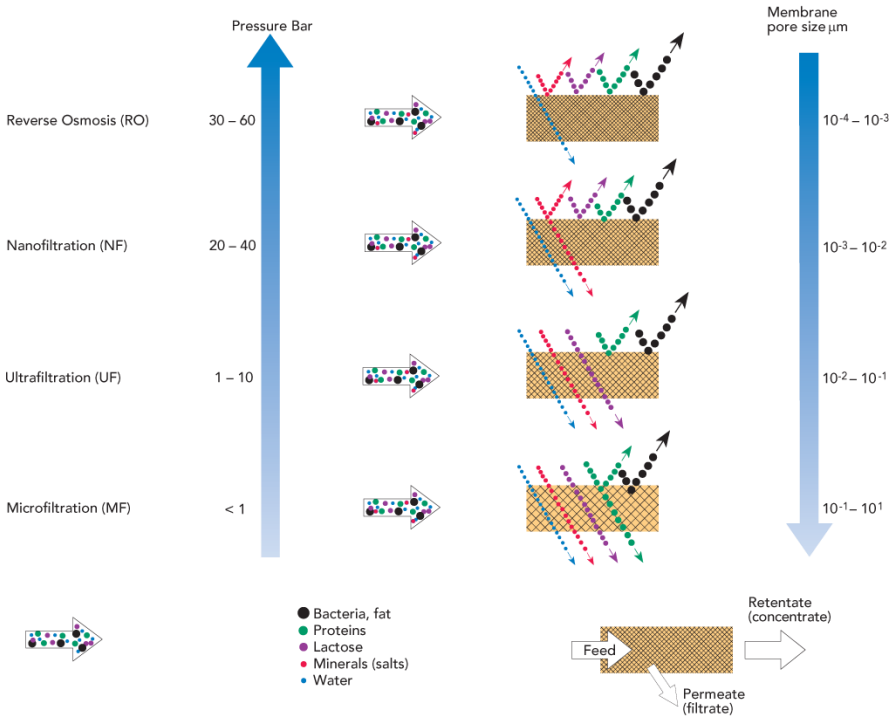

Fig. 1.6. Principles of membrane filtration (Bylund, 2003).

MF, with a pore size of 0.08-0.20 μm, allows serum proteins, lactose, minerals, and other small molecules to pass through the membrane with the pressure range of 1-3 bar (Ayral et al., 2008; Koros, Ma, & Shimidzu, 1996; Xia et al., 2020). The retentate of MF can be used to standardise cheese milk (Brandsma & Rizvi, 1999) or to purify, enrich, and recover micellar casein (Schuck et al., 1994). The main content of MF permeate is serum protein and is considered as native whey protein that is free from CMP, bacterial activity, fat, suspended cheese, and casein micelles compared to cheese whey (Bacher & Kønigsfeldt, 2000). MF can also be used to remove bacteria from cheese milk (Saboyainsta & Maubois, 2000).

UF is a process that uses higher pressures (3-10 bar) to force a permeate through a semi-permeable membrane (Ayral et al., 2008). The pore size of UF is within the range of 2 nm to 0.1 μm, which allows lactose, minerals, non-protein nitrogen and water in milk to pass through the membrane (Ayral et al., 2008; Koros et al., 1996). The concentration factor is the ratio between the initial feed and the final retentate. Low concentration factor UF (1.51 maximum) process or addition of UF retentate can concentrate the cheese-milk, standardise the casein to fat ratio to defined levels, and increase yield per vat. Higher concentration factor UF has been used for Camembert cheese and cast-Feta cheese manufacture (Kosikowski, 1974).

NF with a pore size less than 2 nm using a pressure of 10-40 bar can selectively remove small minerals (Ayral et al., 2008). RO uses a dense membrane and high applied pressure, greater than that used for osmotic pressure, which allows RO to remove water from milk (Ayral et al., 2008; Koros et al., 1996).

1.6.2 Effect of membrane filtration on milk

Besides the concentration of fat and protein, the physiochemical properties of the UF-, RO- or MF-processed milk are different to those of skim milk. Key properties affected by membrane filtration of significance to cheese-making include buffering capacity, rennet coagulation properties and rheological characteristics of the cheese milk (Smith, 2012).

Buffering capacity of milk is increased after skim milk is ultrafiltered or microfiltered, as the casein and colloidal salts like Ca, Mg and P are concentrated The consequence of this includes changes in acidification kinetics by LAB, rennet coagulation properties, enzymatic activity, rheological properties of the curd, the final pH of cheese (Saboya, Goudédranche, Maubois, Lerayer, & Lortal, 2001), growth of spoilage bacteria (Rash & Kosikowski, 1982), and water activity of the ripening cheese (Mistry, 2012). Therefore, more lactic acid from LAB is required to achieve the final pH of cheese (~ 5.2 for hard cheese and 4.6 for soft and fresh cheeses) for concentrated milk (Mistry & Kosikowski, 1985), which results in sour-tasting cheese (Maubois, 1979). The acid dissolves calcium phosphate salts and releases calcium ions into the aqueous phase during acidification of cheese manufacture, which influences casein micelle aggregation. The resulting cheese can have a sandy and brittle texture (Brulé et al., 1975) and the spreadability and stretching properties of the cheese are unsatisfactory (Green & Grandison, 1993).

In terms of rennet coagulation properties, if the same quantity of rennet is added to the same volume of milk and UF retentate, the RCT is not influenced by the increased protein content, while the time from coagulation to cutting is reduced (Dalgleish, 1980; Garnot, 1988; Upreti, Mistry, & Acharya, 2011). The

rate of enzymatic coagulation of milk increases with the substrate (casein) concentration (Garnot, 1988). At pH 6.6, about 80-90% of the κ-CN of milk is hydrolysed at the rennet coagulation point, while only 50% of hydrolysed κ-CN is required for coagulation of milk concentrated four-fold by UF (Dalgleish, 1980). The increase in protein content results in a dramatic increase in the velocity of casein micelle aggregation (Garnot, 1988) and directly relates to the final gel strength (Ferron-Baumy, Maubois, Garric, & Quiblier, 1991; Korolczuk, Maubois, & Fauquant, 1986; Kosikowski, 1986; Maubois & Mocquot, 1975; Maubois et al., 1971). The unique rennet coagulation properties of UF milk are due to the reduction in the surface hydrophobicity of casein during the UF process (Erdem, 2000).

1.6.3 Application of membrane filtration in cheese manufacture

In the context of cheese manufacture, UF can be used for protein standardisation of milk, production of milk concentrates and production of liquid pre-cheeses (i.e., concentrating milk to the total solids composition of the cheese to be made prior to manufacture). The use of UF for protein standardisation of milk may be used for cheese manufacture and may be applied to most types of cheese. The benefits of this method are daily standardisation of cheese milk, higher rennet gel strength decreases casein loss in whey, increased cheese yield due to higher protein recovery, more cheese made per vat, and no specialised cheese manufacture equipment is required except for UF equipment (Mistry, 2012). Protein standardisation by low concentration factor UF is now an increasingly common practice in very large cheese factories.

Medium-concentration factor (from 2:1 to 5:1) UF retentates have been used for several types of cheese ranging from soft to hard. Cheese made from two-fold to five-fold concentrated UF retentates requires a special machine to cut and handle the firm curd and diafiltration is needed with pure, salted, or acidified water (Ayral et al., 2008). The concept was first used in the manufacture of Camembert cheese (Maubois, Mocquot, & Vassal, 1969) and was developed further to be applied to the manufacture of fresh cheese like Quarg, as well as certain semi-hard cheeses (Guinee et al., 1995; Lucey, 2011). The method is now mainly used for the manufacture of white brined ("cast Feta")-type cheese (Hansen, 1985).

RO can concentrate cheese milk, like thermal evaporation although with less damage to whey proteins, which makes it more attractive for cheese manufacture. Cheddar cheese was manufactured using whole milk preconcentrated by RO (20-25% solid) used the same traditional equipment and had identical gross composition to that of cheese made from standardised milk (Agbevavi, Rouleau, & Mayer, 1983; Barbano & Bynum, 1984), while the dosages of rennet and starter used were reduced by 60% and 50% respectively (Agbevavi et al., 1983). With a reduction of milk volume of 20% by RO, it is possible to increase cheese yield by 2-3% (Barbano & Bynum, 1984). The volume reduction of 15% is found to be most desirable (Barbano, Bynum, & Senyk, 1983). With an 8% reduced volume of milk by RO, 5% additional cheese yield can be achieved for Cottage cheese manufacture (Barbano, 1986). This increase in yield is attributed to the entrapment of whey protein, lactose and minerals by the casein network, while the incorporation of whey protein in cheese inhibits the proteolysis of cheese during ripening (Harper, Iyer, Knighton,

& Lelievre, 1989); the increase in yield from whey protein can be off-set by a reduction in the amount of whey products that can be made. In cheese manufactured using RO-treated milk, the residual lactose content is higher than that of normal cheese; this may cause additional lactic acid fermentation by the ripening bacteria after a period of ripening when adequate lactic acid has been metabolised. This phenomenon may impair the sensory qualities of cheese (Eck & Gillis, 2000).

The applications of microfiltration in cheese manufacture are relatively novel in the dairy industry. The use of MF in the dairy field is attracting increasing attention as the range of accessible pore size became wider, which enables MF to fractionate any milk protein particles (Mistry & Maubois, 2017). MF can be used to remove bacteria from raw milk, enrich casein before the manufacture of cheese, and selectively fractionate globular milk fat.

Pasteurisation is generally used to control the microbiology of raw milk. With various temperature and time combinations, the desired microbiological effects can be achieved. However, heat treatment can induce physiochemical changes to the milk, therefore, potentially negatively affecting the functionality and cheesemaking properties of milk (Singh & Waungana, 2001). With the use of MF for microbiological control, the temperature or time of heat treatment can be reduced. The degree of bacterial removal can achieve 99.9% using MF with a pore size of 0.8 or 1.4 μm membrane, which also retains fungal cells (yeasts and moulds) (Saboyainsta & Maubois, 2000).

MF of skim milk using 0.1-μm pore size membranes allows enrichment of micellar casein (Hurt & Barbano, 2010; Maubois, Fauquant, Famelart, & Caussin, 2001). The casein content of the retentate increases as the amount of

microfiltrate increases. The applications of MF retentates are like that of UF retentates and give cheese manufacturers another pathway to manufacture cheese and recover cheese co-products (Maubois et al., 2001). The rennet coagulation properties of 20% casein-enriched cheese milk are enhanced (Maubois et al., 2001), resulting in a significant reduction of fat loss and curd loss in the whey, and increases cheese yield by 2-4% (Daviau, 2000). The stream that passes through 0.1-μm pore size membranes and undergoes diafiltration with water is a novel dairy product called micellar casein concentrate (MCC) (Schuck et al., 1994). MCC has higher heat stability than skim milk because of the lower levels of whey protein (Renhe & Corredig, 2018). MCC can be used for fortifying yoghurt and cheese milk, for the manufacture of processed cheese instead of cheese curd, for recovery of individual casein or GMP (Maubois et al., 2001), or the manufacture of Cheddar cheese (Li et al., 2020; Xia et al., 2020). Cheddar cheese manufactured from MCC has been found to have higher levels of proteolysis, plasmin and chymosin activity than that of Cheddar cheese made from standardised cheese milk, and shows higher springiness, cohesiveness and meltability (Li et al., 2020).

The enrichment of casein through MF has been used for the manufacture of Cheddar and Mozzarella cheese. With a 0.2 μm membrane MF, a retentate with 17.9% casein concentration was obtained for Mozzarella cheese manufacture (Brandsma & Rizvi, 1999). For Cheddar cheese manufacture, cheese milk was concentrated twofold to 4.2% casein, resulting in 87% casein as a percentage of true protein following MF (Neocleous, Barbano, & Rudan, 2002a, 2002b). MF influences protein recovery but does not affect the fat recovery of cheese. The use of MF for casein enrichment for low-fat Cheddar

cheese was found to give softer cheese that lacked flavour compared to full-fat Cheddar cheese (Amelia, Drake, Nelson, & Barbano, 2013). Cheese milk standardised using a MF retentate stream to a typical casein content (2.87%) had similar rennet coagulation properties, Cheddar cheese composition, yield, and texture to that of standard cheese milk (Xia et al., 2020). Cheese milk supplemented with MCC to 1.5 times casein content showed a faster curd firming rate, and the Cheddar cheese produced had lower cheese moisture, and higher pH, hardness, and cheese yield (Xia et al., 2020).

In a cold environment (< 5 °C), β-casein can partially dissociate from the casein micelle because of the weakening of hydrophobic interactions. When the temperature of the milk is decreased to 5 °C or less, β-casein will partition into the serum (Zulewska, Kowalik, & Dec, 2018). The solubilised β-casein can be fractionated from milk using MF with a 100 kDa cut-off UF membranes (Murphy & Fox, 1991a). The retentate of such membrane filtration is MCC with a higher α_S/β-casein ratio while the microfiltrate is the mixture of whey protein and β-casein (Terre et al., 1987). This technology makes it possible to manufacture cheese from a base material with an adjusted α_S/β-casein ratio in the future (Murphy & Fox, 1991b; Terre et al., 1987). In diafiltration with the removal of whey protein, the concentration of inhibitors of both plasmin and plasminogen activators decreases, which promotes the conversion from plasminogen to plasmin and thus increases plasmin activity (Aaltonen and Ollikainen, 2011). The change in enzymatic activity of diafiltered stream may affect the proteolysis in cheese manufactured therefrom. There is thus potential to make various new cheese as the hydrolysis of each casein affects the generation of cheese flavour (Mistry & Maubois, 2017).

1.7 Conclusion

Cheese manufacture involves many steps to achieve or improve the final cheese product and different varieties can be classified on the basis of rennet- and acid-based coagulation principles. The protocols of both types of cheese manufacture are summarised, and the critical point is identified as controlling the syneresis and acidification of cheese curd. A range of technologies may be applied to monitor or promote coagulation of milk, cheese manufacture and cheese properties. In recent decades, new technological approaches have been applied to cheese manufacture, such as membrane filtration, which fractionates milk components according to their size, and which may be used to enhance cheese manufacture and increase cheese yield. Overall, the processes of cheese manufacture from milk are well developed, while some novel techniques involving modified raw materials, coagulants and procedures may change the yield and functionality of cheese products, leaving many areas of the future of cheese manufacture to be explored; the future for applications of these technologies for cheese manufacture holds much promise.

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Chapter 1

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CHAPTER 2

Evaluation of rennet and acid coagulation properties of micellar casein concentrate

Bozhao Li, Thomas C. France, James A. O'Mahony, Alan L. Kelly and Paul L. H. McSweeney

School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Declaration

This chapter was written by author BL and reviewed by his co-authors. BL co-designed the study, participated in sample preparation, and was solely responsible for coagulation properties, texture analysis, and data collection, TCF contributed to the production of liquid microfiltration retentates and analysed streams for composition.

Chapter 2

Abstract

The rennet and acid coagulation properties of micellar casein concentrate manufactured using cold or warm microfiltration (MF) at the same casein content were investigated; low heat skim milk powder (LHSMP) manufactured from the same skim milk stream was used for comparison. MF retentates had higher casein content in total protein compared to LHSMP. Whey protein-casein aggregates formed during heat treatment were only found in LHSMP. With lower level of native whey protein, all MF retentates showed shorter rennet coagulation time and higher gel strength compared to LHSMP. At same casein content, longer rennet coagulation times were found in cold MF retentates compared to warm MF retentates, as the level of κ-CN increases with the depletion of β-CN. As for acid-induced coagulation, all MF retentates coagulated at a higher pH (>5) than the gelation pH (4.7-4.9) of LHSMP. An inflection point in G' value was found during the acidification of warm MF retentates, while was not presented in that of cold MF retentates. Textural and microscopy analysis at pH 5 and 4.6 confirmed the gelation of MF retentates at pH 5. This was due to the structure arrangement initiated by releasing of colloidal calcium phosphate and compacting of structure of warm MF retentates during the decrease of pH value. Both warm and cold MF retentates exhibited different rennet- and acid-induced coagulation properties compared to LHSMP, which might be possible to be utilised for the manufacture of cheese or yogurt with tailored functionalities.

Chapter 2

2.1 Introduction

The principle of cheese manufacture involves concentrating the casein and fat in milk about 10-fold and separating the whey protein and much of the lactose and minerals into the whey. Cheese can be roughly divided into two major families based on either rennet- or acid-induced coagulation of the milk. The rennet and acid coagulation properties of milk are essential in evaluating the cheese manufacturing potential of milk (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015).

Rennet coagulation is a two-stage biochemical process. In the first stage, κ-CN is cleaved into *para*-κ-CN and glycomacropeptide by the action of a proteinase, usually chymosin (Morr, 1975) and, with the consequently reduced negative charge on the surface and reduced steric stabilisation, *para*-casein micelles aggregate in the second stage to form a three-dimensional network (Cheryan, Van Wyk, Olson, & Richardson, 1975).

The principle of acid-induced coagulation involves decreasing the pH of the milk to the isoelectric point of casein (pH 4.6). At a pH of around 4.6, the negative charges on the surface of casein are neutralised, as the protein reaches its isoelectric point, and this initiates the aggregation of casein micelles (Mulvihill & Grufferty, 1995). This typically happens due to fermentation by lactic acid bacteria. To study the acid-coagulation properties of milk, glucono-δ-lactone (GDL) can be used for slow acidification to mimic the action of lactic acid bacteria by producing gluconic acid (Lucey, 2016).

There is an interest in the manufacture of cheese using membrane separation produced material. Membrane separation technology has been used in many aspects of dairy processing; for example, microfiltration (MF) with pore size of

0.1-0.5 μm allows serum protein, lactose, minerals, and other small molecules in milk to pass through the membrane (Carter, Cheng, Kapoor, Meletharayil, & Drake, 2021). The permeate of MF is considered as "ideal" whey since its purity is higher than that of whey recovered from cheese manufacture. MF retentate, which is mainly micellar casein and milk serum, is commonly called micellar casein concentrate (MCC) following drying.

The common operating temperatures of MF for separating casein and serum protein is around 45-55 ℃, which decrease the viscosity of permeate thus maintain a higher flux level (McCarthy, Wijayanti, Crowley, O'Mahony, & Fenelon, 2017; Zulewska & Barbano, 2014). When operating MF at cold temperatures (≤ 20 ℃), microbial growth in the sample is better controlled and fouling is reduced compared to warm MF (France et al., 2021; Schiffer & Kulozik, 2020). However, at a temperature of 5 ℃ or less, β-CN partitions from casein micelle into the serum phase (Zulewska, Kowalik, & Dec, 2018), and the β-CN in serum permeates through MF membranes rather than being retained in retentates (France et al., 2021; McCarthy et al., 2017; O'Mahony et al., 2014; Seibel et al., 2015). Performing MF at low temperatures generates β-CN-enriched whey permeate and higher α_s/β-CN ratio retentate products. Colloidal calcium phosphate (CCP) undergoes a similar partitioning as β-CN, in that CCP dissociates from casein micelles at low temperatures and both total and ionic calcium contents increase in the permeate of skim milk (France et al., 2021; Luo et al., 2015; Méthot-Hains et al., 2016).

Due to the differences mentioned above, warm and cold MF may produce retentates with different properties. Many studies have researched the feasibility of using MCC for the manufacture of different dairy products including cheese, high-protein bars and Greek-style yoghurt (Bong & Moraru, 2014; Hogan, Chaurin,

O'Kennedy, & Kelly, 2012; Li et al., 2020). The cheese manufactured from milk with lower β-CN content showed higher meltability on heating and less bitterness during ripening (O'Mahony, McSweeney, & Lucey, 2008). Seibel et al. (2015) reported that MCC with lower levels of β-CN formed weaker rennet-induced gels compared to MCC processed at warm MF temperatures. The cheese manufactured with cold MF retentate resulted in lower pH than that of warm MF retentate due to the lower ash content and buffering capacity in the cold MF retentate sample, and the rennet coagulation properties were not affected by the β-CN level of MCC (Xia et al., 2022).

Solid membranes can be produced from inorganic or organic materials. Inorganic materials include glass, metals, pyrolyzed carbon, and ceramics. Organic materials are consist of cellulose, cellulose acetate and other polymeric materials (Hausmann, Duke, & Demmer, 2012). Ceramic and polymeric membranes are commonly used for microfiltration (Xia et al., 2022; France et al., 2022). For dairy industry uses, ceramic membranes can be used for more than 8 years while polymeric membranes are changed every 2 years (Schier & Paar, 2007). Ceramic membranes can maintain high water flux while are relatively sensitive to operating temperatures compared to polymeric membrarnes, therefore is usually used for warm microfiltrartion (Xia et al., 2022; Smith, 2012).

To determine the coagulation properties of dairy material, various methods can be used. The Berridge method is a convenient and fast method to measure the rennet coagulation time (RCT) of milk, which is the time at which a sample starts to coagulate (IDF, 2007). Dynamic low-amplitude oscillatory shear rheology using a rheometer applies sinusoidal oscillatory shear to a coagulating sample and continuously measures the storage modulus (G', the energy stored by the sample

during each oscillatory shear cycle), loss modulus (G", the energy transformed to heat during each oscillatory shear cycle) and other parameters that reflect the gel strength of the sample throughout coagulation (Foegeding & Drake, 2007; Guinee, Feeney, Auty, & Fox, 2002; Gunasekaran & Ak, 2003; Tunick, 2011).

The coagulation properties for cheese manufacture of MF retentates have not been characterised comprehensively before. This study investigated the coagulation properties of several dairy streams including low heat skim milk powder, liquid warm and cold MF retentate manufactured from lab-scale membrane filtration, and spray-dried warm and cold MF retentate powder manufactured from pilot plant-scale membrane filtration. The objective of this study was to explore the differences in rennet- and acid-induced coagulation properties and hence suitability for cheese manufacture between those materials.

2.2 Material and methods

2.2.1 Pilot plant-scale membrane filtration

Pilot plant-scale low-heat skim milk powder and cold and warm MF retentate powder were produced at Teagasc (Moorepark, Fermoy Co. Cork, Ireland) in the studies of Xia et al. (2020, 2022). Pasteurised skim milk was obtained from a local dairy company. One portion of pasteurised skim milk was evaporated at 65 ℃ and spray dried to low heat skim milk powder (LHSMP1) (Fig. 2.1). Another portion of pasteurised skim milk was chilled and microfiltered using an 800kDa polyvinylidene fluoride (PVDF) membrane (Model: FR-2B-3838, Synder Filtration, Vacaville, CA, USA) and diafiltered twice with reverse osmosis (RO) water at 3 ℃. The manufacture process temperature was held at 8.5 ℃ to partition some β-CN to the MF permeate and obtain β-CN-depleted casein micelles in the

cold MF retentate. The pilot plant-scale polymeric membrane cold MF retentate was denoted PpCR. The PpCR stream was then spray-dried by a pilot-scale single-stage spray-dryer (Anhydro Laboratory Spray Dryer, SPX Flow Technology, Kolding, Denmark) to powder, with inlet and outlet temperatures of 185 and 85 ℃, respectively. Another portion of pasteurised skim milk was microfiltered using the same polymeric membrane at 47 ℃ and diafiltered twice with RO water at 47 ℃. The pilot plant-scale polymeric membrane warm MF retentate was named PpWR and was spray-dried to powder. All powder samples were packaged in bags, sealed and held at 4 ℃ (Xia et al., 2022).

In separate experiments (Fig. 2.2), raw skim milk was microfiltered using a 1.4-μm membrane (Tami Isoflux® ceramic membranes, Tami Industries, Nyons, France) to retain bacteria and spores. The bacteria-free permeate was heated to 50 ℃ and microfiltered using a 0.14-μm ceramic membrane (Tami Isoflux® ceramic membranes, Tami Industries, Nyons, France), followed by diafiltration (DF) twice with 50 ℃ RO water. The pilot plant-scale ceramic membrane warm MF retentate was named PcWR and was spray-dried to powder, packaged, sealed and held at 4 ℃ (Xia et al., 2020).

2.2.2 Lab-scale membrane filtration

Lab-scale cold and warm MF retentate streams were produced as described by France et al. (2022). Commercial low heat skim milk powder (LHSMP2) obtained from a local dairy company was rehydrated with ultrapure water at 20 ℃ over 2 h to achieve 3.2% protein (Fig. 2.3). The skim milk was gently stirred at 4 ℃ for 48 h before filtration or analysis. The reconstituted skim milk was filtered using Whatman No 113 filter paper and then microfiltered with a lab-scale filtration device (Crowley, O'Callaghan, Kelly, Fenelon, & O'Mahony, 2015) using a 1000-

kDa polyethersulfone membrane (Biomax, Merck Millipore, Merck KGaA, Darmstadt, Germany) at 4 or 50 ℃ (France et al., 2022). The lab-scale liquid cold (4 ℃) and warm (50 ℃) MF retentates were denoted LCR and LWR respectively.

2.2.3 Composition of powders and liquid samples

The concentrations of total protein and non-casein nitrogen and non-protein nitrogen of the LHSMP1, PcWR, PpWR and PpCR powders were measured by the method described by Niro (1978). The total nitrogen content and non-casein nitrogen of lab-scale streams were determined by using the Kjeldahl method (IDF, 2001) and the method of Wojciechowski and Barbano (2015) as modified by France et al. (2022), respectively.

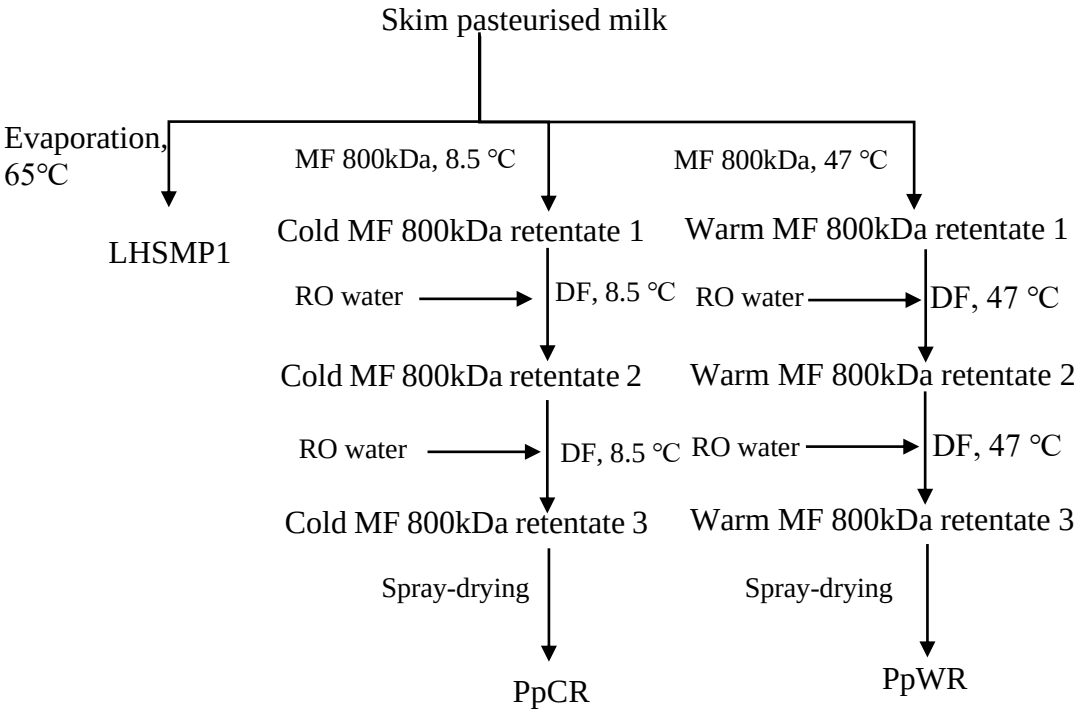

Fig. 2.1. Flow chart of pilot plant-scale polymeric membrane filtration applied in manufacture of cold and warm microfiltration (MF) retentate powder, modified from Xia et al. (2022). DF, diafiltration; RO, reverse osmosis; PpCR, pilot plant-scale cold MF retentate; PpWR, pilot plant-scale polymeric membrane warm MF retentate.

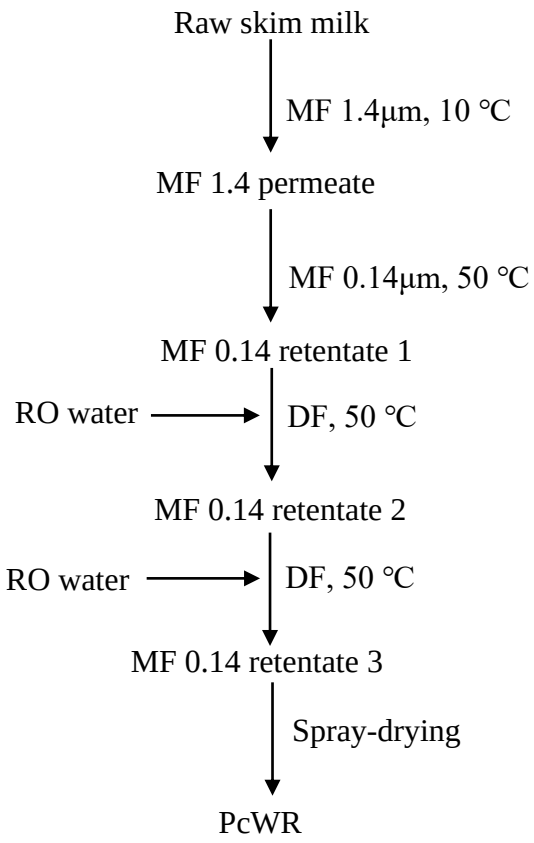

Fig 2.2. Flow chart of pilot plant-scale ceramic membrane filtration applied in manufacture of warm microfiltration (MF) retentate powder, modified from Xia et al. (2020). DF, diafiltration; RO, reverse osmosis; PcWR, pilot plant-scale ceramic membrane warm MF retentate.

2.2.4 Sample preparation

Powders LHSMP1, LHSMP2, PcWR, PpWR and PpCR were rehydrated with deionised water at 50 ℃ over 2 h and stored at 4 ℃ overnight with constant magnetic stirring. After rehydration, the composition of samples was measured by MilkoScan™ Mars (Foss, Hilleroed, Denmark) and the casein content was calculated by multiplying the fraction of casein in total protein by the protein content of each sample. Both reconstituted samples and liquid retentate samples were standardised to a casein content of 2.59%; 0.05% NaN_3 (v/w) and 0.09% $CaCl_2$ (v/w) were added to all samples.

2.2.5 SDS-PAGE

The protein profile of the powder and liquid samples was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) as modified by France et al. (2020). The gels were stained with Coomassie Brilliant Blue R250 and destained with a water, methanol, and acetic acid mixture (50:40:10).

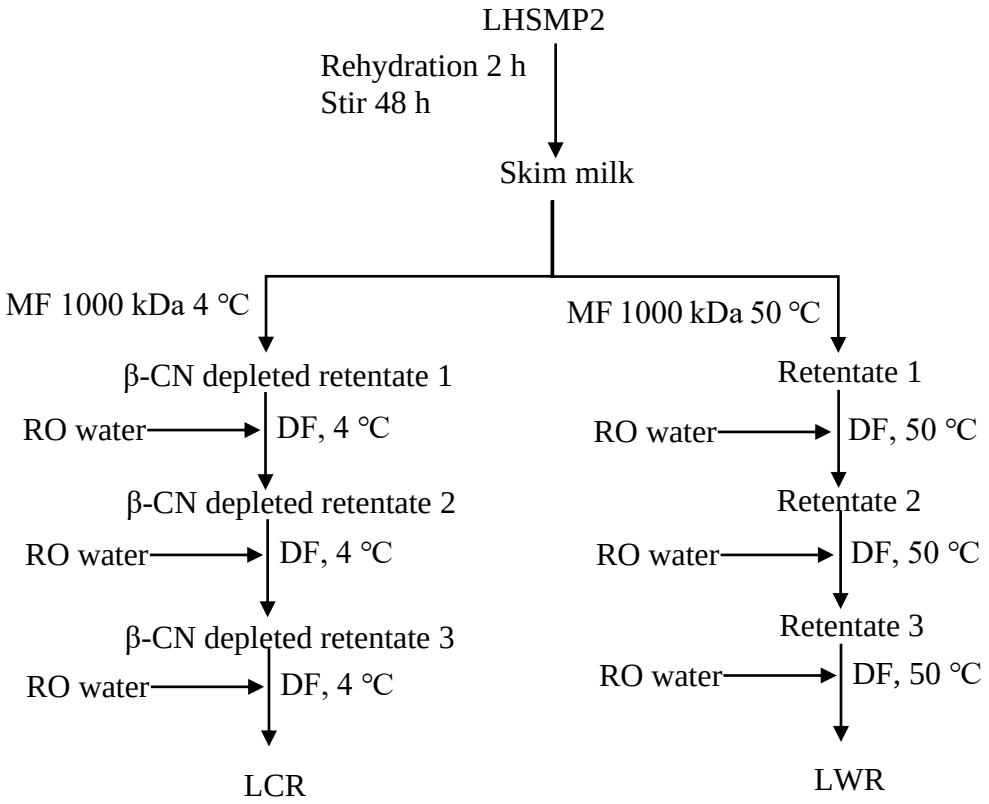

Fig 2.3. Flow chart of lab-scale polyethersulfone membrane filtration applied in manufacture of cold and warm microfiltration (MF) retentate stream, modified from France et al (2022). DF, diafiltration; DI, deionised; LCR, lab-scale cold MF retentate; LWR, lab-scale warm MF retentate.

2.2.6 Urea-PAGE

Urea-polyacrylamide gel electrophoresis (urea-PAGE) was applied directly to the powder and liquid samples on equal protein bases according to the method of Andrews (1983) as modified by (Shalabi & Fox, 1987). The gels were stained with Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977) and destained with deionised water several times.

2.2.7 Rennet Coagulation Properties

Before rennet coagulation tests, reconstituted LHSMP1, LHSMP2, PcWR, PpWR and PpCR and liquid retentates LCRL and LWR were standardised to a pH of 6.65 and pH readjusted after 30 min at room temperature. Rennet coagulation time (RCT) was determined using the modified Berridge method (IDF, 1987) using 0.2 international milk clotting units (IMCL) mL^{-1} commercially available fermentation-produced bovine chymosin (CHY-MAX® Extra, 600 IMCU mL^{-1}, Christian Hansen A/S, Hørsholm, Denmark). Two mL of each sample was placed in transparent glass tubes and warmed to 32 ℃ in a water bath. After chymosin was added, the tubes were gently rocked in the water bath, and RCT was recorded when visible flocculation was observed (IDF, 1987).

Dynamic oscillatory analysis (small-amplitude oscillatory measurement) of all samples was performed using an AR-G2 controlled-stress rheometer (Waters TA Instruments, Leatherhead, Surrey, UK). Aliquots (25 mL) of each sample were pre-warmed in a water bath at 32℃ for 15 min, and 0.2 IMCU mL^{-1} bovine chymosin was added and mixed with the sample. The sample was immediately placed in the preheated concentric cylinder (32 ℃) and oscillated by a conical rotor at a frequency of 1 Hz, and the storage modulus, G', of the sample was recorded

continuously as a function of time at a low-amplitude shear strain (0.01) over 60 min (Ibáñez, Waldron, & McSweeney, 2016). Each sample was analysed in triplicate.

2.2.8 Glucono-δ-lactone-induced acid coagulation properties

Various amounts of GDL were added to each sample at 30 ℃ to determine the GDL level required to decrease the pH value of the sample to 4.6 within 4 h (Meletharayil, Patel, Metzger, & Huppertz, 2016). Dynamic oscillatory analysis of all samples was performed by the rheometer as described above. Aliquots (50 mL) of each sample were pre-warmed in a water bath at 30 ℃ for 15 min, and predetermined levels of GDL were added and mixed to decrease the pH of the sample to ~4.4-4.6 within 4 h (Meletharayil et al., 2016). Half of the mixture was held in a water bath and the pH was measured by a pH meter every 20 min. The other half of the mixture was placed in a preheated concentric cylinder (30 ℃) and oscillated under the same conditions mentioned above. Each sample was analysed in triplicate.

2.2.9 Texture analysis

The hardness and cohesiveness of acid-induced gels of LHSMP1, PcWR, PpWR and PpCR at pH 5 and pH 4.6 were measured through back-extrusion by a Texture Analyser TA-XTplusC (Stable Micro Systems, Godalming, Surrey, UK). Aliquots (100 mL) of each reconstituted sample were placed in a 150-mL beaker and pre-warmed in a water bath at 30 ℃ for 15 min. The same level of GDL as determined above (Section 2.2.8) was added and mixed to initiate acid-induced coagulation. The pH of the sample was monitored and, when that decreased to the desired pH, the sample was analysed immediately. For the back-extrusion test, a

back extrusion rig (A/BE; Stable Micro Systems, Godalming, Surrey, UK) was used for compression test mode, the speed was 1.00 mm s^{-1} and the distance was 30 mm (Joon, Mishra, Brar, Singh, & Panwar, 2017). The hardness of samples was measured by the texture analysis. Three acid gel samples were measured for each treatment.

2.2.10 Confocal Laser Scanning Microscopy

Reconstituted samples LHSMP1, PcWR, PpWR and PpCR were pre-warmed at 30 ℃ for 15 min and 5% (v/w) of Fast Green was added to each sample to stain the protein. Each sample was acidified by the same level of GDL as section 2.2.8. After the mixture, two aliquots (240 μL) of each sample at pH 5 or 4.6 respectively were transferred onto the microscopy slide and held at 30 ℃ in an incubator. The remainder of each sample was retained for pH measurement. Prior to microstructure analysis, acidification was stopped by refrigerating the sample at 4 ℃ for at least 10 min when the pH of that reached 5 or 4.6. Confocal Laser Scanning Microscopy (Olympus FV1000 confocal laser scanning inverted microscope, Olympus Corporation, Tokyo, Japan) was used to observe the microstructure of acid-induced gels. Helium/neon (633 nm) lasers were used for the excitation of Fast Green FCF (Auty et al., 2001).

2.2.11 Statistical analysis

To determine significant differences between samples, statistical analysis was performed using one-way analysis of variance (ANOVA) with R project for Windows version i386 3.4.0 (the R Foundation for Statistical Computing, University of Auckland, Auckland, New Zealand). For statistical significance, the probability level employed was $P < 0.05$.

2.3 Results and discussion

2.3.1 Composition

The composition of all powder and liquid samples are shown in Table 2.1. All MF retentates had a higher content of casein in total protein than that of LHSMP. MF separates whey protein from the feed and increases the casein content of total protein in MF retentates (Carter et al., 2021). The content of casein in total protein in all liquid MF retentates was higher than that of all powder MF retentates. This might due to the different MF process for the lab- and pilot plant-scale manufacture of samples. Three times of diafiltration was applied on lab-scale samples while only twice diafiltration was applied for pilot plant-scale sample. The levels of reduction in β-CN were 4.25% and 40.3% in PpCR and LCR, respectively (Xia et al., 2022; France et al., 2022), which may be attributed to the different degree of β-CN dissociation under different MF temperature.

Table 2.1. Protein content and casein content of total protein of original and standardised low heat skim milk powder (LHSMP1, LHSMP2), cold and warm MF retentate stream (LCR, LWR), cold and warm polymeric MF retentate powder (PpCR, PpWR), warm ceramic MF retentate powder (PcWR).

	Casein % of total protein	Original protein %	Standardised[1] protein %
LHSMP1	81.1	37.1	3.20
LHSMP2	80.1	32.2	3.23
LCR	97.6	8.29	2.65
LWR	97.1	8.40	2.67
PpCR	88.1	60.6	2.94
PcWR	91.2	80.4	2.84
PpWR	88.9	64.2	2.91

[1]Reconstituted and standardised to a casein content of 2.59% for all samples.

2.3.2 Protein profiles

The protein profiles of all samples analysed by SDS-PAGE are shown in Fig. 2.4. Major bands including α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, β-lactoglobulin (β-Lg), α-lactalbumin (α-La) and lactoferrin were identified in samples with and without 2-mercaptoethanol, i.e. under reducing or non-reducing conditions, respectively (Fig. 2.4), which is consistent with the previous research for reconstituted skim milk (Jovanovic, Barac, Macej, Vucic, & Lacnjevac, 2007). No obvious differences can be found in the bands of α_{S1}-CN, α_{S2}-CN, and β-CN of all samples. The relative levels of β-Lg and α-La were lower in all MF retentates compared to that of both samples of LHSMP, as either cold or warm MF retains casein and permeates whey protein (Carter et al., 2021). During heat treatment, aggregates between κ-CN, β-Lg, and α-La were formed through disulphide interactions while, under reducing conditions, disulphide interactions that form aggregates are dissociated (Jovanovic et al., 2007). The levels of β-Lg and α-La were lower in both LHSMP samples under non-reducing conditions compared to that under reducing conditions, which indicates that some heat-induced aggregates were formed during the manufacture of LHSMP. The aggregates in LHSMP2 were removed through filtration by filter paper prior to MF. For all MF retentates, no obvious differences were found in the levels of β-Lg and α-La between samples under reducing and non-reducing conditions, which indicates that the heat-induced aggregates were not formed during the manufacture of MF retentates.

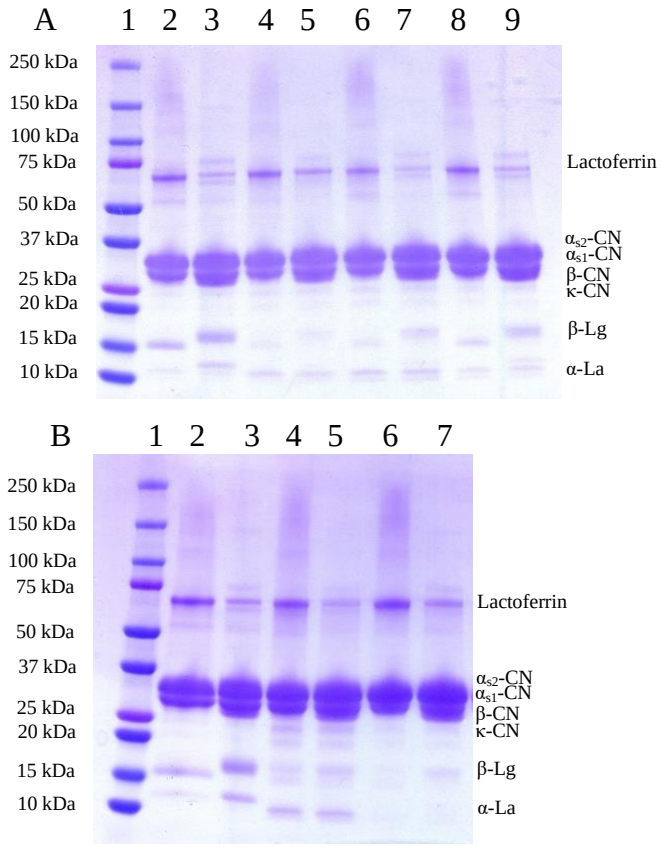

Fig 2.4. SDS-polyacrylamide gel electrophoretograms of LHSMP, pilot plant-scale MF retentate powder, and lab-scale MF retentate streams samples under non-reducing and reducing conditions. In gel A: 1. Molecular weight standards, 2 and 3. LHSMP1, 4 and 5. PcWR, 6 and 7. PpWR, 8 and 9. PpCR. Lanes 2, 4, 6, and 8 were samples under non reducing conditions, 3, 5, 7, 9 were samples under reducing conditions. In gel B: 1. Molecular weight standards, 2 and 3. LHSMP2, 4 and 5. LWR, 6 and 7. LCR. Lanes 2, 4, and 6 were samples under non reducing conditions, 3, 5, and 7 were samples under reducing conditions. The figure corresponds to data for one of the three replicates, all replicates were similar.

The casein profiles of all samples analysed by urea-PAGE are shown in Fig. 2.5. Both samples of LHSMP showed similar levels of α_{S1}-CN and β-CN, while different levels of the breakdown of β-CN were found between LHSMP and all MF retentates samples. B-CN can be hydrolysed to fragments β-CN (f29-209), β-CN (f106-209), and β-CN (f108-209) by the action of plasmin (Farrell et al., 2004). Higher levels of β-CN (f106-209) and β-CN (f108-209) were found in warm MF

retentates compared to cold MF retentates, which may be attributed to higher plasmin activity during warm MF processing. The optimum temperature of plasmin is around 37 ℃ (Vélez, Perotti, Candioti, Bergamini, & Hynes, 2016), which is closer to the temperature of warm MF processing. The plasmin activity of UF retentates produced at 5 ℃ was lower than that in retentates produced at 50 ℃ (Puri, Singh, & O'Mahony, 2020). In the manufacture of cold MF retentates, β-CN dissociates from casein micelles into the serum phase of milk (Zulewska et al., 2018), and β-CN fragments might partition from casein micelle under the same condition, which may cause the lower levels of β-CN (f106-209) and β-CN (f108-209) in cold MF retentates compared to warm MF retentates.

2.3.3 Rennet coagulation properties

The rennet coagulation properties measured through the water bath and rheometer of all samples are shown in Table 2.2. At the same casein content, all MF retentates showed significantly lower ($P < 0.05$) RCT and time to G' > 1 Pa and a significantly higher ($P < 0.05$) G' value after 60 min after rennet addition than that of both samples LHSMP, which was in agreement with the findings of Xia et al. (2022). The rennet coagulation properties of milk deteriorate when milk undergoes heat treatment as the level of κ-CN/β-Lg aggregates increases due to heat denaturation of β-Lg (Lin, Kelly, O'Mahony, & Guinee, 2018), resulting in longer gelation time and lower gel strength. From the result of SDS-PAGE, the levels of κ-CN/β-Lg aggregates were higher in both samples of LHSMP than that of all MF retentates. During rennet coagulation, although κ-CN in κ-CN/β-Lg aggregates can be hydrolysed by chymosin, the aggregates remain soluble and may hinder the formation of the *para*-casein network (Mollé, Jean, & Guyomarc'h, 2006). MF partitions whey protein from casein which decreases the whey protein

content in the retentates; therefore, less whey protein is left and denatured during the manufacture of MF retentates compared with LHSMP (Carter et al., 2021), which may lead to a decreased level of κ-CN/β-Lg aggregates. Other than denatured whey protein, the existence of native whey protein impedes rennet-induced coagulation of milk as native whey protein produces a physical barrier that prevents the gelation of *para*-casein micelles (Gamlath, Leong, Ashokkumar, & Martin, 2018). No significant differences ($P > 0.05$) were found for the rennet coagulation properties of warm MF retentates produced by ceramic or polymeric membranes.

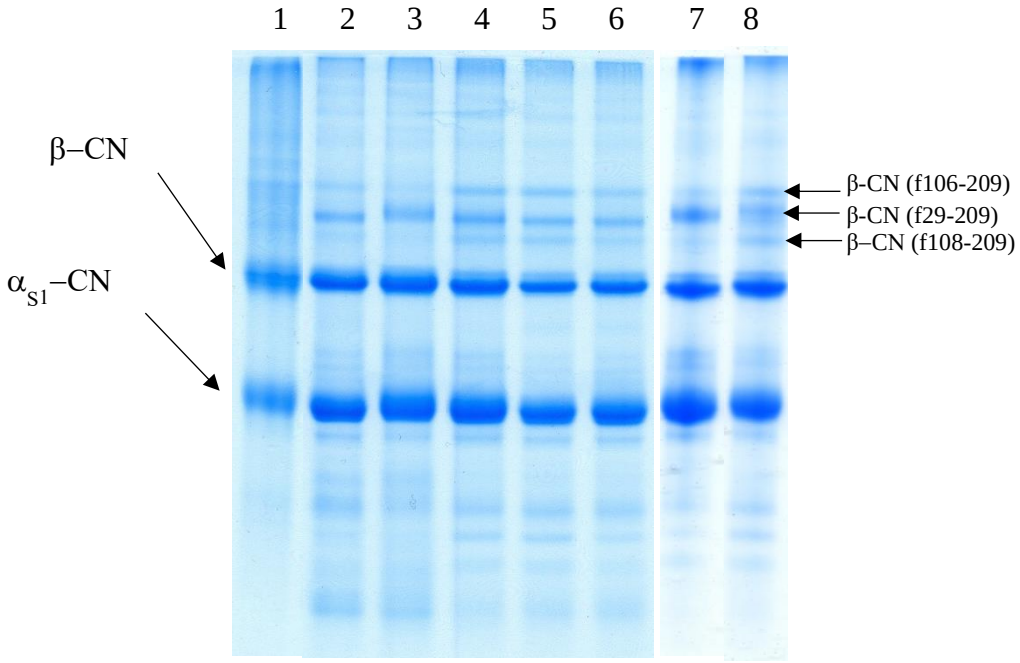

Fig. 2.5. Urea-polyacrylamide gel electrophoretograms of LHSMP, pilot plant-scale MF retentate powder, and lab-scale MF retentate streams samples. 1. Sodium caseinate standard, 2. LHSMP1, 3. LHSMP2, 4. PcWR, 5. PpWR, 6. PpCR, 7. LCR, 8. LWR (note bands from different gels were presented). The figure corresponds to data for one of the three replicates, all replicates were similar.

Table 2.2. Rennet coagulation properties of low heat skim milk powder (LHSMP1, LHSMP2), cold and warm MF retentate stream (LCR, LWR), reconstituted cold and warm polymeric MF retentate powder (PpCR, PpWR), reconstituted warm ceramic MF retentate powder (PcHR).

	RCT (s)[1]	Time G' > 1 Pa (s)	G' after 60 min (Pa)
LHSMP1	306^{d} (9.61)	439.97^{d} (7.04)	108.92^{a} (6.88)
LHSMP2	308^{d} (13.32)	438.20^{d} (58.37)	90.66^{a} (12.52)
LCR	108^{c} (7.00)	104.93^{ab} (5.30)	181.19^{d} (10.35)
LWR	81^{a} (6.18)	86.07^{a} (5.53)	156.54^{c} (6.93)
PpCR	120^{c} (2.52)	181.20^{c} (3.50)	133.40^{b} (10.42)
PcWR	92^{ab} (3.06)	93.73^{ab} (6.19)	140.31^{bc} (9.13)
PpWR	103^{bc} (7.76)	122.85^{b} (8.63)	148.09^{bc} (6.92)

[1]RCT, rennet coagulation time. Values are means (standard deviation) from three independent trials; means within the same column not sharing a common superscript differ significantly ($P < 0.05$).

Either liquid warm MF retentates or reconstituted warm MF retentates showed relatively lower RCT and time G' > 1 Pa compared with cold MF retentates. No significant differences ($P > 0.05$) were found in the final G' values between all reconstituted MF retentates, while significantly higher ($P < 0.05$) final G' values in liquid cold MF retentates were found compared to that of liquid warm MF retentates. β-CN dissociates from casein micelle at low temperature and part of β-CN is separated from retentate during cold MF (France, Bot, et al., 2021; McCarthy et al., 2017; O'mahony et al., 2014; Seibel et al., 2015; Zulewska et al., 2018). In the second stage of rennet coagulation, when around 85% of κ-CN is hydrolysed by the enzyme, the colloidal stability of *para*-casein micelles is reduced, which initiates the aggregation of *para*-casein micelles (Fox et al., 2015). Since the β-CN content in cold MF retentates is lower than that in warm MF retentates, at the same casein content, the relative κ-CN content in cold MF

retentates may be increased compared to that in warm MF retentates; therefore, the time when micelles became unstable with the reaction of chymosin may be delayed.

Liquid cold MF retentates showed significantly shorter ($P < 0.05$) time G' > 1 Pa and significantly higher ($P < 0.05$) G' value after 60 min than that of reconstituted cold MF retentates. This may due to the higher casein content of total protein in liquid cold MF retentates compared to reconstituted cold MF retentates, since less native whey protein in liquid cold MF retentates inhibits rennet coagulation (Table 2.1). As for warm MF retentates, sample LWR showed significantly shorter ($P < 0.05$) RCT and time G' > 1 Pa compared with warm MF retentate processed using the polymeric membrane, which might be attributed to the higher casein content of total protein in the liquid samples (Table 2.1).

2.3.4 Acid coagulation properties

The changes of storage modulus (G') as a function of pH values of all samples are shown in Fig. 2.6 and the acid coagulation properties of all samples are shown in Table 2.3. Due to the differences in buffering capacities, the levels of GDL addition to the samples for acidification were adjusted to decrease the pH of samples to 4.4-4.6 within 4 h at 30 ℃. All samples showed acid-induced gelation. Both samples of LHSMP started gelation at a pH value below 5, which is consistent with previous studies (Donato et al., 2007; Li & Corredig, 2020), while all MF retentate samples gelled at a pH value over 5. Native whey protein produces a physical barrier that prevents the gelation of *para*-casein micelles during rennet-induced coagulation of milk (Gamlath et al., 2018). κ-CN molecules on the surface of casein micelle is negatively charged and this prevents casein micelles from aggregating with each other; when the pH of milk is close to 4.6, κ-CN hairs are neutralised and collapse, thereby the steric and electrostatic stabilisations between

casein micelles are decreased (De Kruif, 1998; Horne, 2002; Lucey & Singh, 1997). The presence of native whey protein may also impede the formation of a casein micelles network in acid gelation. At the same casein content, the significantly higher ($P < 0.05$) gelation pH value of MF retentates might be attributed to the relatively lower whey protein content compared to that of LHSMP, as native whey protein might impede the aggregation of casein particles. The increased ionic strength may screen the charged groups on casein and weaken the electrostatic interactions between casein micelles (Lucey et al., 1997b; Roefs & Van Vliet, 1990). DF removes colloidal calcium phosphate from MF retentates, which affects the gel network rearrangements between pH 5.3 to 4.8 and increases the gelation pH of the sample (Li & Corredig, 2020).

No significant differences ($P > 0.05$) were found for the acid coagulation properties between ceramic and polymeric membrane warm MF retentates. All warm MF retentates gelled at a significantly higher ($P < 0.05$) pH compared with both cold MF retentates. In the milk system, casein micelles are stabilised through the steric repulsion between casein particles and the effect of negative charge on the κ-CN on the surface of micelles (Mulvihill & Grufferty, 1995; Walstra, 1990). When the negative charge on the surface of casein micelles is neutralised by acidification, casein particles aggregate to form a network (Mulvihill & Grufferty, 1995). At the same casein content, the relative κ-CN content in cold MF retentates was increased compared to that of warm MF retentates; therefore, the pH value required to neutralise the negative charge of micelles may be lower.

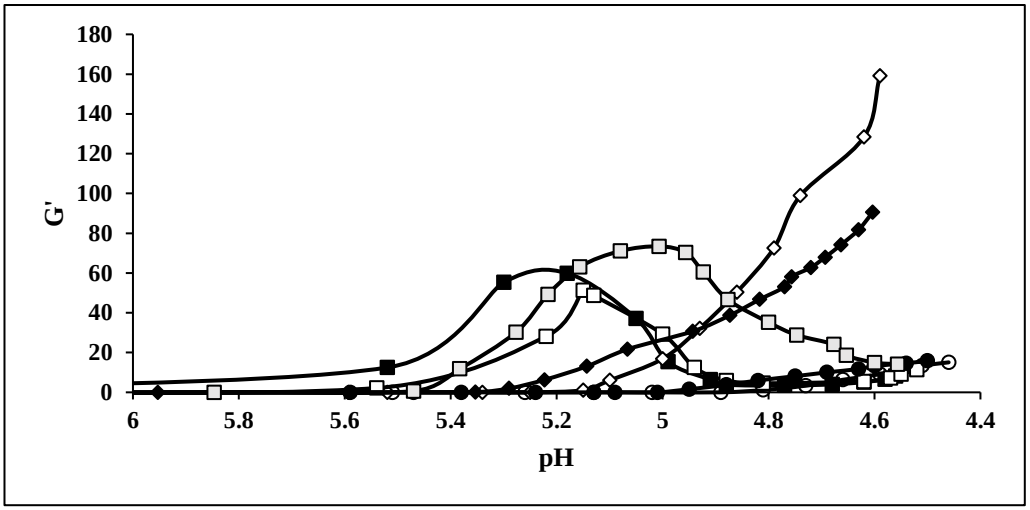

Fig 2.6. Storage modulus (G') as a function of pH during acid-induced coagulation of LCR (black diamonds), LWR (grey squares), and reconstituted LHSMP1 (empty circles), LHSMP2 (black circles), PpCR (empty diamonds), PcWR (black squares), and PpWR (empty squares). The data shown are representative of at least three replicates.

Table 2.3. Acid coagulation properties of low heat skim milk powder (LHSMP1, LHSMP2), cold and warm MF retentate stream (LCR, LWR), reconstituted cold and warm polymeric MF retentate powder (PpCR, PpWR), reconstituted warm ceramic MF retentate powder (PcWR).[1]

	Gelation pH	pH value at G' inflection point	G' at inflection point (Pa)	G' at pH 4.6 (Pa)
LHSMP1	4.78^{a} (0.07)	N/A	N/A	15.07^{a} (0.68)
LHSMP2	4.97^{b} (0.03)	N/A	N/A	15.83^{a} (0.66)
LCR	5.27^{d} (0.05)	N/A	N/A	90.52^{b} (15.49)
LWR	5.45^{e} (0.04)	5.05^{a} (0.06)	73.45^{b} (4.09)	6.92^{a} (3.33)
PpCR	5.15^{c} (0.06)	N/A	N/A	159.83^{c} (12.16)
PcWR	5.52^{ef} (0.05)	5.24^{b} (0.06)	71.97^{ab} (12.10)	6.61^{a} (1.19)
PpWR	5.53^{f} (0.04)	5.15^{b} (0.02)	56.31^{a} (14.52)	9.12^{a} (2.84)

[1]Abbreviation is: N/A, not applicable. Gelation pH is defined as the pH value when G' > 1. Values are means (standard deviation) from three independent trials; means within the same column not sharing a common superscript differ significantly ($P < 0.05$).

Significantly higher ($P < 0.05$) G' value at pH 4.6 was found in sample PpCR, followed by LCR, followed by the other samples, which might due to the lower whey protein content in MF retentates and the absence of inflection point in G' during acid coagulation. The differences in G' value at pH 4.6 between two cold MF retentates might be attributed to the use of different membranes and processes. All warm MF retentates showed an inflection point (~ pH 5.1) at which G' value stopped increasing and decreased before the pH value of the sample reached 4.6 (Fig. 2.6). The appearance of the inflection point has been reported in studies on acid coagulation of heat-treated UF retentates (Li & Corredig, 2020) and milk protein concentrate (Lin et al., 2018). The occurrence of the inflection point may be linked to the rearrangements of the early-formed whey protein-casein aggregates network, which restricts the mobility of casein micelles and promotes casein micelle aggregation; as the pH decreases closer to the isoelectric point of casein, the network rearranges, along with the release of CCP, disaggregation and hydration of casein, and compacting of structure (Li & Corredig, 2020; Lin et al., 2018; Lucey, 2016; Meletharayil et al., 2015). The inflection point may also reflect the differences between samples regarding factors that affect gel structural rearrangements e.g., serum Ca, P (Lin et al., 2018) and CCP (Anema, 2009) levels. Those parameters were not measured in this study; further analysis of the content of ionic minerals and CCP is required to understand the rearrangements differences between cold and warm MF retentates. When CCP was progressively removed from milk, the phenomenon that G' does not increase uniformly with the decrease of pH was less noticeable (Anema, 2009). The absence of an inflection point in cold MF retentates might be attributed to the significantly lower CCP content in cold MF retentates compared to warm MF retentates (Xia et al., 2022).

As for the gels made from reconstituted LHSMP, aggregated casein micelles form dense clusters instead of the network from cross-linking, which may result in the lower G' value (Lucey et al., 1997a).

2.3.5 Texture analysis

To confirm the gelation of MF retentate samples at pH 5 and the decrease in hardness of all warm MF retentate sample from pH 5 to pH 4.6, the texture of all samples at pH 5 and pH 4.6 was analysed. The hardness change of acid-induced coagulation of reconstituted LHSMP1, PcWR, PpWR and PpCR at pH 5 and 4.6 measured by back-extrusion are shown in Fig. 2.7.

At pH 5, the hardness of sample LHSMP1 was significantly lower ($P < 0.05$) than that of sample PpCR, followed by sample PcWR and PpWR. The hardness of sample LHSMP1 and PpCR at pH 5 was significantly lower ($P < 0.05$) than that at pH 4.6, while the hardness of sample PcWR at pH 5 was significantly higher ($P < 0.05$) than that at pH 5. Although no significant differences ($P > 0.05$) were found in the hardness of sample PpWR between pH 5 and 4.6, numerically lower hardness at pH 4.6 in sample PpWR was observed. At pH 4.6, no significant differences ($P > 0.05$) were found in the hardness between samples LHSMP1 and PpCR. Significantly lower ($P < 0.05$) hardness was found in both warm MF retentates compared to that of LHSMP1. The texture analysis showed that, at pH 5, relatively stronger acid-induced gels were formed in all MF retentates compared to LHSMP1 which is consistent with gelation pH (> pH 5) measured by dynamic oscillatory analysis.

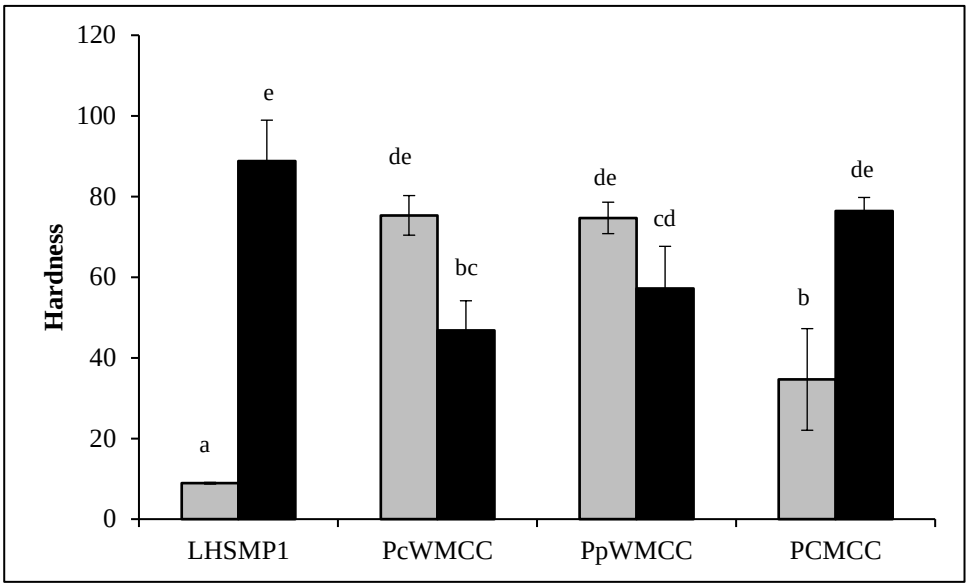

Fig 2.7. Hardness of acid-induced gels prepared from reconstituted LHSMP1, PcWR, PpWR and PpCR at pH 5 (grey bars) and 4.6 (black bars). Means at the sample not sharing a common superscript differ ($P < 0.05$). Values are means of three independent trials. Error bars indicate standard deviation.

The lower hardness of acid gels of all pilot plant-scale warm MF retentates at pH 4.6 was found compared to that at pH 5; the results are comparable with the decreased G' value measured by dynamic oscillatory analysis from the inflection point (~ pH 5.1) to pH 4.6, which may be attributed to the structural rearrangements of the gel network (Lin et al., 2018). No significant differences ($P > 0.05$) were found in the hardness of acid gel formed from warm MF retentates produced by ceramic or polymeric membranes. Both LHSMP1 and PpCR presented a higher hardness at pH 4.6 than that at pH 5, which is consistent with the increasing G' value of LHSMP1 and PpCR during acid-induced coagulation, while the G' value of LHSMP1 at pH 4.6 was significantly lower ($P < 0.05$) than that of PpCR. The storage modulus, G', was determined through small deformation rheology, while the hardness was determined by large deformation behaviour

through the texture analyser, and those two factors do not necessarily correlate with each other (Meletharayil et al., 2015).

2.3.6 Microstructure

The microstructures of the acid-induced coagula made from reconstituted LHSMP1, PcWR, PpWR and PpCR at pH 5 and 4.6 as determined by CLSM are shown in Fig. 2.8. At pH 5, only both warm MF retentates presented a network structure, which confirms the higher hardness and higher G' value at pH 5 than that of sample LHSMP1 and PpCR. At pH 4.6, a fine network was formed in sample LHSMP1, in line with previous reports about acid skim milk gel (Sanchez, Zuniga-Lopez, Schmitt, Despond, & Hardy, 2000). A higher porosity for the acid gel of both warm MF retentates at pH 4.6 was observed than that at pH 5, which may be attributed to the compacting of the structure due to rearrangements of the casein aggregates network (Li & Corredig, 2020; Lin et al., 2018; Lucey, 2016; Meletharayil et al., 2015). A coarse network was observed in the acid gel of sample PpCR at pH 4.6, and larger holes were found in the structure of sample PpCR than that of other treatments, the reason for which is unknown.

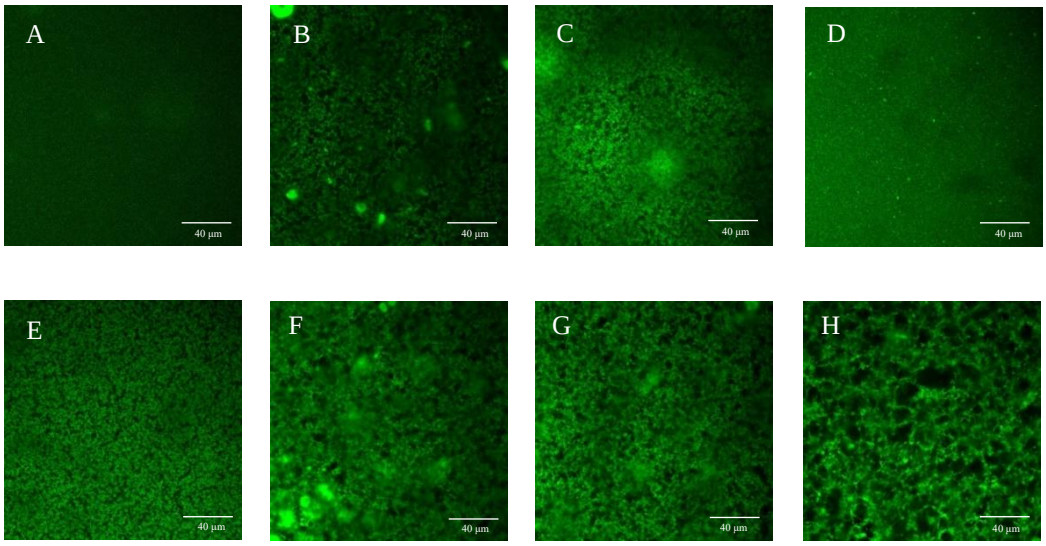

Fig 2.8. Representative CLSM images of acid-induced coagulation of reconstituted LHSMP1, PcWR, PpWR and PpCR at (A-D) pH 5 and (E-H) 4.6 respectively. Images were taken using a 60x objective. Scale bar = 40 µm.

2.4 Conclusions

MF retentates had higher casein in total protein content compared to LHSMP. Whey protein-casein aggregates formed during heat treatment were only found in LHSMP. With less presence of native whey protein, all MF retentates showed shorter rennet coagulation time and higher gel strength compared to LHSMP. Longer rennet coagulation times were found in cold MF retentates compared to warm MF retentates, as the level of κ-CN increases with the depletion of β-CN. Warm MF retentates may be more suitable for manufacture of rennet cheese compared to cold MF retentates. As for acid-induced coagulation, the gelation pH value of all MF retentates was higher than 5. Both ceramic and polymeric membrane warm MF retentates showed an inflection point of G' at around pH 5.1, while cold MF retentates did not. Both warm and cold MF retentates exhibited potential for the manufacture of cheese or yogurt with tailored functionalities. For

the manufacture of rennet cheeses, MF retentates forms firmer gel compared to that of LHSMP, which may be comparable with the gel formed by milk. For the manufacture of acid cheese or yogurt, at pH 4.6, the gel formed by cold MF retentates is firmer than that of LHSMP. Warm MF retentates forms firm gel at higher pH, which may be another opportunity to modify the manufacture of acid cheese or yogurt.

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CHAPTER 3

Evaluation of manufacture of Cheddar cheese from micellar casein concentrate

Bozhao Li[1], David S. Waldron[1], John T. Tobin[2], Surabhi Subhir[2], Alan L. Kelly[1], Paul L. H. McSweeney[1]

[1]*School of Food and Nutritional Sciences, University College Cork, Co Cork, Ireland.*

[2]*Department of Food Chemistry and Technology, Teagasc Food Research Centre, Fermoy, Co. Cork, Ireland.*

Declaration

This chapter was written by author BL and reviewed by his co-authors. The chapter has been published as: Li, B., Waldron, D. S., Tobin, J. T., Subhir, S., Kelly, A. L., & McSweeney, P. L. H. (2020). Evaluation of production of Cheddar cheese from micellar casein concentrate. *International Dairy Journal*, *107*, 104711. BL co-designed the study, manufactured cheese, determined rennet coagulation properties, composition, proteolysis, and functionalities and analysed the data. DSW contributed to cheese manufacture. JTT and SS provided the micellar casein concentrate material for cheese manufacture.

Chapter 3

Abstract

The objective of this study was to evaluate the production of Cheddar cheese using micellar casein concentrate (MCC), a novel milk ingredient powder with a high casein content (~92%). Four types of Cheddar cheese were manufactured and ripened for 180 days from the following starting materials: standardized control milk (control), skim milk with cream (SC), reconstituted MCC with cream (MC) and reconstituted low-heat skim milk powder with cream (PC). Only minor differences were found in composition between treatments, but MC cheese showed higher levels of proteolysis compared to other treatments, linked to significantly higher plasmin and chymosin activities. No differences were observed in hardness between treatments (60, 120 and 180 days), but the springiness and cohesiveness of MC and PC cheeses were significantly higher than that of the control and SC cheeses at 60, 120 and 180 days. In conclusion, the use of casein-dominant dairy streams thus has the potential for production of Cheddar cheese with tailored functionality.

Chapter 3

3.1 Introduction

Global milk production was approximately 843 million tonnes in 2018 (FAO, 2019); among dairy products, cheese production used the highest proportion of milk. Whey protein powder, production of which is traditionally associated with the manufacture of cheese, represents 57% of the market for global protein supplement for exercising and nutrition, while there is a huge market for whey protein powders in infant formula. Cheese output is increasing at a rate of 2% yearly while the demand for whey protein has been growing at 6-7% yearly (Hoogwegt Group, 2019). Nowadays, whey protein with high quality is required as it can be used to manufacture a range of food ingredients or products with nutritional and functional properties (Boland, 2011). Kelly (2019) reported that the ultrafiltration (UF) properties of sweet and acid whey are influenced by their high or low pH, respectively. However, microfiltration (MF) permeate made directly from skim milk is considered to be an ideal whey source for whey protein ingredients production. Therefore, recovering whey protein from milk rather than cheese whey could improve the whey quality and is an option for whey protein ingredients manufacture.

During membrane filtration of skim milk, native micellar casein is concentrated in the retentate, which could be recovered and concentrated to produce micellar casein concentrate (MCC). This is a novel dairy ingredient powder with a high casein fraction of 85-95% of total protein which may be used in functional and nutritional applications (Crowley et al., 2018). As the traditional way to manufacture Cheddar cheese is followed by whey protein

manufacture, the concept of recovering whey protein before Cheddar cheese manufacture is of interest.

In terms of the protein ingredients being used for Cheddar cheese manufacture, low heat skim milk powder (LHSMP) can be used to enhance cheese yield giving a constant cheese production throughout the year (Freeman, Bucy, & Hassan, 1970). LHSMP is produced from skim milk using low temperatures during manufacture and is mostly used for condensed milk, UHT-treated fluid milk and ice-cream (Augustin & Margetts, 2003). Unlike reconstituted medium- and high-heat skim milk powder that have impaired rennet coagulation ability, the rennet coagulation properties of LHSMP are good as the whey protein is not highly denatured (Ménard, Camier, & Guyomarc'h, 2005). With only physical separation processing, the micellar casein in MCC may have better rennet coagulation properties and cheese-making potential compared to LHSMP.

Two main factors that influence cheese yield are lactation and seasonality. The gross composition of bovine milk varies with the stage of lactation. Kuchtík Sustova, Urban, & Zapletal (2008) reported that protein and casein content increase and lactose content decreases through the lactation. After 200 days lactation, cows are in the late lactation stage, which requires the udder tissue to repair and recover for next lactation. During late lactation, milk yield decreases dramatically, which may influence cheese yield, unless milk is standardised. Also, at specific times of the year, the milk volume decreases, and the composition and rennet coagulation properties of milk are significantly influenced (Freeman et al., 1970; O'Brien, Mehra, Connolly, & Harrington, 1999). Both cheese yield and manufacturing efficiency are influenced by

seasonal variation of milk protein and fat composition (Barbano & Sherbon, 1984).

To solve the problem of low cheese yield, one solution is adding LHSMP to low-protein milk to increase the protein composition (Freeman et al., 1970). Another possibility may be making Cheddar cheese with MCC that is manufactured as a co-product of high-quality whey protein. At the same casein content, warm microfiltrated (~ 50 ºC) MCC is found more suitable for rennet cheese manufacture than cold microfiltrated (~ 8 ºC) MCC (Chapter 2).

This study aimed to evaluate the production of Cheddar cheese from micellar casein concentrate, with LHSMP used for comparison. The consequence of this manufacture was evaluated concerning composition, proteolysis, texture and functionality.

3.2 Materials and Methods

3.2.1 Preparation of cheese-milk

Micellar casein concentrate (MCC) powder was obtained from Teagasc (Moorepark, Fermoy Co. Cork, Ireland) as follows: pasteurised bovine skim milk was microfiltered at 50 ºC using 0.14-µm TAMI Isoflux ceramic membranes (TAMI, Lyons, France). MF was performed in a batch mode involving two diafiltration steps to produce a final 3X MF retentate (liquid MCC) which was evaporated at 65 ºC using a pilot plant single-effect falling-film evaporator (Anhydro F1 Laboratory; Copenhagen, Denmark). This was followed by spray-drying of the evaporated liquid MCC in a single-stage spray dryer (Anhydro Laboratory Spray Dryer, SPX Flow Technology, Kolding, Denmark) equipped with nozzle atomisation at inlet and outlet temperatures of

185 and 85°C, respectively. The protein content (91.6% casein) of MCC was determined by the Kjeldahl method (IDF, 1986). MCC powder and low heat skim milk powder (LHSMP; WPNI=6.0) (Uelzena, Uelzen, Germany) were rehydrated using a Silverson mixer at 55 ℃ for 2 h.

Raw milk was obtained from a local farm in Cork, Ireland. Skim milk and cream were separated from raw milk using a separator. Composition of milk samples was measured by MilkoScan™ Mars (Foss, Hillerød, Denmark) and casein content was calculated by multiplying the casein percentage of protein of the milk sample (an estimated percentage of 78% for milk and LHSMP milk and 91.6% for MCC). Control milk was prepared by combining raw milk and skim milk to a casein: fat ratio of 0.7:1. Both reconstituted MCC and reconstituted LHSMP were made up to the same casein level as skim milk. Reconstituted MCC and LHSMP and skim milk were blended with cream and lactose according to the same standardization ratio (casein: fat of 0.70:1.00) and lactose content (~5%) as control milk to obtain three more vats: skim milk with cream (SC), reconstituted MCC milk with cream (MC) and reconstituted LHSMP milk with cream (PC). All milk samples were pasteurized at 72 ℃ for 15 s (Microthermics Inc., Raleigh, NC, USA) and stored at 4 ℃ until analysis and cheese-making.

3.2.2 Rennet Coagulation Properties

Dynamic oscillatory analysis (small amplitude oscillatory measurement) of renneted control milk, SC milk, MC milk and PC milk was performed using a Peltier concentric cylinder geometry, which comprised of an aluminium conical rotor [42.01 mm (h) by 28.02 mm (d)], on an AR-G2 controlled stress

rheometer (Waters TA Instruments, Leatherhead, Surrey, UK). Aliquots (25 mL) of each sample were pre-warmed in a water bath at 32℃ for 15 min, and 80 µL of a 1:10 (v/v) dilution of Maxiren™ (Chr Hansen A/S, Hørsholm, Denmark) was added. The sample was placed immediately in the preheated cup (32 ℃), the frequency of oscillation was set at 0.6283 rad s^{-1}, and the storage modulus, G' of the sample was recorded continuously as a function of time at a low-amplitude shear strain (0.01) over 90 min. Each sample was analysed in triplicate (Ibáñez, Waldron, & McSweeney 2015).

3.2.3 Cheese Manufacture

Cheddar cheeses were made from 20 L control, SC, MC and PC milks which were prepared, pasteurised and stored at 4 ℃ overnight before cheese-making. Cheddar-type cheeses were manufactured according to a standard Cheddar cheese manufacture protocol (Fox, Guinee, Cogan, & McSweeney, 2000). An aliquot of 6 g (0.03% w/w) of Cheddar cheese starter culture (R604Y Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added into each vat and allowed ripen for 30 min, followed by 0.09% (v/w) of 1 mol L^{-1} $CaCl_2$ and 60 IMCU L^{-1} rennet being added to all samples. Whey was drained when the pH dropped to 6.2 and curd was cheddared. When the pH decreased to 5.4, the curd was milled into small pieces and salted at a level of 2.5% (w/w) NaCl. The curds were then wrapped in cheesecloth and moved to 2-kg circular moulds, which were pressed at a pressure of 2.5 kg cm^{-2} for 14 h. The cheeses were then removed, vacuum-packed and ripened at 8 ℃ for 180 days.

3.2.4 Compositional analysis

Gross composition of cheeses was analysed at 14 days old. Moisture was determined by oven-drying method (IDF 1982), protein (%N×6.38) by the Kjeldahl method (IDF 1986), fat was analysed by the Gerber method (IS, 1955) and salt was analysed by titration with $AgNO_3$ (Fox, 1963). The pH was measured at 14, 30, 60, 120 and 180 days of ripening by using a calibrated pH probe placed in contact with dry grated cheeses. All results were determined in triplicate.

3.2.5 Microbiological analysis

Enumeration of starter lactic acid bacteria (LAB) was performed using LM 17 agar plates (Terzaghi and Sandine 1975), incubated for 3 days at 30 ℃. Enumeration of non-starter lactic acid bacteria (NSLAB) was performed on Rogosa agar plates (Rogosa, Mitchell, & Wiseman 1951), incubated anaerobically for 5 days at 30 ℃. Enumeration of LAB and NSLAB were performed in duplicate after 60 and 90 days of ripening.

3.2.6 Analysis of proteolysis

Urea-polyacrylamide gel electrophoresis (urea-PAGE) of cheese samples was used to study the proteolysis of α_{S1}- and β-casein (CN) during ripening using the method of Andrews (1983) with modifications of Shalabi and Fox (1987). The pH 4.6-soluble and insoluble fraction were prepared (Kuchroo & Fox, 1982), and peptide profiles of pH 4.6-soluble fractions filtered through 0.22-μm cellulose acetate filters (Sartorious GmbH, Gottingen, Germany) were

analysed by reverse-phase high-performance liquid chromatography (HPLC) using an ultra-performance liquid chromatography (UPLC) Waters Acquity UPLC H-Class Core System (Waters, Milford, MA, USA), with a Waters Acquity UPLC TUV Detector (dual-wavelength; Waters) operated by Empower 3 software (Waters Corp., Milford, MA, USA), following the method of Mane, Ciocia, Beck, Lillevang, & McSweeney (2019).

For determination of free amino acids (FAA), frozen pH 4.6-soluble fractions were de-proteinised by mixing equal volumes of 24% (w v^{-1}) trichloroacetic acid (TCA) and sample and following the method of Fenelon and Guinee (2000).

3.2.7 Plasmin activity

The plasmin activity of cheese samples at 180 days of ripening was measured using the coumarin peptide method (Richardson & Pearce, 1981). A standard curve of the emission intensity at 460 nm was constructed using 7-amido-4-methyl coumarin (AMC), and results expressed in nmol AMC min^{-1} mL^{-1}, which was defined as one unit of plasmin activity.

3.2.8 Residual coagulant assay

Grated cheese samples (50 mg) were extracted by dissolving cheese in 1 mL of 0.1 M trisodium citrate, followed by 30 minutes incubation at 37 °C. Fat was separated by centrifugation at 1000 *g* (Sigma 1-16K, Harz, Germany) for 1 min, and the aqueous layer was used for analysis. An aliquot of 70 μL citrate dispersion of cheese was incubated with 30 μL of 1 mg mL^{-1} aqueous solution of a synthetic heptapeptide substrate (Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu) in

400 μL 0.1 M sodium formate buffer, at 37 °C, pH 3.2, for 24 h. The mixture was heated at 70 °C for 10 min to stop the reaction, followed by centrifugation at 16,000g for 10 min, and the supernatant was used for UPLC analysis. Substrate and product levels were determined using the reversed-phase HPLC system described above, following the method of Hurley, O'Driscoll, Kelly, & McSweeney (1999).

3.2.9 Texture profile analysis

Texture profile analysis was performed using a Texture Analyzer TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) at 60, 120, and 180 days of ripening. Cheese samples were cut into 20 mm height, 20 mm diameter cylinders and kept at 4 ℃ overnight. Cheese cylinders were compressed to 25% of the initial height in two continuous compressions with a speed of 1 mm s^{-1}. Hardness, cohesiveness and springiness were measured (Truong, Daubert, Drake, & Baxter, 2002), and five cheese samples were measured for each treatment.

3.2.10 Meltability

Meltability was analysed using the Schreiber meltability test as described by Altan, Turhan, & Gunasekaran (2005). Cheese samples were heated at 232 ℃ for 5 min, and meltability was measured as the percentage increase in diameter of the original samples. Analyses were performed in triplicate at 60, 120, 180 days of ripening.

3.2.11 Dynamic small amplitude oscillatory rheology

The rheological properties of meltability of cheese after 180 days ripening were analysed with a controlled stress AR-G2 rheometer (TA Instruments, Waters LLC, Leatherhead, Surrey, UK) according to the method of Ibáñez et al. (2015). Storage modulus (G'), loss modulus (G''), and loss tangent (LT) were measured during heating. The maximum LT (LT_{max}) and the temperature where LT=1, which are indicators of melting, were also recorded. Each sample was analysed in triplicate.

3.2.12 Colour measurements

Colour values were measured using a Konika-Minolta colourimeter CR400 (Konika-Minolta Optics Inc., Osaka, Japan) at 14, 30, 60, 120, 180 days of ripening. The measurement used the CIELAB system based on illuminant D65 and a visual angle of 2°. Five random readings were taken on fresh-cut cheese at 20°C. The Eluclidean distance between the colour of control cheese and that for other treatments was calculated by $\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$ where $\Delta L^* = L^*_{sample} - L^*_{control}$, $\Delta a^* = a^*_{sample} - a^*_{control}$ and $\Delta b^* = b^*_{sample} - b^*_{control}$ (Mahy, Van Eyckden, & Oosterlinck, 1994).

3.2.13 Statistical analysis

Control, SC, MC and PC cheeses were made in three independent trials, 4 × 3 blocks. Statistical analysis was carried out using one-way analysis of variance (ANOVA) with R project for Windows version i386 3.4.0 (the R Foundation for Statistical Computing, University of Auckland, Auckland, New

Zealand) to establish significant differences between samples. The probability level used for statistical significance was $P < 0.05$.

3.3 Results and Discussion

3.3.1 Rennet coagulation properties

The rennet coagulation properties of milk samples are shown in Table 3.1. During the first 10 min, G' value of all the treatments was low and constant, after which the G' value of all samples increased, with the MC and PC samples exhibiting a slower rate of increase compared to other samples (Fig. 3.1). The gel strength (G' value) at 90 min of MC and PC samples was significantly ($P < 0.05$) lower than that of other samples. The gel strength of MC and PC kept increasing and did not reach a constant level during the analysis, while control and SC reached stable values at 45 min. The loss tangent at 90 min of MC sample was significantly ($P < 0.05$) higher than that of the other treatments. Martin, Williams, & Dunstan (2010) reported that, during the manufacture of milk protein concentrate (MPC), casein micelles were not damaged; however, the minerals removed through diafiltration and decreased calcium ionic strength could depress rennet coagulation. The rennet coagulation ability of reconstituted MPC could be restored by adding extra calcium ions. The production of MCC involves two diafiltration steps, which may reduce soluble calcium levels; thus, 0.09% (v/w) of 1 mol L^{-1} $CaCl_2$ was added to all of the batches before cheese manufacture.

Table 3.1. Rheological properties of rennet-induced coagulum made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC)

	Gelation time (min)[1]	G' at 90 min (Pa)	Loss Tangent (LT) at 90 min
Control	7.81^{a} (0.58)	143.3^{bc} (31.66)	0.26^{a} (0.003)
SC	7.78^{a} (0.71)	169.5^{c} (17.68)	0.27^{b} (0.028)
MC	9.29^{a} (0.73)	74.23^{a} (6.71)	0.29^{c} (0.002)
PC	8.79^{a} (1.15)	91.04^{ab} (10.26)	0.27^{ab} (0.001)

[1]Gelation time refers to the time when G' value was higher than 1.
Data are means (standard deviation) of three replicate trials; means within the same column not sharing a common superscript differ significantly (P < 0.05).

3.3.2 Cheese composition and pH

The composition of cheese at 14 days of ripening and pH of cheese samples at 14, 30, 60, 120 and 180 days of ripening are shown in Table 3.2; the results presented are means of data from the three trials. Guinee, Mulholland, Kelly, & O'Callaghan (2007) stated that different casein to fat ratio can influence the protein and fat content of cheese. As the casein: fat ratio of milk was adjusted to 0.7 for all samples, no statistically significant differences ($P > 0.05$) were found in cheese protein and fat contents between all treatments, and the use of MCC or LHSMP for Cheddar cheese manufacture did not affect these parameters.

The moisture content of PC cheese was significantly ($P < 0.05$) higher than that of SC and MC cheese; no significant differences ($P > 0.05$) were found for moisture content between control, SC, and MC cheese. The salt content of MC cheese was significantly ($P < 0.05$) higher than that of control and SC cheese. The pH of MC cheese was significantly ($P < 0.05$) higher than that of other treatments, the reason for which is not clear. No significant differences ($P >$

0.05) were found for moisture in non-fat substances (MNFS) and fat in dry matter (FDM) for all treatments; MNFS is an important parameter for Cheddar cheese quality, so it is important that all batches of cheese sample had similar levels of MNFS (Fox et al., 2000; Bogenrief & Olson, 1995).

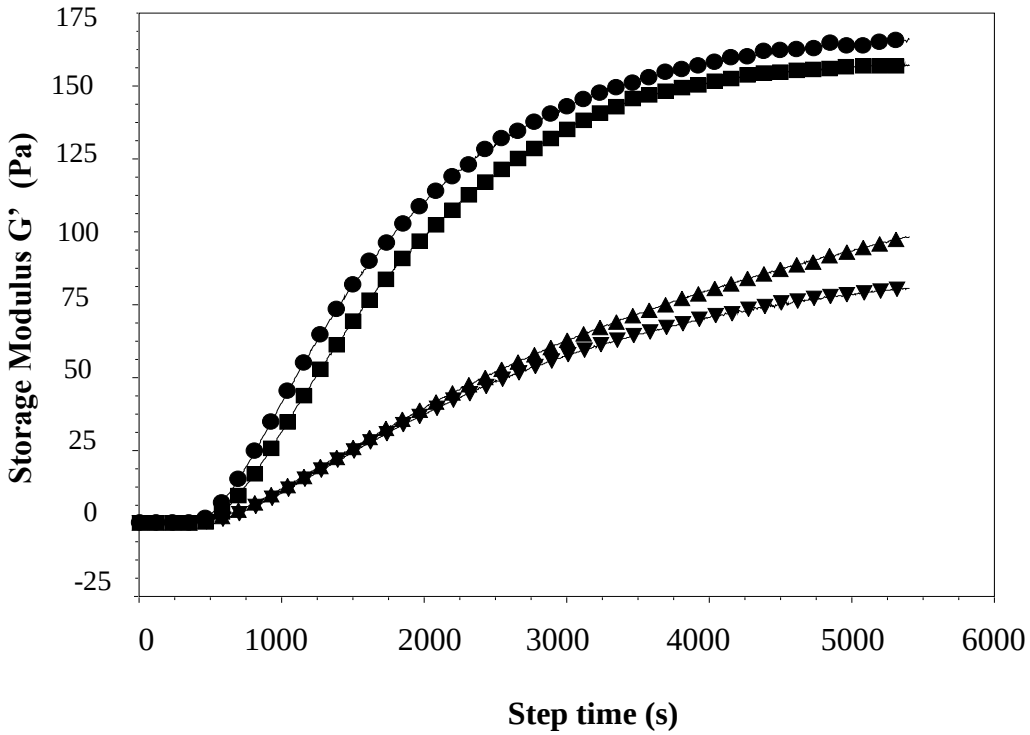

Fig. 3.1. Storage modulus (G') of rennet gel of control (●), SC (■), MC (▼) and PC (▲) samples, under strain of 0.01 and frequency of 1 Hz.

Table 3.2. Composition of cheeses made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) at 14 days of ripening, pH values at 14, 30, 60, 120 and 180 days of ripening, and pH 4.6-SN/TN levels at 180 days of ripening.

	Control	SC	MC	PC
Moisture%	38.41^{ab} (0.41)	37.41^{a} (0.41)	37.80^{a} (1.43)	39.31^{b} (1.14)
Fat%	35.25^{a} (2.47)	35.75^{a} (2.13)	35.50^{a} (2.75)	34.84^{a} (2.12)
Protein%	23.85^{a} (0.71)	24.12^{a} (0.95)	23.44^{a} (0.95)	23.73^{a} (1.47)
Salt%	1.57^{ab} (0.11)	1.48^{a} (0.10)	1.70^{c} (0.10)	1.66^{bc} (0.07)
MNFS%	59.33 (0.65)	58.22 (0.65)	58.61 (2.21)	60.33 (1.75)
FDM%	59.50 (3.00)	58.14 (1.87)	59.22 (2.94)	58.31 (2.59)
pH at 14 days	5.03^{a} (0.09)	5.05^{a} (0.07)	5.23^{b} (0.14)	5.06^{a} (0.11)
pH at 30 days	5.07^{ab} (0.04)	5.01^{a} (0.04)	5.26^{c} (0.04)	5.14^{b} (0.08)
pH at 60 days	5.07^{a} (0.07)	5.11^{ab} (0.07)	5.20^{b} (0.07)	5.12^{ab} (0.03)
pH at 120 days	5.13^{a} (0.09)	5.17^{a} (0.06)	5.40^{b} (0.04)	5.25^{ab} (0.03)
pH at 180 days	5.04^{a} (0.03)	5.07^{a} (0.04)	5.31^{b} (0.10)	5.17^{ab} (0.07)
pH 4.6-SN/TN	23.01^{bc} (1.22)	22.21^{ab} (1.23)	25.89^{c} (1.65)	19.71^{a} (2.03)

Data are means (standard deviation) from three independent trials; means within the same row not sharing a similar superscript differ significantly ($P < 0.05$). There were no significant differences found for MNFS or FDM between means within a row.

Abbreviations: MNFS, moisture in non-fat substance. FDM, fat in dry matter.

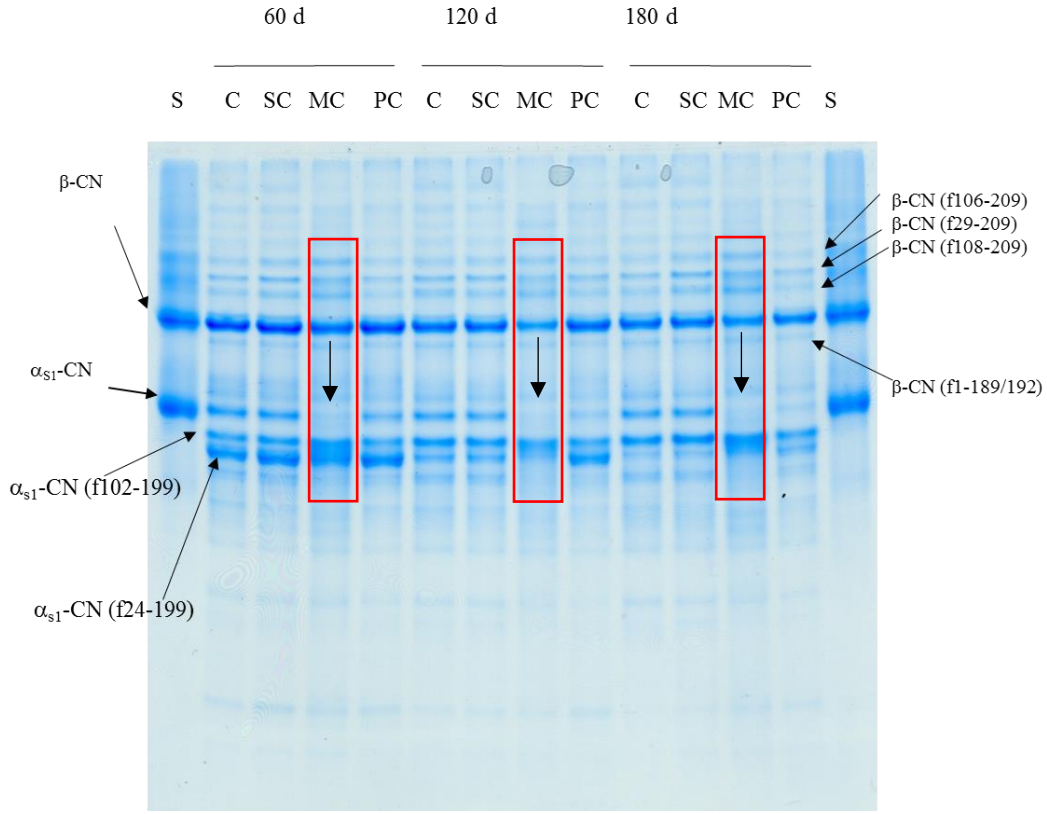

Fig. 3.2. Urea-polyacrylamide gel electrophoretograms of control (C), skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) cheeses at 60, 120 and 180 days of ripening. Sodium caseinate (S) was used as standard on both sides of the gel. The figure corresponds to data for one of the three cheese replicates; all replicates were similar.

3.3.3 Proteolysis

Table 3.2 shows the level of pH 4.6-SN/TN (%) of control, SC, MC and PC cheeses at 180 days of ripening; pH 4.6-SN/TN is an index of proteolysis (Sousa, Ardo, & McSweeney, 2001). Significantly ($P < 0.05$) higher pH 4.6-SN/TN levels were found in MC cheese than in SC and PC cheese. In addition, the levels of pH 4.6-SN/TN were significantly ($P < 0.05$) lower in PC than control cheese. The value (23%) of control sample found at 180 days of ripening in this study was comparable with previous studies on Cheddar cheese (Lucey, Mishra, Hassan, & Johnson, 2005; O'Mahony, Lucey, & McSweeney, 2005). The main agent

producing pH 4.6-soluble nitrogen is the coagulant (O'Keeffe, Fox, & Daly, 1978), while Farkye & Fox (1992) reported that plasmin, the principal indigenous milk proteinase, is also important for primary proteolysis in Cheddar cheese during ripening.

Urea-PAGE electrophoretograms of cheese samples during ripening are shown in Fig. 3.2. All cheese samples showed break-down of β- and α_{S1}-CN, but lower levels of intact β-and α_{S1}-CN were apparent during ripening in MC cheese (highlighted by red frames and black arrows). Due to the action of plasmin, β-CN is hydrolysed to β-CN (f29-209), β-CN (f106-209) and β-CN (f108-209) (Eigel et al., 1984). The levels of β-CN (f106-209) and β-CN (f108-209) of MC cheese were higher than other treatments during ripening, which suggests higher plasmin activity. Also, in Fig. 3.2, the MC cheese showed the lowest level of intact α_{S1}-CN level, followed by PC cheese, control and SC cheese from 60 to 180 days of ripening; at 180 days of ripening, no intact α_{S1}-CN was found in MC and PC samples. Residual chymosin in cheese hydrolyses α_{S1}-CN to α_{S1}-CN (f24-199) and α_{S1}-CN (f102-199) and, as the residual chymosin activity increases, the breakdown of α_{S1}-CN increases (Sheehan, Wilkinson, & McSweeney, 2008). The faster breakdown of α_{S1}-CN of MC and PC samples may indicate higher residual chymosin activity in these samples.

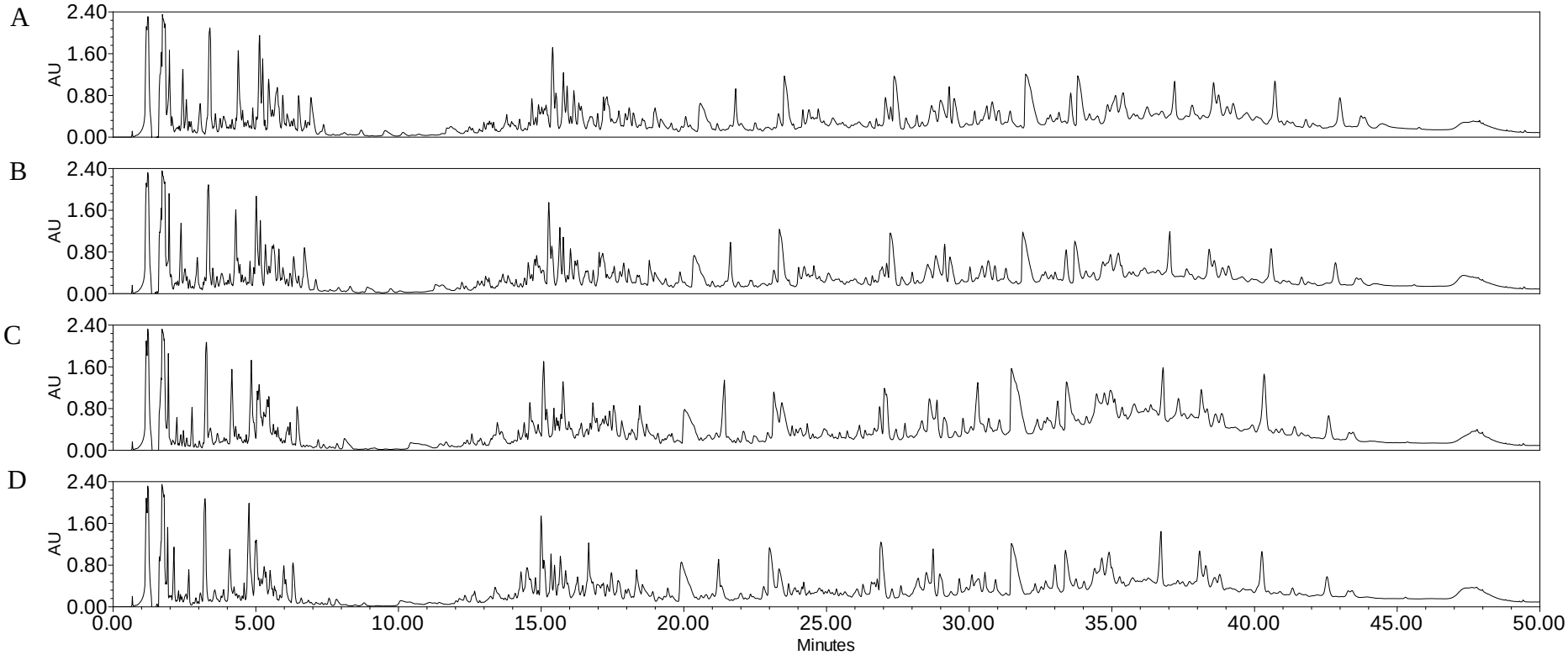

Fig. 3.3. Ultra-performance liquid chromatograms (UPLC profiles, C_8 column) of pH 4.6-soluble extracts from (A) control, (B) SC, (C) MC and (D) PC cheeses at 180 days of ripening, at wavelength of 214 nm. The figure corresponds to data for one of the three cheese replicates; all replicates were similar.

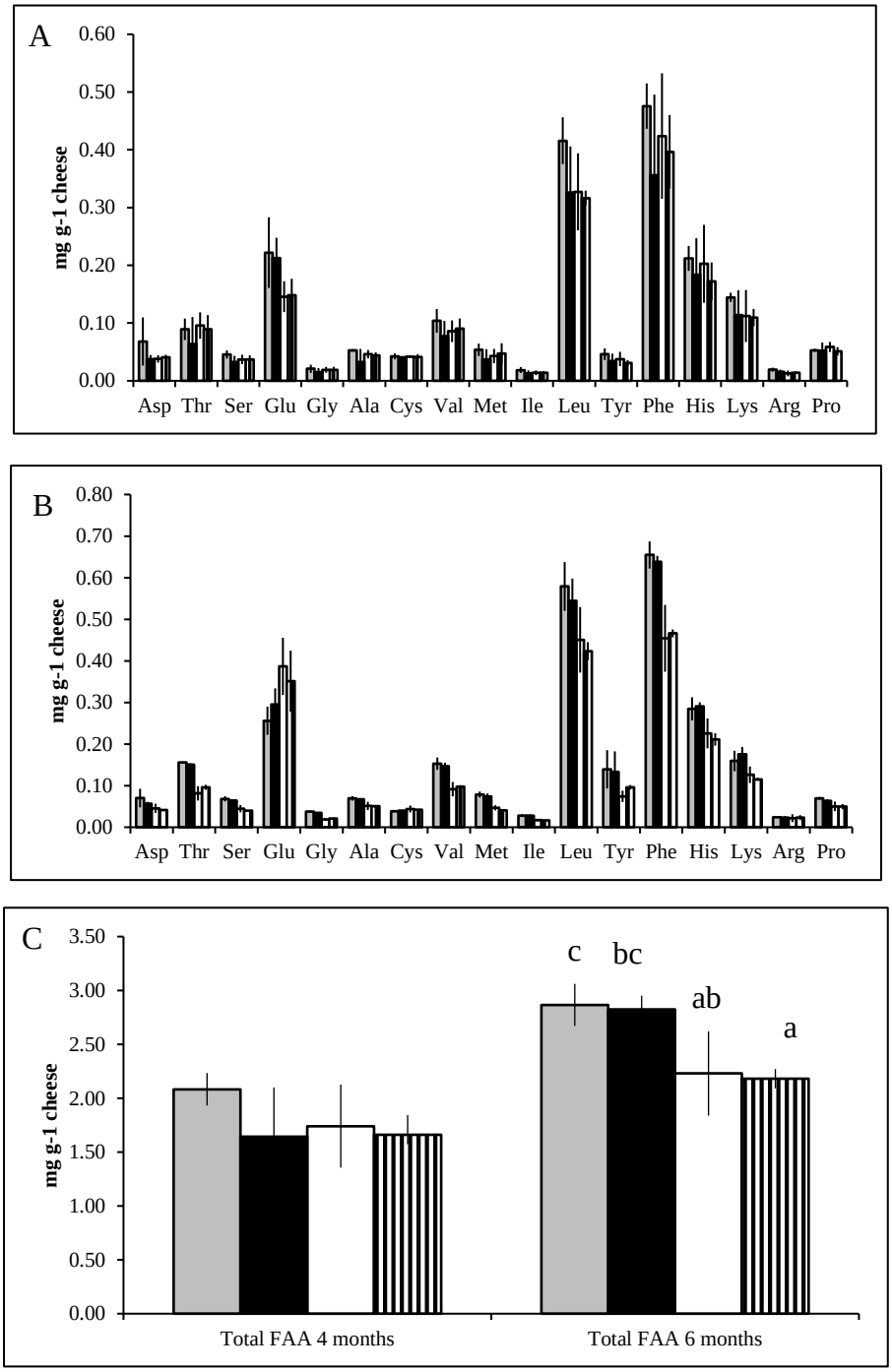

Fig. 3.4. Individual FAA results of cheese samples at (A) 120 and (B) 180 days of ripening and (C) total FAA results of cheese samples at 120 and 180 days of ripening. Control (*grey bars*), SC (*black bars*), MC (*white bars*) and PC (*white bars filled with straight lines*). Values are means of three independent trials; means not sharing a common letter differ ($P < 0.05$). Error bars indicate standard deviation.

The peptide profiles of the pH 4.6-soluble extracts for control, SC, MC and PC cheeses at 180 days of ripening were generated by ultra-performance liquid chromatography (Fig. 3.3). The highest peak areas were observed at the retention time of 1 to 5 min for all treatments. The peak area of peptides that were eluted later (28-45 min retention time) was higher in MC sample compared to the other treatments. Peptides in the pH 4.6-soluble extracts reflect the effect of proteinases and peptidases of starter (Fox & McSweeney, 1997). The number of peptide peaks for all treatments was similar, which suggests that the proteolysis for all treatments broadly followed similar pathways, but differences in peak area indicate that there was more extensive proteolysis in MC cheese compared to the other treatments.

The individual FAA levels of all treatments at 120 and 180 days of ripening are shown in Fig. 3.4 (A and B). The major FAA determined in cheese were glutamic acid, valine, leucine, phenylalanine, histidine and lysine; Bansal et al. (2009) also reported that glutamic acid, valine, leucine, phenylalanine histidine and lysine are the principal FAA in Cheddar cheese after 180 days of ripening. No significant differences ($P > 0.05$) were found between treatments at 120 days of ripening. At 180 days of ripening, significantly ($P < 0.05$) higher concentrations of threonine, serine, glycine, alanine, valine, methionine, isoleucine, phenylalanine, histidine and proline were found in control and SC cheese than that of MC and PC cheese. No significant differences ($P > 0.05$) were found between treatments in the levels of aspartic acid, glutamic acid, leucine, tyrosine, and arginine. From 120 to 180 days of ripening, the concentrations of glutamic acid, alanine, leucine, tyrosine, and phenylalanine increased significantly ($P < 0.05$) for all treatments, while only control and SC cheese had a significant increase in the concentration of threonine, glycine, valine and isoleucine. The total FAA levels of

all treatments at 120 and 180 days of ripening are shown in Fig. 3.4 (C). No significant differences ($P > 0.05$) were found between all treatments at 120 days of ripening, but significantly higher ($P < 0.05$) levels of total FAA were found in control cheese than in MC and PC cheeses at 180 days of ripening. No significant differences ($P > 0.05$) were found between control and SC cheese at 180 days of ripening.

Fox and McSweeney (1996) reported that peptidases of starter and non-starter lactic acid bacteria are the principal agents releasing FAA in Cheddar cheese during ripening. No significant ($P > 0.05$) differences were found for the numbers of starter bacteria and NSLAB between treatments in this study (result not shown). Therefore, the release of the FAA by starter and NSLAB should not have been an influence in this regard, and so the reason for this difference is not clear.

3.3.4 Plasmin and residual chymosin activities in cheese

The plasmin activity of MC cheese was significantly ($P < 0.05$) higher than that of the other cheese batches (Table 3.3) which is consistent with the result of urea-PAGE electrophoresis. The plasmin activity of PC cheese was significantly ($P < 0.05$) lower than that of the other cheese samples. As the pH increases, the hydrolysis of β-CN increases since plasmin has an alkaline optimum pH (Watkinson et al., 2001).

Aaltonen and Ollikainen (2011) reported that in diafiltration, with the removal of whey protein, the concentration of inhibitors of both plasmin and plasminogen activators decreases, which promotes the conversion from plasminogen to plasmin and thus increases plasmin activity. In this study, MCC was made using microfiltration, which may have enhanced the plasmin activity of retentate by

removing inhibitors in the permeate, resulting in the higher plasmin activity and more β-CN breakdown.MC cheese showed significantly ($P < 0.05$) higher residual chymosin activity compared to the other treatments (Table 3.3), which is consistent with the faster breakdown of α_{S1}-CN in that cheese. No significant differences ($P > 0.05$) were found between the residual chymosin activities of control and SC cheese, so the recombination of fat and skim milk did not affect the retention of coagulant during cheese manufacture. The residual chymosin activity of PC cheese was significantly ($P < 0.05$) lower than that of control cheese. The MC cheese apparently retained more coagulant compared to control, SC and PC cheeses; the percentage of retained chymosin in cheese curd depends on the pH at the curd-cutting stage, pH at whey drainage, cooking temperature and method (Hurley et al., 1999). Both MC and PC cheeses were drained at a lower pH compared to the rest of the treatments which might resulted in increased retention of coagulant in the curd.

Table 3.3. Plasmin activity and residual chymosin activity (performed as % of that in the control cheese) of cheeses made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) at 180 days of ripening.

Treatment	Plasmin activity (nmol AMC mL^{-1} min^{-1})	Residual chymosin activity (% of control)
Control	0.572^{b} (0.121)	100^{b}
SC	0.656^{b} (0.185)	106.04^{b} (4.89)
MC	1.116^{c} (0.024)	132.58^{c} (3.81)
PC	0.396^{a} (0.042)	80.74^{a} (6.61)

Data are means (standard deviation) from three independent trials; means within the same column not sharing a similar superscript differ significantly ($P < 0.05$).

3.3.5 Texture profile analysis

No significant changes ($P > 0.05$) were found in the hardness of MC and PC cheeses from 60 to 180 days of ripening (Fig. 3.5). The hardness of control cheese significantly ($P < 0.05$) decreased between 60 and 120 days of ripening, while that of SC cheese significantly ($P < 0.05$) increased. At 60 days of ripening, the hardness of control cheese was significantly ($P < 0.05$) higher than that of SC and PC cheeses. No significant differences ($P > 0.05$) were observed in the hardness of the cheeses made by different treatments at 120 and 180 days of ripening. Chevanan and Muthukumarappan (2007) reported that the contents of calcium, phosphate, and residual lactose affect the texture profile of Cheddar cheese. The cheese-milk of SC, MC and PC samples was reconstituted according to the composition of control cheese-milk, which would not influence lactose content. In this study, extra calcium chloride was added to all treatments, but the addition of calcium chloride does not cause significant changes to the texture of ripened Cheddar cheese (Soodam, Ong, Powell, Kentish, & Gras, 2015). The differences in hardness might be attributed to the microstructure of different types of cheese (Ong, Dagastine, Kentish, & Gras, 2013).

From 60 days to 180 days of ripening, the springiness and cohesiveness of MC cheese decreased, and the springiness and cohesiveness of MC and PC cheeses were significantly ($P < 0.05$) higher than that of control and SC cheese at 60 and 180 days of ripening. At 120 days of ripening, the springiness of MC cheese was significantly ($P < 0.05$) higher than that of the other treatments and the cohesiveness of MC and PC cheeses was significantly ($P < 0.05$) higher than that

of control and SC cheese. Everard et al. (2006) found that higher pH of Cheddar cheese was associated with increased springiness and cohesiveness; the pH of MC cheese was significantly ($P < 0.05$) higher than that for the other treatments (Table 3.2), which may explain the increases in springiness and cohesiveness. O'Mahony et al. (2005) reported that, as levels of secondary proteolysis increase, the charged groups released from peptides would associate with free water, which may lead to increased cohesiveness and springiness during ripening.

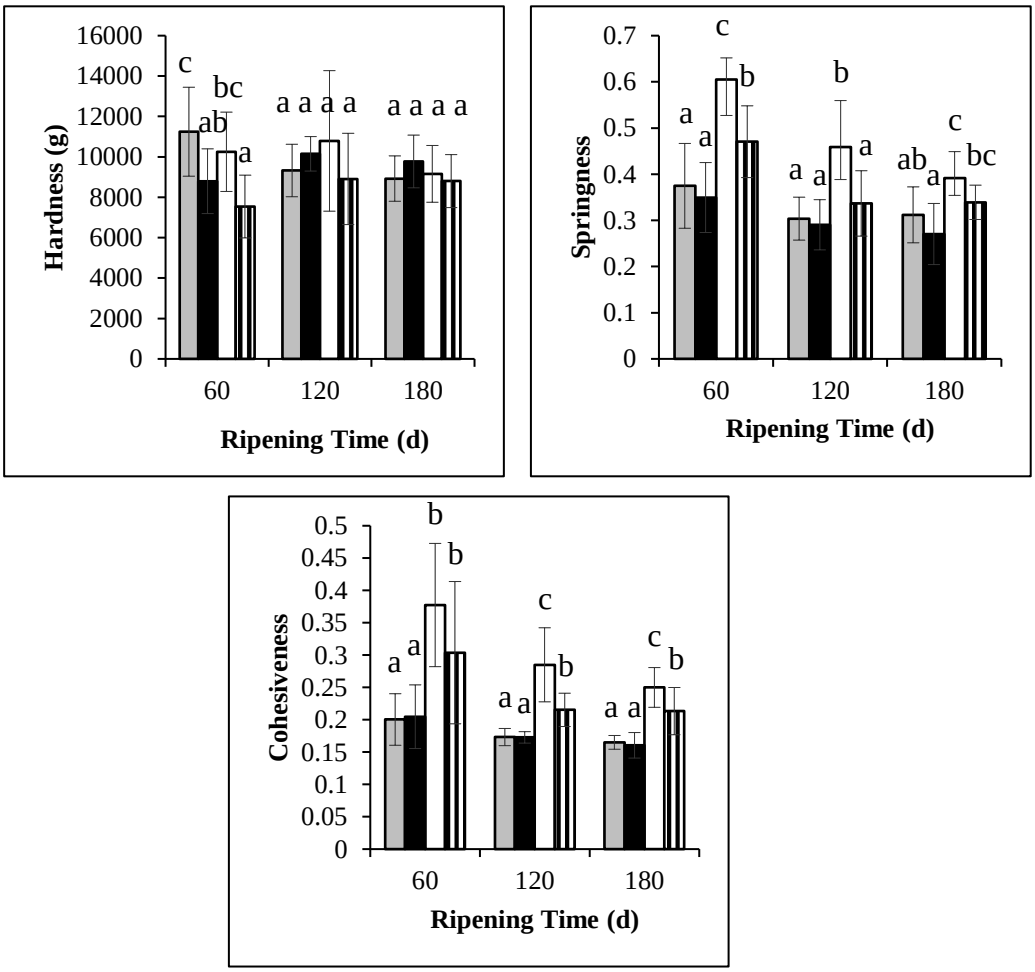

Fig. 3.5. Changes in TPA hardness, springiness and cohesiveness of cheese samples from 60 to 180 days of ripening. Control (*grey bars*), SC (*black bars*), MC (*white bars*) and PC (*white bars filled with straight lines*). Values are means of three independent trials; means at the same time point not sharing a common superscript differ ($P < 0.05$). Error bars indicate standard deviation.

3.3.6 Meltability

Meltability (percentage increase in diameter) of Cheddar cheeses was determined by the Schreiber method; results are shown in Fig. 3.6. The four types of cheeses showed increases in meltability between 60 and 180 days. At 60 and 120 days of ripening, no significant differences ($P > 0.05$) for meltability were found between control and SC cheese. At 180 days of ripening, significantly ($P < 0.05$) higher meltability was found in control cheese compared to SC cheese. At 60, 120 and 180 days of ripening, the meltability of MC cheese was significantly higher ($P < 0.05$) than that of cheese from the other treatments. At 120 and 180 days of ripening, the meltability of PC cheese was significantly lower ($P < 0.05$) than that of cheese from the other treatments.

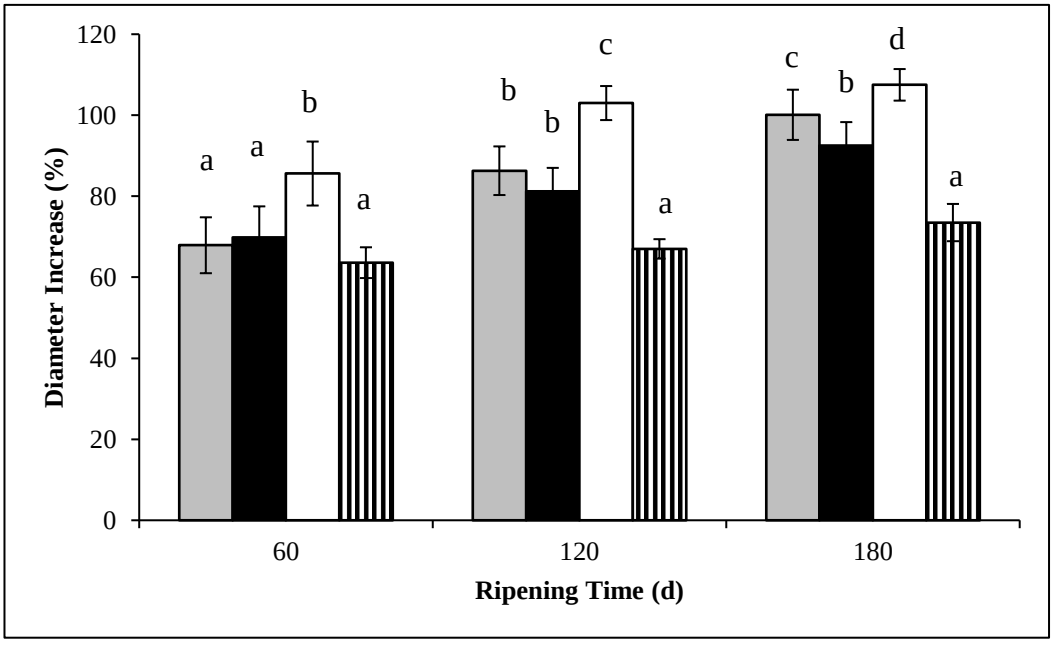

Fig. 3.6. Meltability (diameter increase %) of cheese samples from 60 to 180 days of ripening Control (*grey bars*), SC (*black bars*), MC (*white bars*) and PC (*white bars filled with straight lines*). Values are means of three independent trials; means at the same time point not sharing a common superscript differ ($P < 0.05$). Error bars indicate standard deviation.

The lower meltability of PC cheese may be caused by the changes of casein in protein during drying of milk powder. Moiseev, Suchkova, & Iakovchenko (2017) reported that Mozzarella cheese made with reconstituted non-fat milk powder has lower meltability than the control, due to the drying process of non-fat milk powder decreasing the stability and dispersity of casein micelles and promoting demineralization of calcium salts. Dave, McMahon, Oberg, & Broadbent (2003) found that the meltability of Mozzarella cheese is related to the breakdown of α_{S1}-CN and α_{S1}-CN (f24-199) and breakdown of intact β-casein, while Bogenrief and Olson (1995) reported that hydrolysis of β-casein increases the meltability of Cheddar cheese. Hydrolysis of β-casein is primarily related to the action of plasmin (Eigel, McMahon, Oberg, & Broadbent, 1984) and, although both MC and PC cheeses were made from powder, and showed a higher breakdown of α_{S1}-casein and β-casein, the meltability of MC cheese was significantly higher than that of PC.

Table 3.4. Rheological properties of cheeses made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) at 180 days of ripening, as assessed by dynamic small amplitude oscillatory rheology.

	LT_{max}	Temperature at LT_{max} (°C)	Temperature at LT=1 (°C)
Control	1.25^{a} (0.31)	70.55^{a} (0.70)	62.02^{c} (0.58)
SC	1.42^{a} (0.33)	71.36^{a} (2.49)	61.80^{b} (2.88)
MC	2.93^{b} (0.33)	77.76^{b} (1.91)	55.24^{a} (1.57)
PC	1.92^{c} (0.18)	75.64^{b} (4.75)	58.93^{b} (1.58)

Data are means (standard deviation) from three independent trials; means within the same column not sharing a similar superscript differ significantly ($P < 0.05$).

3.3.7 Dynamic small amplitude oscillatory rheology

The results of the rheology of meltability of all treatments at 180 days of ripening are shown in Table 3.4. Higher LT value indicates a higher extent of melting (Lucey, Johnson, & Horne 2003). MC cheese showed significantly higher ($P < 0.05$) LT_{max} values than PC cheese, followed by control and SC cheese. The temperature at LT_{max} of MC and PC cheeses was significantly higher ($P < 0.05$) than that of control and SC cheese. The LT_{max} is an indicator of cheese meltability (Mounsey & O'Riordan, 1999); the more thermal energy needed to melt cheese, the higher the LT_{max} temperature of cheese will be.

In the Schreiber test for cheese meltability, MC cheese also exhibited the highest percentage increase in diameter (Fig. 3.6). There were differences between Schreiber melting test and dynamic small amplitude rheology for control, SC and PC cheese. Cooke, Khosrowshahi, & McSweeney (2013) stated that differences between results generated by those tests may be linked to the more complete fat melting during the higher temperature of the Schreiber melting test. MC cheese exhibited the lowest temperature when LT=1 compared to the rest of the treatments ($P < 0.05$). At the point of LT=1, cheese is considered to start transforming from a solid to a viscous form (Gunasekaran & Ak, 2002). MC cheese thus commenced melting at the lowest temperature and had the highest meltability compared to the other treatments, which may be linked to the more extensive proteolysis of casein.

3.3.8 Cheese colour

The colour values of samples at 180 days of ripening are shown in Table 3.5. Whiteness (L* values) of control, SC cheese was significantly higher ($P < 0.05$) than that of MC cheese at 180 days of ripening. The greenness of MC and PC cheeses was significantly lower (higher a* values) ($P < 0.05$) than that of control or SC cheese at 180 days of ripening. The yellowness (b* values) of MC cheese was significantly lower ($P < 0.05$) than that of SC cheese at 180 days of ripening. Ibáñez et al. (2015) reported that the whiteness of cheese may be related to proteolysis; lower whiteness of MC cheese may thus be attributed to higher proteolysis. The ΔE^*_{ab} values are also shown in Table 3.5. Sharma (2003) reported that ΔE^*_{ab} of above 2.3 leads to a just noticeable difference (JND) and, on this basis, overall, no JND was found between control cheese and the other treatments. Overall, no visible difference was found between the cheeses made from protein ingredient and control and SC cheese.

Table 3.5. CIE LAB colour values of cheese made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) after 180 days of ripening, and ΔE^*_{ab} between control and each treatment after 180 days of ripening.

	L*	a*	b*	ΔE^*_{ab}
Control	81.05^{b} (1.81)	-4.44^{a} (0.23)	36.72^{ab} (2.42)	-
SC	80.91^{b} (1.19)	-4.36^{a} (0.32)	37.43^{b} (1.53)	1.82
MC	79.34^{a} (1.56)	-3.88^{b} (0.29)	35.56^{a} (1.43)	0.15
PC	79.74^{ab} (0.85)	-3.40^{c} (0.54)	36.07^{ab} (0.89)	1.47

Data are means (standard deviation) of five replicate analyses from three independent trials; means within the same column not sharing a similar superscript differ significantly ($P < 0.05$). ΔE^*_{ab} data are calculated from CIELAB results. The just noticeable differences (JND) of ΔE^*_{ab} were approximately 2.3.

3.4 Conclusions

Cheddar cheeses were made from standardised cheese-milk, skim milk with cream, reconstituted MCC with cream and reconstituted LHSMP with cream according to the same casein, fat and lactose content. Only minor differences in composition were found with cheese made from reconstituted MCC with added cream compared to control cheese. The cheese manufactured with MCC had significantly higher ($P < 0.05$) levels of pH 4.6-SN/TN than cheese manufactured with skim milk or LHSMP with cream. The level of intact β-and α_{S1}-CN of MC cheese was lower than rest of the treatments, consistent with significantly ($P < 0.05$) higher plasmin and chymosin activity. No significant differences ($P > 0.05$) were found in hardness between all treatments from day 120 onwards. Significantly higher ($P < 0.05$) springiness and cohesiveness were found in cheese manufactured from MCC and LHSMP powder, meltability and maximum loss tangent in MC cheese were significantly ($P < 0.05$) higher than that of the other treatments. No overall differences of colour were found between all treatments. Use of novel casein-dominant dairy streams such as MCC has potential for production of Cheddar cheese with tailored functionality. The results of this study suggest that using reconstituted LHSMP with cream for the manufacture of Cheddar cheese may result in changes in functionality, while using reconstituted MCC with cream for the manufacture of Cheddar cheese may be more feasible.

3.5 Acknowledgements

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Chapter 3

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CHAPTER 4

Evaluation of manufacture of Quarg cheese from micellar casein concentrate

Bozhao Li, Alan L. Kelly and Paul L. H. McSweeney

School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Declaration

This chapter was written by author BL and reviewed by his co-authors. BL co-designed the study, prepared sample, manufactured cheese, analysed composition, proteolysis, coagulation properties, rheology and texture of samples, and collected and analysed the data.

Chapter 4

Abstract

The production of Quarg cheese using warm and cold microfiltration micellar casein concentrate (MCC) was evaluated, cheese made using low heat skim milk powder (LHSMP) was used for comparison. Materials were pasteurised (72 ℃ for 15 s) or high-heat treated (90 ℃ for 10 min) before cheese manufacture; centrifugation or a cheesecloth method were used to drain whey. High-heat treatment affected the composition of cheese manufactured from LHSMP, while such treatment showed little effect on cheese manufactured from MCC. The use of centrifugation or the cheesecloth method did not affect the composition of Quarg cheese. The use of MF retentates for Quarg cheese manufacture resulted in lower protein content in cheese whey than that of LHSMP. High-heat treatment reduced the level of proteolysis of cheese during ripening compared to pasteurisation due to heat-induced inactivation of enzymes. The GMP content of cheese whey from MF retentates was relatively higher than that of LHSMP. The use of MF retentates has the potential for manufacture of Quarg cheese with modified properties compared with the use of LHSMP.

Chapter 4

4.1 Introduction

In traditional cheese manufacture, milk is coagulated to cheese curd as the casein aggregates to form a network under the effect of rennet or acid (Cheryan et al., 1975; Lucey, 2016). When the curds are formed, whey is separated from the curds and may be used for whey protein recovery (Boland, 2011). The demand for whey protein is increasing faster than the increasing rate of cheese output (Li et al., 2020). For cheese whey, the addition of chymosin and the pH change during cheese manufacture may affect the quality and purity of resultant whey protein manufactured from ultrafiltration (UF; Kelly, 2019).

Membrane filtration is used to fractionate whey protein and casein in milk according to the molecular size of the protein (Carter et al., 2021). So-called native whey protein, which has higher purity than the whey recovered from cheese manufacture, can be recovered from the permeate of microfiltration (MF). MF retentate, which is mainly micellar casein, is commonly called micellar casein concentrate (MCC) following drying.

To achieve and maintain a high flux level during MF, an operating temperature of around 45-55 ℃ is commonly used to separate the caseins and whey proteins as this decreases the viscosity of permeate (McCarthy et al., 2017; Zulewska & Barbano, 2014). The use of cold MF (temperatures ≤ 20 ℃) can, however, better control bacterial growth and reduces membrane fouling compared to warm MF (France et al., 2021a; Schiffer & Kulozik, 2020). At a temperature of 5 ℃, β-CN dissociates from casein micelle into the serum phase (Zulewska et al., 2018) and, instead of being retained in retentates, part of the β-CN in the milk serum permeates through MF membranes (France et al., 2021b; McCarthy et al., 2017;

O'Mahony et al., 2014; Seibel et al., 2015). Thus, cold MF produces whey permeate with enriched β-CN and micellar casein concentrate retentate with an increased α_s/β-CN ratio. Colloidal calcium phosphate (CCP) undergoes similar disassociation from casein micelles as β-CN at low temperatures, which increases both total and ionic calcium contents in the permeate of skim milk (France et al., 2021b; Luo et al., 2015; Méthot-Hains et al., 2016).

These different compositions of warm and cold MF retentates may influence products subsequently made from such streams. Many studies have investigated the different applications of warm MF retentate in the manufacture of dairy products including protein bars, Greek-style yoghurt and cheese (Bong & Moraru, 2014; Hogan et al., 2012; Li et al., 2020; Chapter 2). Fewer studies have been done on the application of cold MF MCC. With lower β-CN content, the cheese manufactured from cold MF retentate showed increased spreadability on heating and decreased bitterness during ripening (O'Mahony et al., 2008). Weaker rennet-induced gels were formed from reconstituted MCC with lower levels of β-CN compared to that produced by warm MF (Seibel et al., 2015). At the same casein content, depletion of β-CN from MCC was found to delay rennet coagulation compared to warm MF MCC (Chapter 3). The levels of β-CN in MCC were not found to affect the rennet coagulation properties while the cheese made from cold MF MCC had a lower pH value as the ash content and buffering capacity was reduced compared to that from warm MF MCC (Xia et al., 2022). Few studies have investigated the acid coagulation properties and acid cheese manufacture possibilities of cold MF MCC.

According to the principle of acid coagulation, when the pH of milk drops close to 4.6, i.e., the isoelectric point of casein, negative charges on the surface of

the casein micelle are neutralized, decreasing steric and electrostatic stabilisation between casein micelles. Without such stabilisation, casein micelles aggregate to form a gel network (De Kruif, 1998; Horne, 2002; Lucey & Singh, 1997). The acid coagulation of milk can be used to make many kinds of acid cheeses, such as Cottage cheese and Quarg cheese.

Quarg cheese is a variety of acid-coagulated cheese manufactured from whole milk or skim milk through a combination of acid and rennet coagulation (Farkye, 2017; Kelly & O'Donnell, 1998). The addition of low levels of rennet for acid coagulation of milk makes the gel firmer and reduces the loss of casein during whey drainage (Fox et al., 1996; Kelly & O'Donnell, 1998). Two methods of whey drainage for Quarg cheese manufacture, centrifugation and cheesecloth filtration, may be used (Kelly & O'Donnell, 1998; Lepesioti et al., 2021) depending on the scale of production. The differences between those two methods have been studied in small-scale cream cheese manufacture; although both methods resulted in similar microstructure, cheese manufactured from centrifugation retained more fat and was closer to commercial cheese (Ong et al., 2018). Unlike most types of rennet-coagulated cheese that undergo 4 weeks to 2 years of ripening to develop their texture, flavour and aroma, acid-coagulated cheese is ripened for a very short period or no ripening period and is commonly consumed fresh (Fox, 1989).

Manufacture of Cheddar cheese using MF retentates is feasible (Chapter 3), and MF retentates may be suitable for the manufacture of acid cheese. This study evaluated the manufacture of lab-scale and pilot-scale Quarg cheese from warm and cold microfiltration micellar casein concentrate, with low heat skim milk powder (LHSMP) for comparison. The cheese and cheese whey composition,

proteolysis, texture, and microstructure of cheese were analysed to assess the potential use of these streams for Quarg manufacture.

4.2 Material and methods

4.2.1 Sample preparation

Low heat skim milk powder (LHSMP), ceramic membrane warm microfiltered retentate (cWR), polymeric membrane warm microfiltered retentate (pWR) and polymeric membrane cold microfiltered retentate (pCR) were produced by Teagasc (Moorepark, Fermoy Co. Cork, Ireland) as described by Xia et al. (2020, 2022). Samples were rehydrated at a level of 2.59% casein at 50 ℃ for at least 2 h and held at 4 ℃ overnight. After rehydration, the composition of samples was measured by MilkoScan^{TM} Mars (Foss, Hilleroed, Denmark) and reconstituted samples were standardised to the same casein (2.59%); extra lactose was blended in retentate samples to achieve the lactose content (4.45%) relative to the composition of LHSMP. Samples were then pasteurised (72 ℃ for 15 s) or high-heat treated (90 ℃ for 10 min) in a water bath and cooled to 30 ℃ before cheese manufacture.

4.2.2 Culture-induced acid coagulation properties

Pasteurised samples were used for the analysis of culture-induced acid coagulation properties. Various amounts of mesophilic starter (XT-302, Christian Hansen A/S) were added to all samples at 30 ℃ to determine the starter level required to decrease the pH value of the sample to 4.6 within 4 h. Dynamic oscillatory analysis of all samples was performed using an AR-G2 controlled-

stress rheometer (Waters TA Instruments, Leatherhead, Surrey, UK). Aliquots (50 mL) of each sample were pre-warmed in a water bath at 30 ℃ for 15 min, and levels of starter predetermined to decrease the pH of the sample to ~4.4-4.6 within 4 h were added and mixed. Half of the mixture was held in a water bath and the pH was measured by a pH meter every 20 min. The other half of the mixture was placed in a preheated concentric cylinder (30 ℃) and oscillated by a conical rotor at a frequency of 1 Hz, and the storage modulus, G', of the sample was recorded continuously as a function of time at a low-amplitude shear strain (0.01) over 240 min (Ibáñez et al., 2016). Each sample was analysed in triplicate.

4.2.3 Cheese manufacture

An aliquot of 0.01% mesophilic starter as described above was added to all samples (200 mL). After 60 min of incubation at 30 ℃ (pH value ~6.5), 3.33 μL L^{-1} of cheese rennet (CHY-MAX® Extra, 600 IMCU mL^{-1}, Christian Hansen A/S) was added to each sample and kept incubated at 30 ℃. When the pH of the sample reached 4.4 - 4.6, samples were cut with a knife and centrifuged at 1500 g for 15 min at 20 ℃. Whey was poured off after centrifugation, the curds were cooled to 4 ℃ and further drained, and samples were stored at 4 ℃ until further analysis (Kelly & O'Donnell, 1998). Quarg cheese samples manufactured through centrifugation from pasteurised starting materials were denoted $PLHSMP_{ct}$, $PcWR_{ct}$, $PpWR_{ct}$, and $PpCR_{ct}$, and from high-heat treated materials were denoted $HSMP_{ct}$, $HcWR_{ct}$, $HpWR_{ct}$, and $HpCR_{ct}$ (Table 4.1).

In separate trials, performed on a larger pilot scale (1 L), an aliquot of 0.01% mesophilic starter as described above was added to all pasteurised samples. After 60 min of incubation, 10 μL L^{-1} of standard cheese rennet (200 IMCU/ml) was

added to each sample and kept incubated at 30 ℃. When the pH of the sample reached 4.4 - 4.6 (within 18-20 h), samples were cut with a knife and drained with cheesecloth at 20 ℃ for 1 h instead of centrifugation. Samples were stored at 4 ℃ until further analysis. Quarg cheese samples manufactured through cheesecloth were denoted $LHSMP_{cc}$, cWR_{cc}, pWR_{cc}, and pCR_{cc}.

In all, twelve treatments were used for Quarg cheese manufacture (Table 4.1).

Table 4.1. Experimental treatments used for Quarg cheese manufacture.

Code	Material
$PLHSMP_{ct}^{a,c}$	Low heat skim milk powder
$PcWR_{ct}^{a,c}$	Ceramic warm MF retentate
$PpWR_{ct}^{a,c}$	Polymeric warm MF retentate
$PpCR_{ct}^{a,c}$	Polymeric cold MF retentate
$HSMP_{ct}^{b,c}$	Low heat skim milk powder
$HcWR_{ct}^{b,c}$	Ceramic warm MF retentate
$HpWR_{ct}^{b,c}$	Polymeric warm MF retentate
$HpCR_{ct}^{b,c}$	Polymeric cold MF retentate
$PLHSMP_{cc}^{b,d}$	Low heat skim milk powder
$PcWR_{cc}^{b,d}$	Ceramic warm MF retentate
$PpWR_{cc}^{b,d}$	Polymeric warm MF retentate
$PpCR_{cc}^{b,d}$	Polymeric cold MF retentate

[a]Heat treatment at 72 ℃ for 15 s

[b]Heat treatment at 90 ℃ for 10 min

[c]Centrifugation method for whey separation

[d]The cheesecloth method for whey separation

4.2.4 Compositional analysis and analysis of proteolysis

The gross composition of cheeses was analysed at 2 days of ripening. Quarg cheese moisture content was determined by the oven-drying method (IDF,

1982). Quarg cheese protein content and protein levels in cheese whey were determined by the Kjeldahl method (IDF, 1986).

Urea-polyacrylamide gel electrophoresis (urea-PAGE) was applied directly on cheese samples manufactured by centrifugation at 2, 30 and 60 days of ripening according to the method of Andrews (1983) with modifications as described by Shalabi & Fox (1987). The gels were stained with Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977) and destained with deionised water several times.

In addition, pH 4.6-soluble fractions of cheese samples manufactured by centrifugation at 2 and 60 days of ripening were prepared as described by Kuchroo & Fox (1982) and filtered through 0.22-μm cellulose acetate filters (Sartorious GmbH, Gottingen, Germany). The peptide profiles of these extracts were analysed by reverse-phase high-performance liquid chromatography (RP-HPLC) using ultra-performance liquid chromatography (UPLC) Waters Acquity UPLC H-Class Core System (Waters Corp, Milford, MA, USA), with a Waters Acquity UPLC TUV Detector (dual-wavelength; Waters) operated by Empower 3 software (Waters Corp., Milford, MA, USA). A C18 Peptide CSH™ ACQUITY UPLC column (2.1 x 100 mm, 1.7 μm particles size, 130 Å pore size; Waters Corp, Milford, MA, USA) was used for protein separation. The eluate was monitored at 215 nm with a mobile phase of two solvents: A: 0.1% (v v^{-1}) trifluoroacetic acid (TFA) in Milli-Q water and B: 0.1% (v v^{-1}) TFA in acetonitrile. Samples were eluted at a flow rate of 200 μL min^{-1} at 30 °C, with a gradient of 97% solvent A and 3% solvent B at 0.0 min, going to 60% solvent A and 40% solvent B at 45 min; then to 15 % solvent A and 85% solvent B at 47 min and holding for 3 minutes. The eluent then reduced to 97% solvent A

and 3% solvent B at 52 min and was held for 8 minutes to ensure the column was re-equilibrated before the next injection. The injection volume of samples was 10 μL.

A sodium caseinate (NaCN) sample digested by bovine chymosin without culture addition was used as a standard for the peptide profile produced by bovine chymosin. A solution of NaCN, (10 mg mL^{-1}) was prepared by dissolving commercial NaCN (Kerry Group, Listowel, Ireland) in 100 mM Na acetate buffer, pH 5.2, containing 0.05% NaN_3 and 5% NaCl. An aliquot of 0.21 IMCU mL^{-1} of bovine chymosin as described above was added to the NaCN solution and incubated at 37 ℃ for 48 hours. The pH 4.6-soluble extract of the NaCN sample was prepared as described by Huppertz, Fox, and Kelly (2004) and filtered through 0.22-μm cellulose acetate filters. The UPLC method used was as described above.

4.2.5 Texture analysis

The texture of the cheese was measured through a penetration test using a Texture Analyser TA-XTplusC (Stable Micro Systems, Godalming, Surrey, UK). Aliquots (40 g) of each Quarg cheese sample manufactured with cheesecloth were placed in a polystyrene cup (70 mm width and 45 mm height) and pressed gently to smooth the surface. The height of each sample for texture analysis was 20 mm. Using a cylinder probe P/6 (Stable Micro Systems, Godalming, Surrey, UK), cheese samples were penetrated to a depth of 10 mm at the speed of 1.00 mm s^{-1}. The hardness of the sample was measured by the penetration test 7 days after the Quarg cheese samples were made. At least five cheese samples were measured for each treatment.

4.2.6 Rheological properties

The rheological properties of all Quarg cheese samples manufactured with cheesecloth were measured as described by Vaziri et al. (2010) with minor modification. A serrated parallel plate geometry was used. Quarg cheese discs (40 mm diameter, ~8 mm height) were placed on the bottom plate at an initial temperature of 4 °C. A force of 1.0 N was applied to the cheese sample before the measurement. Small-deformation oscillatory measurements of G', G'', and tan δ were measured using a controlled-stress AR-G2 rheometer (TA Instruments, Waters LLC, Leatherhead, Surrey, UK) with frequency in the range from 0.1-10 rad s^{-1} at a fixed strain of 0.1%. The rheological properties of the sample were measured 7 days after Quarg cheese samples were made.

4.2.7 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) was conducted at the National Food Imaging Centre (Teagasc Food Research Centre, Fermoy, Ireland) following a method adapted from that of Lourenco et al. (2022). Internal sections of samples were cut with a blade, placed on glass microscopy slides, and 200 µL of 0.1% Fast Green FCF (aq., Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) were gently deposited on their surface. The stain was allowed to diffuse in the samples for 30 min under refrigeration at 4°C. Then, the samples were covered with 0.13 mm coverslips and observed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) with a 63×/1.4 oil immersion objective and maintaining the pinhole diameter at 1 Airy Unit. Fast green FCF was excited at 633 nm using a He/Ne

laser, and the emission filter was set at 650-720 nm. Z-stacks of the samples were acquired covering a depth of 15 µm with a step size of 1 µm (16 individual images per z-stack), and projections based on average intensity were obtained for each stack using Leica LAS AV software (v 2.7.3.9723).

4.2.8 Glycomacropeptide in whey

Spray-dried glycomacropeptide (GMP) powder (GMP-SD, BiPRO® GMP 9000, Agropur Ingredients, Eden Prairie, MN, USA) was prepared as standard as described by Panthi et al. (2021). Contents of GMP of cheese whey samples and GMP-SD were analysed using the RP-HPLC (Bonfatti et al., 2008). Four main peaks of the standard were identified in cheese whey samples and the peak area of the four peaks was summed to analyse the level of GMP in cheese whey samples.

4.2.9 Statistical analysis

$PLHSMP_{ct}$, $PcWR_{ct}$, $PpWR_{ct}$, $PpCR_{ct}$, $HSMP_{ct}$, $HcWR_{ct}$, $HpWR_{ct}$, $HpCR_{ct}$, $PLHSMP_{cc}$, $PcWR_{cc}$, $PpWR_{cc}$, and $PpCR_{cc}$ cheeses were made in triplicate in independent trials (i.e., 12 × 3). Statistical analysis was carried out using one-way analysis of variance (ANOVA) with R project for Windows version i386 3.4.0 (the R Foundation for Statistical Computing, University of Auckland, Auckland, New Zealand) to establish significant differences between samples. The probability level used for statistical significance was $P < 0.05$.

4.3 Results and discussion

4.3.1 Acid coagulation properties

The culture-induced acid coagulation properties of all MF retentates and LHSMP under pasteurisation or high heat treatment determined are shown in Fig. 4.1. During cheese manufacture (20 h) in this study, the standard dosage (0.01%) of culture was used. To shorten the duration (from 20 h to 4 h) of the analysis, a higher concentration (0.6%) than the common dosage of culture was used to study the acid-induced coagulation properties of the materials using rheometer; the profile of storage modulus (G') change as a function of pH was used to simulate that in cheese manufacture.

Pasteurised LHSMP showed significantly lower ($P < 0.05$) G' value at pH 4.6 and significantly ($P < 0.05$) lower gelation pH than the other treatments. This is consistent with previous studies in which milk was acidified using glucono-δ-lactone (GDL) (Donato et al., 2007; Li & Corredig, 2020).

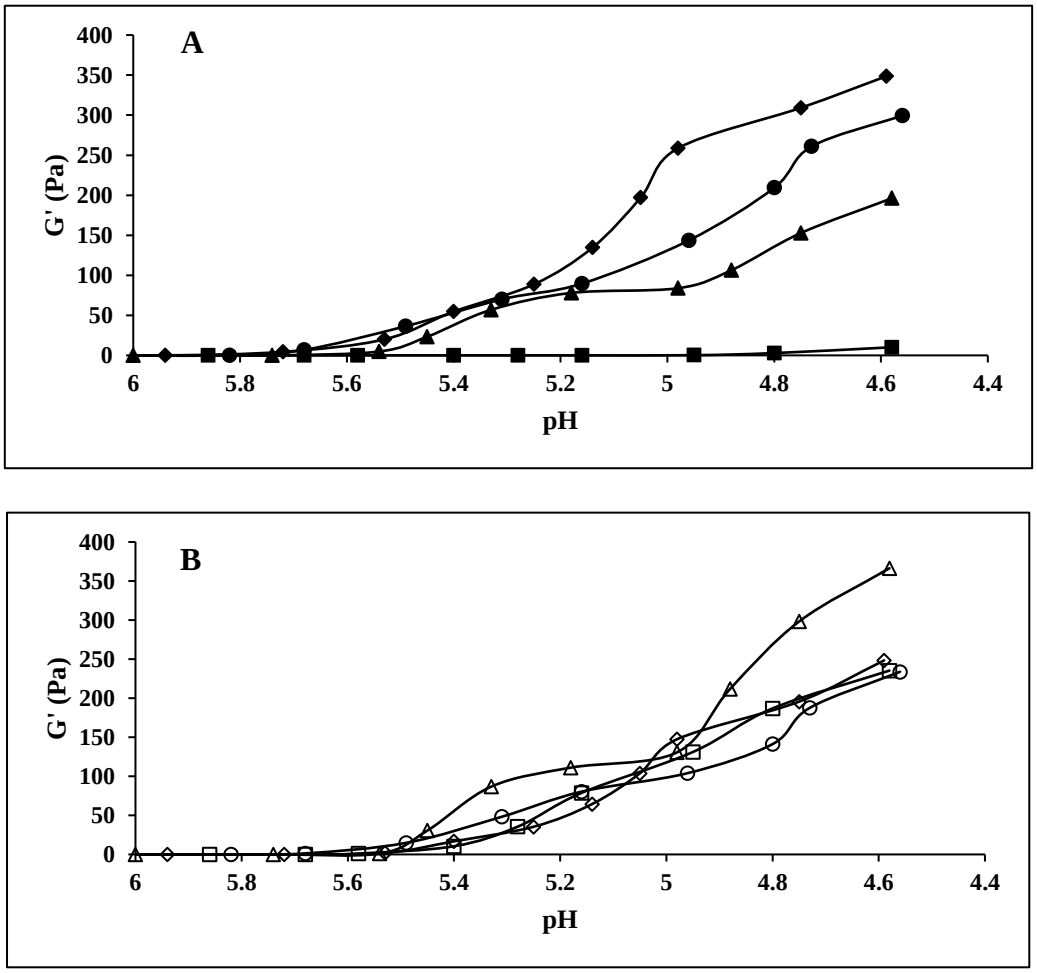

Fig. 4.1. Storage modulus (G') as a function of pH during acid-induced coagulation of (A) PLHSMP (filled square), PcWR (filled triangle), PpWR (filled diamond), PpCR (filled circle), (B) HSMP (empty square), HcWR (empty triangle), HpWR (empty diamond), and HpCR (empty circle) using the cheesecloth method. Values are means of data from three independent trials. For explanation of codes, see Table 4.1.

Aggregated casein micelles in the gels created from reconstituted LHSMP form dense clusters rather than cross-linking networks, which may be the cause of the low G' value (Lucey et al., 1997). High-heat treated skim milk powder (SMP) formed a gel with a significantly higher ($P < 0.05$) G' value than that of pasteurised LHSMP, which is consistent with the results of Lucey et al. (1997). This may be attributed to increased bridging due to whey protein denaturation and entanglement with casein micelles through disulphide bonds during the

high-heat treatment (Lucey et al., 1998; Lucey et al., 1997). No significant differences ($P > 0.05$) were found in the G' value between high-heat treated SMP and all MF retentates. Significantly higher ($P < 0.05$) G' value was found in acid gel formed by all MF retentates compared to that formeded by pasteurised LHSMP. During the production of MF retentates, part of whey protein was fractionated into permeate, which lead to lower whey protein content in all MF retentate samples (Carter et al., 2021). Native whey protein impedes the formation of a network of *para*-casein micelles by producing a physical barrier in the rennet-induced gelation of milk (Gamlath et al., 2018). In acid-induced coagulation of milk, with negatively charges, κ-CN on the surface of casein micelle inhibit casein micelles from aggregating with each other; when the pH of milk is around 4.6, κ-CN fall off and neutralised, reducing the steric and electrostatic stabilisations between casein micelles (De Kruif, 1998; Horne, 2002; Lucey & Singh, 1997). The presence of native whey protein may inhibit the pH-induced aggregation of casein micelles (Chapter 3).

4.3.2 Composition, pH, and yield

For reconstituted LHSMP, high-heat treatment before Quarg cheese manufacture significantly increased ($P < 0.05$) yield and moisture and significantly decreased ($P < 0.05$) protein and protein content in cheese whey (Table 4.2), consistent with a previous study of Quarg and thermoquarg cheese (Kelly & O'Donnell, 1998). Heat treatment of milk increases the recovery of whey protein in the curd as heat-denatured whey protein interacted with casein micelles (Lucey et al., 1998; Lucey et al., 1997).

For all MF retentates, high-heat treatment prior to Quarg cheese manufacture did not affect ($P > 0.05$) the yield, adjusted yield, and protein content. The moisture content of $HpWR_{ct}$ was significantly lower than that of $PpWR_{ct}$ and $PpWR_{cc}$. High-heat treatment of the sample significantly decreased ($P < 0.05$) the whey protein content of all MF retentate samples. The use of centrifugation or cheesecloth filtration for cheese manufacture did not affect ($P > 0.05$) the yield, adjusted yield, moisture, protein, or cheese whey protein content of Quarg cheese. No differences in moisture or protein content were found between the use of batch centrifugation or the cheesecloth method in the manufacture of cream cheese (Ong et al., 2018).

Quarg cheese manufactured from polymeric warm MF retentate (PpWR and HpWR) had significantly lower ($P < 0.05$) yield and moisture level and significantly higher ($P < 0.05$) protein content compared to that from LHSMP. This indicates that higher levels of syneresis took place during the manufacture of cheese PpWR and HpWR. Greater syneresis of acid milk gels may be linked to the alteration of the gel network (Cayot et al., 2003; Gastaldi et al., 2003). Rearrangement of casein aggregates network occurs at a pH value of around 5, along with the release of CCP, hydration and disaggregation of casein, and contraction of the gel structure (Li & Corredig, 2020; Lin et al., 2018; Lucey, 2016; Meletharayil et al., 2015).

No significant differences ($P > 0.05$) were found in yield, adjusted yield, moisture, or protein content between cheese PLHSMP, PcWR and PpCR. Quarg cheese manufactured from high-heat treated MF retentate showed a significantly lower ($P < 0.05$) yield than that from high-heat treated SMP. All MF retentates had lower whey protein than LHSMP (Table 2.1); the effect of

whey protein denaturation on acid coagulation (Lucey et al., 1998; Lucey et al., 1997) is weakened, which might be the reason high-heat treatment did not affect the yield adjusted yield, moisture, or protein content of MF retentate.

The protein content in cheese whey manufactured from all pasteurised MF retentate was significantly lower ($P < 0.05$) than that from pasteurised LHSMP. The whey protein content in all MF retentates material was lower than that in LHSMP (Table 2.1), which may result in the decreased whey protein in cheese whey of all MF retentates. No significant differences ($P > 0.05$) were found in the pH of all treatments.

4.3.3 Proteolysis

Urea-PAGE electrophoretograms of cheese samples at 2, 30, and 60 days of ripening are shown in Fig. 4.2. Small differences were observed in the gel between each sample at 2, 30, and 60 days of ripening, which is consistent with the previous study of Hurley et al. (2000). This may be attributed to the low ripening temperature or short ripening time (Hurley et al., 2000). High-heat treatment did not affect the protein profiles of each treatment. Both warm MF retentates showed higher levels of β-CN (f29-209), β-CN (f106-209) and β-CN (f108-209), which may be attributed to the higher plasmin activity during the manufacture of warm MF retentates (Chapter 3). β-CN is hydrolysed by plasmin and produces β-CN (f29-209), β-CN (f106-209) and β-CN (f108-209) (Farrell et al., 2004).

The protein profiles in Quarg cheese samples were consistent with that in the raw materials (Chapter 2). The temperature of warm MF processing is close to the optimal temperature of plasmin – 37 ℃ (Vélez et al., 2016). UF retentates

produced at 5 ℃ showed lower plasmin activity than those retentates produced at 50 ℃ (Puri et al., 2020). During the manufacture of cold MF retentates, a portion of β-CN fractionates from casein micelles into the serum phase of milk (Zulewska et al., 2018), and the β-CN fragments may act the same under low temperatures.

The peptide profiles of the pH 4.6-soluble extracts for all Quarg cheese manufactured using centrifugation at 2 and 60 days of ripening were generated by ultra-performance liquid chromatography (Fig. 4.3). Slight differences can be found in the pH 4.6-soluble extract profiles of all cheeses at 2 days of ripening. A higher peak area of peptides was observed in all cheeses at 60 days of ripening than that at 2 days of ripening, which indicates that same proteolysis occurred during the ripening of cheese, which is contrast with the results of urea-PAGE.

Table 4.2. Composition, pH, yield in Quarg cheese and protein content in cheese whey. For explanation of codes, see Table 4.1.

Treatment	Yield[1]	$Yield_{adj}$[1,2]	Moisture (%)	Protein (%)	Protein content in cheese whey (%)	pH
$PLHSMP_{ct}$	25.17^{c}	26.54^{cd}	80.95^{cde}	13.21^{ad}	0.90^{d}	4.50
$PcWR_{ct}$	24.45^{c}	24.39^{ac}	82.00^{cdf}	12.16^{ab}	0.44^{b}	4.39
$PpWR_{ct}$	18.85^{ab}	21.82^{ab}	79.12^{b}	16.13^{ef}	0.56^{c}	4.54
$PpCR_{ct}$	22.62^{bc}	25.09^{ad}	81.32^{cde}	14.12^{bde}	0.48^{bc}	4.50
$HSMP_{ct}$	31.47^{d}	29.42^{d}	83.09^{f}	11.24^{a}	0.52^{bc}	4.49
$HcWR_{ct}$	25.17^{c}	26.01^{bcd}	81.35^{cdf}	12.72^{abc}	0.33^{a}	4.42
$HpWR_{ct}$	17.41^{a}	22.18^{ac}	77.06^{a}	17.67^{f}	0.46^{b}	4.52
$HpCR_{ct}$	24.20^{c}	24.85^{ad}	80.62^{bd}	14.74^{cde}	0.34^{a}	4.53
$PLHSMP_{cc}$	24.22^{c}	23.40^{ac}	82.60^{ef}	12.40^{abc}	0.90^{d}	4.53
$PcWR_{cc}$	21.35^{ac}	21.31^{a}	82.04^{df}	13.50^{ad}	0.44^{b}	4.40
$PpWR_{cc}$	18.85^{ab}	20.64^{a}	80.25^{bc}	15.54^{df}	0.55^{c}	4.54
$PpCR_{cc}$	20.64^{ac}	20.92^{a}	81.75^{cdf}	14.09^{bde}	0.49^{bc}	4.50

[1] (g 100 ml^{-1} milk). [2] Adjusted to a constant 82% moisture.
Values are means of data from three independent trials; means within the same column not sharing a common superscript differ significantly ($P < 0.05$).

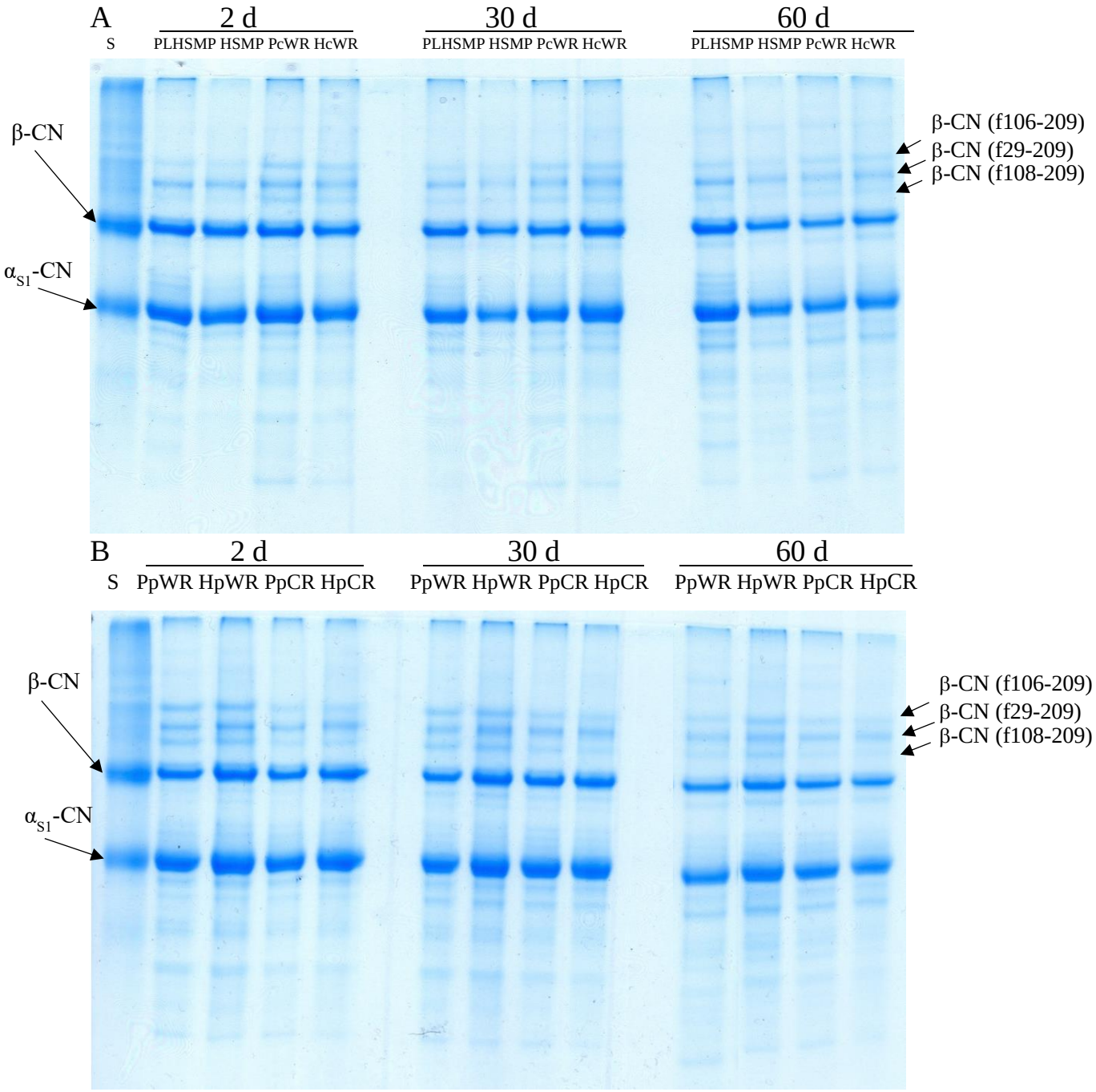

Fig. 4.2. Urea-polyacrylamide gel electrophoretograms of Quarg cheeses made from (A) LHSMP (PLHSMP and HSMP) and warm ceramic MF retentate (PcWR and HcWR), (B) warm polymeric MF retentate (PpWR and HpWR) and cold polymeric MF retentate (PpCR and HpCR) using centrifugation at 2, 30 and 60 days of ripening. Sodium caseinate (S) was used as a standard. The figure corresponds to data for one of the three cheese replicates; all replicates were similar. For explanation of codes, see Table 4.1.

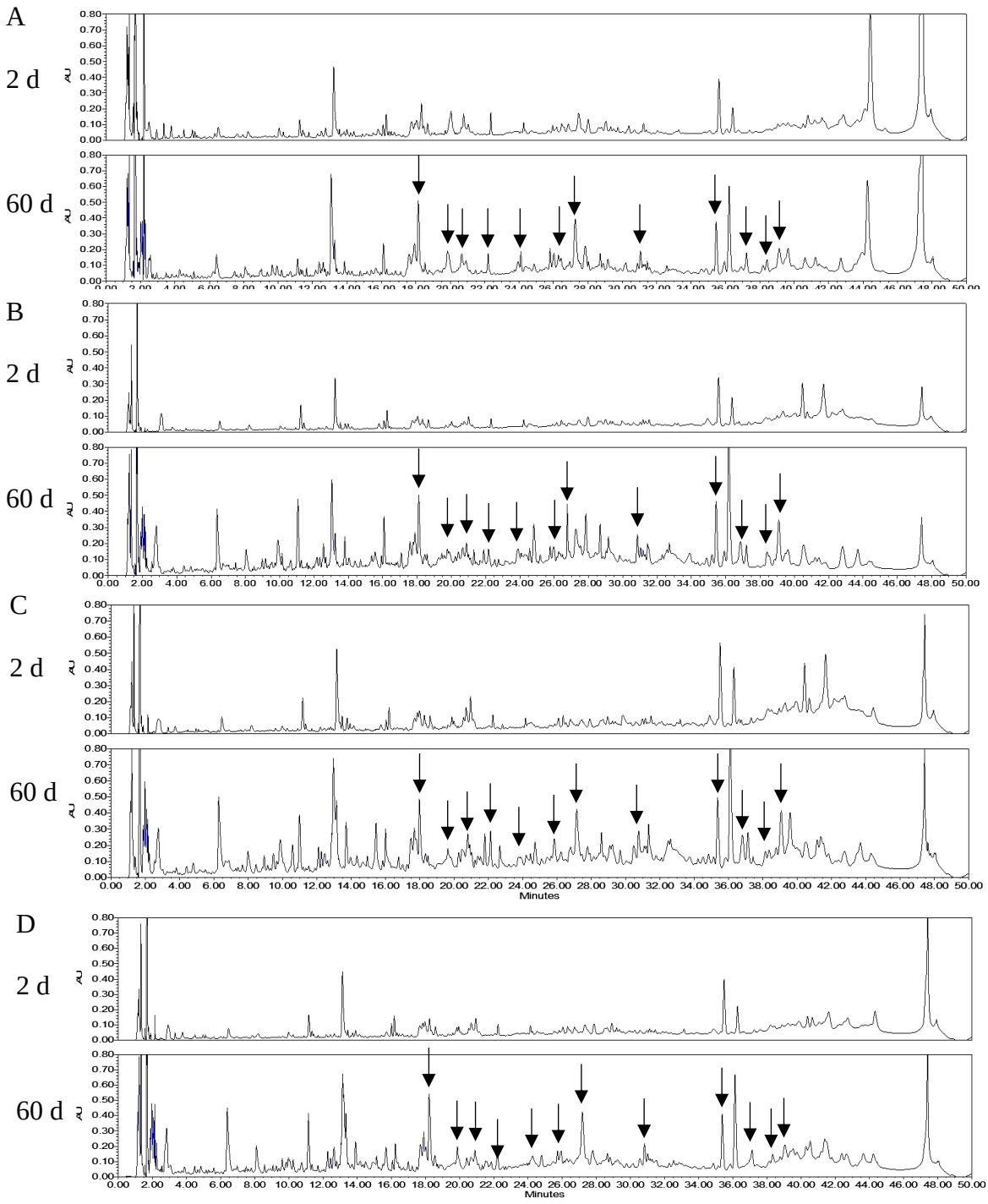

Fig. 4.3. Ultra-performance liquid chromatography (UPLC profiles, C_{18} column) of pH 4.6-soluble extracts from (A) PLHSMP$_{ct}$, (B) PcWR$_{ct}$, (C) PpWR$_{ct}$, (D) PpCR$_{ct}$, (E) HSMP$_{ct}$, (F) HcWR$_{ct}$, (G) HpWR$_{ct}$, and (H) HpCR$_{ct}$ cheeses at 2 and 60 days of ripening, and (I) NaCN digest at the wavelength of 214 nm. The figure corresponds to data for one of the three cheese replicates; all replicates were similar. For explanation of codes, see Table 4.1. Black arrows indicate peptides that may be produced by bovine chymosin during ripening.

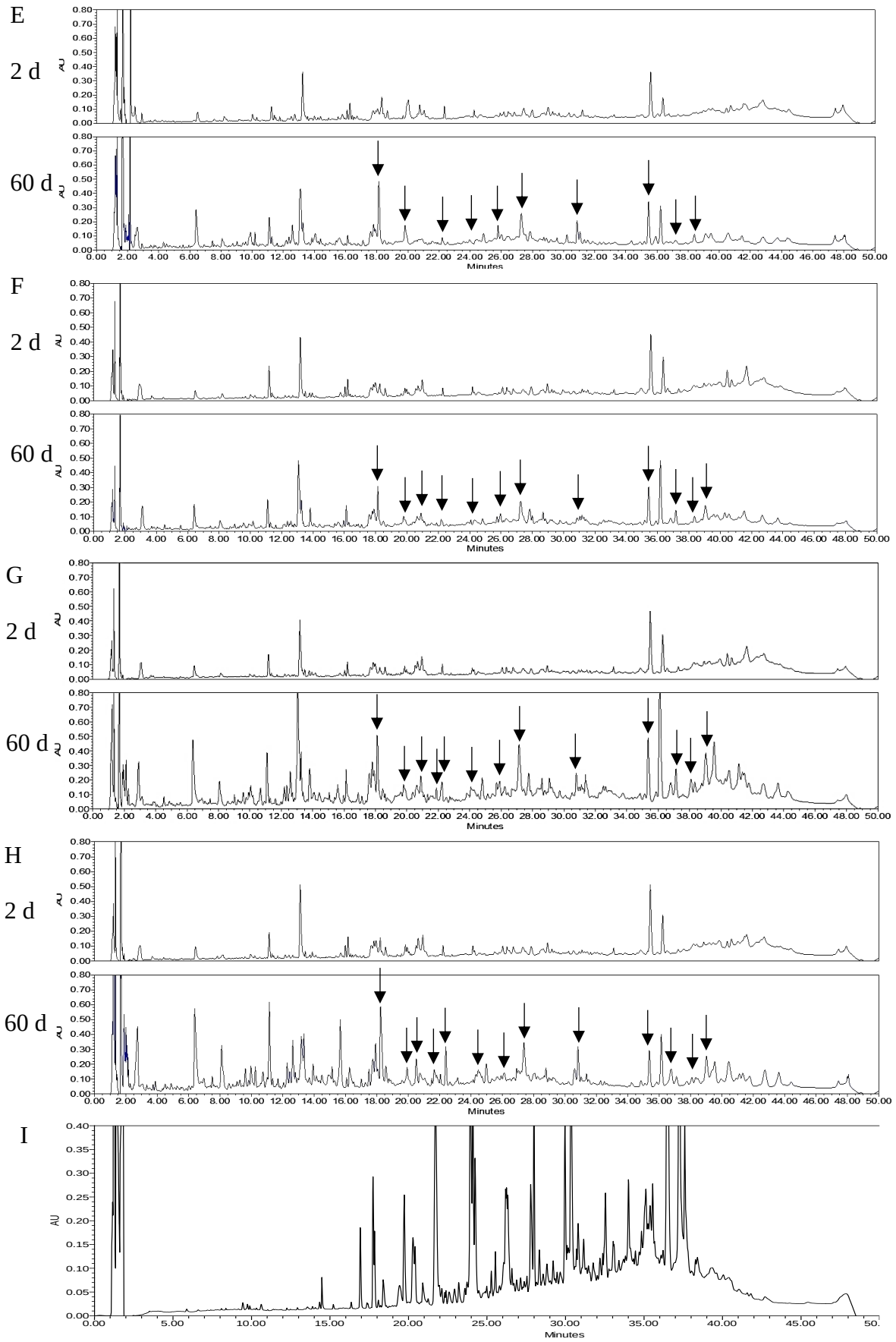

Fig. 4.3 Continued.

Compared to Quarg cheese manufactured from pasteurised samples, Quarg cheese manufactured from high-heat treated samples showed less whey protein content (in the region 43-49 min), which is consistent with the findings of Mara & Kelly (1998). Heating milk at 90 °C for 10 min destroys most non-spore-forming bacteria and causes whey protein-*κ*-casein complexation, which is more extensive than pasteurisation (IDF, 2018). At 60 days of ripening, the peptide profile of Quarg cheese manufactured from pasteurised and high-heat treated samples were similar, while the peak area was relatively lower in high-heat treated samples, which is also in agreement with the findings of Mara & Kelly (1998). This suggests that the proteolysis occurred at a lower rate in Quarg cheese manufactured from high-heat treated samples. Denatured whey protein bonds to casein, producing a steric impediment to casein digestion (Benfeldt et al., 1997). Indigenous enzymes e.g., cathepsin D and plasmin that cause proteolysis in Quarg cheese may be inactivated more extensively during high-heat treatment of samples, resulting in lower levels of proteolysis (Hayes et al., 2001; Hurley et al., 2000; Kelly & Foley, 1997).

Cheeses manufactured from warm MF retentates showed relatively higher levels of proteolysis compared to that of LHSMP or cold MF retentates at 60 days of ripening. Microbial growth at 55 °C is faster than that at 10 °C (Schiffer & Kulozik, 2020), which may result in a higher microbial level in warm MF retentates compared to that in LHSMP and cold MF retentates, which might cause higher levels of proteolysis during the storage of Quarg cheese.

By comparison with the pH 4.6-soluble extract profile of NaCN (Fig. 4.3I), the peptides eluting at 18.2, 19.8, 20.2, 21.9, 24.2, 26.3, 27.9, 31.1, 35.5, 37.3, 38.2, and 38.5 min in the pH 4.6-soluble extract profiles of all Quarg cheese

samples are potentially produced by bovine chymosin. The eluting of pH 4.6-soluble extract profile of NaCN started from 15 min, which indicates that enzymes other than chymosin also contributed to proteolysis in Quarg cheese. Chymosin is the primary proteolytic enzyme in Quarg and the residual starter enzymes, plasmin, and cathepsin D contribute to proteolysis at a lower extent during ripening (Mara & Kelly, 1998).

4.3.4 Texture analysis and rheological properties

No significant differences ($P > 0.05$) were found in hardness between cheeses $PLHSMP_{cc}$, $PcWR_{cc}$, and $PpCR_{cc}$. The hardness of cheese $PpWR_{cc}$ was significantly higher ($P < 0.05$) than that of the other treatments (Table 4.3). This may be attributed to the higher level of syneresis and lower moisture content in cheese $PpWR_{cc}$ compared to that of the other treatments. Acid gels with a higher level of syneresis previously showed higher hardness than those with a lower level of syneresis (Sinaga et al., 2017), which is consistent with the results in this study.

Dynamic rheological properties of Quarg cheese manufactured using the cheesecloth method were used to characterise the gel strength of samples (Fig. 4.4). Within the tested frequency range, G' value was higher than G" value for all analysed cheese samples, which indicates that the Quarg cheese analysed are solid-like (Bong & Moraru, 2014). Loss tangent (tan δ), which reflects the viscosity of the sample, was also measured by dynamic rheological testing. The tan δ value of all cheese samples within the applied frequency range was within

0.27-0.32 (results not shown). Samples with an observed tan δ value that is greater than 0.1 are considered as weak gels (Ikeda & Nishinari, 2001).

The separation between G' and G" values at 0.1 Hz of cheese $PcWR_{cc}$ was significantly higher ($P < 0.05$) than that of cheese $PLHSMP_{cc}$. No significant differences ($P > 0.05$) were found in the separation between G' and G" values at 10 Hz. When the frequency-dependent G' and G" values are high, the smaller the difference between both moduli is, the weaker gel is formed (Ross-Murphy & Chan, 1984). This indicates that the gel strength of cheese $PcWR_{cc}$ was stronger than that of cheese $PLHSMP_{cc}$ at 0.1 Hz, while all samples had the same gel strength at 10 Hz.

4.3.5 Microstructure

The microstructure of all Quarg cheese samples is shown in Fig. 4.5. Various microstructure was observed in Quarg cheese manufactured using centrifugation. Quarg cheese manufactured from high-heat treated SMP tends to have smaller pores and a less dense protein network compared to that of pasteurised LHSMP. This may be attributed to heat-induced whey protein association with casein micelles that act as bridges to connect protein networks (Vaziri et al., 2010). A homogeneous structure was found in Quarg cheese $PpWR_{ct}$, $PpCR_{ct}$, $HSMP_{ct}$, and $HcWR_{ct}$.

Table 4.3. Hardness of Quarg cheese manufactured using the cheesecloth method. For explanation of codes, see Table 4.1.

Treatment	Hardness (g)	Separation between G' and G" at 0.1 Hz (Pa)	Separation between G' and G" at 10 Hz (Pa)
$PLHSMP_{cc}$	57.69^{a}	5374^{a} (450)	10786 (889)
$PcWR_{cc}$	65.55^{a}	6567^{b} (673)	12467 (1320)
$PpWR_{cc}$	86.96^{b}	6210^{ab} (807)	11911 (1479)
$PpCR_{cc}$	60.72^{a}	6017^{ab} (748)	11465 (1285)

Values are means (standard deviation), from three independent trials; means within the same column not sharing a common superscript differ significantly ($P < 0.05$).

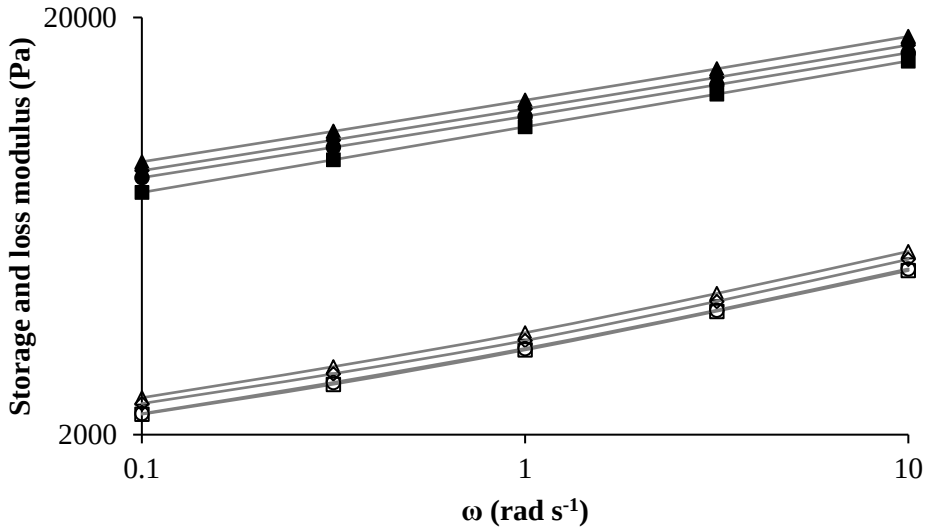

Fig. 4.4. Frequency dependence of storage modulus (G', filled symbol) and loss modulus (G", empty symbol) of Quarg cheese manufacture from $PLHSMP_{cc}$ (square), $PcWR_{cc}$ (triangle), $PpWR_{cc}$ (diamond), and $PpCR_{cc}$ (round) using the cheesecloth method. Values are means of three independent trials. For explanation of codes, see Table 4.1.

The protein network observed in all Quarg cheese manufactured using the cheesecloth method was similar. Small pores and regular protein networks were found in Quarg cheese manufactured using the cheesecloth method. This is consistent with the results of dynamic rheological properties. A similar protein

network was also observed in cream cheese manufactured using the cheesecloth method (Ong et al., 2018).

4.3.6 Glycomacropeptide in cheese whey

The rennet coagulation time of all MF retentates was lower ($P < 0.05$) than that of LHSMP (Chapter 3), indicating that chymosin is more active against *κ*-casein in MF retentates compared to that in LHSMP, which may relate to the higher GMP content in cheese whey. Four peaks were identified by reverse-phase high-performance liquid chromatography of GMP standard. The peak area of the four GMP peaks was summed. The total GMP peak area of $PLHSMP_{ct}$ was considered as 100% for comparison with other treatments (Table 4.4). For each material used for Quarg cheese manufacture, the use of high-heat treatment or pasteurisation did not affect ($P > 0.05$) the GMP content in cheese whey, and nor did the use of centrifugation or the cheesecloth method.

Quarg cheese whey samples $PpWR_{ct}$ and $PpWR_{cc}$ showed the highest total GMP peak area of all pasteurised Quarg cheese whey samples. The total GMP peak area of all samples of high-heat treated MF retentates was significantly higher ($P < 0.05$) than that of high-heat treated SMP. Chymosin hydrolysis κ-casein into GMP and *para*-κ-casein, where GMP is the C-terminal part of κ-casein (Visser, 1993). GMP is released into cheese whey during cheese manufacture (Thomä-Worringer et al., 2006). GMP is an ingredient with biological properties relevant to human health and which is simple to recover from whey, as GMP mainly partitions into the whey during cheese manufacture (Jauregui-Rincón, Salinas-Miralles, Chávez-Vela, & Jiménez-Vargas, 2018).

At pH 4.5-5.0, up to 98% of GMP can be recovered through anion-exchange chromatography from cheese whey (Tek et al., 2005). The higher GMP content in cheese whey of MF retentates compared to that of LHSMP may be an advantage for the recovery of GMP.

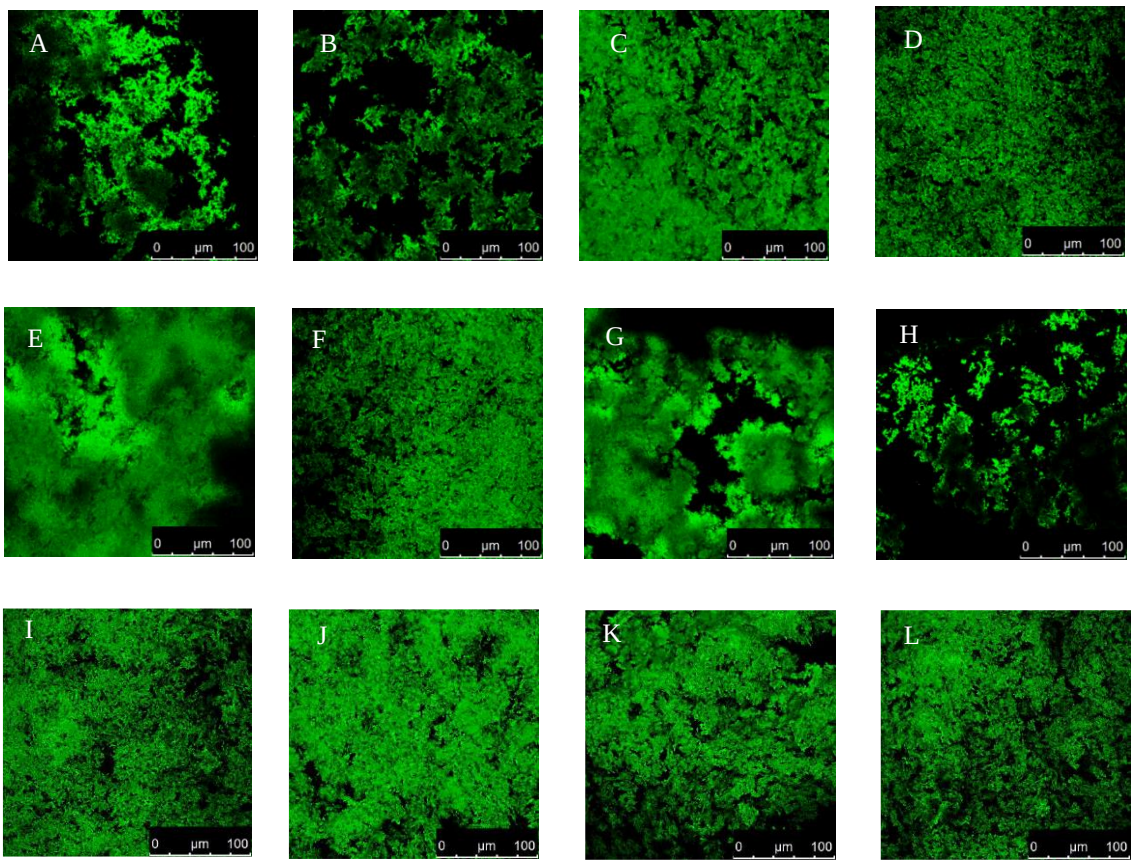

Fig. 4.5. Representative CLSM images of Quarg cheeses (A) $PLHSMP_{ct}$, (B) $PcWR_{ct}$, (C) $PpWR_{ct}$, (D) $PpCR_{ct}$, (E) $HSMP_{ct}$, (F) $HcWR_{ct}$, (G) $HpWR_{ct}$, (H) $HpCR_{ct}$, (I) $PLHSMP_{cc}$, (J) $PcWR_{cc}$, (K) $PpWR_{cc}$, and (L) $PpCR_{cc}$ respectively. Images were taken using a 63x objective. Scale bar = 100 µm. For explanation of codes, see Table 4.1.

Table 4.4. Total glycomacropeptide (GMP) peak area identified by reverse-phase high-performance liquid chromatography (RP-HPLC) for all Quarg cheese whey samples. For explanation of codes, see Table 4.1.

	Total GMP peak area	Relative GMP content (% of $PLHSMP_{cc}$)
$PLHSMP_{ct}$	10978^{ac} (1254)	100
$PcWR_{ct}$	15103^{de} (1108)	138
$PpWR_{ct}$	19288^{f} (1065)	176
$PpCR_{ct}$	12911^{acd} (781)	118
$HSMP_{ct}$	10460^{ab} (373)	95
$HcWR_{ct}$	14237^{ce} (428)	130
$HpWR_{ct}$	17461^{ef} (2388)	159
$HpCR_{ct}$	14318^{ce} (442)	130
$PLHSMP_{cc}$	9502^{a} (674)	87
$PcWR_{cc}$	12484^{acd} (1111)	114
$PpWR_{cc}$	16710^{ef} (1546)	152
$PpCR_{cc}$	13155^{bcd} (1532)	120

Values are means (standard deviation) from three independent trials; means within the same column not sharing a similar superscript letter differ significantly ($P < 0.05$).

4.4 Conclusions

The composition, proteolysis, and functionalities of Quarg cheeses manufactured from pasteurised (72 ℃ x 15 s) or high-heat treated (90 ℃ x 10 min) LHSMP, warm and cold MF retentates using centrifugation or cheesecloth method were evaluated. Overall, high-heat treatment may help the cheese manufactured from LHSMP, while such treatment may not be necessary for cheese manufactured from MF retentates. The use of centrifugation or the cheesecloth method did not affect the composition of Quarg cheese, meaning that the choice of separation method is not important for Quarg cheese manufacture. Cheeses manufactured from most MF retentates showed comparable hardness and rheological properties to that from LHSMP, suggesting that MF retentates are capable to form a gel with structure like LHSMP. The use of MF retentates for Quarg cheese manufacture

resulted in lower protein content in cheese whey than that of LHSMP, while the GMP content of cheese whey from MF retentates was relatively higher than that of LHSMP, which might favour GMP recovery. These results suggest that using MF retentates for acid-induced cheese manufacture using centrifugation or the cheesecloth method is feasible and may result in lower moisture and yield, modified texture and higher GMP content in cheese whey compared with the use of LHSMP.

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CHAPTER 5

Comparison of three generations of fermentation-produced chymosins in the production and ripening of Cheddar cheese

Bozhao Li[1], David S. Waldron[1], MaryAnne Drake[2], John Lyne[3], Alan L. Kelly[1*], Paul L. H. McSweeney[1]

[1]*School of Food and Nutritional Sciences, University College Cork, College Road, Cork, Ireland.*

[2]*Department of Food, Nutrition and Bioprocessing, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, USA*

[3]*Chr. Hansen A/S, Hoersholm, Denmark*

Declaration

This chapter was written by author BL and reviewed by his co-authors. The chapter has been published as: Li, B., Waldron, D. S., Drake, M., Lyne, J., Kelly, A. L., & McSweeney, P. L. H. (2022). Suitability of a novel camel (*Camelus dromedarius*) chymosin as a coagulant for Cheddar cheese manufacture. *International Dairy Journal*, *129*, 105346. BL co-designed the study, manufactured cheese, determined rennet coagulation properties, composition, proteolysis, and functionalities and analysed the data. DSW contributed to cheese manufacture. MD provided data for sensory analysis. JL provided all chymosin samples involved in this study and was a collaborating partner.

Chapter 5

Abstract

Cheddar-type cheese was manufactured using fermentation-produced bovine chymosin (BC), fermentation-produced camel chymosin (CC) and a modified fermentation-produced camel chymosin (mCC) and ripened for 180 days. Only minor differences were found in cheese composition and pH between the cheese made with any of the chymosins studied. Proteolysis in cheese made with mCC was reduced compared to cheese made with BC or CC. Major casein-derived peptides and the corresponding cleavage sites produced by bovine and camel chymosins in cheese were confirmed in this study. Significantly higher instrumental and sensory hardness and significantly lower meltability were found in cheeses made using CC or mCC compared with cheese BC after 180 days of ripening. Descriptive sensory analysis results showed that cheese made with CC or mCC had less sulphur and barny flavour; the brothy flavour and bitter taste of cheese made with mCC were also lowest. In conclusion, the modified camel chymosin appears to be suitable for the manufacture of Cheddar cheese with modified functionalities.

Chapter 5

5.1 Introduction

Rennet is the general name for an enzyme preparation used to coagulate milk that originates from the abomasum of ruminant mammals. Chymosin, the principal enzyme in most rennets, cleaves the Phe_{105}-Met_{106} bond of κ-CN, thereby reducing the negative charge of casein micelles and reducing steric stabilisation. Without this stabilisation, a casein micelle network is formed, leading to the separation of whey and casein curds. This property of chymosin is a key aspect of cheese manufacture, and calf chymosin is the most common coagulant used for cheesemaking. Other animal sources of chymosin extracted from lambs and camels are also commercially available, as well as vegetable coagulants, including enzymes from *Cynara cardunculus, Ficus carica, Arctium minus* and *Solanum dubium*, which are sometimes used in traditional cheese production (Scott, Robinson, & Wilbey, 1998). Enzymes naturally produced by microorganisms (e.g., *Endothia parasitica*) can also be used, for example, for Emmental cheese manufacture (Gagnaire, Mollé, Herrouin, & Léonil, 2001).

In cheese manufacture, it is ideal for a coagulant to have low proteolytic activity, with high cleavage specificity at or near the Phe_{105}-Met_{106} bond of κ-CN, and is easy to inactivate in whey (Bansal et al., 2009). The coagulation/proteolysis (C/P) ratio, which is the ratio of milk coagulating activity to the general proteolytic activity, is related to the specificity of enzyme activity (Soodam, Ong, Powell, Kentish, & Gras, 2015). Cheese yield is an important property to consider and may be decreased as the degree of proteolysis increases or C/P ratio decreases during cheese manufacture (Fox & McSweeney, 1997; Soodam et al., 2015).

Camel chymosin has 85% amino acid sequence identity to calf chymosin while its clotting activity is 70% higher than that of calf chymosin, and it has 25% of the hydrolytic activity of calf chymosin (Kappeler et al., 2006; Langholm Jensen et al., 2013). Compared to calf chymosin, camel chymosin has an additional histidine residue with a positive charge on the N-terminal lobe of the enzyme, which attracts the negative charge in κ-CN, and therefore leads to a strong association of camel chymosin with the casein micelles; the higher molecular flexibility and electrostatic interaction of camel chymosin facilitate the association of enzyme and substrate (Kappeler et al., 2006; Langholm Jensen et al., 2013).

With gene recombination technology, once the gene sequence is determined, chymosin may be produced by microbial fermentation rather than extracted from the abomasum of the animal. Calf chymosin is produced through fermentation by transferring the gene for chymosin to *Aspergillus niger* or *Kluyveromyces lactis* (Kappeler et al., 2006). Cheddar cheese manufactured using camel chymosin produced by Chr. Hansen A/S (ChyMax M^{TM}) has been shown to have higher hardness and less bitterness than cheese made from calf chymosin, due to decreased primary proteolysis (Bansal et al., 2009; Møller, Rattray, Sørensen, & Ardö, 2012). Also, Mozzarella cheese manufactured using camel chymosin was found to have less proteolysis, harder texture and longer shelf-life than cheese made from bovine calf chymosin (Moynihan et al., 2014).

A modified camel chymosin enzyme was developed through a structure-based design approach involving bioinformatics (amino acid diversity among chymosin sequence homologues) sequence function analysis (exchange of amino acids) and structure-based design (optimizing substrate binding – κ vs α vs β caseins). Selected enzymes were then expressed in a fungal model to allow production of

enough protein enzyme for application in cheese model and cheese making systems to select for enzyme with optimized characteristics in terms of coagulation activity, relative coagulation versus proteolytic activity and specific casein cleavage; the resultant enzyme (CHY-MAX ® Supreme) has increased specific milk-clotting activity and greatly increased proteolytic specificity compared to the original generation camel chymosin (Jäckel et al., 2017; Jäckel et al., 2019).

The objective of this study was to manufacture Cheddar cheese using commercial fermentation-produced bovine calf chymosin, commercially available fermentation-produced camel chymosin, and commercially available fermentation-produced camel chymosin with altered molecular structure and compare the proteolysis, peptide profiles, texture, and sensory properties of the resulting cheeses.

5.2 Materials and methods

5.2.1 Coagulants

The coagulants used for Cheddar cheese manufacture were commercially available fermentation-produced bovine calf chymosin (BC; CHY-MAX® Extra, 600 international milk clotting units (IMCU) mL^{-1}), commercially available fermented-produced camel chymosin (CC; CHY-MAX® M 200, 200 IMCU mL^{-1}) and commercially available fermentation-produced camel chymosin with structural changes through amino acid substitution (mCC; CHY-MAX® Supreme 200, 200 IMCU mL^{-1}; all from Christian Hansen A/S, Hoersholm, Denmark). To determine the amount of each chymosin used for Cheddar cheese manufacture, the milk coagulation properties of each chymosin were analysed according to the

method described by O'Mahony, Lucey, & McSweeney (2005). Milk was coagulated by each chymosin and the gel strength (G', storage modulus) of the sample formed at a normal cutting time (45 min) was used to determine coagulating activity (Bansal et al., 2009); G' values were measured on an AR-G2 controlled-stress rheometer (Waters TA Instruments, Leatherhead, Surrey, UK).

5.2.2 Cheese manufacture

Whole milk (4.64% fat, 3.68% protein, pH 6.5) was standardised to a casein to fat ratio of 0.7:1.0, pasteurised at 72 ℃ for 15 s and cooled to 32 ℃. Cheddar cheeses were manufactured according to a standard Cheddar cheese manufacturing protocol (Fox, Guinee, Cogan, & McSweeney, 2000) from 100 kg standardised milk. An aliquot of 30 g (0.03% w/w) of Cheddar cheese starter culture (R604Y Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added into each sample and allowed to ripen for 30 min, followed by 60 IMCU L^{-1} of calf chymosin or camel chymosin or modified camel chymosin. The curd was cut after 45 min. Whey was drained when the pH reached 6.2 and curd was cheddared; when the pH decreased to 5.4, the curd was milled into small pieces and salted at a level of 2.5% (w/w) NaCl. The curds were then wrapped in cheesecloth and moved to moulds, which were pressed at a pressure of 2.5 kg cm^{-2} for 14 h. The cheeses were then removed, vacuum-packed (99.9% air extracted); samples were taken periodically during ripening at 8 ℃ for 180 days. The masses of milk, whey, starter, chymosin, salt and cheese were measured to estimate the yield of cheese.

5.2.3 Compositional analysis and analysis of proteolysis

The gross composition of cheeses was analysed at 14 days of ripening. Moisture was determined by the oven-drying method (IDF, 1982), protein (%N × 6.38) by the Kjeldahl method (IDF, 1986), fat was analysed by the Gerber method (IS, 1955) and salt was analysed by titration with $AgNO_3$ (Fox, 1963). The pH was measured at 14 days of ripening by using a calibrated pH probe placed in contact with dry grated cheeses. All results were determined in triplicate.

Urea-polyacrylamide gel electrophoresis (urea-PAGE) was applied directly on cheese samples at 14, 30, 90 and 180 days of ripening according to the method of Andrews (1983) with modifications as described by Shalabi and Fox (1987). pH 4.6-soluble extracts were prepared (Kuchroo & Fox, 1982) and their nitrogen content was determined by the Kjeldahl method (IDF 1986) and expressed as % of the total nitrogen content of the cheese samples.

5.2.4 Analysis of peptides

5.2.4.1 Sample preparation

pH 4.6-soluble extracts of cheese samples at 90 and 180 days of ripening were shipped to Centre of Food and Fermentation Technologies, Tallinn, Estonia within 24 h, packaged with dry ice.

Before peptide analysis, pH 4.6-soluble extracts of three trials of cheeses were diluted with MilliQ® water 1: 20, vortexed and centrifuged for 10 min at 15000 g at room temperature. After centrifugation, the supernatants were transferred into autosampler compatible vial low-volume inserts and capped.

5.2.4.2 Liquid chromatography-mass spectrometry

The peptide profiles of samples (cheese samples: 1 µL, NaCN-chymosin digestion samples: 2 µL) prepared as described in Section 6.2.6.1 were analysed in triplicate using a Waters I-Class Plus (BSM, SM-FL and CM-A) ultra-performance liquid chromatography (UPLC®) (Waters Corporation, Milford, MA, USA) system hyphenated to a Waters Vion ion mobility separation-quadrupole time-of-flight (IMS-QTOF) mass spectrometer (3.1.0) equipped with LockSpray Exact Mass Ionisation Source and controlled by Waters UNIFI (1.9.4), as described by Xia et al. (2023).

5.2.4.3 Raw data processing and analysis

Peptide identification and quantification were carried out using UNIFI Large Molecule Package with Peptide Map (IMS) workflow. Peptide mapping was carried out using the UNIFI Large Molecule Package (LMP) (Waters Corporation, Milford, MA, USA) by searching the peptides against α_{S1}-CN (P02662), α_{S2}-CN (P02663), β-CN (P02666) and κ-CN, P02668) sequences from *Bos taurus* obtained from the UniProt database (Cambridge, UK). Peptide search was set to non-specific digest with no missed cleavages allowed, and minimum sequence length was set to 4. The following filters were applied: mass error between -5 and 5 ppm; matched first gen primary ions greater than or equal to 1; fragment label not containing in-source fragments; loss of H_2O and loss of NH_3. The following variable post-translational modifications were used in the analysis: oxidation (M), acetyl-(protein N-terminal), deamidation (NQ) and phosphorylation (STY).

Processed data from UNIFI were exported into Microsoft Excel for grouping, alignment, and filtration based on the retention time (0.1 min), observed molecular weight (0.01 Da per charge) and collision cross section (10% RSD). The aligned data were then further used to construct a data matrix for revision and removal of outliers and false-positive identifications. Only peptides identified in each of the three technical replicates were considered for further analysis.

The identified and quantified peptides as well as their normalised and averaged intensities were further analysed and visualised with the help of in-house data analysis and visualisation scripts written in the Python™ programming language (Python Software Foundation, Delaware, USA). The resulting data were plotted as peptide maps, cleavage sites and peptide length distribution diagrams. Similarity analysis based on Euclidean distances in a space of peptide intensities was carried out to highlight differences between technical replicates as well as different hydrolysis treatments.

5.2.5 Texture profile analysis and analysis of meltability

Texture profile analysis was performed using a Texture Analyzer TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) at 30, 90, and 180 days of ripening. Cheese samples were cut into cylinders of 20-mm height and 20-mm diameter and kept at 4 °C overnight. Cheese cylinders were compressed to 25% of the initial height in two continuous compressions with a speed of 1 mm s^{-1}. Hardness, cohesiveness, and springiness were also measured; five cheese samples were measured for each treatment (Li et al., 2020; Chapter 3).

Meltability was analysed using the Schreiber meltability test as described by (Altan, Turhan, & Gunasekaran, 2005). Cheese samples were heated at 232 ℃ for 5 min, and meltability was measured as the percentage increase in diameter of the original samples; analyses were performed in triplicate at 30, 90 and 180 days of ripening.

5.2.6 Dynamic small-amplitude oscillatory rheology

The rheological properties of melted cheese after 30, 90 and 180 days of ripening were analysed with a controlled-stress AR-G2 rheometer (TA Instruments, Waters LLC, Leatherhead, Surrey, UK) according to the method described by Ibáñez, Waldron, & McSweeney (2016). The initial temperature of cheese sample was 20 ℃, and samples were heated to 80 ℃ at a heating rate of 2 ℃ per min. Storage modulus (G'), loss modulus (G"), and loss tangent (LT) were measured during heating. The maximum LT (LT_{max}) and the temperature where LT = 1, which are indicators of melting, were also recorded. Each sample was analysed in triplicate (Li et al., 2020; Chapter 3).

5.2.7 Descriptive sensory analysis

Cheese samples were shipped to North Carolina State University within 72 h under refrigerated conditions after 180 days of ripening. Sensory testing was conducted on cheese samples in North Carolina State University in compliance with approval from the North Carolina State University Institutional Review Board for Human Subjects. Cheese samples were cut into 1 x 1 x 1 cm cubes and put into 110-g lidded soufflé cups with random three-digit codes for descriptive

sensory analysis. The cheeses were tempered at 10 ℃ for one hour and were served at this temperature for flavour and texture profiling, with spring water and unsalted crackers for palate cleansing. Descriptive analysis of flavour used a 0 to 15-point universal intensity scale with the Spectrum^{TM} method (Meilgaard, Carr, & Civille, 1999; Drake & Civille, 2003) and a previously established Cheddar cheese flavour sensory language (Drake, McIngvale, Gerard, Cadwallader, & Civille, 2001). Texture evaluation utilized a 0 to 15-point product-specific scale and an established lexicon (Rogers et al., 2009). Flavour and texture were evaluated in separate sessions.

A descriptive sensory panel (n=6, 6 females, ages 22 – 46 years), with more than 200 h collective experience with the descriptive analysis of Cheddar cheese flavour and texture, evaluated the cheeses. Paper ballots were used. Consistent with Spectrum^{TM} descriptive analysis training, panellists were presented with reference solutions of sweet, sour, salty, and bitter tastes to learn to use consistently the universal intensity scale (Meilgaard et al.,1999; Drake & Civille, 2003). Following consistent use of the Spectrum^{TM} scale with basic tastes, panellists learned to identify and scale flavour descriptors using the same intensity scale through presentation and discussion of flavour definitions, references and a wide array of cheeses. Discussion and evaluation of a wide array of cheeses were also conducted during training enabling panellists consistently to differentiate and replicate samples. Analysis of data collected from training sessions confirmed that panel results were consistent and that terms were not redundant, consistent with previous use of the developed language (Drake et al., 2001; Drake et al., 2005). Each panellist evaluated each product in duplicate.

5.2.8 Statistical analysis

BC, CC and mCC cheeses were made in triplicate in independent trials (i.e., 3 × 3 blocks). Statistical analysis was carried out using one-way analysis of variance (ANOVA) with R project for Windows version i386 3.4.0 (the R Foundation for Statistical Computing, University of Auckland, Auckland, New Zealand) to establish significant differences between samples. The probability level used for statistical significance was $P < 0.05$. The data of descriptive sensory analysis were analysed by a general linear model analysis of variance with Fisher's least significant difference (LSD) as a *post hoc* test (SAS version 9.1, Cary, NC, USA).

5.3 Results and discussion

5.3.1 Determination of coagulant performance

To determine the amount of CC and mCC used for Cheddar cheese manufacture, a preliminary rennet coagulation rheology test was performed. At an incubation time of approximately 45 min (2700 s), the storage modulus, G', of milk samples BC, CC and mCC was almost the same (Fig. 5.1). With the same level of IMCU, although there was a significant difference ($P < 0.05$) in the time taken for G' to exceed 1 Pa between BC and CC, there was no statistically significant difference ($P > 0.05$) between the gel firmness at 45 min for all of the coagulants (Table 5.1). Therefore, BC, CC and mCC were added to cheese-milk at a constant level of 0.06 IMCU mL^{-1} milk (i.e., 10, 30 and 30 mL 100 L^{-1} milk, respectively). Lower levels of camel chymosin were used in previous Cheddar cheese studies to obtain the same gel strength of the coagulum at the cutting time (Bansal et al.,

2009; Govindasamy-Lucey, Lu, Jaeggi, Johnson, & Lucey, 2010). Moynihan et al. (2014) used identical levels of BC and CC for Mozzarella cheese manufacture at different coagulating temperatures to obtain the same gel strength at cutting.

5.3.2 Composition, pH, and yield

No significant differences ($P > 0.05$) were found in moisture, fat, and protein contents or pH of cheese samples (Table 5.2). Although the salt content and salt-in-moisture values of cheese mCC were significantly higher ($P < 0.05$) than those of the cheese BC (Table 5.2), the numerical differences were small, and no significant differences ($P > 0.05$) were found between samples CC and mCC. Bansal et al. (2009) and Moynihan et al. (2014) also reported that no significant differences were found in composition and pH between Cheddar or Mozzarella cheeses made with calf or camel chymosin. No significant differences ($P > 0.05$) were found in the yield of cheese (Table 5.2), which is in agreement with the conclusion of Bansal et al. (2009).

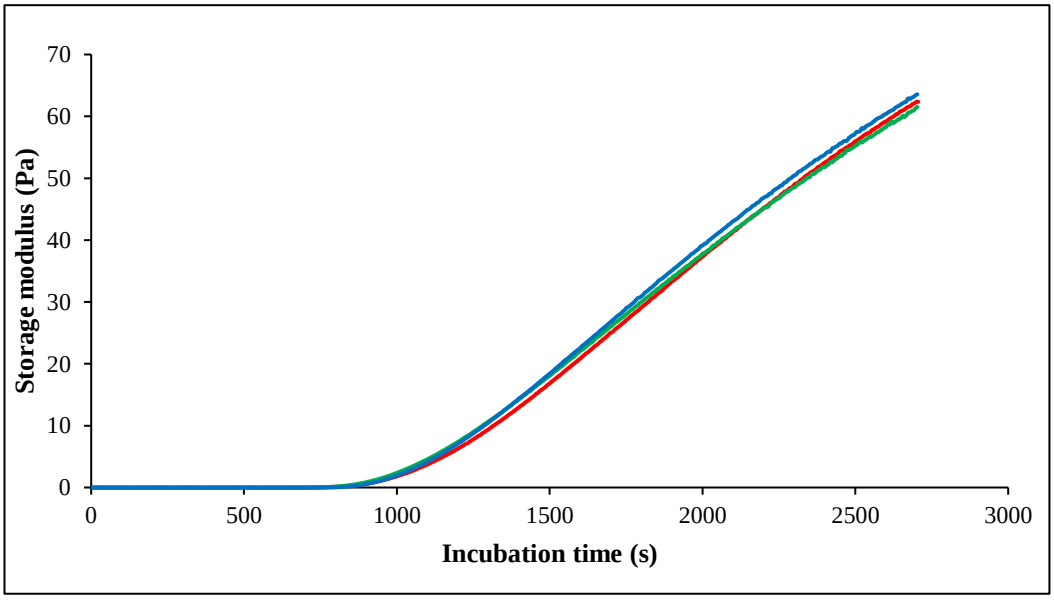

Fig. 5.1. Storage modulus (G') as a function of incubation time of milk renneted by bovine calf chymosin (red), camel chymosin (green) and modified camel

chymosin (blue). Data shown are one of the three replicates; all replicates were similar.

5.3.3 Proteolysis

Urea-PAGE electrophoretograms of cheese samples after 14, 30, 90 and 180 days of ripening are shown in Fig. 5.2. All cheese samples showed the breakdown of β-CN into β-CN (f29-209), β-CN (f106-209) and β-CN (f108-209), consistent with the action of plasmin (Eigel et al., 1984), and no differences in the breakdown of β-CN were found between samples. Bansal et al. (2009) found that the urea-PAGE electrophoretograms of cheese manufactured using calf and camel chymosin showed that hydrolysis of β-CN by calf chymosin was only linked to the hydrolysis of β-CN to β-CN (1-189/192), which is consistent with the urea-PAGE results of our cheeses, and almost no β-CN (1-189/192) was observed in cheese mCC (Fig. 5.2). The complementary peptides, β-CN (190-209) and β-CN (193-209) are hydrophobic and related to the bitterness of cheese (Visser et al., 1983). β-CN (190-209) and β-CN (193-209) were produced to a very small extent in cheese manufactured with mCC, suggesting that using camel chymosin instead of calf chymosin may decrease bitterness in cheese during maturation.

Table 5.1. Rheological properties of milk coagula produced by bovine calf chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC).

	IMCU/L	Time G' > 1 Pa (s)	Gel firmness (G' at 45 min, Pa)
BC	60	1031^{b} (80)	57.7 (4.3)
CC	60	858^{a} (50)	66.0 (4.2)
mCC	60	959^{ab} (26)	61.1 (2.7)

Data are means (standard deviation), from three independent trials; means within the same column not sharing a similar superscript differ ($P < 0.05$).

All cheese samples showed a breakdown of α_{S1}-CN, and the rate of hydrolysis was fastest for the cheese BC, followed by cheese CC and then mCC. After 180 days of ripening, cheese mCC retained some intact α_{S1}-CN while that in cheeses BC and CC was broken down completely. The level of hydrolysis of α_{S1}-CN in cheese mCC at 180 days of ripening was similar to that in cheese BC at 14 days of ripening. McSweeney, Olson, Fox, Healy, & Højrup (1993) reported that, during primary proteolysis, intact α_{S1}-CN is broken into α_{S1}-CN (f1-23) and α_{S1}-CN (24-199). Overall, primary proteolysis of α_{S1}-CN in cheese by the action of mCC was slower than in cheeses BC and CC.

The levels of pH 4.6-soluble nitrogen as a percentage of total nitrogen (pH 4.6-SN/TN) in cheese made using different chymosins at 90 and 180 days of ripening were significantly different ($P < 0.05$) (Table 5.2). At each time point, the level of pH 4.6-SN/TN in cheese BC was significantly higher ($P < 0.05$) than that in cheese CC, followed by that in mCC cheese. After 180 days of ripening, the level of pH 4.6-SN/TN value in mCC cheese (9.86 %) was approximately half of that in cheese BC (18.68 %) and significantly lower ($P < 0.05$) than that in cheese CC (13.64 %).

Moynihan et al. (2014) reported that the levels of pH 4.6-SN/TN for Mozzarella cheeses were made with the same levels of calf and camel chymosins after 84 days of ripening were 12 % and 7 %, respectively. Bansal et al. (2009) reported that the pH 4.6-SN/TN values for Cheddar cheeses manufactured with calf chymosin and camel chymosin after 180 days of ripening were 19.93 % and 14.99 %, respectively, which is close to the values found in this study (18.68 % and 13.64 %), although lower levels of camel chymosin were used in this study.

O'Keeffe, Fox, & Daly (1978) reported that the main factor influencing pH 4.6-soluble nitrogen levels in cheese is the coagulant; therefore, these results indicate that the level of primary proteolysis in cheese mCC was lower than that in cheeses BC and CC, which suggests that, at the same level of usage, coagulant mCC has lower proteolytic activity than calf chymosin and camel chymosin.

Table 5.2. Composition, pH, yield, and levels of soluble N in Cheddar cheeses made from milk coagulated with bovine calf chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC).

	BC	CC	mCC
Moisture (%)	37.8^{a} (0.9)	37.6^{a} (0.7)	37.8^{a} (0.4)
Protein (%)	26.1^{a} (0.3)	26.0^{a} (0.5)	26.0^{a} (0.5)
Salt (%)	1.23^{a} (0.10)	1.26^{ab} (0.06)	1.37^{b} (0.15)
Fat (%)	32.6^{a} (0.7)	32.1^{a} (0.8)	31.9^{a} (0.7)
pH	5.22^{a} (0.03)	5.22^{a} (0.05)	5.22^{a} (0.03)
MNFS (%)	57.8^{a} (2.0)	58.6^{a} (1.1)	57.8^{a} (0.6)
FDM (%)	52.3^{a} (1.1)	51.5^{a} (1.3)	50.9^{a} (1.2)
Salt in moisture (%)	3.26^{a} (0.28)	3.35^{ab} (0.14)	3.67^{b} (0.43)
Yield (kg cheese 100 kg^{-1} milk)	9.92^{a} (0.45)	9.89^{a} (0.32)	9.76^{a} (0.23)
Moisture adjusted[1] yield (kg cheese 100 kg^{-1} milk)	9.83^{a} (0.28)	9.89^{a} (0.27)	9.89^{a} (0.33)
pH 4.6-SN/TN at 90 days (%)	13.8^{c} (1.2)	9.72^{b} (0.83)	6.58^{a} (0.50)
pH 4.6-SN/TN at 180 days (%)	18.7^{c} (0.5)	13.6^{b} (0.8)	9.86^{a} (0.60)

Analysis for moisture, fat, protein, salt, moisture in non-fat substance (MNFS), fat in dry matter (FDM), salt in moisture and pH value was performed at 14 days of ripening and pH 4.6-SN/TN levels at 90 and 180 days of ripening. Yield of cheese was measured at 0 day of ripening. Values are means (standard deviations), from three independent trials; means within the same row not sharing a common superscript differ significantly ($P < 0.05$).
[1]Adjusted to 37.5% moisture.

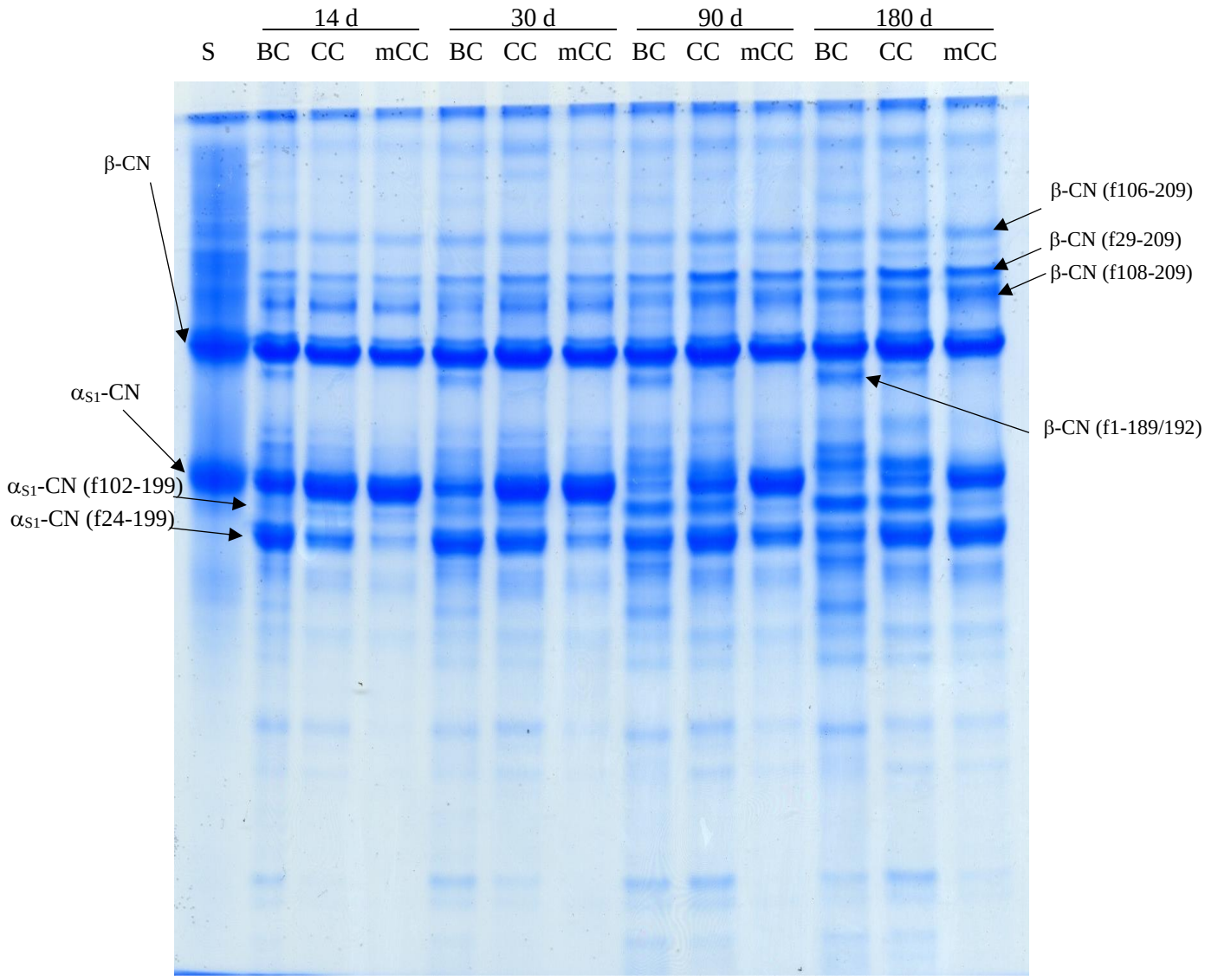

Fig. 5.2. Urea-polyacrylamide gel electrophoretograms of Cheddar cheeses made from milk coagulated with bovine calf chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC) at 14, 30, 90 and 180 days of ripening. Sodium caseinate (S) was used as standard on both sides of the gel. The figure corresponds to data for one of the three cheese replicates; all replicates were similar.

The peptides in the pH 4.6-soluble extracts of cheeses BC, CC and mCC at 90 and 180 days of ripening were separated by ultra-performance liquid chromatography (Fig. 5.3) and identified by mass spectrometry. No significant differences ($P > 0.05$) were found in the number of peptides identified in cheeses BC, CC and mCC at 90 or 180 days of ripening (Table 5.3) while the total identified peptide responses of BC, CC and mCC cheeses increased from 90 to 180 days of ripening (Table 5.3). The total identified peptide intensities decreased in the order BC to CC to mCC at 90 and 180 days of ripening (Table 5.3). Overall, the levels of proteolysis in cheeses decreased from BC to samples CC to mCC, which is consistent with the levels of pH 4.6-SN/TN and urea-PAGE results.

Table 5.3. Numbers and summed intensities of identified peptides in pH 4.6-soluble extracts of cheeses manufactured using bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at 90 and 180 days of ripening.

Ripening time	90 d	180 d
	Number of peptides	
BC	537 (25)	553 (29)
CC	537 (21)	561 (34)
mCC	554 (18)	578 (26)
	Summed intensities of identified peptide (E+09)	
BC	3.18	4.44
CC	2.82	3.19
mCC	1.25	2.41

Data are means with standard deviation in parenthesis, from three independent trials.

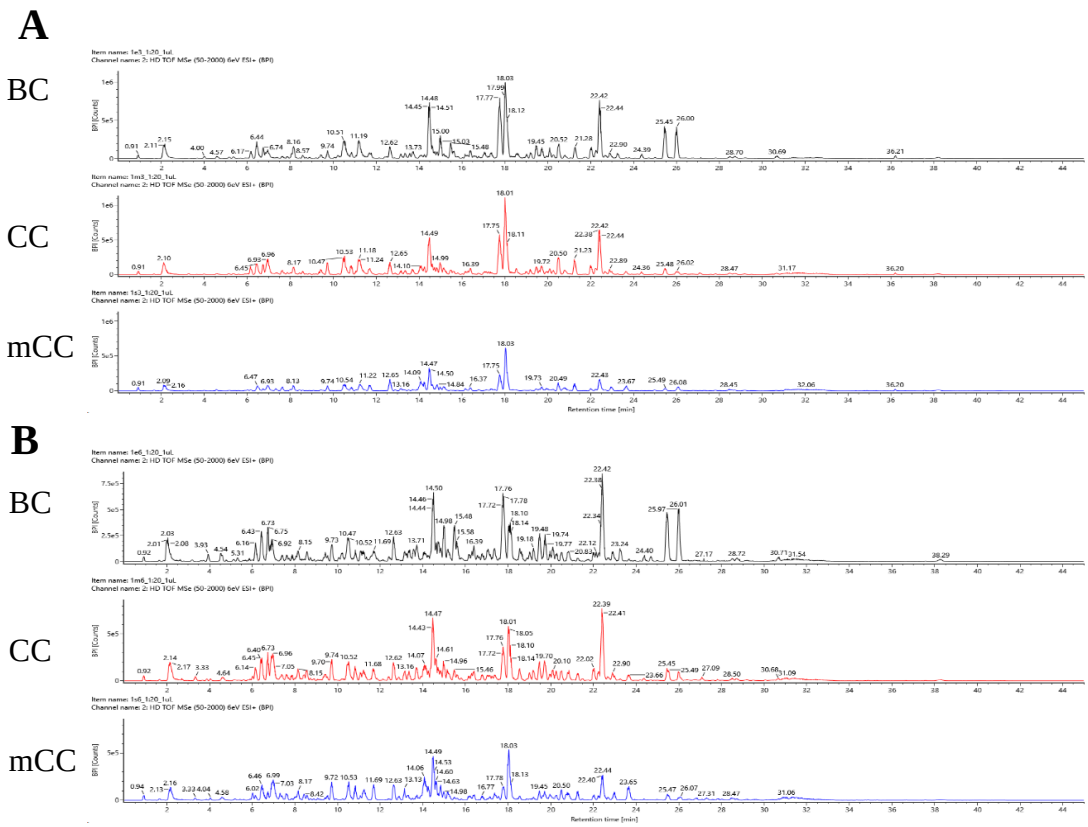

Fig. 5.3. Base peak intensity chromatogram of pH 4.6-soluble extracts of cheeses manufactured using bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at (A) 90 and (B) 180 days of ripening, at a wavelength of 214 nm.

A Venn diagram (Fig. 5.4) was used to visualise the proportions of unique and overlapping peptides identified in cheeses BC, CC and mCC at 90 and 180 days of ripening (Oliveros, 2007; Arju et al., 2020). From 90 to 180 days of ripening, each proportion of the Venn diagram changed by less than 2%. Only 22% of identified peptides were found that existed in all three samples at 180 days of ripening. Sample CC had the lowest number of unique peptides compared to BC and mCC samples at 180 days of ripening. Both samples BC and mCC showed over 210 unique identified peptides at 90 or 180 days of ripening.

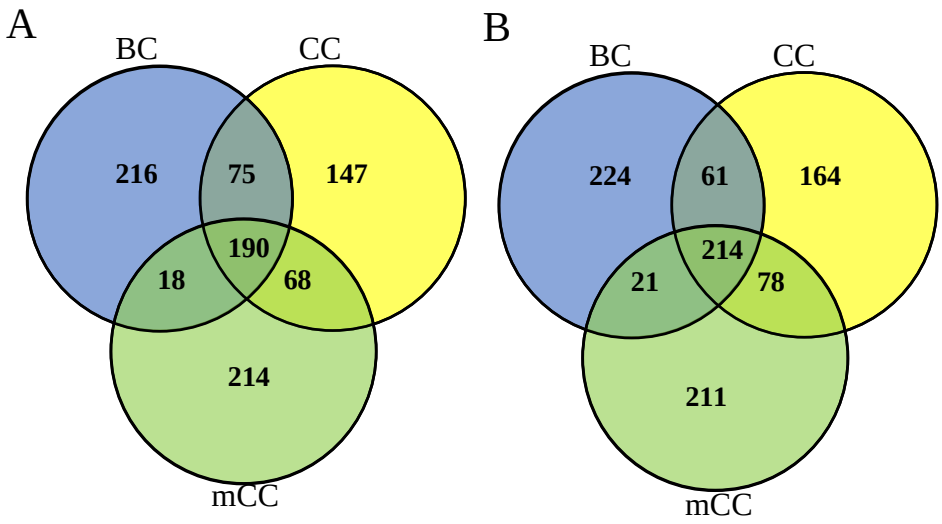

Fig. 5.4. Venn diagrams of peptide distribution for peptides identified in pH 4.6-soluble extracts of cheeses manufactured using bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at (A) 90 and (B) 180 days of ripening.

The concentrations of all peptides identified were summed and plotted according to the sequence of each casein to visualise the proteolytic specificity of chymosins (Appendix). Ten pH 4.6-soluble peptides with the highest response from each cheese samples are listed in Tables 5.4-5.7. Overall, many similar peptides were found from all casein proteins in each treatment while the peptide intensities in cheese mCC were lower than that in cheese BC and CC.

Table 5.4. pH 4.6-soluble peptides with the highest response produced from α_{S1}-CN in each cheese manufactured with bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at 180 days of ripening.

		Response (E+06)		
Amino acid sequence	Peptide	BC	CC	mCC
RPKHPIKHQ	f1-9	83.7	82.9	45.2
RPKHPIKHQGLPQ	f1-13	105.0	92.4	70.7
RPKHPIKHQGLPQE	f1-14	63.8	120.0	97.5
RPKHPIKHQGLPQEVL	f1-16	33.1	7.3	35.9
RPKHPIKHQGLPQEVLN	f1-17	108.9	96.4	3.8
GLPQEVL	f10-16	119.5	69.2	30.1
NENLL	f17-21	69.4	57.4	52.7
NENLLRF	f17-23	38.1	57.5	54.8
ENLLRF	f18-23	45.1	41.3	18.9
FVAPFPE	f24-30	57.3	40.2	19.7
FVAPFPEVF	f24-32	127.1	22.0	5.8
FVAPFPEVFGKE	f24-35	173.2	174.6	59.1
FVAPFPEVFGKEKVNELSKDIGSESTEDQAME	f24-55	67.2	27.3	32.1
KKYKVPQLEIVPN	f89-101	96.3	32.7	9.1
KKYKVPQLEIVPNSAEERLH	f89-106	89.7	34.8	10.5

5.3.3.1 α_{S1}-CN-derived peptides and cleavage sites

In the case of α_{S1}-CN, most of the peptides in all three cheese samples arose from the N-terminal part of the protein, from residues 1 to 60. Fewer peptides with lower intensities originated from the central and C-terminal parts of the sequence, from residues 80 to 125 and from residues 165 to 199. Ten pH 4.6-soluble peptides with the highest response derived from α_{S1}-CN from each cheese at 180 days of ripening are listed in Table 5.4. The intensities of most

identified peptides decreased from BC to CC to mCC. Peptides f1-9, f1-13, f1-14, f1-16, and f1-17 are reported to be hydrolysed by the cell envelope proteinases (CEP) of the lactic acid bacteria starter- *Lc. lactis* at cleavage sites including Gln_{9}-Gly_{10}, Gln_{13}-Glu_{14}, Gln_{14}-Val_{15}, and Leu_{16}-Asn_{17} (Exterkate et al., 1993; Kunji et al., 1996). The precursor peptide for those peptides, f1-23, is produced by chymosin. At the same level of starter addition, less levels of those peptides were found in cheese mCC, which indicates that lower levels of f1-23 were produced by chymosin mCC.

Sample BC showed the highest intensity of peptides corresponding to cleavage at the Phe_{23}-Phe_{24} of α_{S1}-CN, followed by peptide bond Leu_{101}-Lys_{102}, and the two camel chymosins exhibited lower intensity (Appendix), which is consistent with previous research (Børsting et al., 2012; Møller et al., 2013). The intensity of peptides corresponding to the cleavage site at α_{S1}-CN bond Phe_{23}-Phe_{24} of sample mCC was about 25% and 50% of that of samples BC and CC, which indicates that the use of mCC chymosin may decrease the hydrolysis of α_{S1}-CN bond Phe_{23}-Phe_{24}.

5.3.3.2 α_{S2}-CN-derived peptides and cleavage sites

Peptides derived from α_{S2}-CN with lower intensities than that from α_{S1}-CN were identified (Table 5.5), which may be attributed to the decreased accessibility of α_{S2}-CN to proteolytic enzymes in the cheese matrix (Farrell et al., 2009).

Some peptides arose from the N-terminal part of α_{S2}-CN, peptides corresponding to cleavage sites Lys_{21}-Gln_{22}, which are reported to be cleaved by plasmin and are not related to the proteolytic action of chymosin, were detected

in all cheese samples (Le Bars & Gripon, 1989) (Table 5.5). Only a few peptides from the middle part of α_{S2}-CN were identified in cheese samples. Many peptides from the C-terminal part of α_{S2}-CN showed high intensities in cheese BC, while most of those peptides were absent from cheeses CC and mCC e.g., Phe_{174}-Ala_{175}, which has been reported to be cleaved by bovine chymosin in cheese environment (McSweeney et al., 1994). Overall, the intensities of peptides in each sample increased from 90 to 180 days of ripening. The intensities of most peptides decreased from cheese BC to CC to mCC (Table 5.5).

Table 5.5. pH 4.6-soluble peptides with the highest response produced from α_{S2}-CN in each cheese manufactured with bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at 180 days of ripening.

		Response (E+06)		
Amino acid sequence	Peptide	BC	CC	mCC
KNTMEHVSSSEESIISQETYK	f1-21	44.6	41.0	30.1
HVSSSEESIISQETYK	f6-21	10.6	11.1	10.6
SSSEESIISQETYKQEKNMAINPSKGNLC	f8-36	—	—	13.5
SIISQETYK	f13-21	6.5	10.6	15.1
SIISQ…RNANE	f13-49	—	6.5	0.3
LCSTF…ATEEV	f35-69	—	9.0	8.8
NRLNFLKKISQR	f159-170	7.2	6.6	11.4
ALPQYLK	f175-181	9.8	6.3	2.8
ALPQYLKT	f175-182	41.9	26.5	10.9
ALPQY…KVIPY	f175-203	62.5	—	—
ALPQY…YVRYL	f175-207	37.1	—	—
VYQHQKAMKPWIQPKTKVIPY	f183-203	64.1	—	—
VYQHQKAMKPWIQPKTKVIPYVRYL	f183-207	89.9	—	—
KAMKPWIQPKTKVIPY	f188-203	22.0	—	—
AMKPWIQPKTKVIPY	f189-203	18.6	—	9.8
AMKPWIQPKTKVIPYVRYL	f189-207	19.0	—	—
IQPKTKVIPY	f194-203	15.4	16.9	13.7
VRYL	f204-207	10.7	11.1	11.2

—means peptides in corresponding sample were absent.

5.3.3.3 β-CN-derived peptides and cleavage sites

Most β-CN-derived small peptides in cheese were derived from residues 1-40, 45-95, and 165-209 (Appendix). The intensities of peptides of β-CN between residues 163-209 decreased from cheeses BC to CC to mCC at both 90 and 180 days of ripening, while some peptides from the N-terminal region and middle

region an increased intensity from cheeses BC to CC to mCC at 180 days of ripening (Table 5.6).

Peptide bonds Leu_{165}-Ser_{166}, Gln_{167}-Ser_{168}, Ala_{189}-Phe_{190} and Leu_{192}-Tyr_{193} are the main cleavage sites hydrolysed by bovine chymosin (Exterkate et al., 1997; Møller et al., 2012). Peptide β-CN (f193-209) contributes to bitterness in Cheddar cheese and is formed by cleavage at bond Leu_{192}-Tyr_{193} (Børsting et al., 2012). The intensity of peptide f193-209 cheese BC was about 4 and 8 times that of cheeses CC and mCC, respectively.

Table 5.6. pH 4.6-soluble peptides with the highest response produced from β-CN in cheese manufactured with bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at 180 days of ripening.

		Response (E+06)		
Amino acid sequence	Peptide	BC	CC	mCC
RELEEL	f1-6	35.0	37.0	30.8
RELEELNVPGEIVESLSSSEESITRINK	f1-28	64.0	56.4	41.4
RELEELNVPGEIVESLSSSEESITRINKK	f1-29	29.4	—	—
NVPGEIVE	f7-14	29.1	54.7	76.6
NVPGEIVESLSSSEESITRINK	f7-28	61.2	70.5	57.1
NVPGEIVESLSSSEESITRINKK	f7-29	—	39.9	41.8
AQTQS…H_{67}…QPEVM	f53-93[A1]	35.3	21.5	12.7
AQTQS…P_{67}…QPEVM	f53-93[A2]	—	25.7	13.4
VYPFPGPIPN	f59-68[A2]	6.2	12.2	23.9
ELQDKIHPF	f44-52	23.1	22.9	27.2
TQTPVVVPPFLQPE	f78-91	7.9	22.0	45.3
TQTPVVVPPFLQPEVM	f78-93	0.78	22.7	23.7
GVSKVKEAMAPKHKEMPFPKYPVEPF	f94-119	37.8	—	—
SQSKVLPVPQKAVPYPQRDMPIQA	f166-189	28.8	—	—
SKVLPVPQKAVPYPQRDMPIQA	f168-189	26.7	—	—
YQEPVLGPVRGPFPIIV	f193-209	213.8	63.6	30.0

—means peptides in corresponding sample were absent.

Table 5.7. pH 4.6-soluble peptides with the highest response produced from κ-CN in each cheese manufactured with bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at 180 days of ripening.

		Response (E+06)		
Amino acid sequence	Peptide	BC	CC	mCC
QEQPIR	f5-10	—	1.9	2.5
EQPI	f6-9	2.1	—	—
KDERF	f13-17	2.8	—	—
KDERFF	f13-18	1.6	3.6	3.8
SPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPH	f69-102	—	2.1	—
WQVL	f76-79	4.2	—	—
QVLSNTVPA	f77-85	2.1	—	—
SNTVPA	f80-85	2.0	0.59	0.41
NTVPAKSCQAQPTTMAHHPHPHLSFMAI	f81-108	—	9.8	—
NTVPAKSCQAQPTTMAHHPHPHLSFMAIPPKK	f81-112	—	—	11.6
TVPAKSCQAQPTTMARHPHPHLSFM	f82-106	—	—	1.9
TVPAKSCQAQPTTMAHHPHPHLSFMAIPPKKN	f82-113	—	—	10.6
VPAKSCQAQPTTMARHPHPHLS	f83-104	2.5	—	—
PAKSCQAQPTTMARHPHPHLSFMAI	f84-108	—	2.3	—
ARHPHPHLSF	f96-105	16.1	3.5	2.1
GEPTS…VQVTS	f128-166	—	2.5	—
EPTSTPTIEAVESTVATLEASPEVIESPPEIN	f129-160	4.8	—	—
EPTSTPTIEAVESTVATLEASPEVIESPPEINTVQVT	f129-165	—	—	2.1
TPTTEAVESTVATLEDSPEVIEGPP	f133-157	10.3	10.7	11.1
PTTEAVESTVATLEDSPEVIEGPPEINTVQVT	f134-165	—	—	10.0
VIESPPEIN	f152-160	3.0	3.4	2.7

—means peptides in corresponding sample were absent.

5.3.3.4 κ-CN-derived peptides and cleavage sites

The κ-CN-derived peptides from pH 4.6-soluble extracts from cheese samples were present at lower concentrations compared to those from other caseins (Table 5.7). Some minor peptides were found in the GMP region, which indicates that some κ-CN in cheese was not hydrolysed during rennet coagulation, or some GMP was retained in cheese curds on whey drainage.

Kappeler et al. (2006) found that camel chymosin had a higher efficiency for the hydrolysis of κ-CN bond Phe_{105}-Met_{106} than bovine chymosin, which is not consistent with the current results. The reason for this difference may be that the peptide κ-CN (f106-169) was released into the whey during manufacture and was not detected.

5.3.4 Texture profile analysis

Cheeses CC and mCC were significantly harder ($P < 0.05$) than cheese BC after 30, 90 and 180 days of ripening (Fig. 5.3A). Bansal et al. (2009) reported that the hardness of cheese made with camel chymosin was significantly higher ($P < 0.05$) than that of cheese made with calf chymosin after 150 days of ripening which is consistent with these results. No significant differences ($P > 0.05$) were found in springiness between all treatments at 30 and 90 days of ripening, although the springiness of cheese mCC was significantly higher ($P < 0.05$) than that of cheese BC after 180 days of ripening (Fig. 5.3B). The cohesiveness of cheese mCC was significantly higher ($P < 0.05$) than that of cheese BC and CC after 30 or 180 days of ripening (Fig. 5.3C). The results of springiness and cohesiveness of cheese made with bovine or camel chymosins are in agreement with previous research

that no significant differences were found between the springiness and cohesiveness of cheese made with calf or camel chymosins (Bansal et al., 2009). The higher hardness of cheeses made with both camel chymosins may be attributed to the lower level of primary proteolysis caused by the camel chymosins.

5.3.5 Descriptive sensory analysis

After 180 days of ripening, no significant differences ($P > 0.05$) were found in milky flavour, whey flavour, milk-fat flavour, mothball flavour, sour taste, salty taste, sweet or umami tastes among the cheeses. The fruity flavour of cheese CC was significantly lower ($P < 0.05$) than that of cheese BC and mCC. The sulphur and barny flavours of cheeses CC and mCC were significantly lower ($P < 0.05$) than that of cheese BC, which might meet the preferences of consumers. The brothy flavour and bitter taste of cheese mCC were significantly lower ($P < 0.05$) than that of cheese CC, followed by cheese BC (Table 5.8). The intensity of peptide β-CN (f193-209) in cheese BC, which is responsible for the bitterness of cheese (Børsting et al., 2012), was about 4 and 8 times that of cheeses CC and mCC, respectively which is consistent with the sensory results. Bansal et al. (2009) found that cheese made with camel chymosin has less sulphur, brothy flavours and less bitterness than cheese made with bovine calf chymosin.

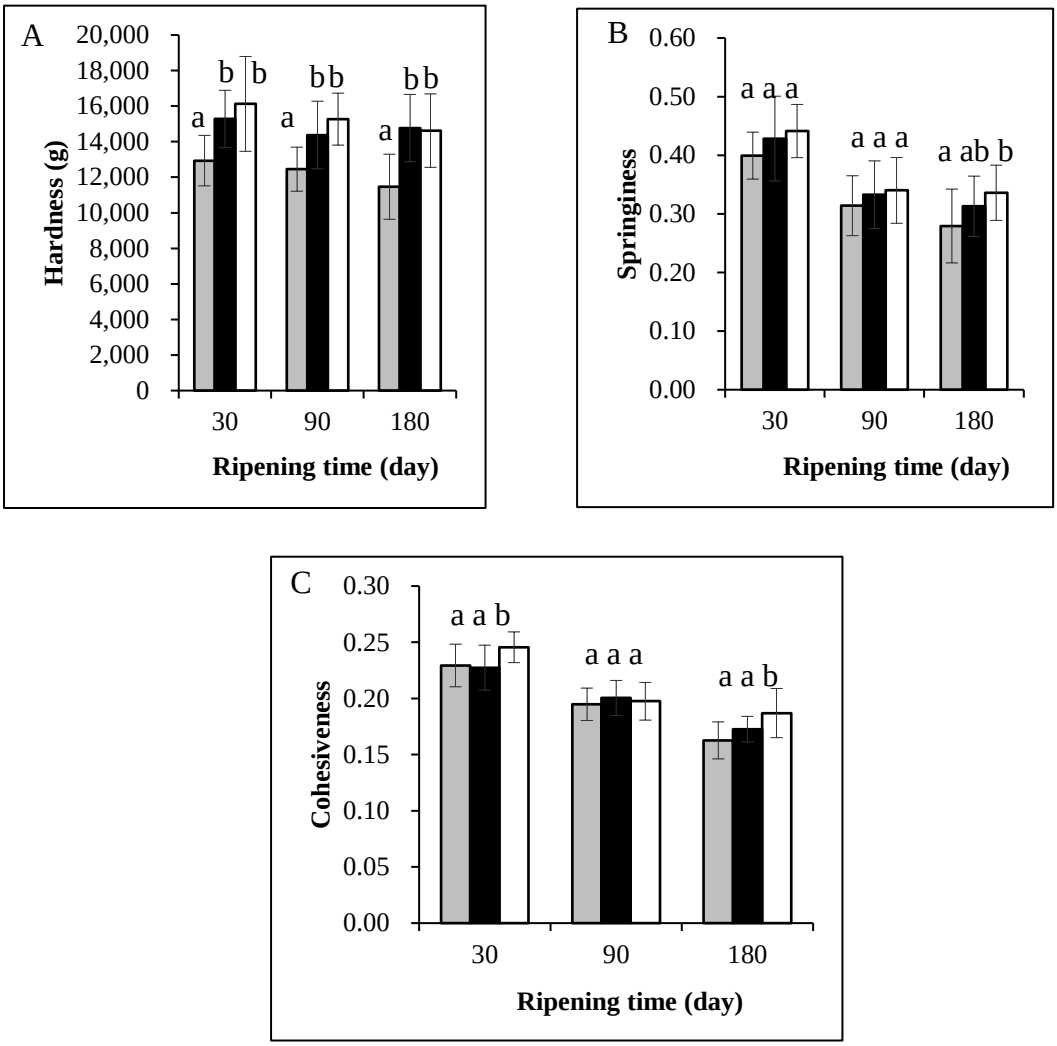

Fig. 5.3. Changes in TPA hardness (A), springiness (B) and cohesiveness (C) of Cheddar cheeses made from milk coagulated with bovine calf chymosin (BC, *grey bars*), camel chymosin (CC, *black bars*) or modified camel chymosin (mCC, *white bars*) at 30, 90 and 180 days of ripening; means at the same time point not sharing a common superscript differ ($P < 0.05$). Values are means of three independent trials. Error bars indicate standard deviation.

Significant differences were also found in sensory texture attributes. The hand firmness of cheese mCC was significantly higher ($P < 0.05$) than that of cheese BC (Table 5.9). This is in agreement with instrumental texture analysis, which showed that the hardness of cheeses CC and mCC was significantly higher than that of cheese BC after 180 days of ripening (Fig. 5.3A). The rate of recovery measured by hand and springiness were not detected in cheese BC or CC, and only

the cheese made with modified camel chymosin (mCC) showed the rate of recovery and springiness (Table 5.9). The TPA results also showed significantly higher springiness ($P < 0.05$) in cheese mCC compared with cheese BC after 180 days of ripening (Fig. 5.3B).

At first bite, cheeses made with both camel chymosin types showed significantly higher ($P < 0.05$) firmness than that of cheese made with calf chymosin; the fracturability of cheese made with modified camel chymosin was significantly higher ($P < 0.05$) than that of cheese made with calf and camel chymosin. During mastication, mCC cheese showed a significantly lower ($P < 0.05$) degree of breakdown, cohesiveness and adhesiveness than that of cheeses BC and CC. In contrast, TPA results showed that the cohesiveness of cheese mCC was significantly higher ($P < 0.05$) than that of the other batches of cheese after 180 days of ripening, the reason for which is unclear. The different results of cohesiveness between the TPA and the sensory test were observed before: Bansal et al. (2009) found no significant differences ($P > 0.05$) in cohesiveness between the cheeses made from bovine or camel chymosins; however, the sensory cohesiveness of the cheese made from camel chymosin in that study was lower than that of the cheese made from bovine chymosin.

Table 5.8. Flavour profiles for Cheddar cheeses made with bovine calf chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC) after 180 days of ripening.

Flavour	Treatments		
	BC	CC	mCC
Cooked/milky	3.1[a] (0.3)	3.1[a] (0.4)	3.3[a] (0.3)
Whey	1.0[a] (0.3)	1.0[a] (0.4)	1.2[a] (0.4)
Milkfat	3.0[a] (0.4)	3.2[a] (0.4)	3.0[a] (0.4)
Fruity	1.5[a] (0.4)	0.7[b] (0.3)	1.2[a] (0.5)
Sulphur	2.3[a] (0.4)	1.5[b] (0.3)	1.6[b] (0.3)
Brothy	2.1[a] (0.4)	1.8[b] (0.5)	1.5[c] (0.4)
Barny	2.4[a] (0.4)	2.0[b] (0.5)	1.9[b] (0.4)
Mothball/fecal	0.5[a] (0.4)	0.5[a] (0.5)	0.5[a] (0.4)
Sour taste	3.1[a] (0.3)	3.2[a] (0.3)	3.2[a] (0.4)
Bitter taste	2.0[a] (0.5)	1.3[b] (0.4)	0.9[c] (0.3)
Salty taste	3.0[a] (0.4)	3.0[a] (0.4)	3.1[a] (0.3)
Sweet taste	1.9[a] (0.3)	1.9[a] (0.4)	1.9[a] (0.4)
Umami taste	3.2[a] (0.5)	3.2[a] (0.4)	3.1[a] (0.5)

Data are means (standard deviation), from duplicate analyses by 6 trained panellists of three independent trials. Attributes not listed were not detected in cheeses. Cheese flavours (Drake et al., 2001) were scored on a 0 to 15-point universal intensity scale (Spectrum method, Meilgaard et al., 1999). Most cheese flavours fall between 0 and 5 (Drake, 2004). Means within the same row not sharing a similar superscript differ significantly ($P < 0.05$).

After expectoration, the smoothness of mouthcoating of cheese made with modified camel chymosin (mCC) was significantly lower ($P < 0.05$) than that of cheeses BC and CC. Bansal et al. (2009) found that cheeses made with calf chymosin showed higher smoothness and mouthcoating and higher cohesiveness than cheeses made with camel chymosin, which is consistent with these results. Differences in sensory flavour and texture between treatments may be related to different extents of proteolysis in cheeses (Bansal et al., 2009).

Table 5.9. Sensory texture profiles for Cheddar cheeses made with bovine calf chymosin (BC), camel chymosin (CC) or highly specific camel chymosin (mCC) after 180 days of ripening.

Attributes	Treatments		
	BC	CC	mCC
Hand firmness	13.3^{b} (0.7)	13.9^{ab} (1.0)	14.3^{a} (0.9)
Hand rate of recovery	ND	ND	7.8 (1.2)
Springiness	ND	ND	7.8 (1.3)
First bite			
Firmness	9.0^{b} (0.4)	9.7^{a} (0.8)	10.1^{a} (1.1)
Fracturability	7.4^{b} (1.0)	7.5^{b} (1.2)	8.4^{a} (1.1)
Mastication			
Degree of breakdown	10.3^{a} (1.2)	9.6^{b} (1.1)	8.8^{c} (1.2)
Cohesiveness	9.8^{a} (1.0)	9.6^{a} (1.0)	8.6^{b} (1.2)
Adhesiveness	9.7^{a} (1.2)	9.6^{a} (0.9)	8.7^{b} (1.3)
Smoothness of mass	9.8^{a} (0.9)	9.8^{a} (1.2)	9.7^{a} (1.0)
After expectoration			
Smoothness mouthcoat	10.4^{a} (1.3)	9.9^{a} (1.0)	8.8^{b} (0.9)

Data are means (standard deviation) from duplicate analyses by 6 trained panellists of three independent trials. Attributes not listed were not detected in cheeses. Cheese texture attributes (Rogers et al., 2009) were scored on a 0 to 15-point product-specific intensity scale (Spectrum method, Meilgaard et al., 1999). ND – not detected. Means within the same row not sharing a similar superscript differ significantly ($P < 0.05$).

5.3.6 Meltability

At 30 days of ripening, no significant differences ($P > 0.05$) in meltability were found between treatments, but the meltability of both cheeses CC and mCC were significantly lower ($P < 0.05$) than that of cheese BC at 90 and 180 days of ripening. No significant differences were found between the meltability of cheeses CC and mCC at 30, 90 and 180 days of ripening (Fig. 5.5). The meltability of cheese increases with the breakdown of α_{S1}-CN and β-CN (Dave, McMahon, Oberg, & Broadbent, 2003; Bogenrief & Olson, 1995). No differences in breakdown of β-CN were found between samples, while lower extents of breakdown of α_{S1}-CN were found in cheeses CC and mCC compared to cheese

BC. The lower meltability of cheeses CC and mCC may thus be linked to their lower levels of proteolysis.

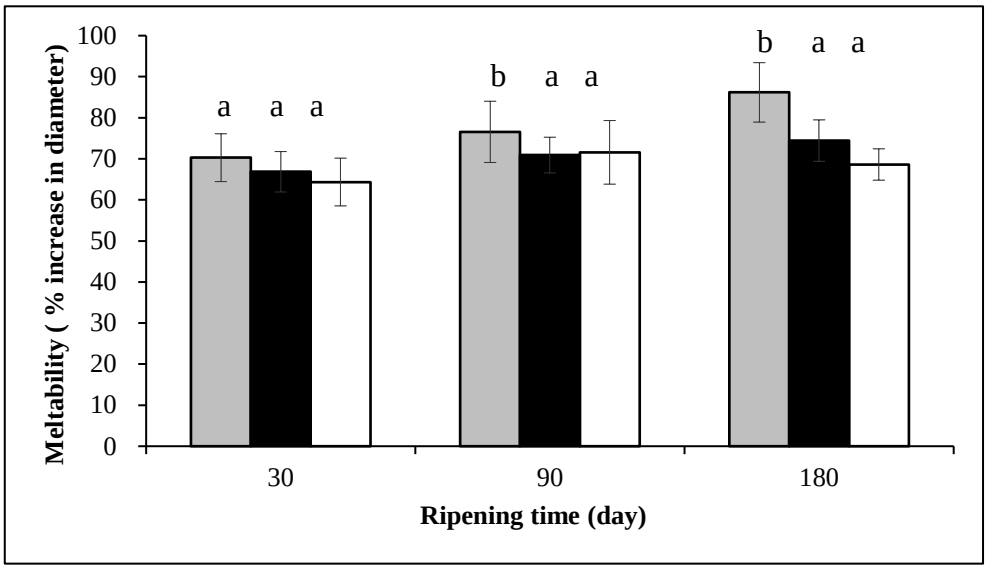

Fig. 5.5. Meltability (diameter increase %) of Cheddar cheeses made from milk coagulated with bovine calf chymosin (BC, *grey bars*), camel chymosin (CC, *black bars*) or modified camel chymosin (mCC, *white bars*) at 30, 90 and 180 days of ripening; means at the same time point not sharing a common superscript differ ($P < 0.05$). Values are means of three independent trials. Error bars indicate standard deviation.

5.3.7 Dynamic small-amplitude oscillatory rheology

No significant differences ($P > 0.05$) were found in the temperature at LT=1 between all treatments after 30, 90 and 180 days of ripening (Table 5.10). The temperature at LT=1 is the temperature at which the cheese started to melt (Gunasekaran & Ak, 2002). After 30 days of ripening, no significant differences ($P > 0.05$) were found in LT_{max} values between all the treatments; the temperature at LT_{max} of sample mCC was significantly higher ($P < 0.05$) than that of the sample BC. After 90 and 180 days of ripening, the LT_{max} of cheese mCC was significantly higher ($P < 0.05$) than that of cheeses BC and CC and no significant differences

($P > 0.05$) were found between cheeses BC and CC; the temperature at LT_{max} of cheese mCC was significantly higher ($P < 0.05$) than that of cheese BC. Moynihan et al. (2014) did not find any significant difference in the LT_{max} values between Mozzarella cheese made with BC and CC, which is consistent with the results of this study. The higher LT_{max} relates to higher meltability (Lucey, Johnson, & Horne, 2003), which indicates that the meltability of cheese mCC was significantly higher ($P < 0.05$) than that of cheeses BC and CC at the temperature between 20 and 80 ℃. Proteolysis affects the rheology properties of cheese during ripening (Lucey et al., 2003). Cheese mCC showed the lowest proteolysis, while the meltability of cheese mCC at the temperature between 20 and 80 ℃ was not the lowest, the reason of which is unknown. In contrast, the results of the Schreiber test showed that cheeses made with bovine calf chymosin had the highest meltability at high temperatures (232 ℃). Cooke, Khosrowshahi, & McSweeney (2013) found that the differences between the results of the Schreiber test and dynamic small-amplitude rheology may be linked to the different extent of temperature exposure and different speed of moisture, fat, and protein separation at different temperatures. At lower temperatures (<80 ℃), the cheeses made with mCC showed higher meltability, while cheeses made with BC showed greater meltability at high temperatures (232 ℃).

Table 5.10. Rheological properties of cheeses made from bovine calf chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at 30, 60 and 180 days of ripening, as assessed by dynamic small amplitude oscillatory rheology.

30 d	LT_{max}	Temperature at LT_{max} (°C)	Temperature at LT=1 (°C)
BC	2.32^{a} (0.22)	71.6^{a} (2.0)	55.6^{a} (1.7)
CC	2.36^{a} (0.23)	73.4^{ab} (1.3)	54.5^{a} (1.9)
mCC	2.55^{a} (0.23)	74.9^{b} (1.7)	54.7^{a} (1.1)
90 d			
BC	1.67^{a} (0.15)	64.7^{a} (2.0)	56.6^{a} (1.5)
CC	1.80^{a} (0.20)	67.6^{b} (1.3)	57.9^{a} (1.2)
mCC	2.37^{b} (0.34)	70.6^{c} (3.1)	57.2^{a} (1.4)
180 d			
BC	1.66^{a} (0.18)	66.6^{a} (1.3)	58.2^{a} (2.1)
CC	1.79^{a} (0.28)	67.5^{ab} (1.3)	58.7^{a} (0.9)
mCC	2.29^{b} (0.37)	70.2^{b} (3.8)	58.7^{a} (1.3)

Data are means (standard deviation), from three independent trials; means within the same column not sharing a similar superscript differ significantly ($P < 0.05$).

5.4 Conclusion

Cheddar cheeses were manufactured using the same IMCU level of fermentation-produced bovine or fermentation-produced camel or fermentation-produced camel chymosin with structural changes as a coagulant. The same curd strength was obtained at the same time from three different chymosins during manufacture. Minor differences were found in the composition and pH of the 14 days old cheeses made with fermentation produced calf chymosin, camel chymosin or modified camel chymosin. Levels of pH 4.6 SN/TN and urea-PAGE analysis showed that the proteolysis of cheese mCC may be lower than that of cheeses BC and CC, especially lower in the hydrolysis in α_{S1}-CN and β-CN. Major casein-derived peptides and the corresponding cleavage sites produced by bovine and camel chymosins in cheese reported in the literature were confirmed in this study. The peptide profiles in cheese manufactured using modified camel

chymosin were identified. Cheeses manufactured using modified camel chymosin (mCC) showed significantly higher hardness, springiness and cohesiveness and significantly lower meltability compared to the cheese manufactured using calf chymosin (BC). Descriptive sensory analysis suggested that cheese made with both camel chymosins had lower ($P < 0.05$) sulphur, brothy, barny flavours and bitter taste than cheese made with calf chymosin. The sensory firmness of cheese made with both camel chymosins was significantly lower ($P < 0.05$) than that of cheese BC which is consistent with the texture profile analysis results. The springiness, cohesiveness and smoothness of mouthcoating of cheese mCC were significantly lower ($P < 0.05$) than that of cheeses BC and CC. At low temperature (80 °C), cheese mCC showed significantly higher meltability than cheeses BC and CC, while at high temperature (232 °C) cheese BC showed greater meltability than that of cheeses CC and mCC. These results suggest that using the modified camel chymosin for Cheddar cheese manufacture is feasible and results in limited levels of primary proteolysis, modified texture and different flavour compared to the use of calf and camel chymosin, which provides another option for consumers.

5.5 Acknowledgements

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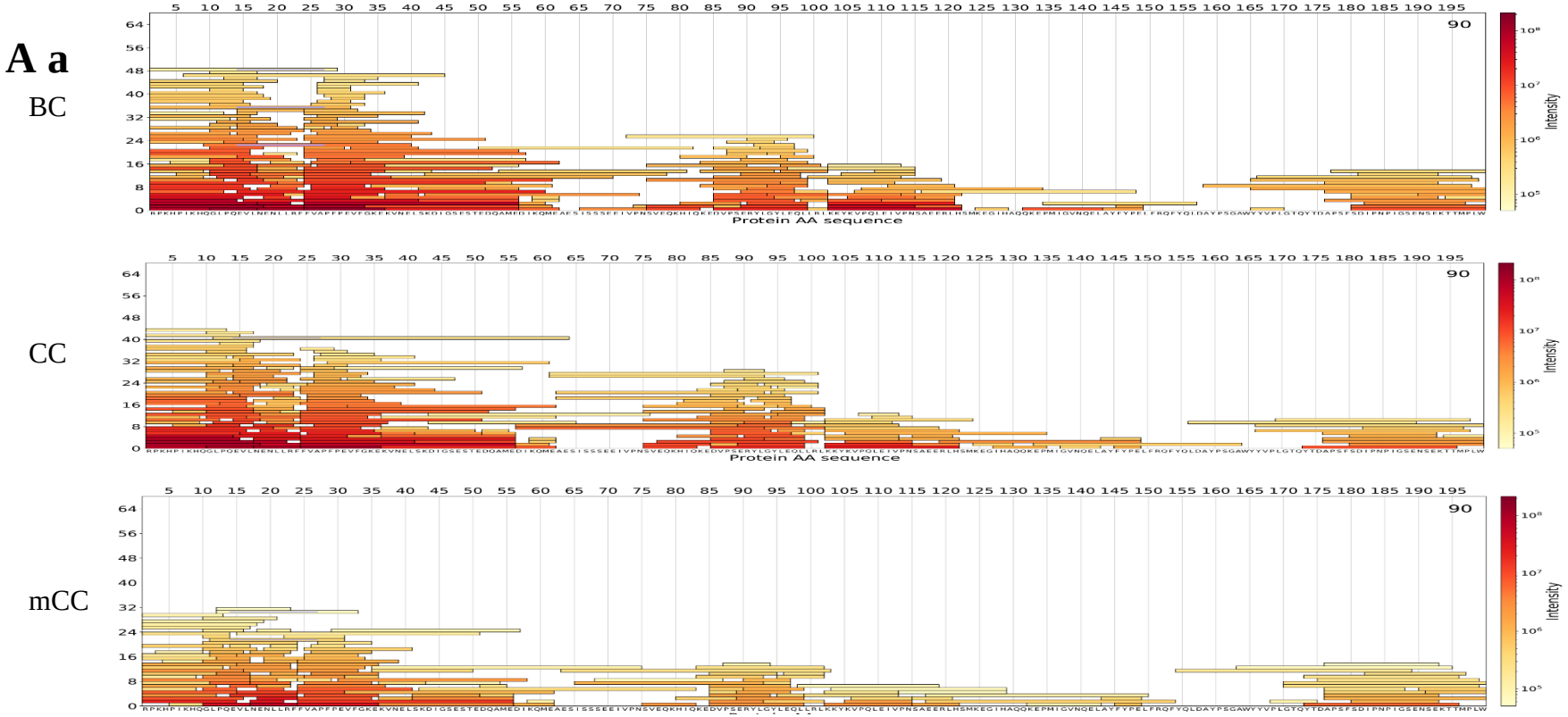
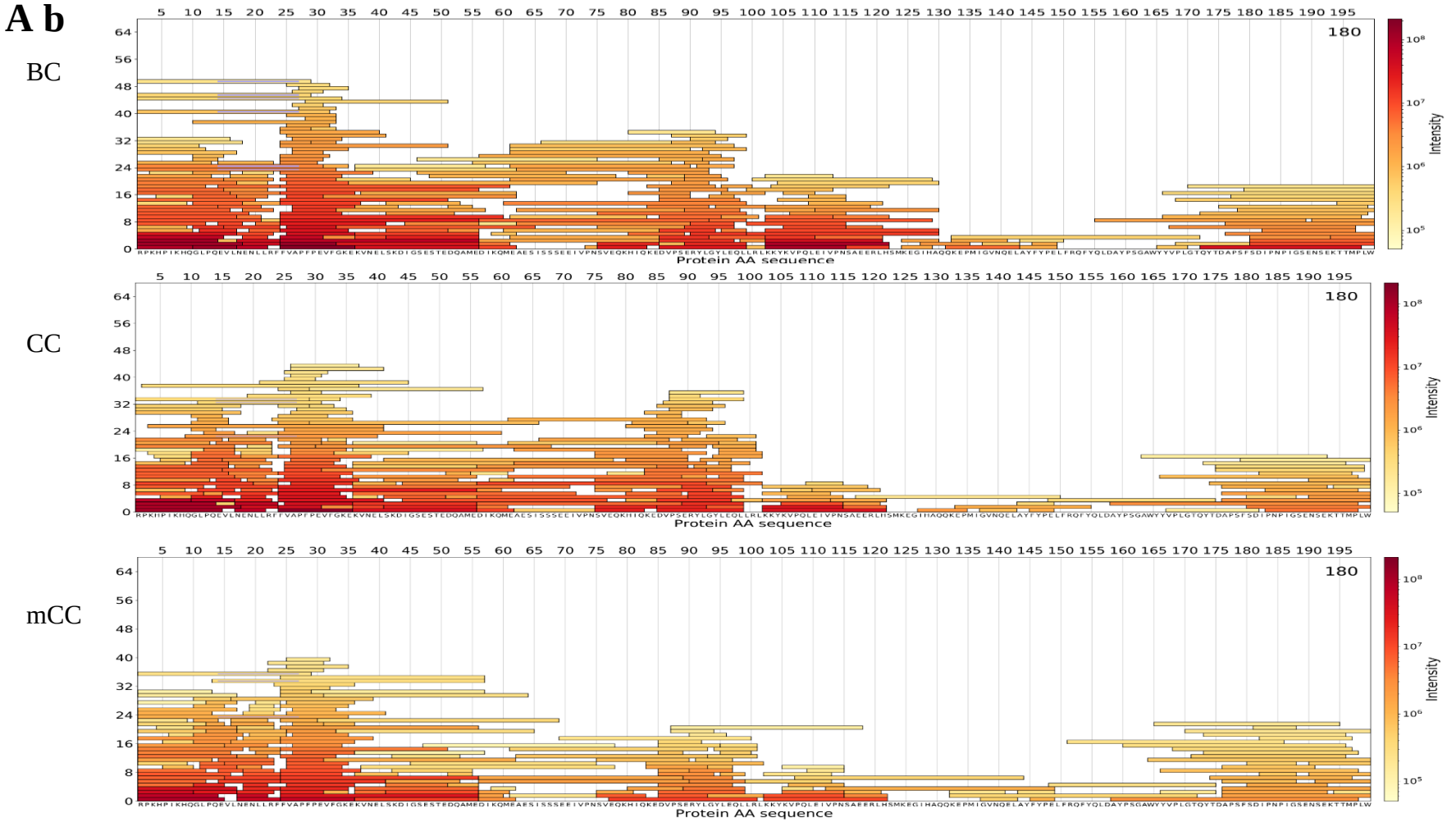
Appendix



Supplemental Fig. S1. Peptide coverage map of the (A) α_{S1}-casein, (B) α_{S2}-casein, (C) β-casein and (D) κ-casein sequence of pH 4.6 soluble extracts of cheeses manufactured using bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at (a) 90 and (b) 180 days of ripening. Blue lines and one-letter abbreviations of amino acids on the peptides indicate the differences in the peptide sequences in genetic variants of α_{S1}-CN.



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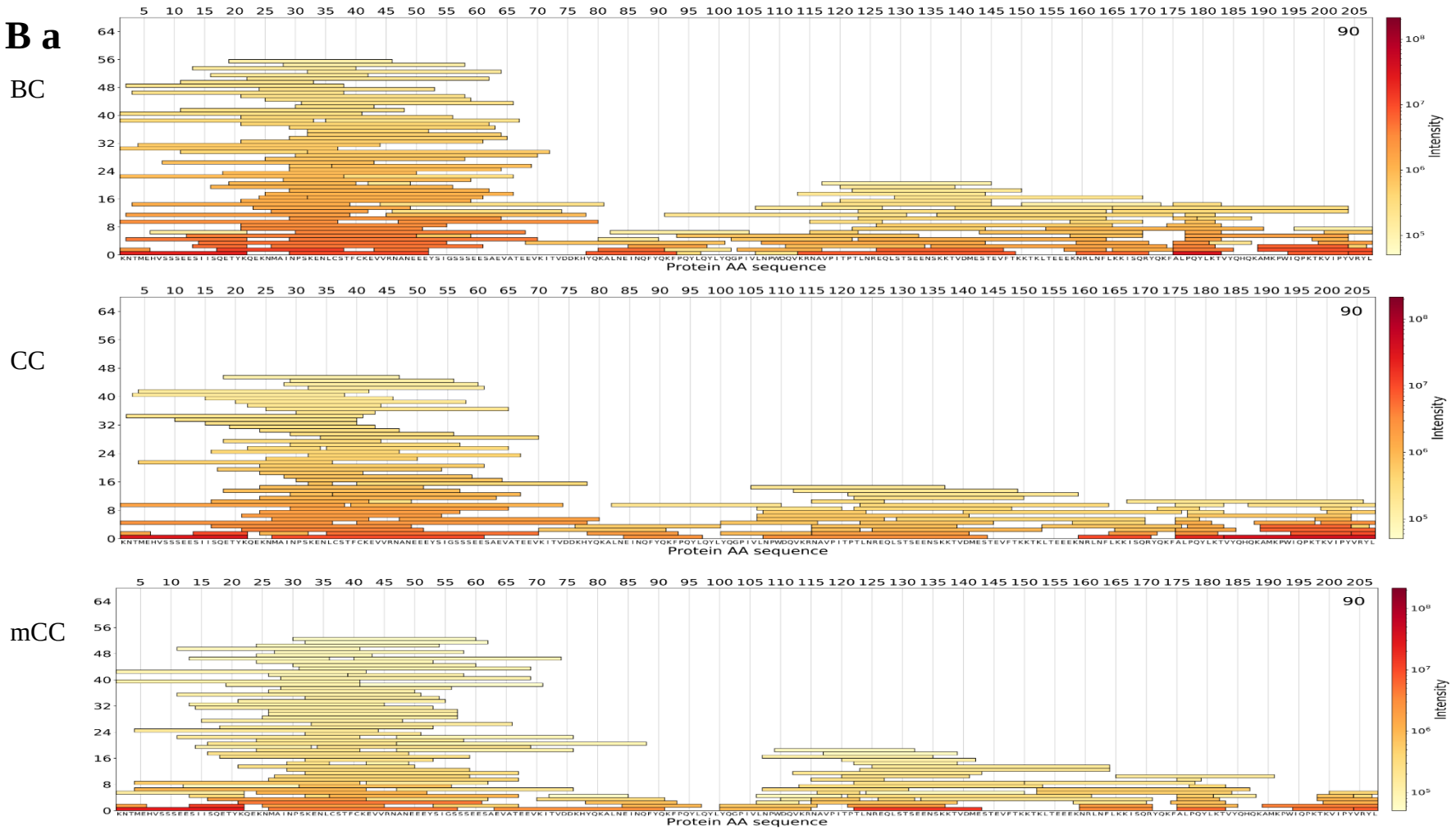

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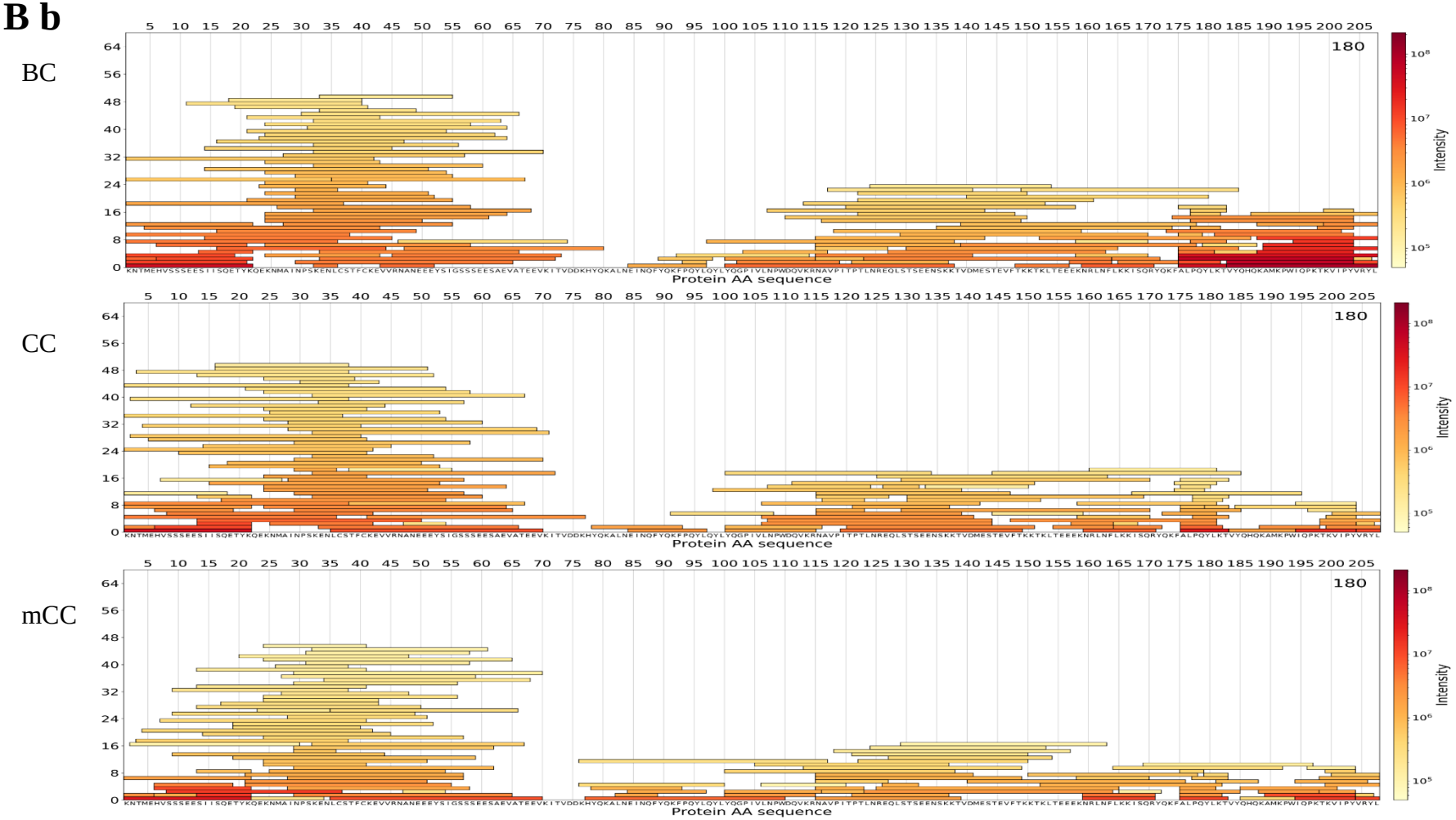
B b



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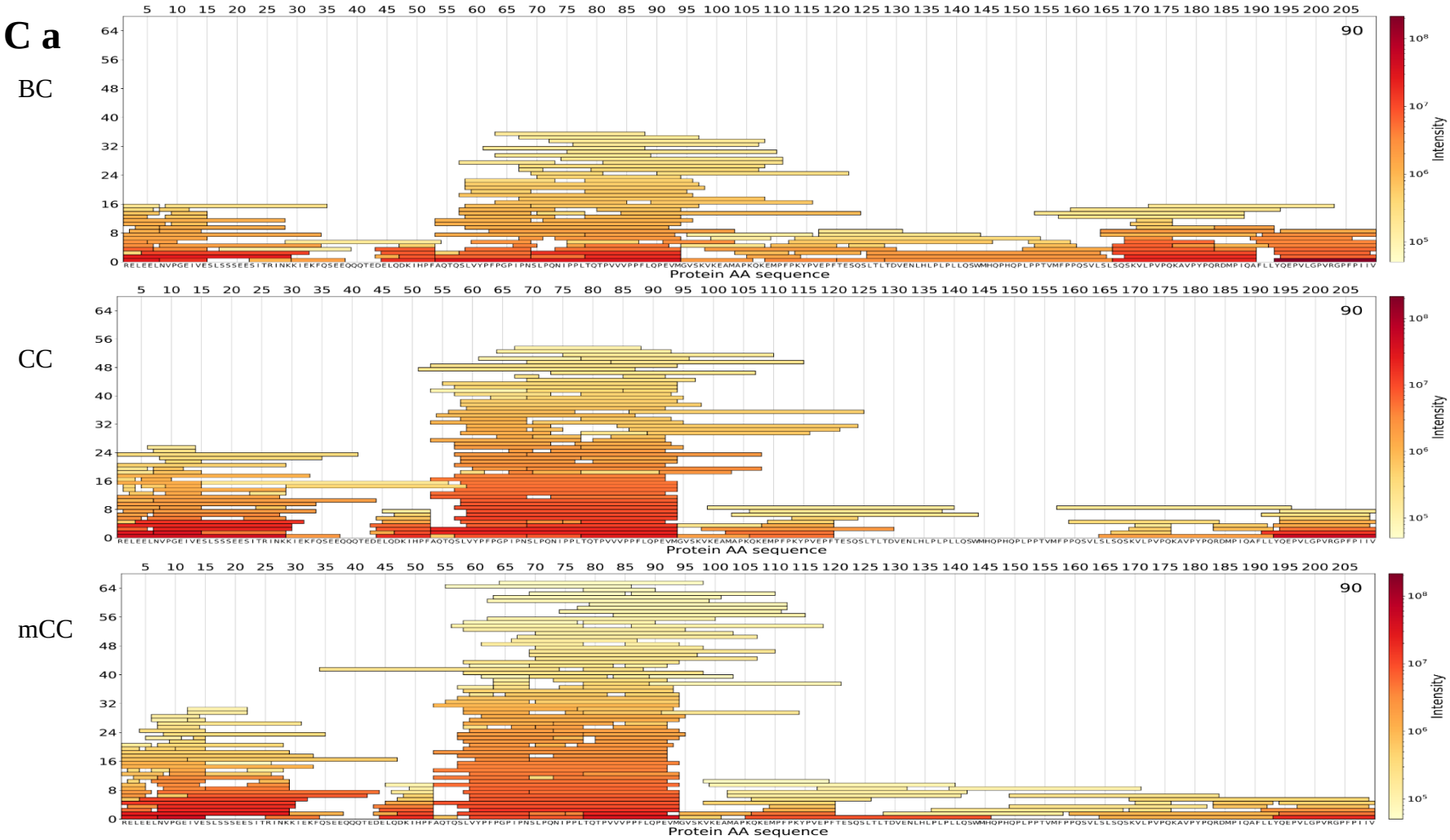

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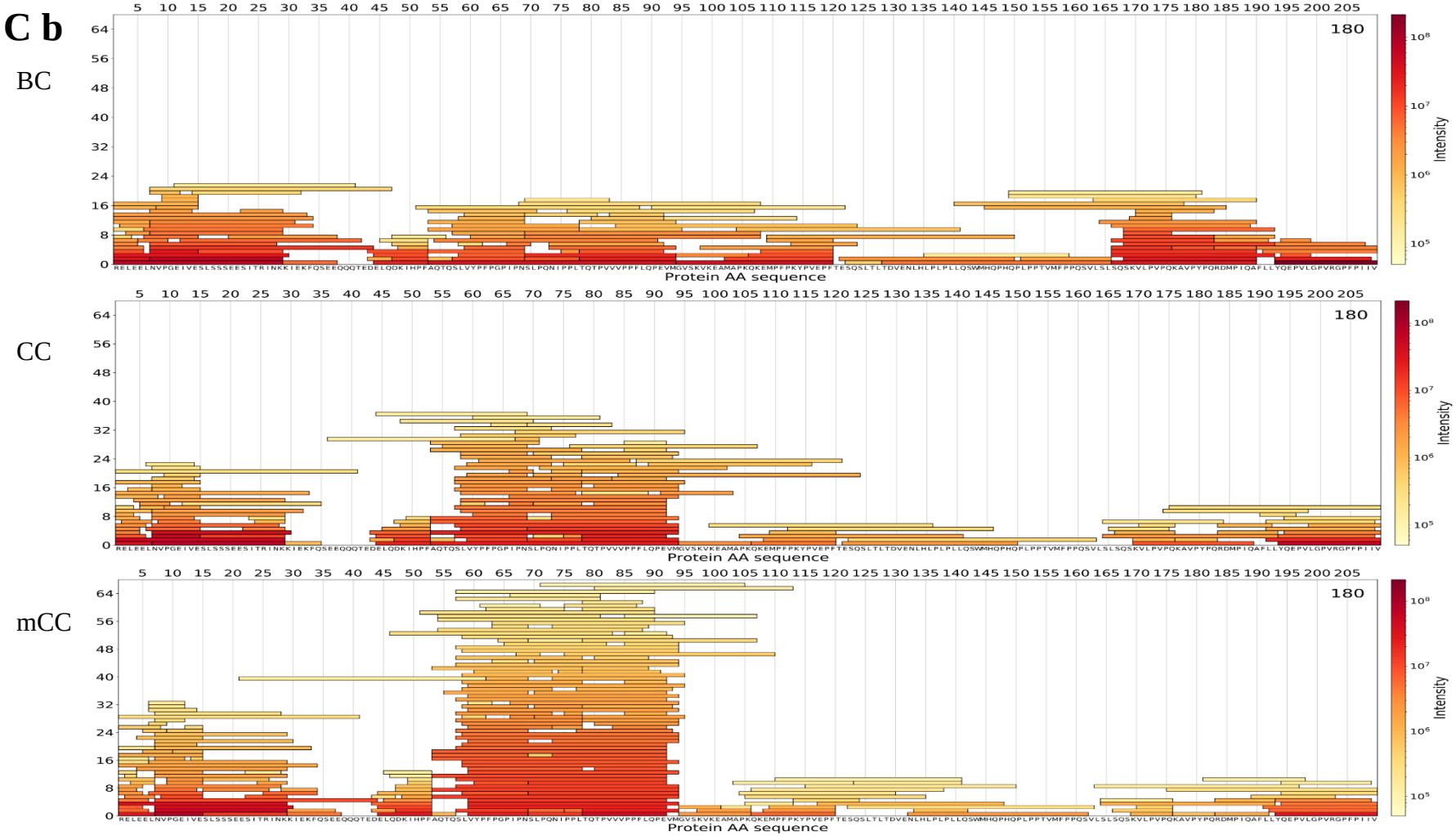

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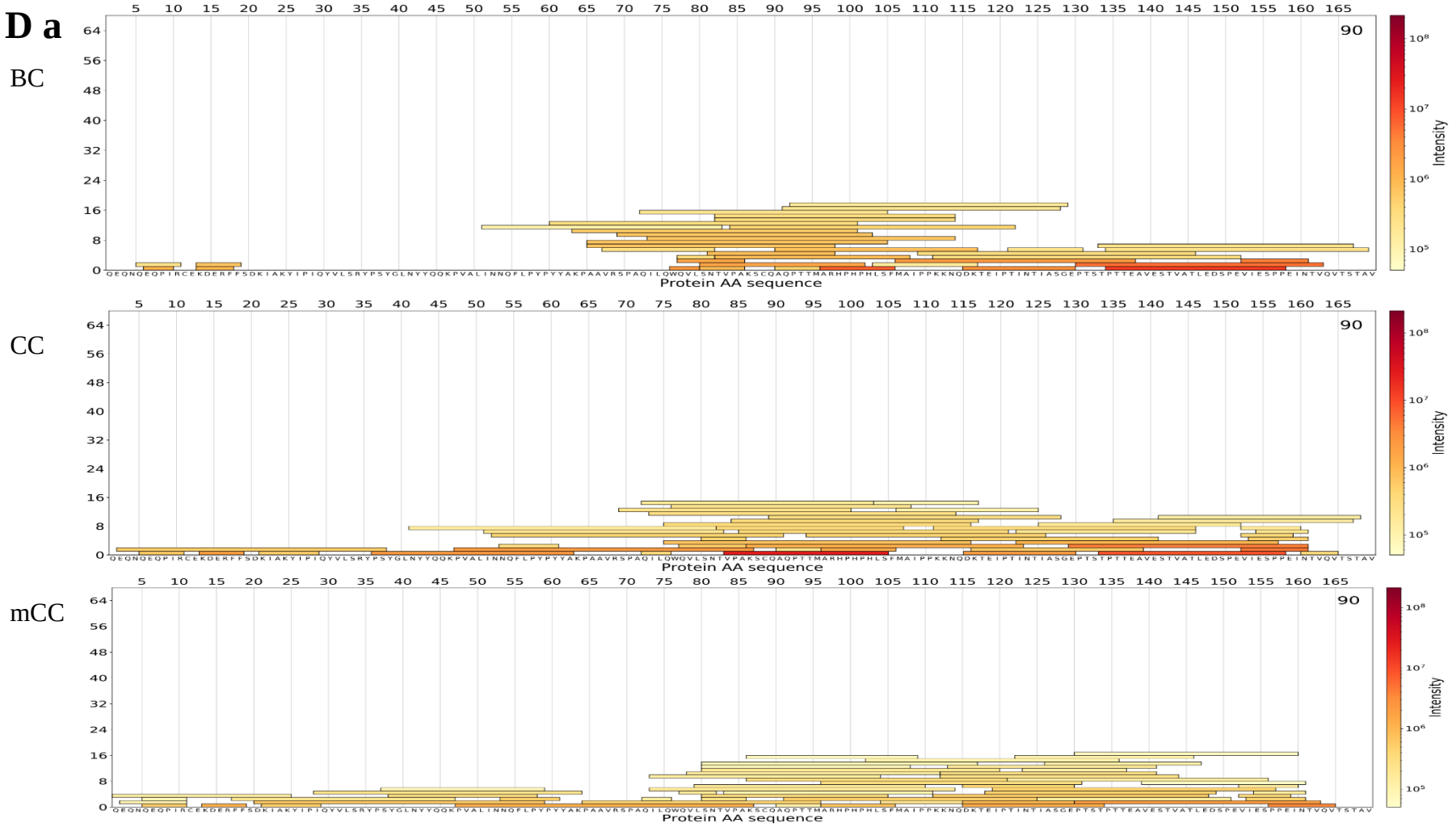

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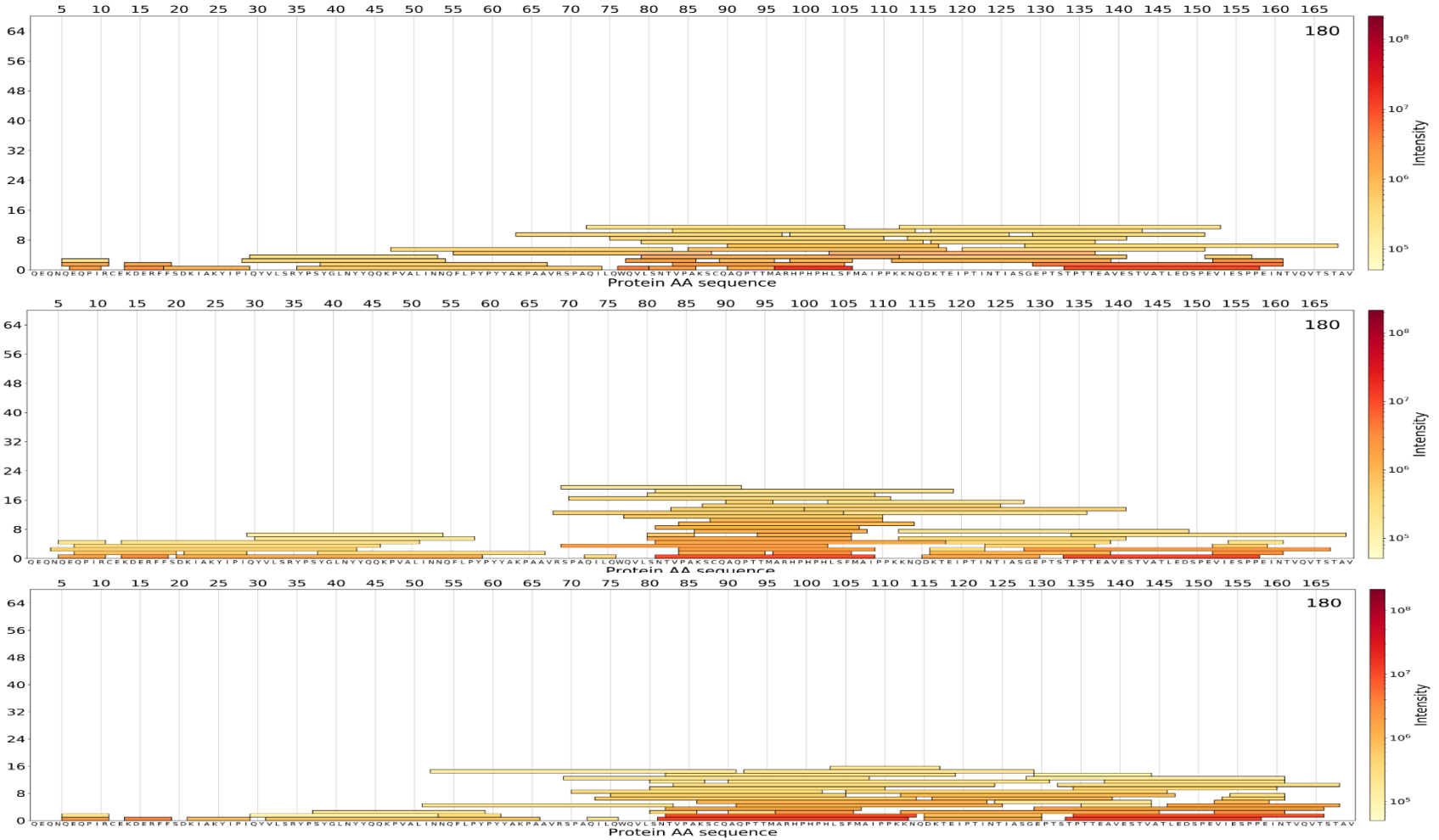
D b

BC

CC

mCC



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CHAPTER 6

Proteolysis of sodium caseinate and in Cheddar cheese by three generations of fermentation-produced chymosin

Bozhao Li[1], Georg Arju[2], Raivo Vilu[2], Alan L. Kelly[1] and Paul L. H. McSweeney[1]

[1]School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

[2] Department of Chemistry and Biotechnology, School of Science, Tallinn University of Technology, Tallinn, Estonia

Declaration

This chapter was written by author BL and reviewed by his co-authors. BL co-designed the study, participated in sample preparation, analysis of urea-PAGE, and data analysis. GA and RV contributed to analysis of LC-MS and data collection.

Chapter 6

Abstract

The proteolytic activities and specificities of three generations of fermentation-produced chymosin were investigated in sodium caseinate (NaCN) digests using these coagulants and were compared with the peptide profiles in cheese. The highest level of enzyme required to hydrolyse all intact α_{S1}-CN in NaCN solution over 24 h was for modified camel chymosin (mCC), followed by camel chymosin (CC) and bovine chymosin (BC). Many peptides were separated and identified through liquid chromatography-mass spectrometry (LC-MS) from both NaCN digests produced using each chymosin. In addition, some new peptides and the corresponding cleavage sites were also identified. Most of the peptides observed in NaCN digests were also identified in Cheddar cheese samples. The overall proteolytic activity of mCC was relatively lower than that of BC and CC, which is linked to the modified functionalities in Cheddar cheese. The proteolytic specificity of the three coagulants in NaCN solution was determined.

Chapter 6

6.1 Introduction

Rennet, the general name for a proteinase preparation that traditionally originated in the abomasum of ruminant mammals, is used to coagulate milk for the manufacture of dairy products, principally cheese. Chymosin, the major proteinase in most rennets, has its primary proteolytic specificity on the Phe_{105}-Met_{106} bond of κ-CN. This cleavage by chymosin splits κ-CN into *para*-κ-CN and glycomacropeptide (GMP), reducing the negative charge and steric stabilisation of casein micelles, and thereby initiating the aggregation of casein micelles and triggering the coagulation of milk. Other sources of chymosin are occasionally used in cheese production, such as lamb and camel chymosins and vegetable enzymes from *Cynara cardunculus*, *Ficus carica*, *Arctium minus*, and *Solanum dubium* (Scott et al., 1998). Enzymes generated naturally by microorganisms (e.g., *Rhizomucor miehei* or *Cryphonectria parasitica*) are also employed in the production of cheese (Gagnaire et al., 2001).

In the production of cheese, a coagulant with high proteolytic activity at or around the Phe_{105}-Met_{106} bond of κ-CN, low general proteolytic activity, and ease of inactivation in whey is preferred (Bansal et al., 2009). The coagulation/proteolysis (C/P) ratio, which is the proportion of milk coagulating activity to overall proteolytic activity, is related to enzyme specificity (Soodam et al., 2015). Effect on cheese yield is another essential attribute of a coagulant, since cheese yield might be reduced if a coagulant with a higher proteolytic activity or a lower C/P ratio was used during cheese production (Fox & McSweeney, 1997; Soodam et al., 2015).

Chapter 6

The proteolytic specificity of bovine chymosin on the four caseins has been studied. The peptide bond Phe_{23}-Phe_{24} of α_{S1}-casein (α_{S1}-CN) is the bond in that protein most susceptible to bovine chymosin during ripening. For other peptide bonds in α_{S1}-CN, the peptide bond Leu_{101}-Lys_{102} is hydrolysed by bovine chymosin later, followed by Phe_{32}-Gly_{33}, Leu_{98}-Lys_{99} and Leu_{109}-Glu_{110} (Mooney et al., 1998). Eight sites in the hydrophobic part of α_{S2}-CN were reported to be preferentially cleaved by bovine chymosin (McSweeney et al. 1994), while peptide bonds Leu_{165}-Ser_{166}, Gln_{167}-Ser_{168}, Ala_{189}-Phe_{190} and Leu_{192}-Tyr_{193} of β-CN is slowly hydrolysed by bovine chymosin at the ionic strength of the aqueous phase in cheese during ripening (Exterkate et al., 1997).

Proteolysis caused by chymosin during cheese manufacture and cheese ripening contributes to the texture, and indirectly to the aroma and flavour of ripened Cheddar cheese (Mooney et al., 1998). As for the chymosins from other ruminants, the proteolytic activity of ovine and caprine chymosins on ewes' and goats' milk, respectively, is greater than on bovine milk (McSweeney, 2022). Camel chymosin has higher activity clotting bovine milk than that of bovine chymosin. A commercial recombinant camel chymosin (CHY-MAX ® M) has a 70% greater specific milk-clotting activity for bovine milk than bovine chymosin, but only 20% of the latter's overall proteolytic activity (Kappeler et al., 2006; Langholm Jensen et al., 2013). For the initial cleavage sites in α_{S1}-CN (Phe_{23}-Phe_{24}) and β-CN (Leu_{192}-Tyr_{193}), camel chymosin only showed about 36 and 7%, respectively, of the proteolytic activity compared to that of bovine chymosin (Møller, Rattray, & Ardö, 2012a).

A structure-based design technique has been used to create modified camel chymosin (CHY-MAX ® Supreme; Chr Hansen, Hoersholm, Denmark) with

increased specific milk-clotting activity and more restricted proteolytic specificity compared to the original form of the enzyme (Jäckel et al., 2017; Jäckel et al., 2019). A lower level of proteolysis, correlated with higher hardness value, lower meltability at high temperatures and altered flavour profile was found in Cheddar cheese manufactured with the modified camel chymosin compared to commercial bovine and camel chymosins (Li et al. 2022; Chapter 5). The peptide profile in Cheddar cheese were studied using liquid chromatography-mass spectrometry (LC-MS) in Chapter 5.

The objective of this study was to further analyse the differences in the proteolytic specificity between commercially available fermentation-produced bovine chymosin (CHY-MAX ® Extra), commercially available fermentation-produced camel chymosin (CHY-MAX ® M), and commercially available fermentation-produced camel chymosin with an altered molecular structure (CHY-MAX ® Supreme). Sodium caseinate (NaCN)-chymosin digested samples were analysed using LC-MS to compare the peptide profiles of the cheeses with the *in vitro* hydrolysates.

6.2 Material and methods

6.2.1 Chemicals

The coagulants used for Cheddar cheese manufacture and incubation analysis were commercially available fermentation-produced bovine chymosin (BC; CHY-MAX® Extra, 600 international milk clotting units (IMCU) mL^{-1}), commercially available fermented-produced camel chymosin (CC; CHY-MAX® M 200, 200 IMCU mL^{-1}) and commercially available fermentation-produced camel chymosin with structural changes through amino acid substitution (mCC; CHY-MAX®

Supreme 200, 200 IMCU mL^{-1}; all from Christian Hansen A/S, Hoersholm, Denmark). Hi3 *E.coli* STD (p/n: 186006012) and leucine-enkephalin (p/n: WT186006013) were purchased from Waters Corporation (Milford, MA, USA). Ultrapure water (18.2 MΩ.cm) was prepared with MilliQ® IQ 7000 and equipped with LC-Pak (Merck KGaA, Darmstadt, Germany). Acetonitrile (MeCN; LiChrosolv, hypergrade for LC-MS) and formic acid (FA; LC-MS grade) were acquired from Sigma-Aldrich (Darmstadt, Germany).

6.2.2 Chymosin-induced hydrolysis of sodium caseinate

Solutions of sodium caseinate (10 mg mL^{-1}) were prepared by dissolving sodium caseinate (Kerry Group, Listowel, Ireland) in 100 mM K phosphate buffer, pH 6.5, containing 0.05% NaN_3, or 100 mM Na acetate buffer, pH 5.2, containing 0.05% NaN_3 and 5% NaCl. Levels of bovine chymosin, camel chymosin or modified camel chymosin sufficient to hydrolyse all intact α_{S1}-CN in sodium caseinate within 24 h at 37 °C were determined in preliminary trials, following which various levels of chymosins were applied to incubated with sodium caseinate solution for 0, 3, 9, 24 and 48 h at 37 °C (McSweeney, Fox, and Olson 1995). The incubation was stopped at each time point by heating samples at 60 °C for 10 min (Hayes et al., 2002).

6.2.3 Preparation of the pH 4.6-soluble extracts of samples

The pH 4.6-soluble extracts of cheese samples were prepared by the method of Kuchroo & Fox (1982). Samples of the pH 4.6-soluble extracts of NaCN hydrolysed by chymosin and NaCN blank (sodium caseinate solution, pH 6.5 mentioned above incubated for 24 h at 37 °C) were prepared according to the

method of Huppertz, Fox, and Kelly (2004) and filtered through 0.22-μm cellulose acetate filters (Sartorious GmbH, Gottingen, Germany) for further analysis.

6.2.4 Urea-polyacrylamide gel electrophoresis

Cheese samples for urea-polyacrylamide gel electrophoresis (urea-PAGE) were prepared by dissolving cheese sample in single-strength sample buffer at a ratio of 1 in 100 (w: v) (Shalabi & Fox, 1987). NaCN samples for urea-PAGE were prepared by the addition of an equal amount of double-strength sample buffer (McSweeney et al., 1995). Urea-PAGE was applied to cheese samples at 14, 30, 90 and 180 days of ripening and NaCN samples hydrolysed by chymosin according to the method of Andrews (1983) with modifications as described by Shalabi and Fox (1987). Gels were stained directly with Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977) and destained with deionised water several times.

6.2.5 Analysis of peptides

6.2.5.1 Sample preparation

pH 4.6-soluble extracts of cheese samples at 90 and 180 days of ripening and NaCN samples hydrolysed by chymosin for 3 and 24 h were shipped to Centre of Food and Fermentation Technologies, Tallinn, Estonia within 24 h, packaged with dry ice.

Before peptide analysis, pH 4.6-soluble extracts of three trials of cheeses were diluted with MilliQ® water 1: 20, vortexed and centrifuged for 10 min at 15000 g at room temperature. After centrifugation, the supernatants were transferred into autosampler compatible vial low-volume inserts and capped. The digestion samples (100 µL) were mixed with acetonitrile (100 µL) in Eppendorf® tubes (Eppendorf AG, Germany) to precipitate proteins, vortexed for 30 s and centrifuged at 11,200 g for 15 min. Then, the supernatant (100 µL) and MilliQ® water (900 µL) were transferred into a new Eppendorf® tube and vortexed for 30 s. The mixture obtained (130 µL) was transferred into a low-volume insert and spiked with 20 µL of 1 pmol μL^{-1} of Hi3 *E.coli* STD as housekeeping ions.

6.2.5.2 Liquid chromatography-mass spectrometry

The peptide profiles of samples (cheese samples: 1 µL, NaCN-chymosin digestion samples: 2 µL) prepared as described in Section 6.2.6.1 were analysed in triplicate using a Waters I-Class Plus (BSM, SM-FL and CM-A) ultra-performance liquid chromatography (UPLC®) (Waters Corporation, Milford, MA, USA) system hyphenated to a Waters Vion ion mobility separation-quadrupole

time-of-flight (IMS-QTOF) mass spectrometer (3.1.0) equipped with LockSpray Exact Mass Ionisation Source and controlled by Waters UNIFI (1.9.4), as described by Xia et al. (2023).

6.2.5.3 Raw data processing and analysis

Peptide identification and quantification were carried out using UNIFI Large Molecule Package with Peptide Map (IMS) workflow. Peptide mapping was carried out using the UNIFI Large Molecule Package (LMP) (Waters Corporation, Milford, MA, USA) by searching the peptides against α_{S1}-CN (P02662), α_{S2}-CN (P02663), β-CN (P02666) and κ-CN, P02668) sequences from *Bos taurus* obtained from the UniProt database (Cambridge, UK). Peptide search was set to non-specific digest with no missed cleavages allowed, and minimum sequence length was set to 4. The following filters were applied: mass error between -5 and 5 ppm; matched first gen primary ions greater than or equal to 1; fragment label not containing in-source fragments; loss of H_2O and loss of NH_3. The following variable post-translational modifications were used in the analysis: oxidation (M), acetyl-(protein N-terminal), deamidation (NQ) and phosphorylation (STY).

Processed data from UNIFI were exported into Microsoft Excel for grouping, alignment, and filtration based on the retention time (0.1 min), observed molecular weight (0.01 Da per charge) and collision cross section (10% RSD). The aligned data were then further used to construct a data matrix for revision and removal of outliers and false-positive identifications. Only peptides identified in each of the three technical replicates were considered for further analysis.

The identified and quantified peptides as well as their normalised and averaged intensities were further analysed and visualised with the help of in-house

data analysis and visualisation scripts written in the Python™ programming language (Python Software Foundation, Delaware, USA). The resulting data were plotted as peptide maps, cleavage sites and peptide length distribution diagrams. Similarity analysis based on Euclidean distances in a space of peptide intensities was carried out to highlight differences between technical replicates as well as different hydrolysis treatments.

6.3 Results and discussion

6.3.1 Urea-PAGE analysis of sodium caseinate digestion

NaCN hydrolysed by chymosin at pH 6.5 and 5.2 was analysed by urea-PAGE after 0, 3, 9, 24, and 48 h (Fig. 6.1 and 6.2). Very different levels of BC (0.4 IMCU mL^{-1} in pH 6.5 and 0.21 IMCU mL^{-1} in pH 5.2 plus salt), CC (4 IMCU mL^{-1} in pH 6.5 and 2.1 IMCU mL^{-1} in pH 5.2 plus salt) and mCC (13.34 IMCU mL^{-1} in pH 6.5 and 7 IMCU mL^{-1} in pH 5.2 plus salt) were required to hydrolyse all intact α_{S1}-CN within 24 h, which indicates that the proteolytic activity of BC on α_{S1}-CN is higher than that of CC, followed by mCC, at both pH 6.5 and pH 5.2 plus salt. The α_{S1}-CN breakdown products of all samples at pH 6.5 were different to those at pH 5.2 plus salt. In the area of α_{S1}-CN breakdown products, different bands were observed between samples digested by bovine chymosin and the two camel chymosins at both pH values, which indicates that the proteolytic specificities of bovine chymosin and the two camel chymosins differ.

Although all intact α_{S1}-CN was hydrolysed by each chymosin, the breakdown of β-CN in each sample was different. NaCN hydrolysed by BC showed higher levels of breakdown of β-CN than that of CC while a higher level of intact β-CN

remained in that of mCC, which indicates that the proteolytic activity on β-CN of BC was highest, followed by CC, and then mCC, at both pH 6.5 and pH 5.2 plus salt.

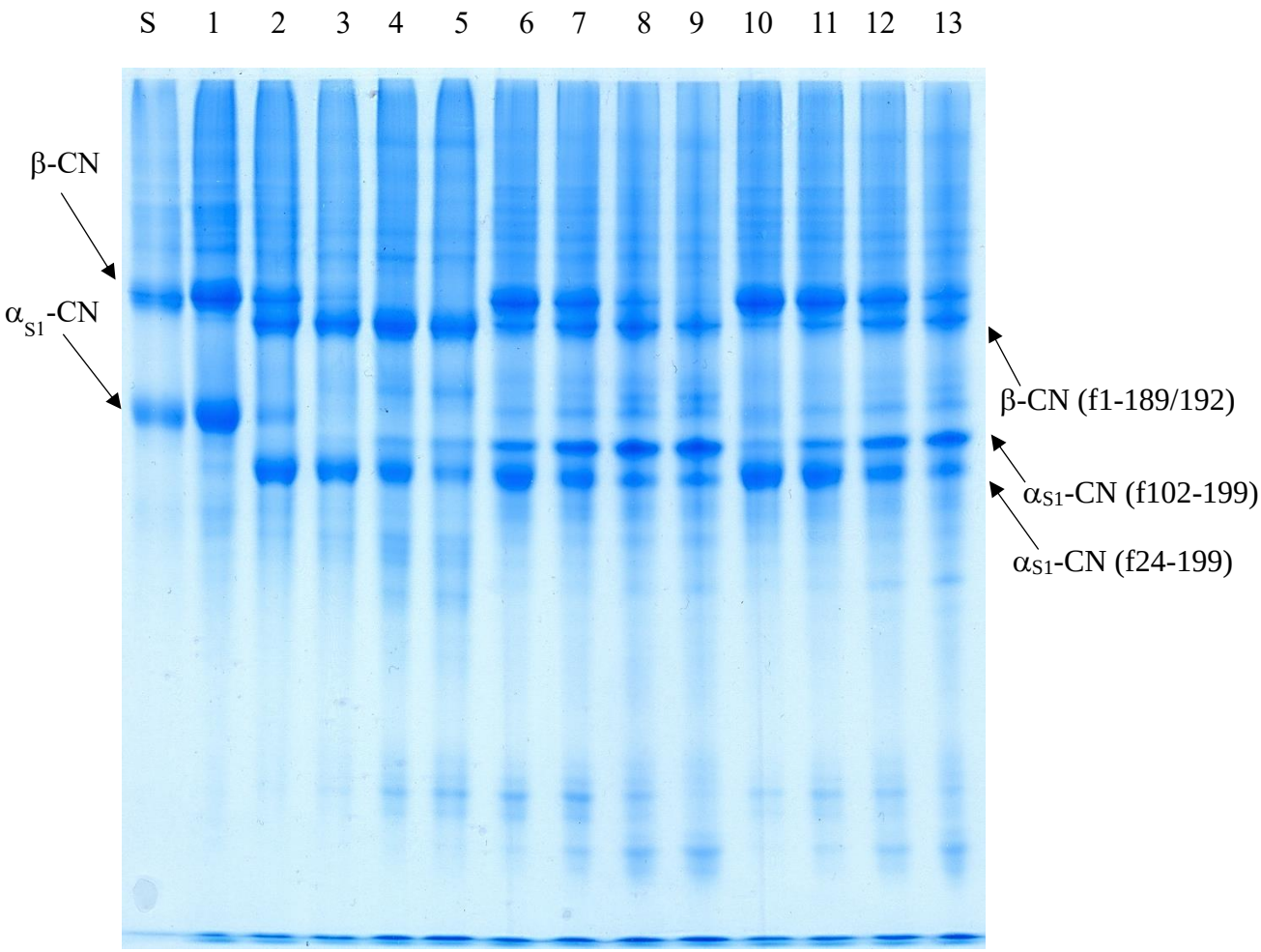

Fig. 6.1. Urea-polyacrylamide gel electrophoretograms of Na caseinate (10 mg mL^{-1}) in 100 mM K phosphate buffer, pH 6.5, hydrolysed by bovine chymosin (0.4 IMCU mL^{-1}) for 0, 3, 9, 24 or 48 h (lanes 1-5) or camel chymosin (4 IMCU mL^{-1}) for 3, 9, 24 or 48 h (lanes 6-9) or modified camel chymosin (13.34 IMCU mL^{-1}) for 3, 9, 24 or 48 h (lanes 10-13) at 37 °C. Sodium caseinate (S) was used as standard on the left side of the gel. The figure shows electrophoretograms for one of the three digestion replicates; all replicates were similar.

6.3.2 Liquid chromatography-mass spectrometry of sodium caseinate digests

The number of peptides identified in the pH 4.6-soluble extracts of NaCN digested by the three chymosins at pH 5.2 plus salt and 6.5 is shown in Table 6.1. A larger number of peptides was found in the samples digested at pH 5.2 plus salt compared to at pH 6.5, which was expected as chymosin has an acidic optimum pH (Nájera et al., 2003).

The total intensities of identified peptide in the NaCN digestion distributed between the different caseins (as well as total) are shown in Table 6.1. α_{S1}-CN is a primary target of chymosin, and accordingly the breakdown of β-CN and α_{S2}-CN was lower compared with the breakdown of α_{S1}-CN. In cheese or solutions similar in ionic composition to the aqueous phase of cheese, the levels of proteolysis of α_{S1}-CN are the highest compared to the other caseins (Exterkate et al., 1997; McSweeney et al., 1994). A significant number of peptides were produced from these caseins under all conditions. Different levels of each chymosin were required to hydrolyse all intact α_{S1}-CN. After 3 h incubation at pH 5.2 plus salt, peptides released by CC showed higher total intensity than that of samples BC and mCC whereas, at pH 6.5, peptides released by mCC were present at the highest concentration. After 24 h incubation, peptides released by CC showed higher total intensity at both pH values. The Cheddar cheese environment is around pH 5.2 plus salt, under which conditions chymosin hydrolyses mainly α_{S1}-CN, while β-CN and α_{S2}-CN remain intact (Farkye & Fox, 1992; Mooney et al., 1998). Nevertheless, substantial chymosin activity towards β-CN and α_{S2}-CN were found in NaCN samples digested by all types of chymosin at pH 5.2 plus salt.

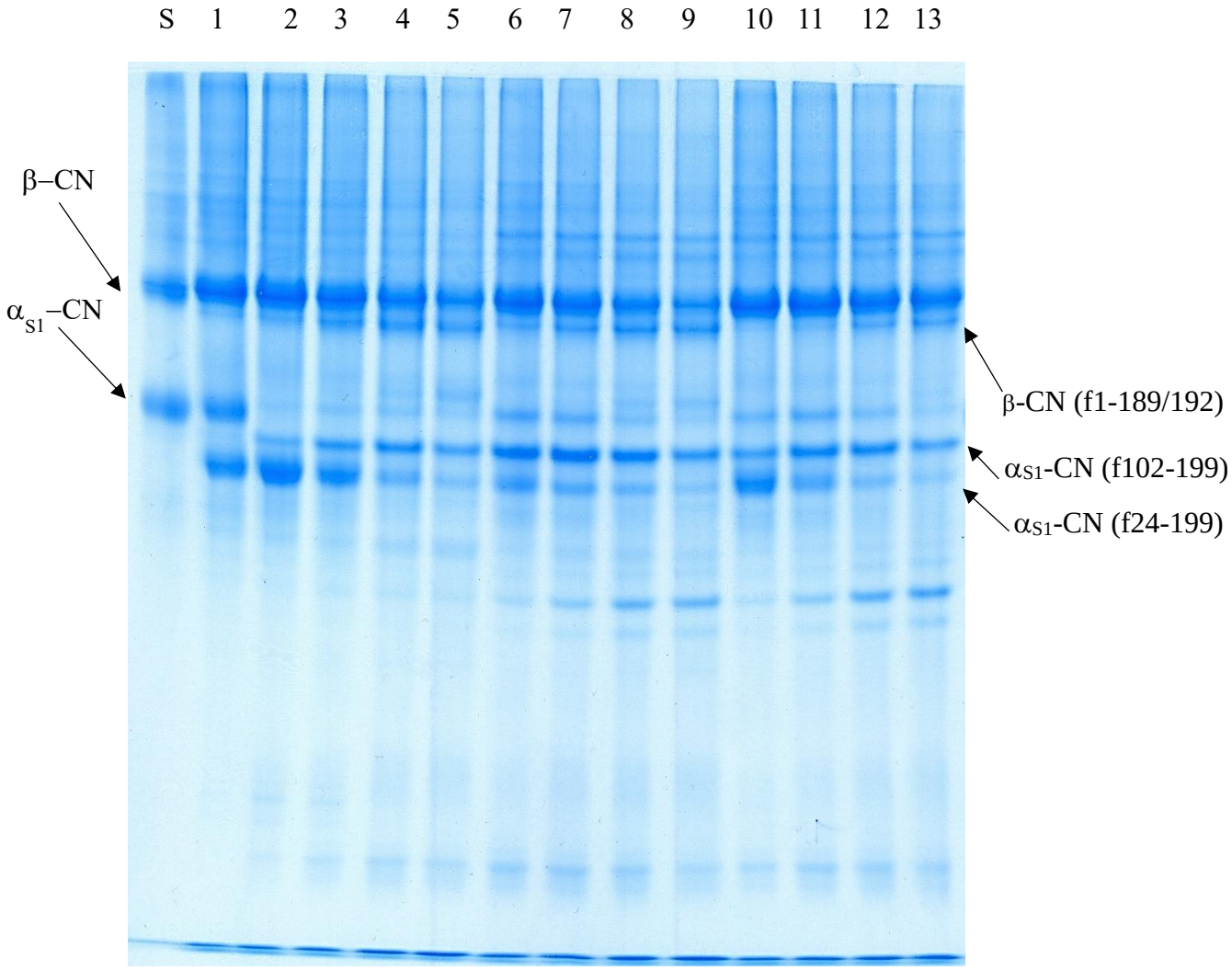

Fig. 6.2. Urea-polyacrylamide gel electrophoretograms of Na caseinate (10 mg mL^{-1}) in 100 mM Na acetate buffer, pH 5.2, with 5% NaCl, hydrolysed by bovine chymosin (0.21 IMCU mL^{-1}) for 0, 3, 9, 24 or 48 h (lanes 1-5) or camel chymosin (2.1 IMCU mL^{-1}) for 3, 9, 24 or 48 h (lanes 6-9) or modified camel chymosin (7 IMCU mL^{-1}) for 3, 9, 24 or 48 h (lanes 10-13) at 37 °C. Sodium caseinate (S) was used as standard on the left side of the gel. The figure shows electrophoretograms for one of the three digestion replicates; all replicates were similar.

Table 6.1. Numbers and summed intensities of identified peptides in pH 4.6-soluble extracts of NaCN digested by bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) for 3 h at pH 5.2 with 5% NaCl, 24 h at pH 5.2 with 5% NaCl, 3 h at pH 6.5, and 24 h at pH 6.5.

Digestion condition	pH 5.2 with 5% NaCl		pH 6.5	
	3 h	24 h	3 h	24 h
	Numbers of identified peptides			
BC	68	112	38	84
CC	95	144	51	111
mCC	71	134	59	99
	Summed intensities of identified peptides (E+08)			
BC	2.01	3.32	1.01	2.09
CC	2.53	4.35	1.47	3.09
mCC	1.93	3.22	1.99	2.01

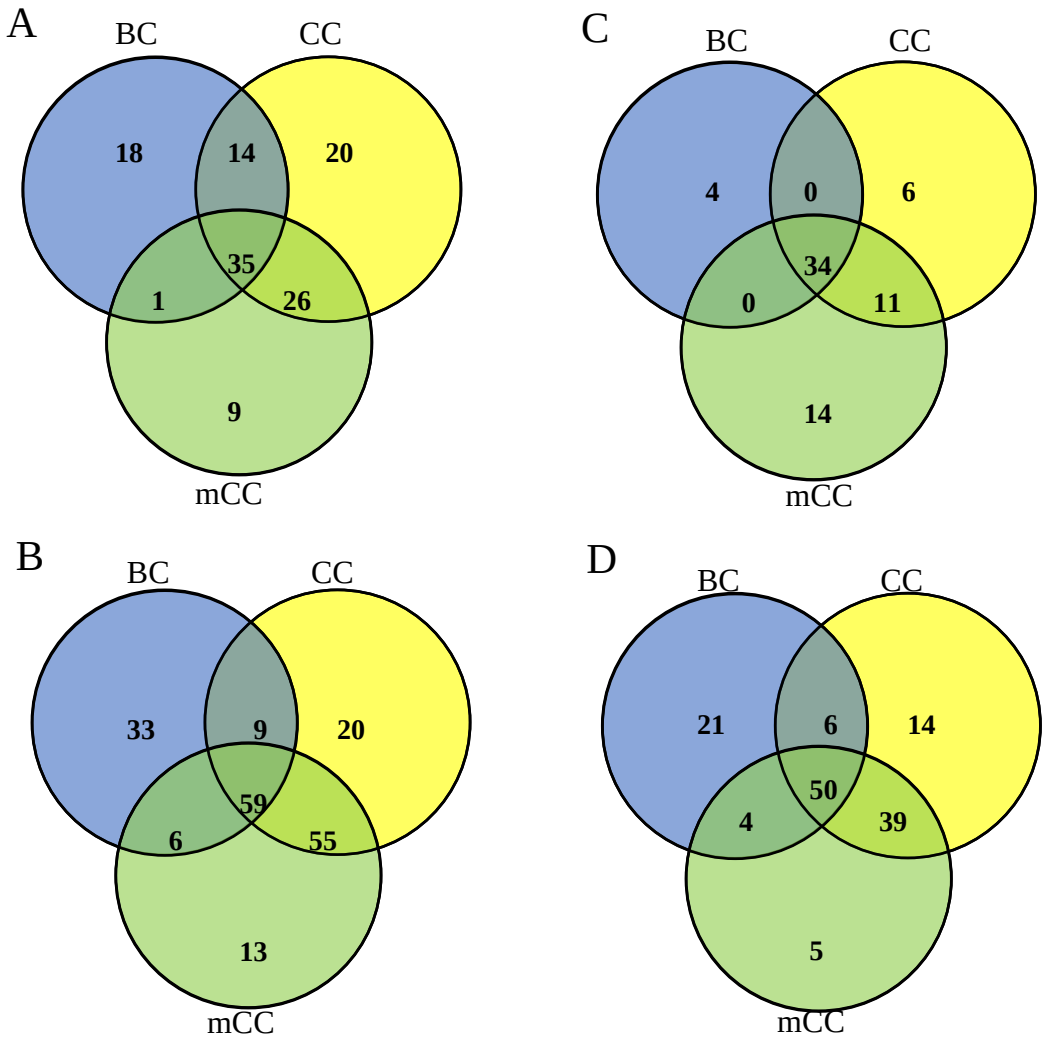

Fig. 6.3. Venn diagrams of peptide distributions for pH 4.6-soluble extracts of NaCN digested by bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) for (A) 3 h at pH 5.2 with 5% NaCl, (B) 24 h at pH 5.2 with 5% NaCl, (C) 3 h at pH 6.5 and (D) 24 h at pH 6.5.

The overlapping and unique peptides identified in NaCN samples digested by each chymosin at pH 6.5 and pH 5.2 plus salt were visualised in a Venn diagram (Fig. 6.3) (Oliveros, 2007; Arju et al., 2020). Irrespectively of pH and duration of hydrolysis, treatments with CC and mCC showed more peptides in common than treatments with BC, while about half the number of peptides identified for each chymosin were in common.

The concentrations of all peptides identified were summed and plotted according to the sequence of each casein to visualise the proteolytic specificity of chymosins (Appendix). Ten pH 4.6-soluble peptides with the highest response derived from α_{S1}-CN, α_{S2}-CN, β-CN of each sample are listed (Table 6.2-6.4). All pH 4.6-soluble peptides derived from κ-CN of each sample are listed in Table 6.5, as the number of peptides identified was less than 10. Cleavage sites from α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN sequences found to be hydrolysed in NaCN samples that corresponded to peptides present in the pH 4.6-soluble extracts of cheese samples were compared (Figs. 6.4-6.7). Most cleavage sites found to be cleaved in NaCN samples were also hydrolysed in cheese samples. A few cleavage sites were found only hydrolysed in NaCN samples.

NaCN digested by all types of chymosin showed a high intensity of peptide α_{S1}-CN f1-23 at both pH 6.5 and 5.2, which indicates that all types of chymosin had high activity on the peptide bond Phe_{23}-Phe_{24} (McSweeney et al., 1993; Møller et al., 2012a).

As for α_{S2}-CN, the peptide f119-130 was found in samples digested by all types of chymosin. Peptide f164-174 of α_{S2}-CN presented a high intensity in the sample digested by bovine chymosin at either pH; the peptide bonds Phe_{163}-Leu_{164} and Phe_{174}-Ala_{175}, that are cleaved to form the peptide f164-174 have previously been reported to be hydrolysed by bovine chymosin (McSweeney et al., 1994). Both samples CC and mCC showed a high intensity of peptide f164-171.

In the case of β-CN, the peptide with the highest intensity in NaCN digested with all types of chymosin was peptide f193-209, corresponding to cleavage of the peptide bond Leu_{192}-Tyr_{193}, as has been reported in the literature (McSweeney et al., 1993; Møller et al., 2012a). The peptide intensity of peptide f193-209 was also

the highest among the peptide identified for pH 4.6-soluble extraction of cheese cBC, while was the second highest for cCC and the seventh highest for cmCC. The number and intensities of peptides from κ-CN detected in all NaCN digests were relatively lower than that for other caseins.

Table 6.2. pH 4.6-soluble peptides from α_{S1}-CN with the highest response present (X) or absent (—) from each NaCN digests over 24 h with bovine chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC).

		pH 5.2 with 5% NaCl			pH 6.5		
Amino acid sequence	Peptide	BC	CC	mCC	BC	CC	mCC
RPKHPIKHQGLPQEVL	f1-16	X	X	X	X	X	X
RPKHPIKHQGLPQEVLNE	f1-18	X	X	X	—	—	—
RPKHPIKHQGLPQEVLNENL	f1-20	X	X	X	X	X	X
RPKHPIKHQGLPQEVLNENLLR	f1-22	—	X	X	X	X	X
RPKHPIKHQGLPQEVLNENLLRF	f1-23	X	X	X	X	X	X
KHPIKHQGLPQEVLNENLLR	f3-22	—	X	X	—	X	X
KHPIKHQGLPQEVLNENLLRF	f3-23	X	X	X	X	X	X
KHPIKHQGLPQEVLNENL	f3-20	X	X	X	—	—	—
NENLLR	f17-22	—	X	X	—	—	—
NENLLRF	f17-23	X	X	X	—	X	X
FVAPFPEVFGKEKVNEL	f24-40	X	X	X	X	X	X
LLRL	f98-101	X	X	—	X	X	X
KKYKVPQ	f102-108	—	X	X	—	—	—
KKYKVPQL	f102-109	X	X	X	—	X	X
KKYKV…EGIHA	f102-129	X	X	X	X	X	—
AYFYPEL	f143-149	X	X	X	—	—	—
FRQF	f150-153	X	X	X	X	—	X

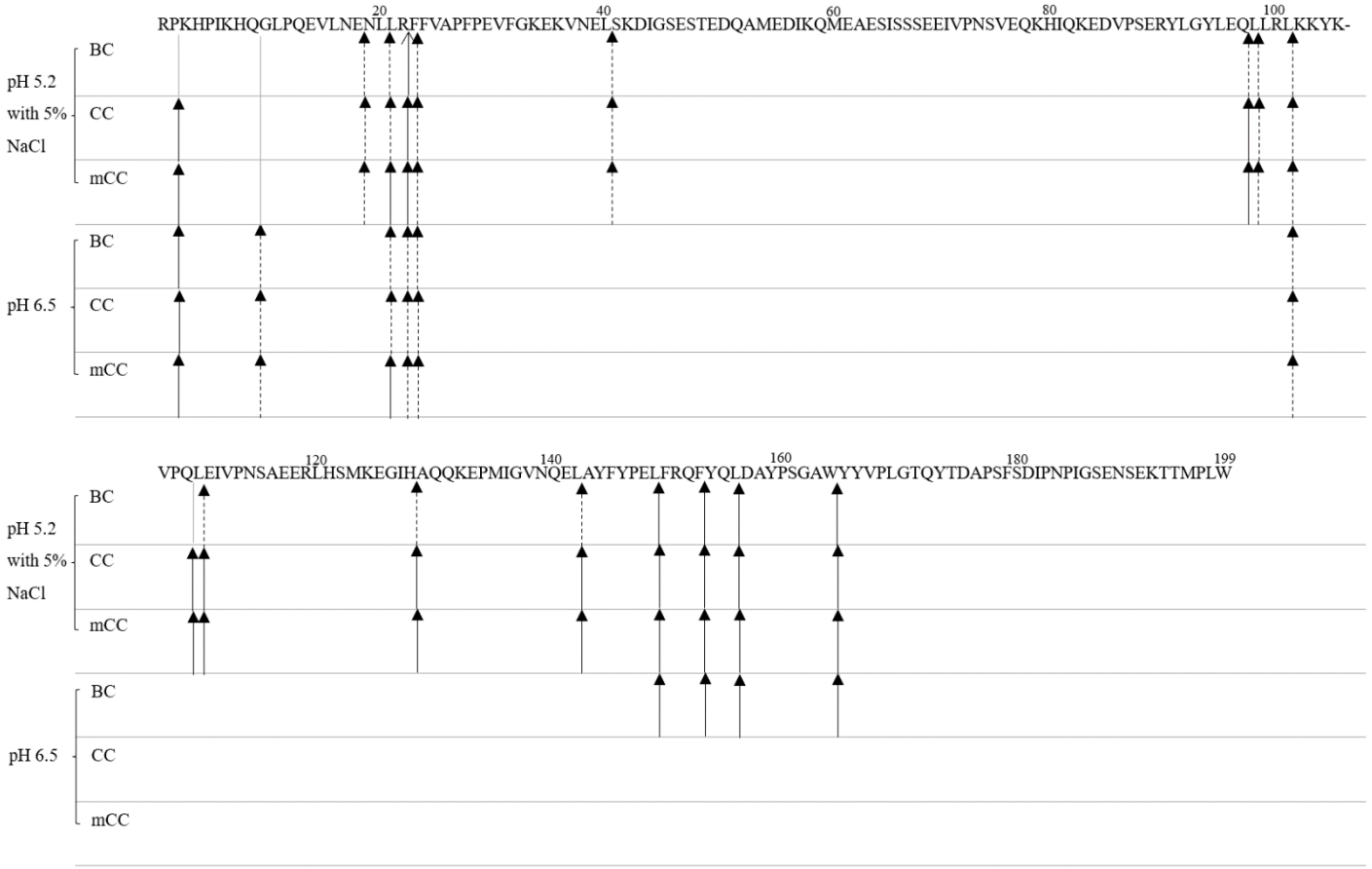

Fig. 6.4. Cleavage sites of bovine chymosin (BC), camel chymosin (CC), and modified camel chymosin (mCC) in α_{S1}-CN hydrolysed at pH 6.5 or 5.2 with 5% NaCl and their appear status in cheese. Straight arrows (↑) indicate cleavage sites hydrolysed only in NaCN digests, straight open arrows (⇑) indicate cleavage sites hydrolysed only in cheese, and dotted arrows (⇡)indicate cleavage sites hydrolysed both in NaCN digests and cheese.

6.3.2.1 α_{S1}-CN-derived peptides and cleavage sites

Ten pH 4.6-soluble peptides derived from α_{S1}-CN with the highest response from each NaCN digest after 24 h are listed in Table 6.2. For α_{S1}-CN, most peptides arose from the N-terminal part of the protein from residues 1 to 40. Fewer peptides, with much lower intensities, originated from the central and C-terminal parts of the sequence (residues 80 to 199). The activity of BC was higher on the central part of α_{S1}-CN, while CC and mCC seemed to preferentially hydrolyse the N-terminal end of the sequence.

The cleavage sites of the enzymes were plotted on the casein sequences by summing up the intensities of N-terminal and C-terminal amino acids of all identified pH 4.6-soluble peptides (Fig. 6.4). Chymosin preferred to cleave bonds containing Phe and Leu, which is consistent with the literature (McSweeney et al., 1993; Møller et al., 2012a). The primary cleavage sites of chymosin in α_{S1}-CN were Phe_{23}-Phe_{24}, and also Leu_{149}-Phe_{150}, Leu_{156}-Asp_{157}, and Trp_{164}-Tyr_{165}, which is consistent with the literature (Exterkate et al., 1997). The peptides produced after the primary cleavage were rapidly converted into several small fragments by chymosin as the result of subsequent cleavages. Other cleavage sites found to be hydrolysed by bovine chymosin were: Glu_{18}-Asn_{19}, Leu_{20}-Leu_{21}, Leu_{40}-Ser_{41}, Gln_{97}-Leu_{98}, Leu_{98}-Leu_{99}, Leu_{101}-Lys_{102}, His_{128}-Ala_{129}, Leu_{142}-Ala_{143}, and Phe_{153}-Tyr_{154}. The sites mentioned above were the same as that found by others (Exterkate et al., 1997; McSweeney et al., 1993; Møller et al., 2012a). Cleavage site Arg_{22}-Phe_{23} was found to be hydrolysed by both camel chymosins, but this was not hydrolysed by bovine chymosin, which is in agreement with the results of Møller et al., (2012a). With the presence of the cleavage site Arg_{22}-Phe_{23}, the hydrolysis of the cleavage site Phe_{23}-Phe_{24} of the sample digested by the

two camel chymosins was reduced compared to bovine chymosin, which indicates that camel chymosins cut the peptide f1-23 from the C-terminal part of the peptide, further producing f1-22 and some smaller peptides. Peptide f1-22 was reported in α_{S1}-CN digested by camel chymosin but not previously in hydrolysates of bovine chymosin (Møller et al., 2012a) which is consistent with our results.

Leu_{16}-Asn_{17} was a cleavage site newly found to be hydrolysed by bovine chymosin which has not been reported in the literature so far. Likewise, Gln_{108}-Leu_{109} was a new cleavage site detected in both samples CC and mCC, and Leu_{109}-Glu_{110} was a new cleavage site that produced peptides found in NaCN treated with all types of chymosin.

Peptide bonds Phe_{23}-Phe_{24}, Leu_{40}-Ser_{41}, Leu_{98}-Leu_{99}, Leu_{101}-Lys_{102}, Leu_{142}-Ala_{143} were hydrolysed in both cheese and NaCN samples treated with BC and CC (Fig. 6.4). Peptide bond Arg_{22}-Phe_{23}, which has been reported to be cleaved by camel chymosin (Møller et al., 2012a), was found to be hydrolysed in all cheese samples, while this peptide bond was not hydrolysed in NaCN hydrolysed by bovine chymosin, which indicates that other proteinase in cheese might hydrolyses peptide bond Arg_{22}-Phe_{23}. Peptide bonds Leu_{149}-Phe_{150}, Leu_{156}-Asp_{157}, and Trp_{164}-Tyr_{165} that were found to be hydrolysed by all types of chymosin in NaCN solution were not hydrolysed in all cheese samples, which might be attributed to the decrease in accessibility of the segment due to a specific structural change of α_{S1}-CN induced by the cheese environment (Exterkate et al., 1997).

6.3.2.2 α_{S2}-CN-derived peptides and cleavage sites

In terms of the peptides identified from pH 4.6 water-soluble peptides of NaCN digestion by the three chymosins, all enzymes showed moderate activities towards α_{S2}-

CN, especially in the C-terminal part of the protein, and showed broader proteolytic activity towards the whole sequence compared to other caseins. Ten pH 4.6-soluble peptides derived from α_{S2}-CN with the highest response from each NaCN digests after 24 h are listed in Table 6.3. The peptide bond Phe_{174}-Ala_{175} is known to be hydrolysed by chymosin in the cheese environment (McSweeney et al., 1994), and was found to be cleaved in hydrolysates in this study (Fig. 6.5). Four peptides with high response that correspond to peptide bond Phe_{174}-Ala_{175} were found in the cheese samples (Table 6.3); two peptides of which showed responses decreasing from cheese BC to CC to mCC and the rest two peptides were absent in the pH 4.6-soluble extraction of cheese CC and mCC. Besides Phe_{174}-Ala_{175}, peptides corresponding to cleavage sites Phe_{88}-Tyr_{89}, Tyr_{95}-Leu_{96}, Gln_{97}-Tyr_{98}, Tyr_{98}-Leu_{99}, Phe_{163}-Leu_{164}, and Tyr_{179}-Leu_{180} were found in NaCN samples hydrolysed by all types of chymosin and have been reported earlier (McSweeney et al., 1994). Several new cleavage sites e.g., Ala_{27}-Ile_{28}, Arg_{45}-Asn_{46} and His_{77}-Tyr_{78} were found to be hydrolysed in digests of all types of chymosin; some cleavage sites e.g., Gln_{94}-Tyr_{95}, Leu_{123}-Asn_{124} and Leu_{153}-Thr_{154} were hydrolysed by both camel chymosins only (Fig. 6.5). Cleavage sites found to be hydrolysed in NaCN digests and their presence in cheese are shown in Fig. 6.5.

Table 6.3. pH 4.6-soluble peptides from α_{S2}-CN with the highest response present (X) or absent (—) from each NaCN digests over 24 h with bovine chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC).

		pH 5.2 with 5% NaCl			pH 6.5		
Amino acid sequence	Peptide	BC	CC	mCC	BC	CC	mCC
INPSKENLCSTFCKEVVR	f28-45	X	X	X	X	X	X
YQKALNEINQFYQKF	f78-92	X	X	X	—	X	X
YQKFPQY	f89-95	X	X	X	X	X	X
YQKFPQYLQ	f89-97	X	X	X	—	—	—
YQKFPQYLQY	f89-98	X	X	X	X	X	X
YQKFPQYLQYL	f89-99	X	X	X	X	X	X
ITPTLNREQLST	f119-130	X	X	X	X	X	X
TKKTKLTEEEKNRLNF	f148-163	X	X	X	—	—	—
TEEEKNRLNF	f154-163	—	X	X	—	X	—
LKKISQR	f164-170	—	X	X	—	X	X
LKKISQRY	f164-171	X	X	X	X	X	X
LKKISQRYQKF	f164-174	X	—	X	X	—	—
QKFALPQY	f172-179	—	X	X	—	—	—
KTVYQ…PYVRY	f181-206	—	X	X	—	X	X
KTVYQ…YVRYL	f181-207	—	X	X	—	X	X

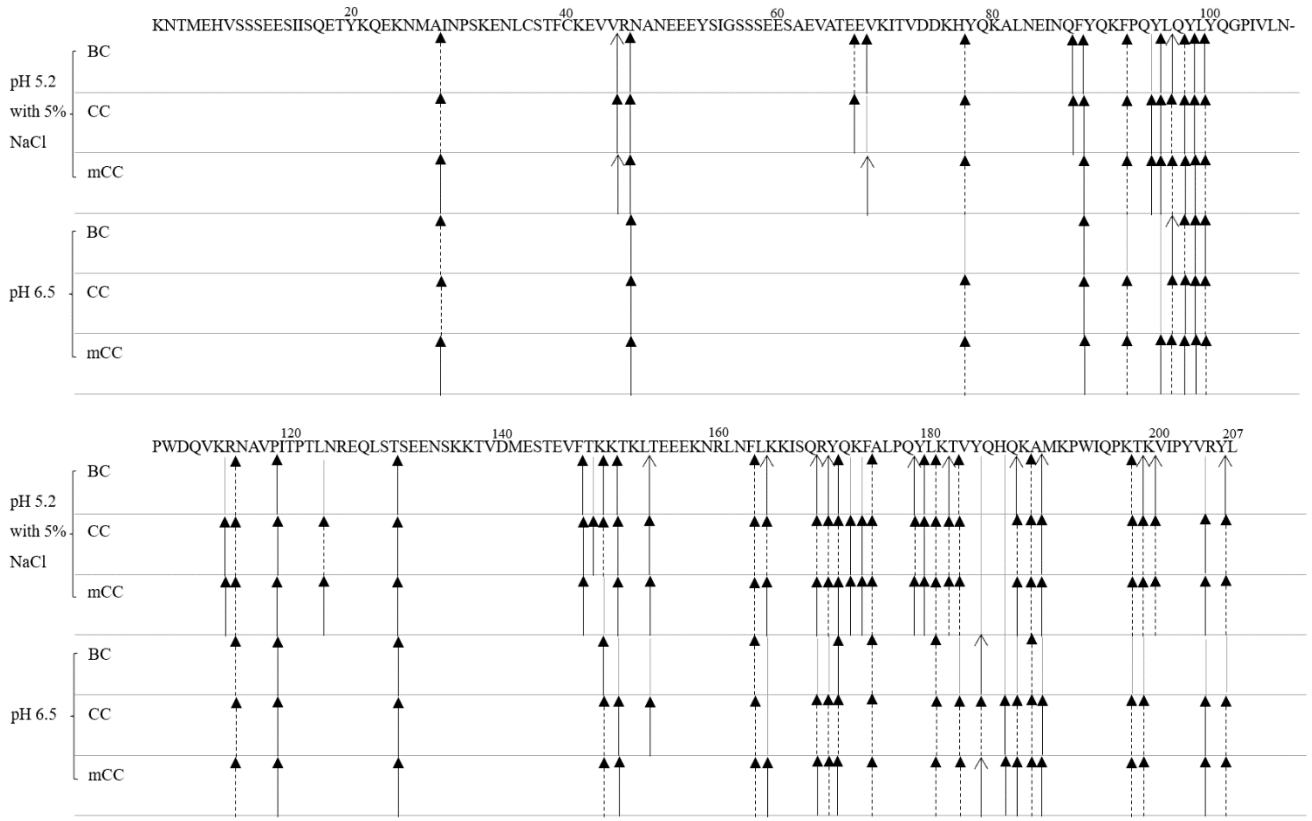

Fig. 6.5. Cleavage sites of bovine chymosin (BC), camel chymosin (CC), and modified camel chymosin (mCC) in α_{S2}-CN hydrolysed at pH 6.5 or 5.2 with 5% NaCl and their appear status in cheese. Straight arrows (↑) indicate cleavage sites hydrolysed only in NaCN digests, straight open arrows (⇑) indicate cleavage sites hydrolysed only in cheese, and dotted arrows (⇡)indicate cleavage sites hydrolysed both in NaCN digests and cheese.

6.3.2.3 β-CN-derived peptides and cleavage sites

Most of the β-CN-derived peptides arose from the C-terminal part, i.e., from residues 163 to 209. Ten pH 4.6-soluble peptides derived from β-CN with the highest response from each NaCN digests after 24 h are listed in Table 6.4. The very bitter peptide f193-209 (Børsting et al., 2012) was less intense in samples hydrolysed by mCC, despite the concentration of mCC used being higher than that for the other chymosins. The intensity of peptide f193-209 cheese BC was about 4 and 8 times that in cheeses CC and mCC, respectively, and the lowest bitterness was found in cheese manufactured using modified camel chymosin (Li et al., 2022; Chapter 5).

Cleavage sites found to be hydrolysed in NaCN digests and their presence in cheese are shown in Fig. 6.6. In the case of the cleavage of β-CN in NaCN digests, Leu_{165}-Ser_{166}, Gln_{167}-Ser_{168}, Ala_{189}-Phe_{190} and Leu_{192}-Tyr_{193} are the peptide bonds found to be cleaved by bovine chymosin in this study that have also been reported in the literature (Exterkate et al., 1997; Møller et al., 2012a), and were the main cleavage sites found to be cleaved in NaCN digests of all types of chymosin (Fig. 6.6). Cleavage site Gln_{167}-Ser_{168} was not cleaved by both camel chymosins, which was consistent with the results of Møller et al. (2012a). Other than the peptide bonds mentioned above, some new cleavage sites were observed to be hydrolysed by chymosin in the NaCN samples digested by BC and CC, while the intensities of the peptides corresponding to those cleavage sites are relatively lower than the peptides have been reported before (Fig. 6.6; Appendix). The peptides relating to cleavage site Leu_{192}-Tyr_{193}, which produces peptide f193-209, reportedly responsible for bitterness in cheese (Børsting et al., 2012), were found at the lowest intensity in sample mCC, although mCC was used at the highest concentration to digest NaCN.

Table 6.4. pH 4.6-soluble peptides from β-CN with the highest response present (X) or absent (—) from each NaCN digests over 24 h with bovine chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC).

		pH 5.2 with 5% NaCl			pH 6.5		
Amino acid sequence	Peptide	BC	CC	mCC	BC	CC	mCC
RELEE	f1-5	X	—	—	X	—	—
RELEEL	f1-6	X	X	X	X	X	X
PFAQT…H_{67}…LPQNI	f51-74^{A1}	X	X	X	X	X	X
PIHNS…PPFLQ	f65-89^{A1}	X	X	X	X	X	X
SKVKE…PKYPV	f96-116	X	X	—	—	X	X
SLSQS…MPIQA	f164-189	X	X	—	X	—	—
SLSQS…PIQAF	f164-190	—	X	X	—	X	—
SQSKV…MPIQA	f166-189	X	X	X	X	X	—
SQSKV…IQAFL	f166-191	—	X	X	—	X	X
SQSKV…QAFLL	f166-192	X	X	X	X	X	X
SKVLP…MPIQA	f168-189	X	—	—	X	—	—
VLPVP…MPIQA	f170-189	X	X	—	X	—	—
YQEPVLGPVRGPFPIIV	f193-209	X	X	X	X	X	X

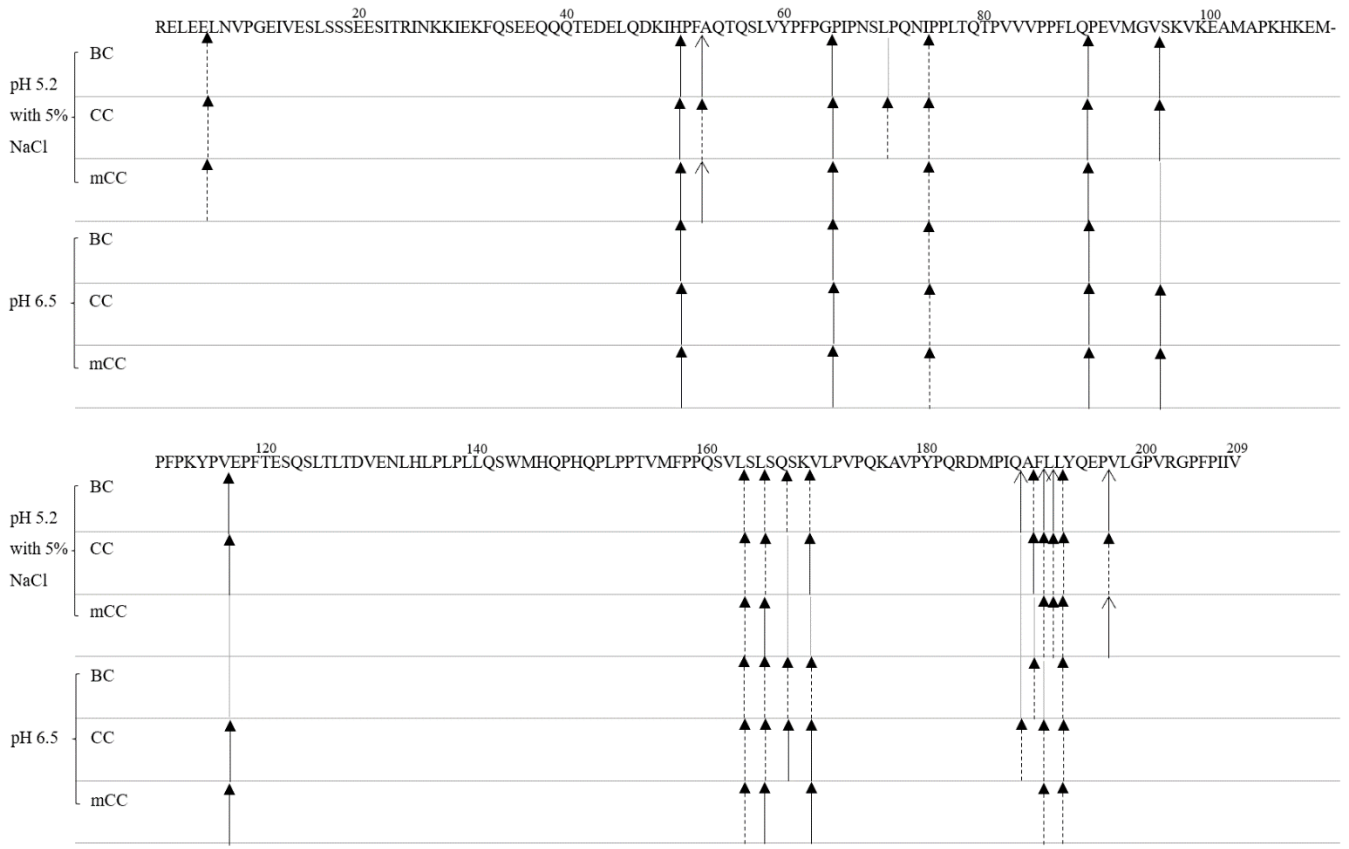

Fig. 6.6. Cleavage sites of bovine chymosin (BC), camel chymosin (CC), and modified camel chymosin (mCC) in β-CN hydrolysed at pH 6.5 or 5.2 with 5% NaCl and their appear status in cheese. Straight arrows (↑) indicate cleavage sites hydrolysed only in NaCN digests, straight open arrows (⇑) indicate cleavage sites hydrolysed only in cheese, and dotted arrows (⇡)indicate cleavage sites hydrolysed both in NaCN digests and cheese.

6.3.2.4 κ-CN-derived peptides and cleavage sites

In the case of the κ-CN-derived peptide coverage map of pH 4.6-soluble extracts of NaCN digests, peptides in all samples were present at very low intensities compared to those from other caseins. All pH 4.6-soluble peptides derived from κ-CN from each NaCN digests over 24 h are listed in Table 6.5. More peptides were identified in sample BC compared to those found in samples CC and mCC. The pH 6.5 NaCN blank without any chymosin applied showed comparable intensities of peptides f161-169 and f164-169 to that of NaCN samples treated with all types of chymosin (data not shown). Therefore, those peptides were not considered in terms of the activity of chymosins. Both bovine chymosin and camel chymosin hydrolyse the Phe_{105}-Met_{106} bond, producing *para*-κ-CN (N-term end of κ-CN) and GMP (C-terminal end κ-CN) (Møller et al., 2012b; Walstra et al., 2005). Proteolysis of κ-casein, both *para*-κ-CN and GMP regions, was more obviously seen in NaCN hydrolysed by BC.

The common and unique cleavage sites from κ-CN found to be hydrolysed by three chymosins at pH 5.2 plus salt and 6.5 and their presence in cheese are shown in Fig. 6.7. Compared to BC, the peptides corresponding to the Phd_{105}-Met_{106} bond were not found in CC and mCC digests, which suggests that peptides f1-105 and f106-169, produced as a result of the primary action of chymosin, were not further broken down by either camel chymosin to the smaller peptides and were too large to be detected by LC-MS/MS. In BC-treated NaCN samples, only one peptide (f95-105) corresponding to cleavage site Phe_{105}-Met_{106} can be seen due to broader hydrolytic activity of BC towards the whole κ-CN sequence and production of smaller peptides detectable by the LC-MS/MS method. Camel chymosin was found to have a higher efficiency

hydrolysing the Phe_{105}-Met_{106} bond of κ-CN compared to bovine chymosin (Kappeler et al., 2006).

Table 6.5. All pH 4.6-soluble peptides from κ-CN present (X) or absent (—) from each NaCN digests over 24 h with bovine chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC).

		pH 5.2 with 5% NaCl			pH 6.5		
Amino acid sequence	Peptide	BC	CC	mCC	BC	CC	mCC
FSDKIAKYIPIQY	f18-30	X	—	—	—	—	—
FSDKIAKYIPIQYVL	f18-32	X	X	—	X	—	—
VLSRY…NNQFL	f31-56	—	X	—	—	—	—
SRYPSYGLNY	f33-42	X	—	—	—	—	—
LNYYQQKPVALINNQFL	f40-56	X	—	—	—	—	—
YQQKPVAL	f43-50	—	X	—	—	—	—
QKPVALINNQFLPYPYYAKPA	f45-65	X	—	—	X	—	—
YYAKP…SNTVP	f60-84	X	—	—	X	—	—
AKPAAVRSPAQILQWQV	f62-78	—	—	—	X	—	X
PAAVR…CQAQP	f64-92	—	X	—	—	X	—
PAKSCQAQPTT	f84-94	—	X	—	—	—	—
KSCQA…FMAIP	f86-109	—	X	—	X	—	X
ARHPHPHLSF	f96-105	X	—	—	X	—	—
TEIPTINTIASGEPTSTPTIE	f117-137[B]	X	—	—	—	—	—
VIESPPEIN	f152-160	X	X	X	—	—	—
VQVTSTAV	f162-169	X	X	—	—	—	—

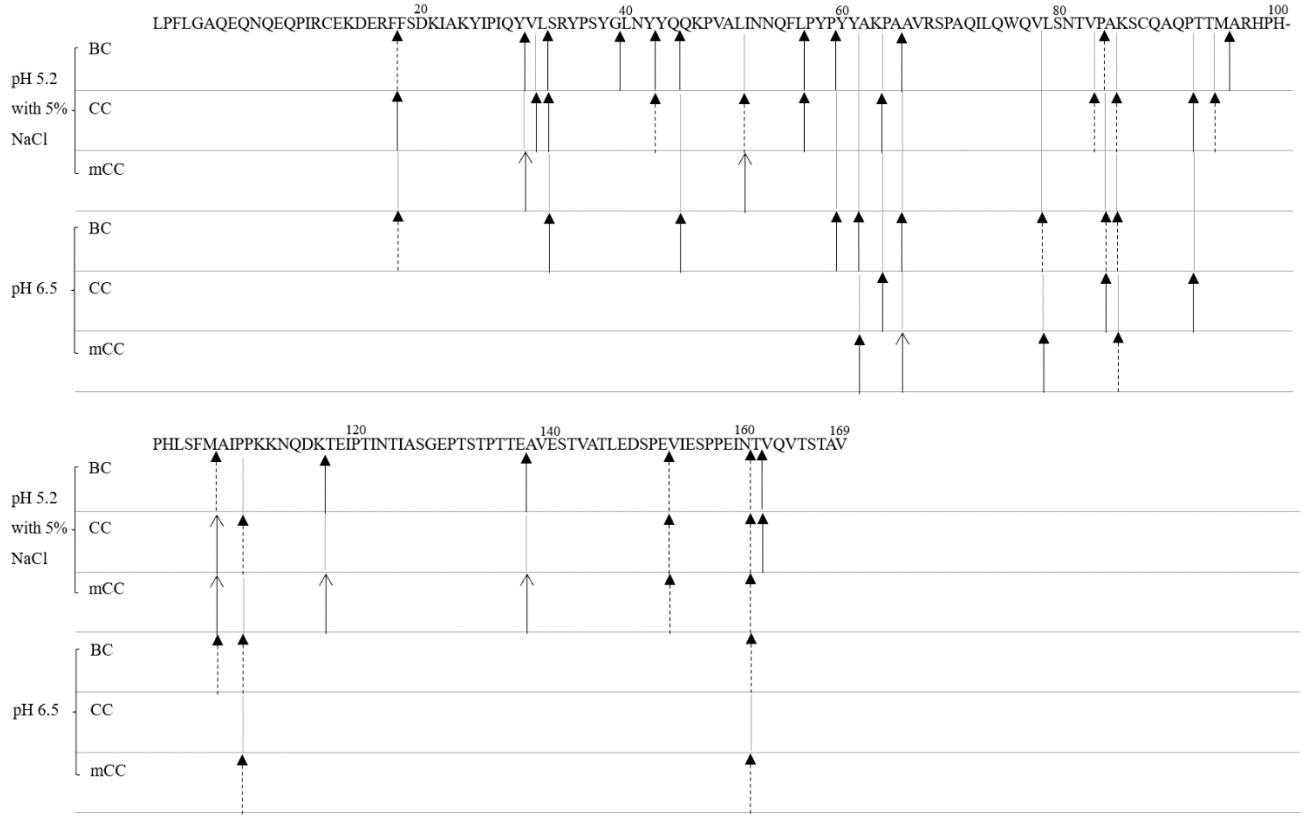

Fig. 6.7. Cleavage sites of bovine chymosin (BC), camel chymosin (CC), and modified camel chymosin (mCC) in κ-CN hydrolysed at pH 6.5 or 5.2 with 5% NaCl and their appear status in cheese. Straight arrows (↑) indicate cleavage sites hydrolysed only in NaCN digests, straight open arrows (⇑) indicate cleavage sites hydrolysed only in cheese, and dotted arrows (⇡)indicate cleavage sites hydrolysed both in NaCN digests and cheese.

6.4 Conclusion

This work investigated the proteolytic specificity of three generations of chymosin and compared that with the *in vitro* proteolysis of Cheddar cheese manufactured therefrom after 90 and 180 days of ripening. Many peptides were determined through LC-MS/MS in NaCN digests produced from each chymosin. In addition, more cleavage sites that produce peptides with lower intensities were found to be hydrolysed by bovine and camel chymosins. Most of the peptides observed in NaCN digests were also identified in Cheddar cheese samples. The proteolytic activity of modified camel chymosin was relatively lower than that of bovine and camel chymosins, which resulted in Cheddar cheese with modified functionality and sensory properties (Li et al., 2022). These results provide a comprehensive understanding of the proteolytic specificity of the three generations of chymosins, which could benefit the dairy industry. Future work should be focused on the influence of the structure of modified camel chymosin on the proteolysis of caseins.

6.5 Acknowledgements

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Chapter 6

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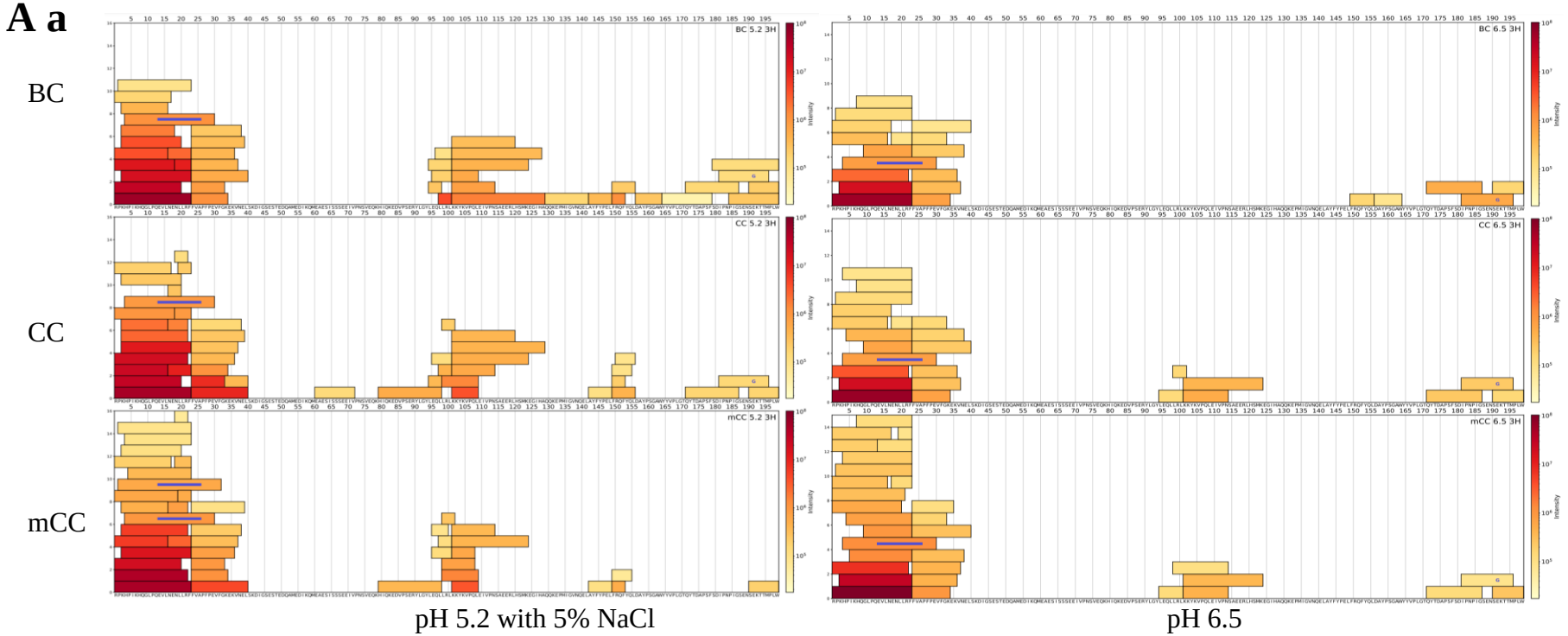
Appendix



Supplemental Fig. S1. Peptide coverage map of the (A) α_{S1}-casein, (B) α_{S2}-casein, β-casein (C) and κ-casein (D) sequence of pH 4.6 soluble extracts of NaCN digested by bovine chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) for (a) 3 and (b) 24 hours at pH 5.2 with 5% NaCl and 6.5. Blue lines and one-letter abbreviations of amino acids on the peptides indicate the differences in the peptide sequences in genetic variants of α_{S1}-CN.

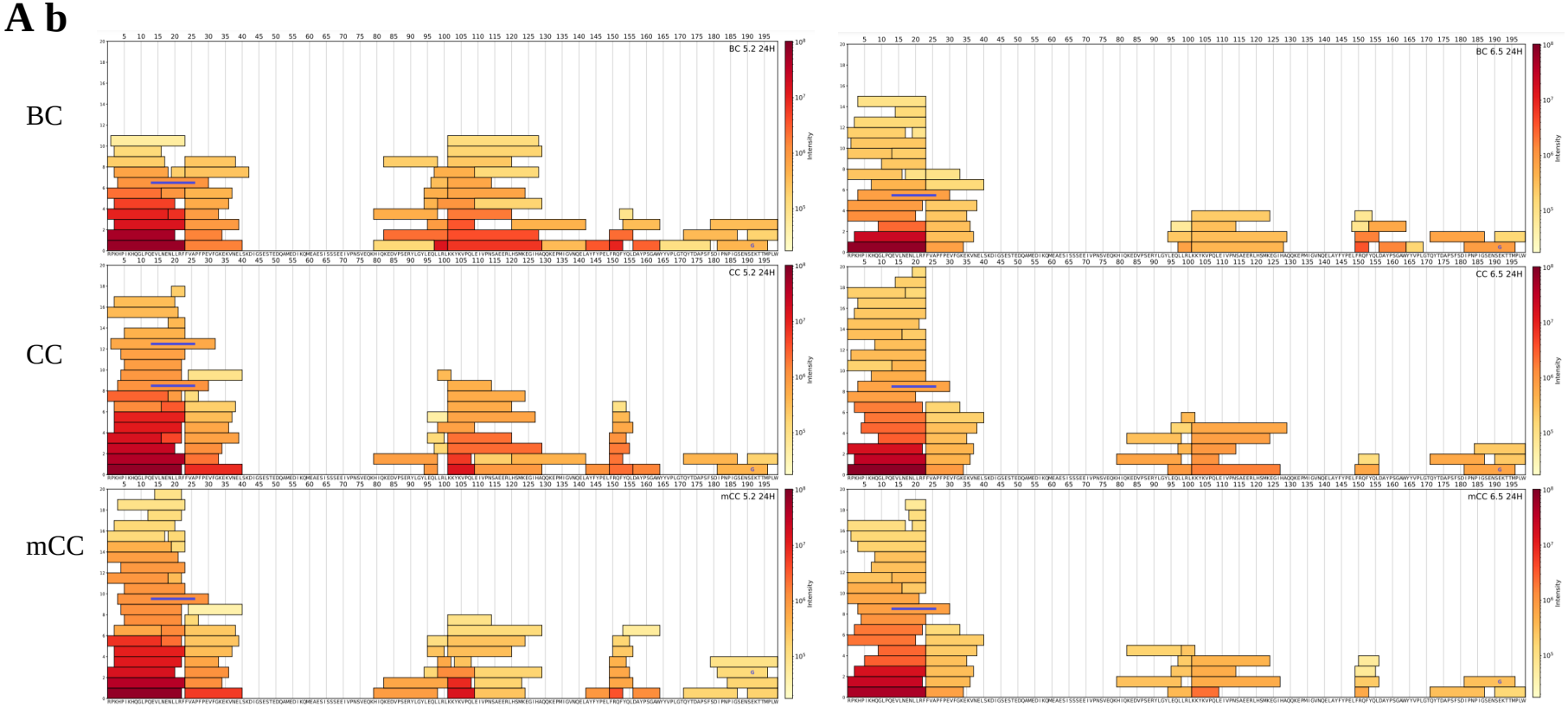


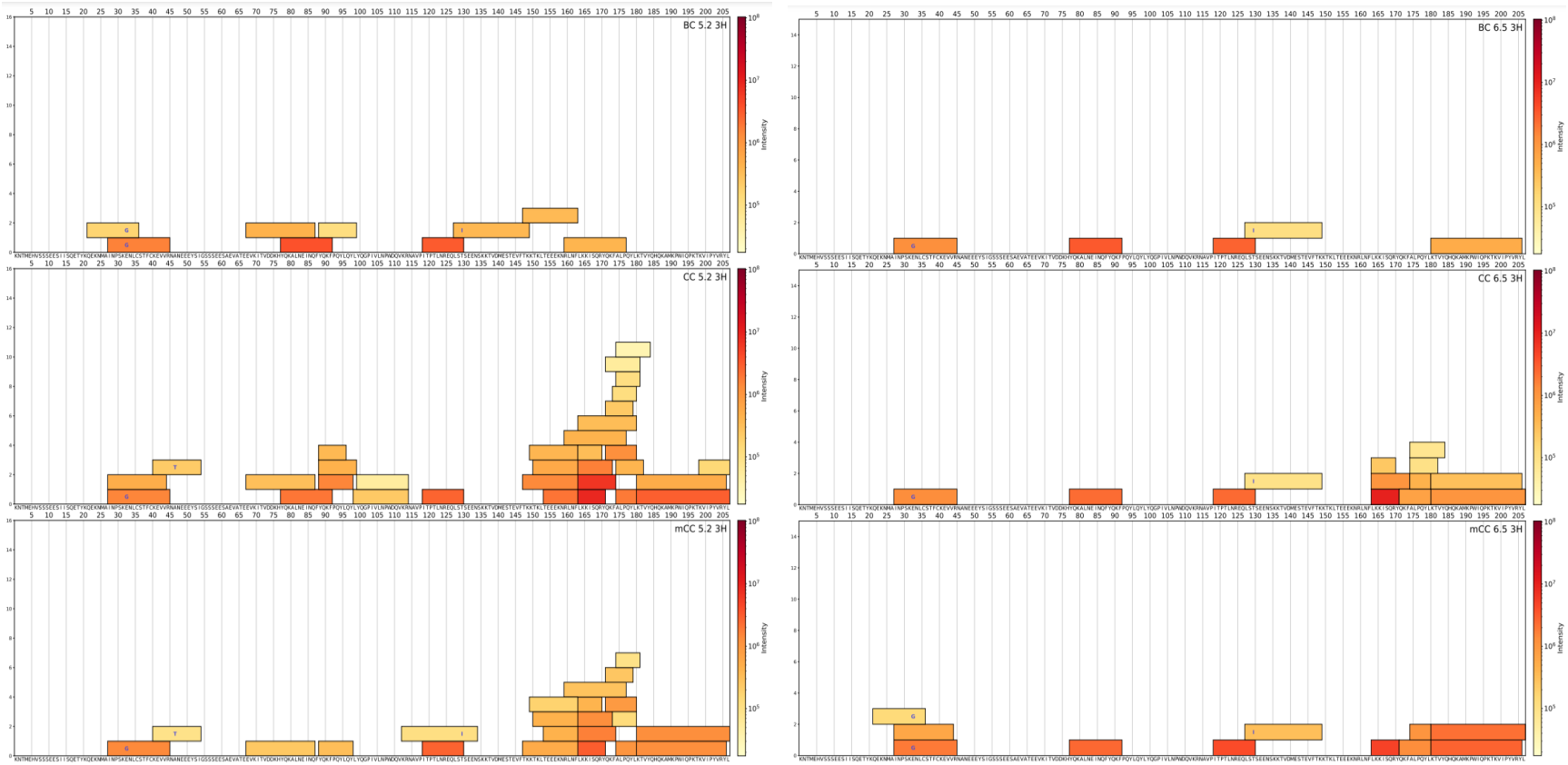
Fig. S1. Continued.

B a

BC

CC

mCC



pH 5.2 with 5% NaCl

pH 6.5

Fig. S1. Continued.

B b

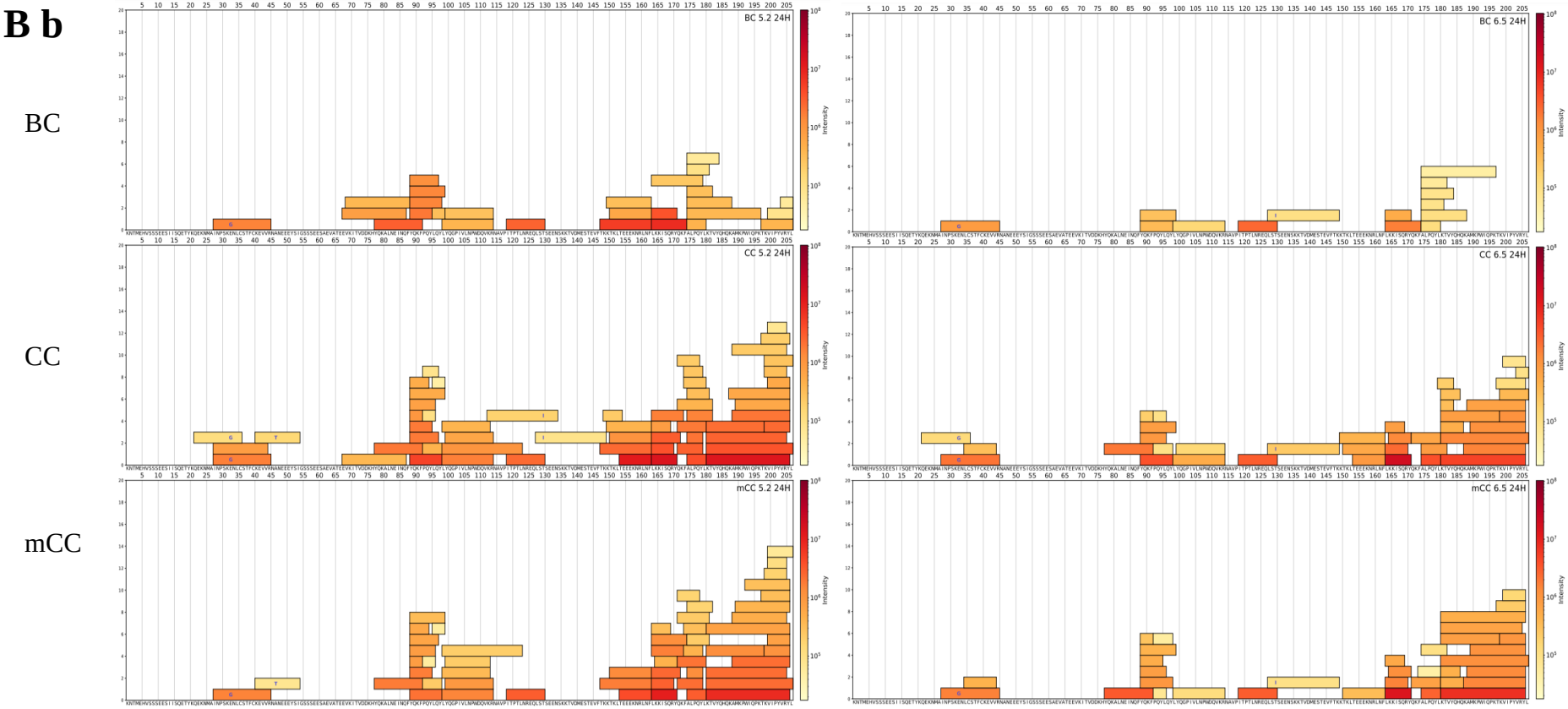
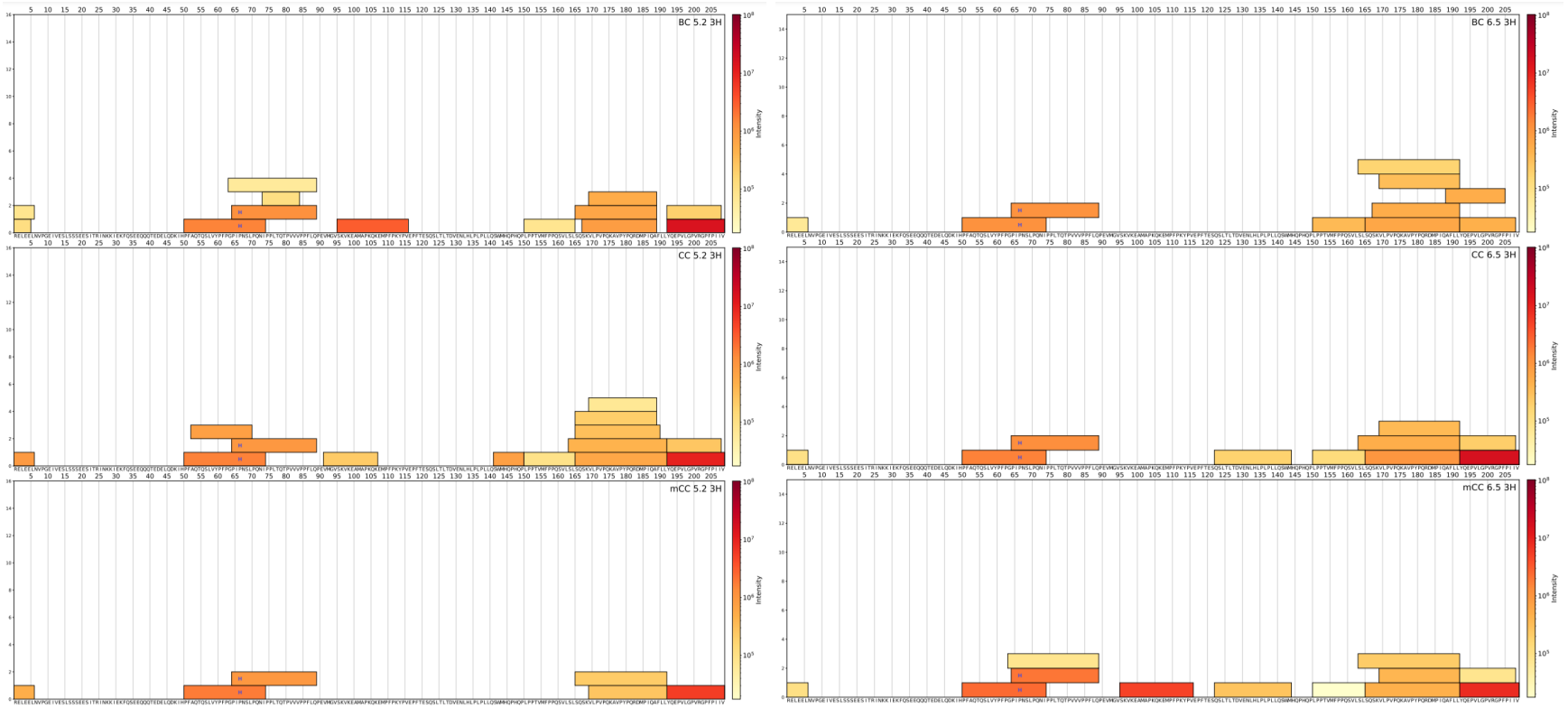


Fig. S1. Continued.

C a



pH 5.2 with 5% NaCl pH 6.5

Fig. S1. Continued.

C b

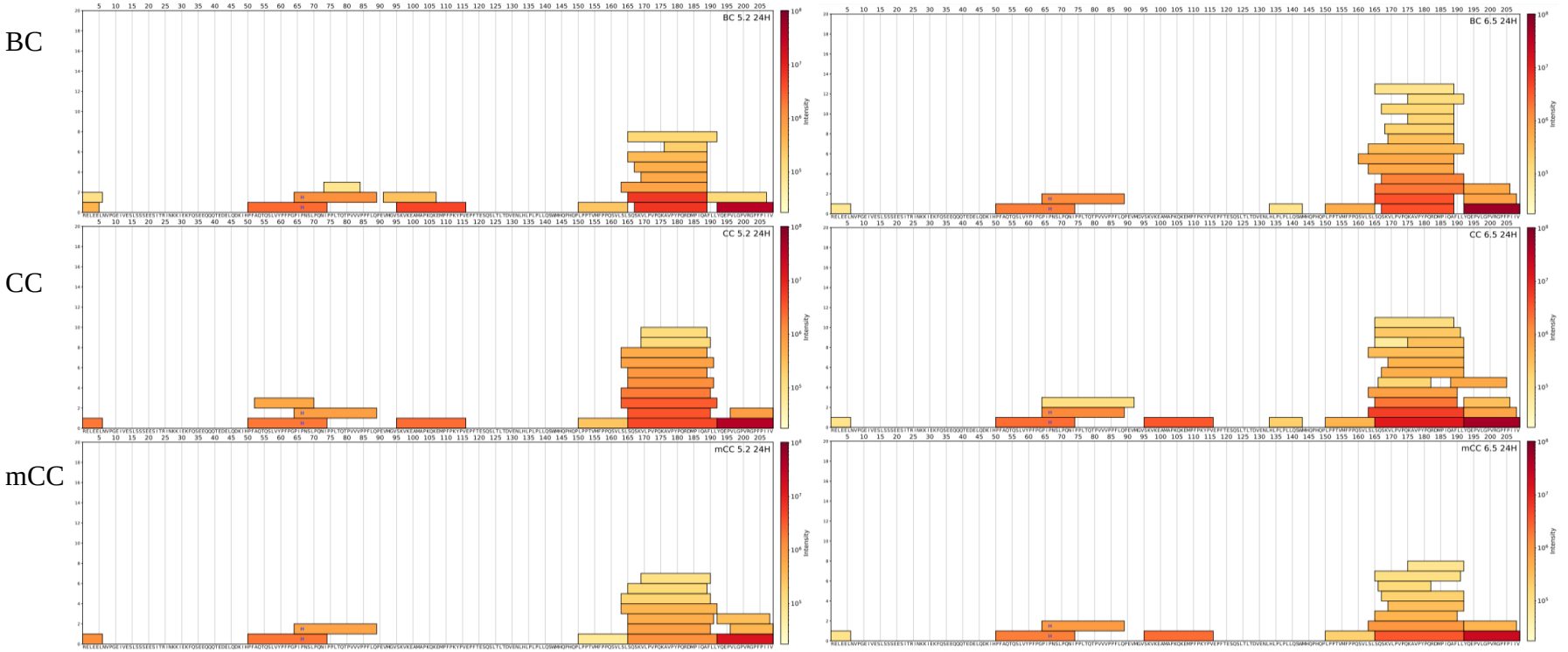
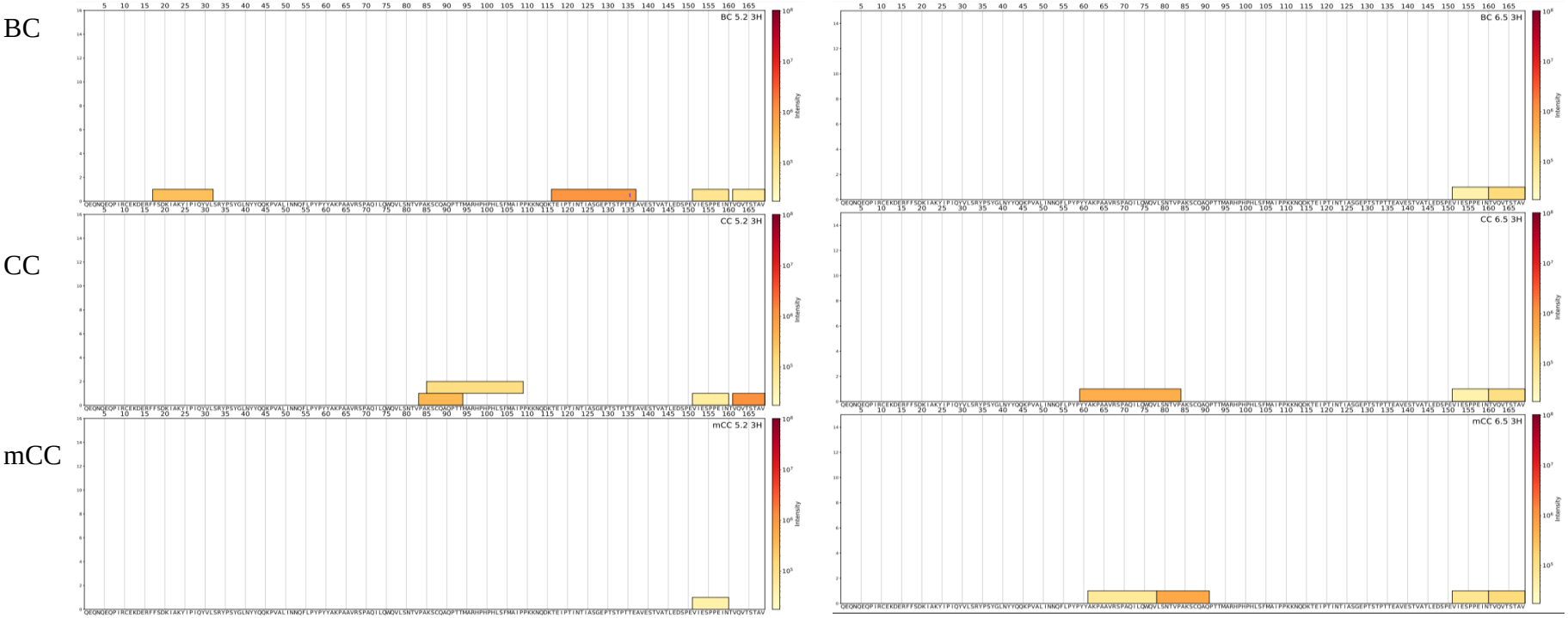


Fig. S1. Continued.

D a



pH 5.2 with 5% NaCl pH 6.5

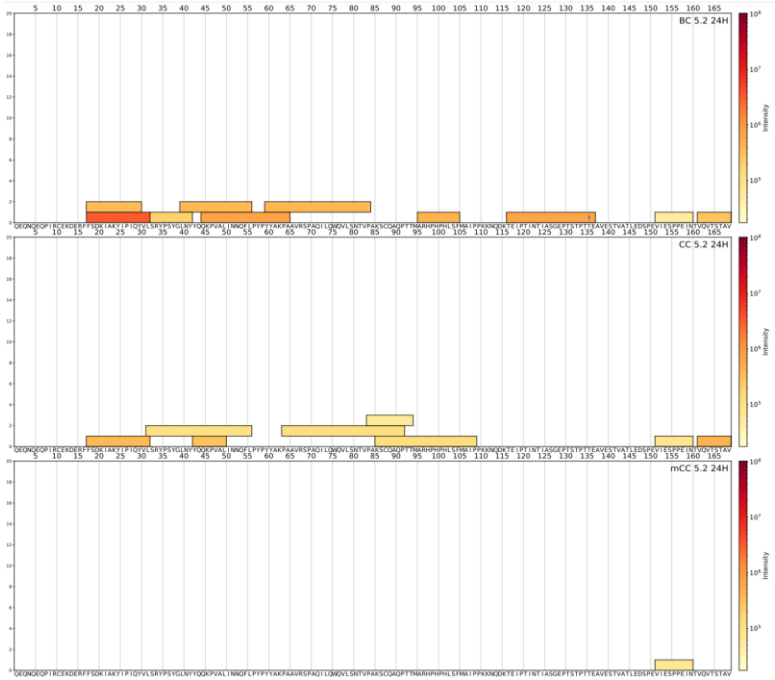
Fig. S1. Continued.

D b

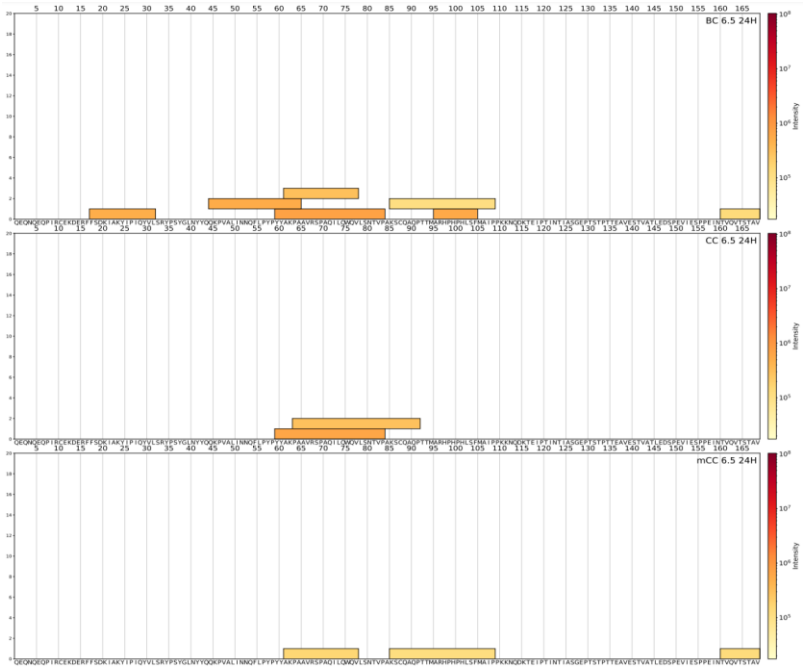
BC

CC

mCC



pH 5.2 with 5% NaCl



pH 6.5

Fig.S1. Continued.

CHAPTER 7

Conclusions and Recommendations

Declaration

This chapter was written by author BL and reviewed by his co-authors.

Chapter 7

7.1 Novel cheese manufacturing material

Cheese manufacture can be traced back to about 8000 years ago and the manufacture procedures for different varieties of cheese are nowadays quite well established. The applications of novel streams are of continuing interest recently and may further promote the manufacture of cheese, or adaptation of cheese manufacture for the recovery or enrichment of whey protein and glycomacropeptides (GMP).

The use of protein ingredients for cheese manufacture has been studied since 1970, when low heat skim milk powder (LHSMP) was used for the fortification of protein content and enhancement of cheese yield to improve inconsistent cheese production caused by seasonality and lactation of bovine milk (Freeman, Bucy, & Hassan, 1970; O'Brien, Mehra, Connolly, & Harrington, 1999). Sodium caseinate (NaCN) powder has also been used for the fortification of milk protein (Moynihan, 2015).

There is an interest in the manufacture of cheese from material produced by membrane separation. Microfiltration (MF) retentates (micellar casein concentrate, MCC) are the co-product of MF permeate, or native whey protein, which is a more suitable material to produce infant formula than whey recovered from cheese manufacture. MCC is used not only in functional and nutritional fields but also for dairy product manufacture, including cheese, high-protein bars and Greek-style yoghurt (Bong & Moraru, 2014; Crowley et al., 2018; Hogan, Chaurin, O'Kennedy, & Kelly, 2012; Li et al., 2020; Chapter 3). Unlike using milk as the starting material for cheese manufacture, the yield of cheese manufactured from

reconstituted MCC may not be affected by the seasonality and lactation of bovine milk due to the consistency of MCC (Sauer & Moraru, 2012).

When assessing milk's potential for making cheese, the rennet and acid coagulation qualities are crucial (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). The rennet and acid coagulation properties of MCC were analysed in this study to evaluate the cheese manufacture potential of this dairy material (Chapter 2). Cheddar- (Chapter 3) and Quarg- (Chapter 4) type cheeses that are representative of rennet and acid cheese, respectively, were manufactured from MCC, and LHSMP was used for comparison.

The presence of denatured whey protein impairs the rennet coagulation properties of milk since the whey protein-casein complex impedes the access of enzymes to casein and inhibits aggregation of renneted micelles (Vasbinder, Rollema, & De Kruif, 2003). Native whey protein in milk also presents a physical barrier to aggregation between *para*-casein micelles (Gamlath, Leong, Ashokkumar, & Martin, 2018). Compared to LHSMP, a higher casein fraction of total protein was presented in MCC (85-95%) as MF fractionates whey protein into permeates (Carter, Cheng, Kapoor, Meletharayil, & Drake, 2021; Crowley et al., 2018; Table 2.1). At the same casein concentration, less whey protein is thus present in reconstituted MCC compared to reconstituted LHSMP; this may be linked to a shorter rennet coagulation time (RCT) and a higher gel strength of MCC compared to LHSMP (Table 2.2). Although no significant differences ($P > 0.05$) were found in hardness between all cheese samples at 180 days of ripening, at 60 days of ripening, the Cheddar cheese manufactured from MCC showed higher hardness compared to the cheese manufactured from LHSMP (Fig. 3.5), which is consistent with the rennet coagulation properties of MCC and LHSMP.

Due to the low whey protein fraction in total protein, reconstituted MCC may be more resistant to changes induced by heating compared to LHSMP, as the potential for association between denatured whey protein and casein is reduced. High-heat treatment (90 ℃ for 15 s) to MCC does not impaire the functionality profiles of cheese manufacture therefrom (Xia et al., 2022a).

The principle of acid-induced coagulation involves decreasing the pH of the milk to the isoelectric point of casein (pH 4.6). At a pH of around 4.6, the negative charges on the surface of casein are neutralised, as the protein reaches its isoelectric point, and this initiates the aggregation of casein micelles (Mulvihill & Grufferty, 1995). The acid coagulation properties of MCC were determined using glucono-δ-lactone (GDL) and lactic acid bacteria (LAB) in Chapter 2 and Chapter 4, respectively.

Unlike LHSMP having a gelation pH of around 4.8, MCC showed initiation of gelation (storage modulus, G' > 1) at a pH greater than 5 (Fig. 2.6; Fig. 4.1). This may be attributed to the reduced impediment to the formation of a casein network by native whey protein in reconstituted MCC compared to LHSMP. A similar phenomenon has been reported in rennet coagulation tests (Gamlath et al., 2018). In addition, the removal of micellar minerals during diafiltration in the manufacture of MCC may increase the gelation pH of the sample (Li & Corredig, 2020).

When acidified using GDL or LAB, the increase in G' value of all warm MF retentates both stopped at a pH around 5.1 (Fig. 2.6; Fig. 4.1), and then decreased, in the analysis using GDL, or increased, in the analysis using LAB. This may be attributed to the different rates of acidification between GDL and LAB. GDL is hydrolysed to gluconic acid and reduces pH rapidly, while LAB changes pH

slowly initially and decreases pH gradually with incubation time afterwards (Lucey, 2016). The interruption of increase in G' value may be caused by bridges between casein micelles and early formed whey protein-casein aggregation or the solubilisation of CCP (Anema, 2009; Li & Corredig, 2020; Lin, Kelly, O'Mahony, & Guinee, 2018). This phenomenon became less notable in cold MF retentates compared to warm MF retentates, as the CCP content was lower in cold MF retentates (Xia et al., 2022b).

Texture analysis was carried out on acid gels and Quarg cheese manufactured from MCC and LHSMP (Fig. 2.7; Table 4.3). The hardness of gels formed from warm MF retentates at pH 5 was significantly higher ($P < 0.05$) than that at pH 4.6, which confirmed the decrease in G' values of warm MF retentates from pH 5 to pH 4.6. The texture analysis showed that, at pH 5, relatively stronger acid-induced gels were formed in all MF retentates compared to LHSMP, which is consistent with the gelation pH (> pH 5) as measured by dynamic oscillatory analysis. Also, at pH 4.6, the typical pH value in Quarg cheese (Kelly & O'Donnell, 1998), the hardness was significantly lower ($P < 0.05$) in both warm MF retentates compared to that in LHSMP.

However, perhaps caused by the different acidification (GDL or starter) and processing (cutting and draining), the textural and rheological properties of treatments in acid gel or Quarg cheese were different (Fig. 2.7; Table 4.3). The hardness of Quarg cheese manufactured from polymeric warm MF retentate was significantly higher ($P < 0.05$) than that of the other treatments and no significant differences ($P > 0.05$) in hardness were found between the other treatments. All Quarg cheese samples showed a loss tangent (tan δ) value within 0.27-0.32, which indicates that the samples were weak gels (Ikeda & Nishinari, 2001). The

differences between G' and loss modulus (G") values at 0.1 Hz of cheese manufactured from warm ceramic membrane MF retentates were significantly higher ($P < 0.05$) than that of cheese made using LHSMP. No significant differences ($P > 0.05$) were found in the separation between G' and G" values at 10 Hz for all treatments. The differences between the texture and rheological properties of cheese manufactured from all treatments were small, which indicates that Quarg cheese manufactured from MCC is comparable with that from LHSMP.

Differences were found in the composition of Quarg cheese manufactured from polymeric warm MF retentate and LHSMP (Table 4.2). The significantly lower ($P < 0.05$) yield and moisture content in cheese manufactured from polymetric warm MF retentate indicate that higher levels of syneresis took place during the manufacture of cheese, which may be linked to the alteration of the gel network (Cayot, Fairise, Colas, Lorient, & Brulé, 2003; Gastaldi et al., 2003). Also, the possibility that rearrangement of the casein aggregate network occurs at a pH value of around 5 was confirmed in the acid coagulation property analysis (Chapter 2), along with the release of CCP, hydration and disaggregation of casein, and condensing of gel structure (Li & Corredig, 2020; Lin et al., 2018; Lucey, 2016; Meletharayil et al., 2015). High heat-treatment increased the yield of cheese manufactured from LHSMP, while it did not affect that of cheese manufactured from MCC, as the whey protein level was relatively lower in MCC compared to LHSMP.

The protein content in cheese whey manufactured from all pasteurised MF retentate was significantly lower ($P < 0.05$) than that from pasteurised LHSMP (Table 4.2) due to the lower whey protein content in all MF retentates material compared to that in LHSMP (Table 2.1), while the GMP content in cheese whey

manufactured from MCC was relatively higher than that from LHSMP (Table 4.4). GMP has many biological properties and anti-inflammatory abilities relevant to several diseases (Córdova-Dávalos, Jiménez, & Salinas, 2019). At pH 4.5-5.0, up to 98 % of GMP can be recovered from cheese whey by anion-exchange chromatography (Tek, Turhan, & Etzel, 2005). Recovering GMP from cheese whey manufactured from MCC may result in a higher yield at the same casein content compared to LHSMP.

The results of using MF rententates as a material for cheese manufacture offer an option for utilising MF retentates. The cost of membrane filtration is probably much more expensive compared to the profit of cheese production. However, the manufacture of whey protein may not avoid membrane filtration and the profit of cheese production may be a bonus benefit of whey protein manufacture. In addition, compared to the regular cheese manufacture (from milk), making cheese using MF retentates could be more consistent, standardised, and is not affected by the lactation or seasonality of bovine milk.

Overall, the use of novel casein-dominant dairy streams such as MCC has the potential for the production of rennet and acid cheese with tailored functionality. Cheddar cheese manufactured from MCC showed higher meltability compared to cheese made using milk or LHSMP. The Quarg cheese manufactured from MF retentates showed modified texture and higher GMP content in cheese whey compared with the use of LHSMP. The results of this study suggest that using reconstituted MCC for the manufacture of cheese may be feasible, and may in addition help the production or recovery of other dairy products.

7.2 Novel milk coagulants

Chapter 7

Originating from the abomasum of ruminant mammals, rennet is an enzyme preparation that coagulates milk. Chymosin is the main enzyme in most rennets that hydrolyses the peptide bond Phe_{105}-Met_{106} of κ-casein, thereby reducing electrostatic stabilisation and steric stabilisation. Without these forms of stabilisation, a casein micelle network is formed, which initiates the coagulation of milk (Cheryan, Van Wyk, Olson, & Richardson, 1975; Morr, 1975). This property of chymosin is a key aspect of cheese manufacture, and bovine chymosin is the most commonly used chymosin for cheese-making.

In cheese manufacture, it is ideal for a coagulant to have low proteolytic activity, with a high preference at or near the peptide bond Phe_{105}-Met_{106} of κ-casein, and that is easy to inactivate in whey (Bansal et al., 2009). Camel chymosin, having 85% amino acid sequence identity to bovine chymosin, is 70% more active at milk clotting than bovine chymosin, while it has only 25% of the hydrolytic activity of bovine chymosin generally (Kappeler et al., 2006; Langholm Jensen et al., 2013). The manufacture of Cheddar and Mozzarella cheese using camel chymosin has been studied, and lower proteolytic activity was found in cheese manufactured using camel chymosin (Bansal et al., 2009; Moynihan et al., 2014). The Cheddar cheese manufacturing properties (Chapter 5) and proteolytic specificity on casein (Chapter 5 and 6) of a fermentation-produced camel chymosin with structural molecular changes (mCC) were studied in this section of the thesis; fermentation-produced bovine chymosin (BC) and camel chymosin (CC) were used for comparison.

Minor differences were found in the composition of cheese manufactured from all the coagulants, indicating the use of different coagulants did not affect the manufacturing process of cheese (Table 5.2).

The coagulation/proteolysis (C/P) ratio, which is the ratio of milk coagulating activity to the general proteolytic activity, is related to the specificity of enzyme activity (Soodam, Ong, Powell, Kentish, & Gras, 2015). Cheese yield is an important property to consider and may be decreased as the degree of proteolysis increases or the C/P ratio decreases during cheese manufacture (Fox & McSweeney, 1997; Soodam et al., 2015). No significant differences ($P > 0.05$) were found in the yield between cheeses manufactured using all the coagulants (Table 5.2), which is in agreement with the results of Bansal et al. (2009).

At the same level of activity (IMCU), no significant differences were found in the gel strength (G') at 45 min between the treatments (Table 5.1; Fig. 5.1), which indicates that the milk coagulating activity is the same for all the coagulants. BC, CC and mCC were therefore used at the same level (0.06 IMCU mL^{-1}) for cheese manufacture. However, different levels of coagulants sufficient to hydrolyse all intact α_{S1}-CN within 24 h were used for NaCN digestion. The general proteolytic activity was analysed through pH 4.6-soluble nitrogen/total nitrogen, urea-polyacrylamide gel electrophoresis and liquid chromatography-mass spectrometry on cheese samples (Table 5.2, Fig. 5.2, Fig. 5.3) and NaCN digests (Fig. 6.1, 6.2, Table 6.1). The lowest levels of proteolysis were found in cheese manufactured using mCC, followed by CC and BC. The highest level of mCC was required to hydrolyse all intact α_{S1}-CN within 24 hours, followed by CC and BC. At equal milk-coagulating activity, the general proteolytic activity of mCC may be the lowest among the three coagulants which indicates that mCC is more specific for milk-coagulation compared to BC and CC.

Major casein-derived peptides and the corresponding cleavage sites by bovine and camel chymosins reported in the literature were confirmed in this study. In

addition, more cleavage sites that produce peptides with lower intensities were found to be hydrolysed by bovine and camel chymosins. The cleavage specificity of modified camel chymosin was determined (Fig. 6.4-6.7). Most of the peptides identified in NaCN digests were also observed in the corresponding cheese samples.

Proteolysis affects the functionalities and flavour of cheese (Børsting et al., 2012; Lucey, Johnson, & Horne, 2003). The overall number of pH 4.6-soluble peptides identified in cheese manufactured from all the coagulants was similar, while the summed intensities of identified peptides decreased from cheese BC to CC to mCC. Peptides with a high response from each casein appeared to be common for each cheese type mostly, while different intensities were observed (Table 5.4-5.7). The different levels of proteolysis may be linked to the differences in the texture and meltability of cheese samples. The lower levels of primary proteolysis in cheese caused by camel chymosin and modified chymosin may be responsible for the higher hardness, springiness, and cohesiveness compared to cheese BC (Bansal et al., 2009; Li et al., 2022; Moynihan et al., 2014; Chapter 5).

Meltability of cheese was analysed using the Schreiber test and dynamic small-amplitude oscillatory rheology at two different temperatures. At lower temperatures (<80 ℃), compared to cheeses BC and CC, the cheeses made with mCC showed a higher maximum loss tangent (LT_{max}) value (Table 5.9), which is correlated to the meltability of cheese (Lucey et al., 2003). Udayarajan, Lucey, & Horne (2005) reported that the index LT_{max} is highly frequency-dependent; therefore, LT_{max} may be used with caution to represent meltability. In contrast, cheeses made with BC showed greater meltability at high temperatures (232 ℃), followed by cheese CC and mCC (Fig. 5.5) as the meltability of cheese increases

with the breakdown of α_{S1}-CN and β-CN (Bogenrief & Olson, 1995; Dave, McMahon, Oberg, & Broadbent, 2003). Higher levels of breakdown of α_{S1}-CN and β-CN in cheese BC compared to cheese CC and mCC were confirmed in Chapter 6.

Peptide β-CN (f193−209), which contributes to bitterness in cheese (Børsting et al., 2012), was present at relatively lower levels in cheese CC (29.7%) and mCC (14.0%) compared to levels in cheese BC (Table 5.6), which may be linked to the decreased bitter taste from cheese BC to CC to mCC (Table 5.9)

Overall, this study investigated the proteolytic specificity of the three generations of chymosin and the proteolysis in cheese manufactured therefrom. With the same level of coagulating activity, the use of modified camel chymosin for cheese manufacture gave lower levels of proteolysis and derived modified functionalities and flavour compared to that of bovine chymosin and camel chymosin, which may be of interest for cheese manufacturers and researchers.

7.3 Recommendations for future work

Novel aspects of cheese manufacture were investigated in this study which might be of interest in fields involving cheese and other dairy products in the future. Several recommendations for future research are proposed

- Quarg cheese whey made from MCC has relatively higher GMP content compared to that from LHSMP. Investigation of GMP recovery therefrom and measurement of GMP content of cheese whey of other types of cheese made from MCC is encouraged;
- The yield measurement was limited by the small scale of pilot-scale cheese manufacture facilities available. Larger scale of Cheddar cheese

and Quarg cheese manufacture using modified camel chymosin or MCC for determination of yield are of interest for industrial manufacture;

- Sensory analysis on cheeses manufactured using MCC was not conducted as it was limited by the scale of cheese manufacture in this study. It is of interest to evaluate the effect of using MCC as a new material for cheese on sensory properties;
- The use of MCC makes it possible to manufacture cheese from material that has undergone heat treatment with a higher temperature and longer time than pasteurisation. Evaluation of rennet-coagulated cheese manufactured from high-heat treated MCC is warranted as the cost and processing time of the production for cheese or other dairy products may be saved when the dairy material can be stored stably for a longer time;
- There may be a potential for manufacturing acid cheeses from MCC at a higher cutting pH value than 4.6, which may shorten the production time;
- Although the proteolytic activity of the three types of chymosins are determined in this study, it may be useful to determine the level of residual chymosin in cheese manufactured from different coagulants as different levels of proteolysis were presented in those cheeses;
- The proteolytic activity of camel chymosin with modified structure is lower than that of bovine chymosin and camel chymosin. Future work may be focused on the influence of the structure of modified camel chymosin on the proteolysis of caseins;
- Since the proteolytic activity of camel chymosin is lower than that of bovine chymosin, using camel chymosin to coagulate reconstituted MCC for cheese manufacture might be useful to decrease the level of

proteolysis in cheese manufactured from MCC.

Overall, more knowledge needs to be learned about novel dairy materials and milk coagulants, as there is a potential for improvement and combination of the production of dairy products like cheese, whey protein, and GMP. The research described in this thesis will support the innovation of new materials for the manufacture of cheese and other dairy products and add to the understanding of the properties of three generations of chymosin when used in cheese manufacture.

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Chapter 7

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Appendix

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Evaluation of production of Cheddar cheese from micellar casein concentrate

Bozhao Li [a], David S. Waldron [a], John T. Tobin [b], Surabhi Subhir [b], Alan L. Kelly [a, *], Paul L.H. McSweeney [a]

[a] School of Food and Nutritional Sciences, University College Cork, College Road, Cork, T12 K8AF, Ireland
[b] Department of Food Chemistry and Technology, Teagasc Food Research Centre, Fermoy, Co. Cork, P61 C996, Ireland

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ABSTRACT

The production of Cheddar cheese using micellar casein concentrate (MCC), a novel milk ingredient powder with a high casein content (~92%), was evaluated. Four types of Cheddar cheese were manufactured and ripened for 180 days from the following starting materials: standardised control milk (control), skim milk with cream (SC), reconstituted MCC with cream (MC) and reconstituted low-heat skim milk powder with cream (PC). Only minor differences were found in composition between treatments, but MC cheese showed higher levels of proteolysis compared with other treatments, linked to significantly higher plasmin and chymosin activities. No differences were observed in hardness between treatments (60, 120 and 180 days), but the springiness and cohesiveness of MC and PC cheeses were significantly higher than that of the control and SC cheeses at 60, 120 and 180 days. The use of casein-dominant dairy streams thus has the potential for production of Cheddar cheese with tailored functionality.

1. Introduction

Global milk production was approximately 843 million tonnes in 2018 (FAO, 2019); among dairy products (i.e., cheese, casein and butter), cheese production used the highest proportion of milk. Whey protein powder, production of which is traditionally associated with the manufacture of cheese, represents 57% of the market for global protein supplement for exercising and nutrition, while there is a huge market for whey protein powders in infant formula. Cheese output is increasing at a rate of 2% yearly while the demand for whey protein has been growing at 6–7% yearly (Hoogwegt Group, 2019). Nowadays, whey protein with high quality is required as it can be used to manufacture a range of food ingredients or products with nutrition and functional properties (Boland, 2011). Kelly (2019) reported that the ultrafiltration (UF) properties of sweet and acid whey are influenced by their high or low pH, respectively. However, microfiltration (MF) permeate made directly from skim milk is considered to be an ideal whey source for whey protein ingredient production. Therefore, recovering whey protein from milk rather than cheese whey could improve the whey quality and is an option for whey protein ingredients manufacture.

During microfiltration of skim milk, native micellar casein is concentrated in the retentate, which could be recovered and concentrated to produce micellar casein concentrate (MCC). This is a novel dairy ingredient powder with a high casein fraction of 85–95% of total protein which may be used in functional and nutritional applications (Crowley et al., 2018). As the traditional way to manufacture Cheddar cheese is followed by whey protein manufacture, the concept of recovering whey protein before Cheddar cheese manufacture is of interest.

In terms of the protein ingredients being used for Cheddar cheese manufacture, low heat skim milk powder (LHSMP) can be used to enhance cheese yield giving a constant cheese production throughout the year (Freeman, Bucy, & Hassan, 1970). LHSMP is produced from skim milk using low temperatures during manufacture and is mostly used for condensed milk, UHT-treated fluid milk and ice-cream (Augustin & Margetts, 2003). Unlike reconstituted medium- and high-heat skim milk powder that have impaired rennet coagulation ability, the rennet coagulation properties of LHSMP are good as the whey protein is not highly denatured (Ménard, Camier, & Guyomarc'h, 2005). With only physical separation processing, the micellar casein in MCC may have better

* Corresponding author. Tel.: +353 21 4903405.
E-mail address: a.kelly@ucc.ie (A.L. Kelly).

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rennet coagulation properties compared to LHSMP. The rennet coagulation properties and cheese-making potential of MCC may be better than those of LHSMP.

Two main factors that influence cheese yield are lactation and seasonality. The gross composition of bovine milk varies with the stage of lactation. Kuchtík, Šustová, Urban, and Zapletal (2008) reported that protein and casein content increase and lactose content decreases through lactation. After 200 days lactation, cows are in the late lactation stage, which requires the udder tissue to repair and recover for the next lactation. During late lactation, milk yield decreases dramatically, which may influence cheese yield, unless milk is standardised. Also, at specific times of the year, the milk volume decreases, and the composition and rennet coagulation properties of milk are significantly influenced (Freeman et al., 1970; O'Brien, Mehra, Connolly, & Harrington, 1999). Both cheese yield and manufacturing efficiency are influenced by seasonal variation of milk protein and fat composition (Barbano & Sherbon, 1984).

To solve the problem of low cheese yield, one solution is adding LHSMP to low-protein milk to increase the protein composition (Freeman et al., 1970). Another possibility may be making Cheddar cheese with MCC that is manufactured as a co-product of high-quality whey protein.

This study evaluated the production of Cheddar cheese from micellar casein concentrate, with LHSMP used for comparison. The consequence of this manufacture was evaluated concerning composition, proteolysis, texture and functionality.

2. Materials and methods

2.1. Preparation of cheese milk

Micellar casein concentrate (MCC) powder was obtained from Teagasc (Moorepark, Fermoy Co. Cork, Ireland) as follows; pasteurised bovine skim milk was microfiltered at 50 °C using 0.14 μm TAMI Isoflux ceramic membranes (TAMI, Lyons, France). MF was performed in a batch mode involving two diafiltration steps to produce a final 3× MF retentate (liquid MCC) which was evaporated at 65 °C using a pilot plant single-effect falling-film evaporator (Anhydro F1 Laboratory; Copenhagen, Denmark). This was followed by spray-drying of the evaporated liquid MCC in a single-stage spray dryer (Anhydro Laboratory Spray Dryer, SPX Flow Technology, Kolding, Denmark) equipped with nozzle atomisation at inlet and outlet temperatures of 185 and 85 °C, respectively. The protein content (91.6% casein) of MCC was determined by the Kjeldahl method (IDF, 1986). MCC powder and low heat skim milk powder (LHSMP; WPNI = 6.0) (Uelzena, Uelzen, Germany) were rehydrated using a Silverson mixer at 55 °C for 2 h.

Raw milk was obtained from a local farm in Cork, Ireland. Skim milk and cream were separated from raw milk using a separator. Composition of milk samples was measured by MilkoScan™ Mars (Foss, Hillerød, Denmark) and casein content was calculated by multiplying the casein percentage of protein of the milk sample (an estimated percentage of 78% for milk and LHSMP milk and 91.6% for MCC). Control milk was prepared by combining raw milk and skim milk to a casein: fat ratio of 0.7:1. Both reconstituted MCC and reconstituted LHSMP were made up to the same casein level as skim milk. Reconstituted MCC and LHSMP and skim milk were blended with cream and lactose according to the same standardization ratio (casein: fat of 0.70:1.00) and lactose content (~5%) as control milk to obtain three more vats: skim milk with cream (SC), reconstituted MCC milk with cream (MC) and reconstituted LHSMP milk with cream (PC). All milk samples were pasteurised at 72 °C for 15 s (Microthermics Inc., Raleigh, NC, USA) and stored at 4 °C until analysis and cheese-making.

2.2. Rennet coagulation properties

Dynamic oscillatory analysis (small amplitude oscillatory measurement) of renneted control milk, SC milk, MC milk and PC milk was performed using a Peltier concentric cylinder geometry, which comprised of an aluminium conical rotor [42.01 mm (h) by 28.02 mm (d)], on an AR-G2 controlled stress rheometer (Waters TA Instruments, Leatherhead, Surrey, UK). Aliquots (25 mL) of each sample were pre-warmed in a water bath at 32 °C for 15 min, and 80 μL of a 1:10 (v/v) dilution of Maxiren™ (Chr Hansen A/S, Hørsholm, Denmark) was added. The sample was placed immediately in the preheated cup (32 °C), the frequency of oscillation was set at 0.6283 rad s^{-1}, and the storage modulus, G′ of the sample was recorded continuously as a function of time at a low-amplitude shear strain (0.01) over 90 min. Each sample was analysed in triplicate (Ibáñez, Waldron, & McSweeney, 2015).

2.3. Cheese manufacture

Cheddar cheeses were made from 20 L control, SC, MC and PC milks which were prepared, pasteurised and stored at 4 °C overnight before cheese-making. Cheddar-type cheeses were manufactured according to a standard Cheddar cheese manufacture protocol (Fox, Guinee, Cogan, & McSweeney, 2000). An aliquot of 6 g (0.03%, w/w) of Cheddar cheese starter culture (R604Y Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added into each vat and allowed ripen for 30 min, followed by 0.09% (v/w) of 1 mol L^{-1} $CaCl_2$ and 60 IMCU L^{-1} rennet being added to all samples. Whey was drained when the pH dropped to 6.2 and curd was cheddared. When the pH decreased to 5.4, the curd was milled into small pieces and salted at a level of 2.5% (w/w) NaCl. The curds were then wrapped in cheesecloth and moved to 2-kg circular moulds, which were pressed at a pressure of 2.5 kg cm^{-2} for 14 h. The cheeses were then removed, vacuum-packed and ripened at 8 °C for 180 days.

2.4. Compositional analysis

Gross composition of cheeses was analysed at 14 days old. Moisture was determined by oven-drying method (IDF, 1982) fat by the Gerber method (1955), protein (%N × 6.38) by the Kjeldahl method (IDF, 1986) and salt was analysed by titration with $AgNO_3$ (Fox, 1963). The pH was measured at 14, 30, 60, 120 and 180 days of ripening by using a calibrated pH probe placed in contact with dry grated cheeses. All results were determined in triplicate.

2.5. Microbiological analysis

Enumeration of starter lactic acid bacteria (LAB) was performed using LM 17 agar plates (Terzaghi & Sandine, 1975), incubated for 3 days at 30 °C. Enumeration of non-starter lactic acid bacteria (NSLAB) was performed on Rogosa agar plates (Rogosa, Mitchell, & Wiseman, 1951), incubated anaerobically for 5 days at 30 °C. Enumeration of LAB and NSLAB were performed in duplicate after 60 and 90 days of ripening.

2.6. Analysis of proteolysis

Urea-polyacrylamide gel electrophoresis (urea-PAGE) of cheese samples was used to study the proteolysis of α_{S1}- and β-casein (CN) during ripening using the method of Andrews (1983) with modifications of Shalabi and Fox (1987). The pH 4.6-soluble and insoluble fraction were prepared (Kuchroo & Fox, 1982), and peptide profiles of pH 4.6-soluble fractions filtered through 0.22 μm cellulose acetate filters (Sartorious GmbH, Gottingen, Germany) were analysed by reverse-phase high-performance liquid

chromatography (HPLC) using an ultra-performance liquid chromatography (UPLC) Waters Acquity UPLC H-Class Core System (Waters Corp, Milford, MA, USA), with a Waters Acquity UPLC TUV Detector (dual-wavelength; Waters Corp) operated by Empower 3 software (Waters Corp), following the method of Mane, Ciocia, Beck, Lillevang, and McSweeney (2019).

For determination of free amino acids (FAA), frozen pH 4.6-soluble fractions were de-proteinised by mixing equal volumes of 24% (w/v) trichloroacetic acid (TCA) and sample and following the method of Fenelon and Guinee (2000).

2.7. Plasmin activity

The plasmin activity of cheese samples at 180 days of ripening was measured using the coumarin peptide method (Richardson & Pearce, 1981). A standard curve of the emission intensity at 460 nm was constructed using 7-amido-4-methyl coumarin (AMC), and results expressed in nmol AMC min^{-1} mL^{-1}, which was defined as one unit of plasmin activity.

2.8. Residual coagulant assay

Grated cheese samples (50 mg) were extracted by dissolving cheese in 1 mL of 0.1 M trisodium citrate, followed by 30 min incubation at 37 °C. Fat was separated by centrifugation at 1000 × *g* (Sigma 1-16K, Harz, Germany) for 1 min, and the aqueous layer was used for analysis. An aliquot of 70 μL citrate dispersion of cheese was incubated with 30 μL of 1 mg mL^{-1} aqueous solution of a synthetic heptapeptide substrate (Pro–Thr–Glu–Phe–[NO_2–Phe]–Arg–Leu) in 400 μL 0.1 M sodium formate buffer, at 37 °C, pH 3.2, for 24 h. The mixture was heated at 70 °C for 10 min to stop the reaction, followed by centrifugation at 16,000 × *g* for 10 min, and the supernatant was used for UPLC analysis. Substrate and product levels were determined using the reversed-phase HPLC system described above, following the method of Hurley, O'Driscoll, Kelly, and McSweeney (1999).

2.9. Texture profile analysis

Texture profile analysis was performed using a Texture Analyzer TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) at 60, 120, and 180 days of ripening. Cheese samples were cut into 20 mm height, 20 mm diameter cylinders and kept at 4 °C overnight. Cheese cylinders were compressed to 25% of the initial height in two continuous compressions with a speed of 1 mm s^{-1}. Hardness, cohesiveness and springiness were measured (Truong, Daubert, Drake, & Baxter, 2002), and five cheese samples were measured for each treatment.

2.10. Meltability

Meltability was analysed using the Schreiber meltability test as described by Altan, Turhan, and Gunasekaran (2005). Cheese samples were heated at 232 °C for 5 min, and meltability was measured as the percentage increase in diameter of the original samples. Analyses were performed in triplicate at 60, 120, 180 days of ripening.

2.11. Dynamic small amplitude oscillatory rheology

The rheological properties of meltability of cheese after 180 days ripening were analysed with a controlled stress AR-G2 rheometer (TA Instruments, Waters LLC, Leatherhead, Surrey, UK) according to the method of Ibáñez et al. (2015). Storage modulus (G′), loss modulus (G″), and loss tangent (LT) were measured during heating. The maximum LT (LT_{max}) and the temperature where LT = 1, which are indicators of melting, were also recorded. Each sample was analysed in triplicate.

2.12. Colour measurements

Colour values were measured using a Konika-Minolta colourimeter CR400 (Konika-Minolta Optics Inc., Osaka, Japan) at 14, 30, 60, 120, 180 days of ripening. The measurement used the CIELAB system based on illuminant D65 and a visual angle of 2°. Five random readings were taken on fresh-cut cheese at 20 °C. The Euclidean distance between the colour of control cheese and that for other treatments was calculated by:

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where $\Delta L^* = L^*_{sample} - L^*_{control}$, $\Delta a^* = a^*_{sample} - a^*_{control}$ and $\Delta b^* = b^*_{sample} - b^*_{control}$ (Mahy, Van Eyckden, & Oosterlinck, 1994).

2.13. Statistical analysis

Control, SC, MC and PC cheeses were made in three independent trials, 4 × 3 blocks. Statistical analysis was carried out using one-way analysis of variance (ANOVA) with R project for Windows version i386 3.4.0 to establish significant differences between samples. The probability level used for statistical significance was $P < 0.05$.

3. Results and discussion

3.1. Rennet coagulation properties

The rennet coagulation properties of milk samples are shown in Table 1. During the first 10 min, G′ value of all the treatments was low and constant, after which the G′ value of all samples increased, with the MC and PC samples exhibiting a slower rate of increase compared to other samples (Fig. 1). The gel strength (G′ value) at 90 min of MC and PC samples was significantly ($P < 0.05$) lower than that of other samples. The gel strength of MC and PC kept increasing and did not reach a constant level during the analysis, while control and SC reached stable values at 45 min. The loss tangent at 90 min of MC sample was significantly ($P < 0.05$) higher than that of the other treatments.

Martin, Williams, and Dunstan (2010) reported that, during the manufacture of milk protein concentrate (MPC), casein micelles were not damaged; however, the minerals removed through diafiltration and decreased calcium ionic strength could depress rennet coagulation. The rennet coagulation ability of reconstituted MPC could be restored by adding extra calcium ions. The production of MCC involves two diafiltration steps, which may reduce soluble calcium levels; thus, 0.09% (v/w) of 1 mol L^{-1} $CaCl_2$ was added to all of the batches before cheese manufacture.

3.2. Cheese composition and pH

The composition of cheese at 14 days of ripening and pH of cheese samples at 14, 30, 60, 120 and 180 days of ripening are shown in Table 2; the results presented are means of data from the three trials. Guinee, Mulholland, Kelly, and O'Callaghan (2007) stated that different casein to fat ratio can influence the protein and fat content of cheese. As the casein: fat ratio of milk was adjusted to 0.7 for all samples, no statistically significant differences ($P > 0.05$) were found in cheese protein and fat contents

Table 1
Rheological properties of rennet-induced coagulum made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC).[a]

Sample	Gelation time (min)	G′ at 90 min (Pa)	Loss tangent at 90 min
Control	7.81^{a} (0.58)	143.3^{bc} (31.66)	0.26^{a} (0.003)
SC	7.78^{a} (0.71)	169.5^{c} (17.68)	0.27^{b} (0.028)
MC	9.29^{a} (0.73)	74.23^{a} (6.71)	0.29^{c} (0.002)
PC	8.79^{a} (1.15)	91.04^{ab} (10.26)	0.27^{ab} (0.001)

[a] Gelation time refers to the time when G′ value was higher than 1. Values are means, with standard deviation in parenthesis, of three replicate trials; means within the same column not sharing a common superscript letter differ significantly ($P < 0.05$).

between all treatments, and the use of MCC or LHSMP for Cheddar cheese manufacture did not affect these parameters.

The moisture content of PC cheese was significantly ($P < 0.05$) higher than that of SC and MC cheese; no significant differences ($P > 0.05$) were found for moisture content between control, SC and MC cheese. The salt content of MC cheese was significantly ($P < 0.05$) higher than that of control and SC cheese. The pH of MC cheese was significantly ($P < 0.05$) higher than that of other treatments, the reason for which is not clear. No significant differences ($P > 0.05$) were found for moisture in non-fat substances (MNFS) and fat in dry matter (FDM) for all treatments; MNFS is an important parameter for Cheddar cheese quality, so it is important that all batches of cheese sample had similar levels of MNFS (Bogenrief & Olson, 1995; Fox et al., 2000).

3.3. Proteolysis

Table 2 shows the level of pH 4.6-SN/TN (%) of control, SC, MC and PC cheeses at 180 days of ripening; pH 4.6-SN/TN is an index of proteolysis (Sousa, Ardo, & McSweeney, 2001). Significantly ($P < 0.05$) higher pH 4.6-SN/TN levels were found in MC cheese than in SC and PC cheese. In addition, the levels of pH 4.6-SN/TN

Table 2
Composition of cheeses made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC).[a]

Parameter	Control	SC	MC	PC
Moisture (%)	38.41^{ab} (0.41)	37.41^{a} (0.41)	37.80^{a} (1.43)	39.31^{b} (1.14)
Fat (%)	35.25^{a} (2.47)	35.75^{a} (2.13)	35.50^{a} (2.75)	34.84^{a} (2.12)
Protein (%)	23.85^{a} (0.71)	24.12^{a} (0.95)	23.44^{a} (0.95)	23.73^{a} (1.47)
Salt (%)	1.57^{ab} (0.11)	1.48^{a} (0.10)	1.70^{c} (0.10)	1.66^{bc} (0.07)
MNFS (%)	59.33 (0.65)	58.22 (0.65)	58.61 (2.21)	60.33 (1.75)
FDM (%)	59.50 (3.00)	58.14 (1.87)	59.22 (2.94)	58.31 (2.59)
pH at 14 days	5.03^{a} (0.09)	5.05^{a} (0.07)	5.23^{b} (0.14)	5.06^{a} (0.11)
pH at 30 days	5.07^{ab} (0.04)	5.01^{a} (0.04)	5.26^{c} (0.04)	5.14^{b} (0.08)
pH at 60 days	5.07^{a} (0.07)	5.11^{ab} (0.07)	5.20^{b} (0.07)	5.12^{ab} (0.03)
pH at 120 days	5.13^{a} (0.09)	5.17^{a} (0.06)	5.40^{b} (0.04)	5.25^{ab} (0.03)
pH at 180 days	5.04^{a} (0.03)	5.07^{a} (0.04)	5.31^{b} (0.10)	5.17^{ab} (0.07)
pH 4.6-SN/TN	23.01^{bc} (1.22)	22.21^{ab} (1.23)	25.89^{c} (1.65)	19.71^{a} (2.03)

[a] Data taken for moisture, fat, protein, salt, moisture in non-fat substance (MNFS) and fat in dry matter (FDM) at 14 days of ripening, pH values at 14–180 days of ripening, and pH 4.6-SN/TN levels at 180 days of ripening. Values are means, with standard deviation in parenthesis, of three independent trials; means within the same row not sharing a common superscript letter differ significantly ($P < 0.05$). There were no significant differences found for MNFS or FDM.

were significantly ($P < 0.05$) lower in PC than control cheese. The value (23%) found at 180 days of ripening in this study was comparable with previous studies on Cheddar cheese (Lucey, Mishra, Hassan, & Johnson, 2005; O'Mahony, Lucey, & McSweeney, 2005). The main agent producing pH 4.6-soluble nitrogen is the coagulant (O'Keeffe, Fox, & Daly, 1978), while Farkye and Fox (1992) reported that plasmin, the principal indigenous milk proteinase, is also important for primary proteolysis in Cheddar cheese during ripening.

Urea-PAGE electrophoretograms of cheese samples during ripening are shown in Fig. 2. All cheese samples showed breakdown of β- and α_{S1}-CN, but lower levels of intact β-and α_{S1}-CN were apparent during ripening in MC cheese. Due to the action of plasmin, β-CN is hydrolysed to β-CN (f29–209), β-CN (f106–209)

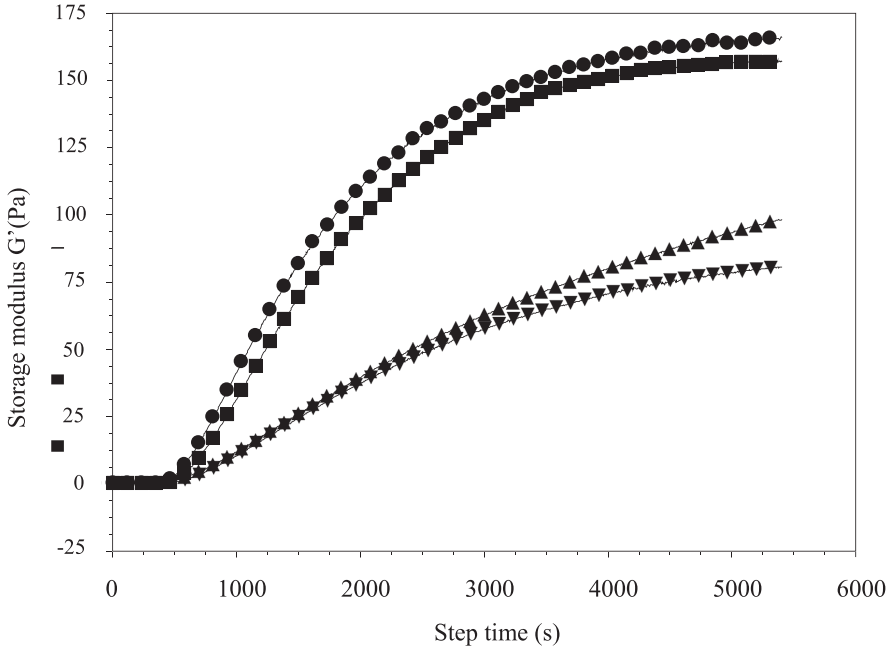

Fig. 1. Storage modulus (G′) of rennet gel of control (●), SC (■), MC (▼) and PC (▲) samples, under strain of 0.01 and frequency of 1 Hz.

and β-CN (f108–209) (Eigel et al., 1984). The levels of β-CN (f106–209) and β-CN (f108–209) of MC cheese were higher than other treatments during ripening, which suggests higher plasmin activity. Also, in Fig. 2, the MC cheese showed the lowest level of intact α_{S1}-CN level, followed by PC cheese, control and SC cheese from 60 to 180 days of ripening; at 180 days of ripening, no intact α_{S1}-CN was found in MC and PC samples. Residual chymosin in cheese hydrolyses α_{S1}-CN to α_{S1}-CN (f24–199) and α_{S1}-CN (f102–199) and, as the residual chymosin activity increases, the breakdown of α_{S1}-CN increases (Sheehan, Wilkinson, & McSweeney, 2008). The faster breakdown of α_{S1}-CN of MC and PC samples may indicate higher residual chymosin activity in these samples.

The peptide profiles of the pH 4.6-soluble extracts for control, SC, MC and PC cheeses at 180 days of ripening were generated by ultra-performance liquid chromatography (Fig. 3). The highest peak areas were observed at the retention time of 1–5 min for all treatments. The peak area of peptides that were eluted later (28–45 min retention time) was higher in MC sample compared to the other treatments. Peptides in the pH 4.6-soluble extracts reflect the effect of proteinases and peptidases of starter (Fox & McSweeney, 1997). The number of peptide peaks for all treatments was similar, which suggests that the proteolysis for all treatments broadly followed similar pathways, but differences in peak area indicate that there was more extensive proteolysis in MC cheese compared to the other treatments.

The individual FAA levels of all treatments at 120 and 180 days of ripening are shown in Fig. 4 (A and B). The major FAA determined in cheese were glutamic acid, valine, leucine, phenylalanine, histidine and lysine; Bansal et al. (2009) also reported that glutamic acid, valine, leucine, phenylalanine histidine and lysine are the principal FAA in Cheddar cheese after 180 days of ripening. No significant differences ($P > 0.05$) were found between treatments at 120 days of ripening. At 180 days of ripening, significantly ($P < 0.05$) higher concentrations of threonine, serine, glycine, alanine, valine, methionine, isoleucine, phenylalanine, histidine and proline were found in control and SC cheese than that of MC and PC cheese. No significant differences ($P > 0.05$) were found between treatments in the levels of aspartic acid, glutamic acid, leucine, tyrosine and arginine. From 120 to 180 days of ripening, the concentrations of glutamic acid, alanine, leucine, tyrosine and phenylalanine increased significantly ($P < 0.05$) for all treatments, while only control and SC cheese had a significant increase in the concentration of threonine, glycine, valine and isoleucine. The total FAA levels of all treatments at 120 and 180 days of ripening are shown in Fig. 4(C). No significant differences ($P > 0.05$) were found between all treatments at 120 days of ripening, but significantly higher ($P < 0.05$) levels of total FAA were found in control cheese than in MC and PC cheeses at 180 days of ripening. No significant differences ($P > 0.05$) were found between control and SC cheese at 180 days of ripening.

Fox and McSweeney (1996) reported that peptidases of starter and non-starter lactic acid bacteria are the principal agents releasing FAA in Cheddar cheese during ripening. No significant ($P > 0.05$) differences were found for the numbers of starter bacteria and NSLAB between treatments in this study (result not shown). Therefore, the release of the FAA by starter and NSLAB should not have been an influence in this regard, and so the reasons for this difference are not clear.

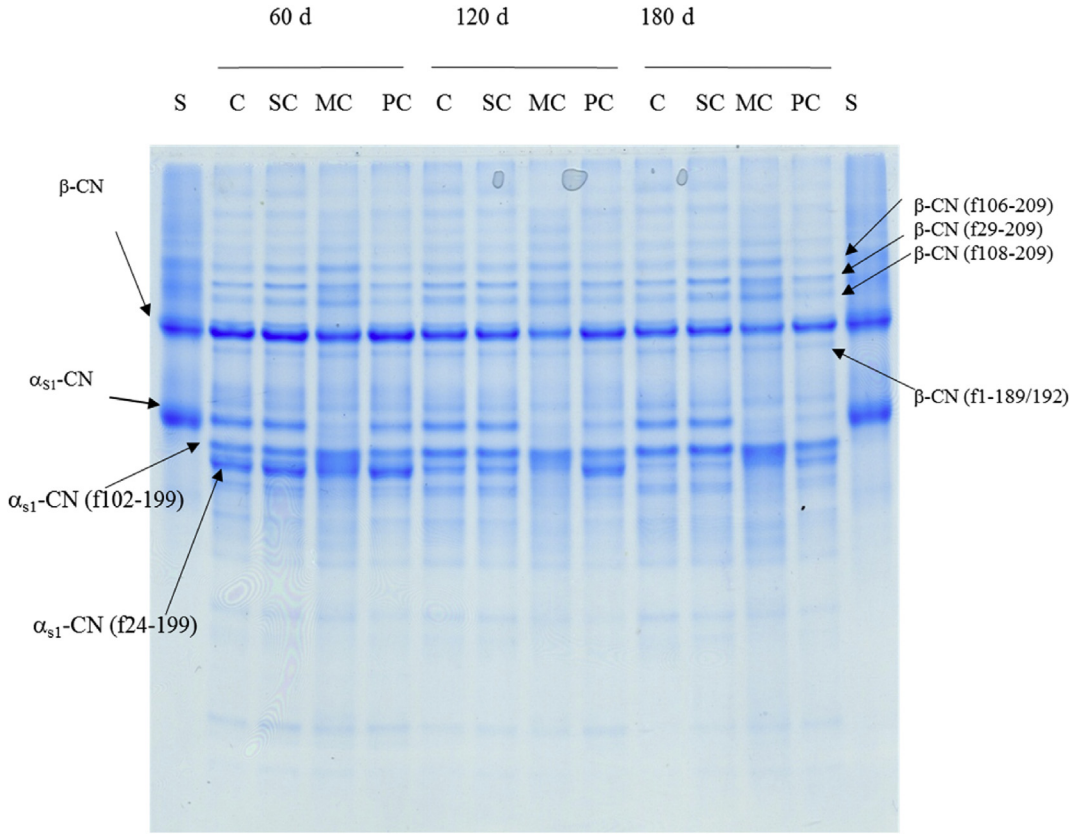

Fig. 2. Urea-polyacrylamide gel electrophoretograms of control (C), skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) cheeses at 60, 120 and 180 days of ripening. Sodium caseinate (S) was used as standard on both sides of the gel. The figure corresponds to data for one of the three cheese replicates; all replicates were similar.

Fig. 3. Ultra-performance liquid chromatograms (UPLC profiles, C_8 column) of pH 4.6-soluble extracts from control (A), SC (B), MC (C) and PC (D) cheeses at 180 days of ripening, at wavelength of 214 nm. The figure corresponds to data for one of the three cheese replicates; all replicates were similar.

3.4. Plasmin and residual chymosin activities in cheese

The plasmin activity of MC cheese was significantly ($P < 0.05$) higher than that of the other cheese batches (Table 3) which is consistent with the result of urea-PAGE electrophoresis. The plasmin activity of PC cheese was significantly ($P < 0.05$) lower than that of the other cheese samples. As the pH increases, the hydrolysis of β-CN increases since plasmin has an alkaline optimum pH (Watkinson et al., 2001).

Aaltonen and Ollikainen (2011) reported that in diafiltration, with the removal of whey protein, the concentration of inhibitors of both plasmin and plasminogen activators decreases, which promotes the conversion from plasminogen to plasmin and thus increases plasmin activity. In this study, MCC was made using microfiltration, which may have enhanced the plasmin activity of retentate by removing inhibitors in the permeate, resulting in the higher plasmin activity and more β-CN breakdown. MC cheese showed significantly ($P < 0.05$) higher residual chymosin activity compared to the other treatments (Table 3), which is consistent with the faster breakdown of α_{S1}-CN in that cheese. No significant differences ($P > 0.05$) were found between the residual chymosin activities of control and SC cheese, so the recombination of fat and skim milk did not affect the retention of coagulant during cheese manufacture. The residual chymosin activity of PC cheese was significantly ($P < 0.05$) lower than that of control cheese. The MC cheese apparently retained more coagulant compared with control, SC and PC cheeses; the percentage of retained chymosin in cheese curd depends on the pH at the curd-cutting stage, pH at whey drainage, cooking temperature and method (Hurley et al., 1999).

3.5. Texture profile analysis

No significant changes ($P > 0.05$) were found in the hardness of MC and PC cheeses from 60 to 180 days of ripening (Fig. 5). The hardness of control cheese significantly ($P < 0.05$) decreased between 60 and 120 days of ripening, while that of SC cheese significantly ($P < 0.05$) increased. At 60 days of ripening, the hardness of control cheese was significantly ($P < 0.05$) higher than that of SC and PC cheeses. No significant differences ($P > 0.05$) were observed in the hardness of the cheeses made by different treatments at 120 and 180 days of ripening. Chevanan and Muthukumarappan (2007) reported that the contents of calcium, phosphate, and residual lactose affect the texture profile of Cheddar cheese. The cheese-milk of SC, MC and PC samples was reconstituted according to the composition of control cheese-milk, which would not influence lactose content. In this study, extra calcium chloride was added to all treatments, but the addition of calcium chloride does not cause significant changes to the texture of ripened Cheddar cheese (Soodam, Ong, Powell, Kentish, & Gras, 2015).

From 60 days to 180 days of ripening, the springiness and cohesiveness of MC cheese decreased, and the springiness and cohesiveness of MC and PC cheeses were significantly ($P < 0.05$) higher than that of control and SC cheese at 60 and 180 days of ripening. At 120 days of ripening, the springiness of MC cheese was significantly ($P < 0.05$) higher than that of the other treatments and the cohesiveness of MC and PC cheeses was significantly ($P < 0.05$) higher than that of control and SC cheese. Everard et al. (2006) found that higher pH of Cheddar cheese was associated with increased springiness and cohesiveness; the pH of MC cheese was significantly ($P < 0.05$) higher than that for the other treatments (Table 2), which may explain the increases in springiness and cohesiveness. O'Mahony et al. (2005) reported that, as levels of secondary proteolysis increase, the charged groups released from peptides would associate with free water, which may lead to increased cohesiveness and springiness during ripening.

3.6. Meltability

Meltability (percentage increase in diameter) of Cheddar cheeses was determined by the Schreiber method; results are shown in Fig. 6. The four types of cheeses showed increases in meltability between 60 and 180 days. At 60 and 120 days of ripening, no significant differences ($P > 0.05$) for meltability were found between control and SC cheese. Significantly ($P < 0.05$) higher meltability was found in control cheese compared to SC cheese. At 60, 120 and 180 days of ripening, the meltability of MC cheese was significantly higher ($P < 0.05$) than that of cheese from the other treatments. At 120 and 180 days of ripening, the

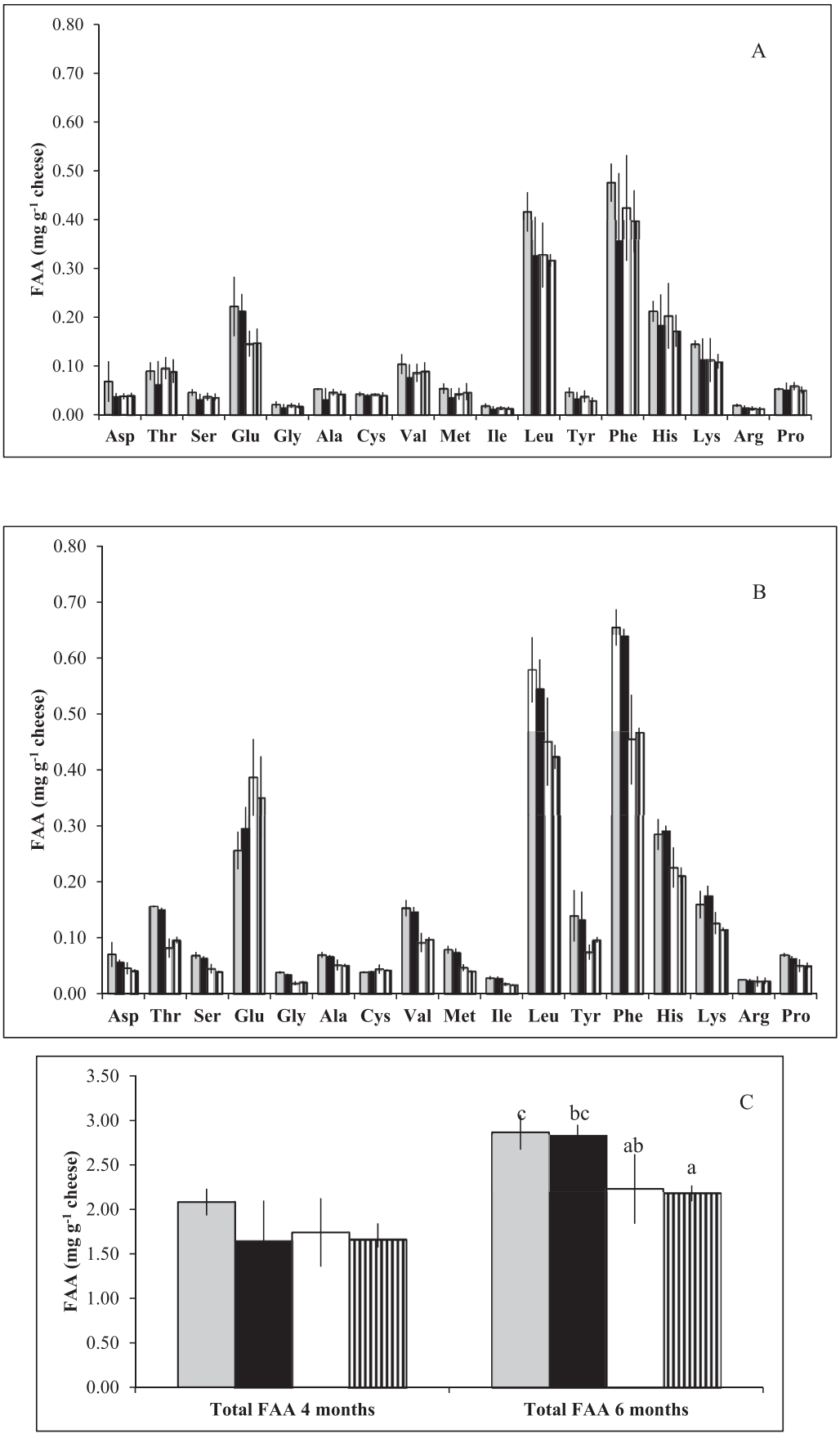

Fig. 4. Individual FAA results of cheese samples at 120 (A) and 180 (B) days of ripening and total FAA results of cheese samples at 120 and 180 days of ripening (C). Control (▩), SC (■), MC (□) and PC (▥). Values are means of three independent trials; means not sharing a common letter differ ($P < 0.05$). Error bars indicate standard deviation.

Table 3
Plasmin activity and residual chymosin activity of cheeses made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) at 180 days of ripening.[a]

Treatment	Plasmin activity (nmol AMC mL^{-1} min^{-1})	Residual chymosin activity (% of control)
Control	0.572[b] (0.121)	100[b]
SC	0.656[b] (0.185)	106.04[b] (4.89)
MC	1.116[c] (0.024)	132.58[c] (3.81)
PC	0.396[a] (0.042)	80.74[a] (6.61)

[a] Data are means, with standard deviation in parenthesis, from three independent trials; means within the same column not sharing a similar superscript letter differ significantly ($P < 0.05$).

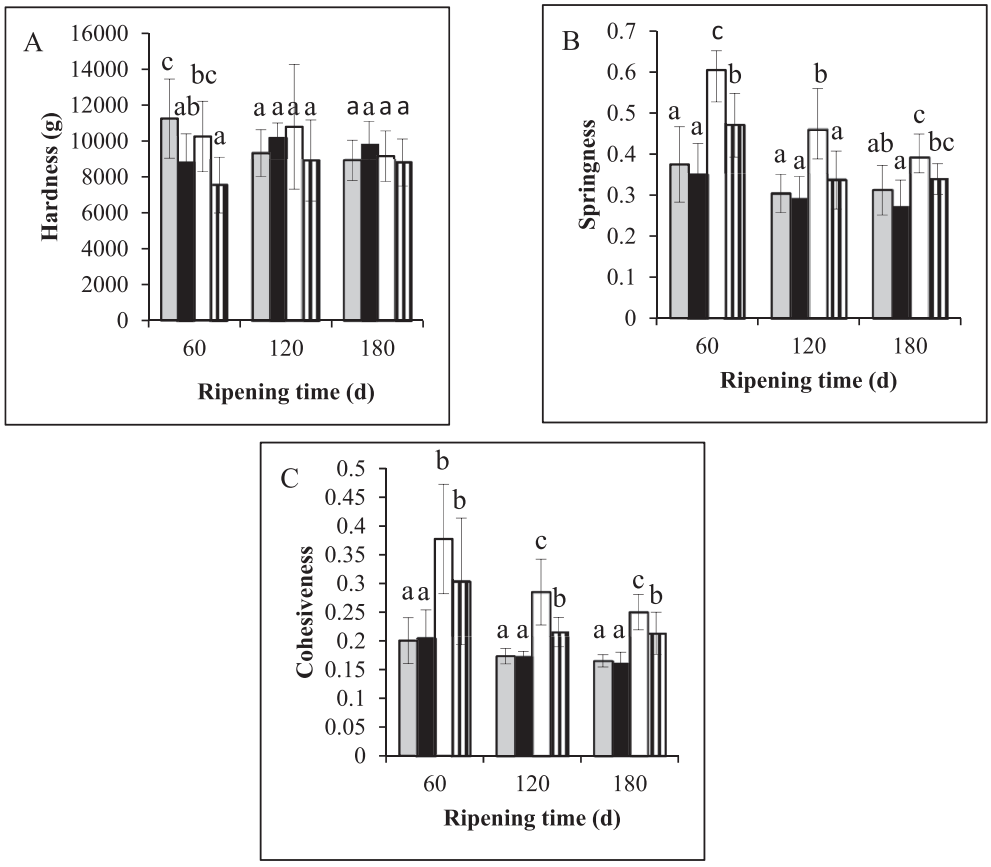

Fig. 5. Changes in (A) TPA hardness, (B) springiness and (C) cohesiveness of cheese samples from 60 to 180 days of ripening. Control (▒), SC (■), MC (□) and PC (▥). Values are means of three independent trials; means at the same time point not sharing a common letter differ ($P < 0.05$). Error bars indicate standard deviation.

meltability of PC cheese was significantly lower ($P < 0.05$) than that of cheese from the other treatments.

The lower meltability of PC cheese may be caused by the changes in functionality of milk proteins during and after powder production. Moiseev, Suchkova, and Iakovchenko (2017) reported that mozzarella cheese made with reconstituted non-fat milk powder has lower meltability than the control, due to the drying process of non-fat milk powder decreasing the stability and dispersity of casein micelles and promoting demineralisation of calcium salts. Dave, McMahon, Oberg, and Broadbent (2003) found that the meltability of mozzarella cheese is related to the breakdown of (α_{S1}-CN and α_{S1}-CN (f24-199) and breakdown of intact β-casein, while Bogenrief and Olson (1995) reported that hydrolysis of β-casein increases the meltability of Cheddar cheese. Hydrolysis of β-casein is primarily related to the action of plasmin (Eigel, McMahon, Oberg, & Broadbent 1984) and, although both MC and PC cheeses were made from powder, and showed a higher breakdown of α_{S1}-casein and β-casein, the meltability of MC cheese was significantly higher than that of PC.

3.7. Dynamic small amplitude oscillatory rheology

The results of the rheology of meltability of all treatments at 180 days of ripening are shown in Table 4. Higher LT value indicates a higher extent of melting (Lucey, Johnson, & Horne, 2003). MC cheese showed significantly higher ($P < 0.05$) LT_{max} values than PC cheese, followed by control and SC cheese. The temperature at LT_{max} of MC and PC cheeses was significantly higher ($P < 0.05$) than that of control and SC cheese. The LT_{max} is an indicator of cheese meltability (Mounsey & O'Riordan, 1999); the more thermal energy needed to melt cheese, the higher the LT_{max} temperature of cheese will be.

In the Schreiber test for cheese meltability, MC cheese also exhibited the highest percentage increase in diameter (Fig. 6).

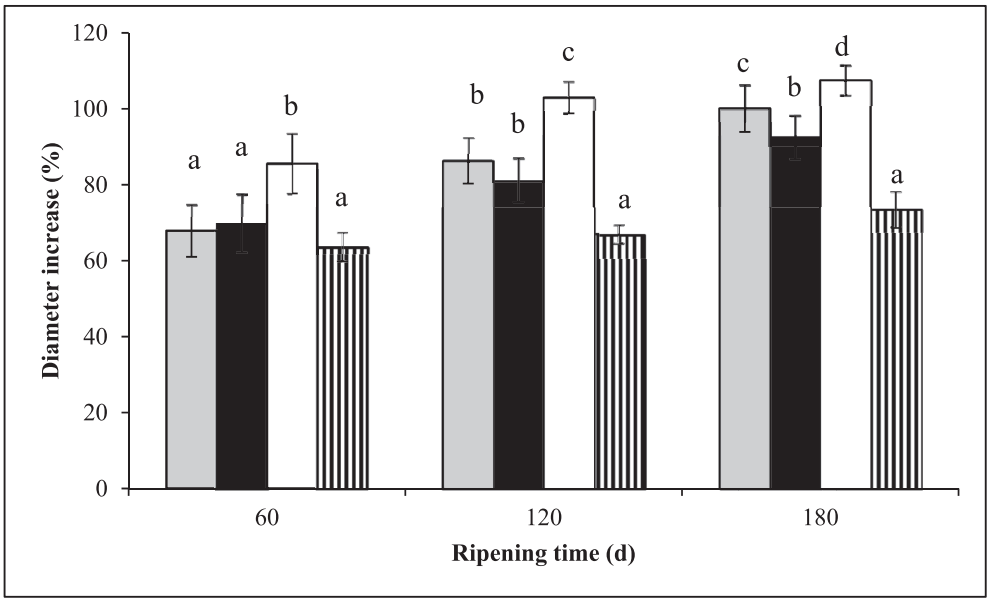

Fig. 6. Meltability (diameter increase, %) of cheese samples from 60 to 180 days of ripening Control (▒), SC (■), MC (□) and PC (▥). Values are means of three independent trials; means at the same time point not sharing a common letter differ ($P < 0.05$). Error bars indicate standard deviation.

Table 4
Rheological properties of cheeses made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) at 180 days of ripening.[a]

Sample	LT_{max}	Temperature at LT_{max} (°C)	Temperature at LT = 1 (°C)
Control	1.25^{a} (0.31)	70.55^{a} (0.70)	62.02^{c} (0.58)
SC	1.42^{a} (0.33)	71.36^{a} (2.49)	61.80^{b} (2.88)
MC	2.93^{b} (0.33)	77.76^{b} (1.91)	55.24^{a} (1.57)
PC	1.92^{c} (0.18)	75.64^{b} (4.75)	58.93^{b} (1.58)

[a] Rheological parameters assessed by dynamic small amplitude oscillatory rheology. Data are means, with standard deviation in parenthesis, from three independent trials; means within the same column not sharing a similar superscript letter differ significantly ($P < 0.05$).

Table 5
CIE LAB colour values of cheeses made from control, skim milk with cream (SC), reconstituted micellar casein concentrate with cream (MC) and reconstituted low heat skim milk powder with cream (PC) after 180 days of ripening.[a]

Sample	L*	a*	b*	ΔE^*_{ab}
Control	81.05^{b} (1.81)	-4.44^{a} (0.23)	36.72^{ab} (2.42)	–
SC	80.91^{b} (1.19)	-4.36^{a} (0.32)	37.43^{b} (1.53)	1.82
MC	79.34^{a} (1.56)	-3.88^{b} (0.29)	35.56^{a} (1.43)	0.15
PC	79.74^{ab} (0.85)	-3.40^{c} (0.54)	36.07^{ab} (0.89)	1.47

[a] CIELAB data are means, with standard deviation in parenthesis, of five replicate analyses from three independent trials with ΔE^*_{ab}, calculated from CIELAB results, between control and each treatment after 180 days of ripening. Means within the same column not sharing a similar superscript letter differ significantly ($P < 0.05$). The just noticeable differences of ΔE^*_{ab} was approximately 2.3.

There were differences between Schreiber melting test and dynamic small amplitude rheology for control, SC and PC cheese. Cooke, Khosrowshahi, and McSweeney (2013) stated that differences between results generated by those tests may be linked to the more complete fat melting during the higher temperature of the Schreiber melting test. MC cheese exhibited the lowest temperature when LT = 1 compared with the rest of the treatments ($P < 0.05$). At the point of LT = 1, cheese is considered to start transforming from a solid to a viscous form (Gunasekaran & Ak, 2002). MC cheese thus commenced this transition at the lowest temperature and had the highest meltability compared to the other treatments, which may be linked to the more extensive proteolysis of casein.

3.8. Cheese colour

The colour values of samples at 180 days of ripening are shown in Table 5. Whiteness (L* values) of control, SC cheese was significantly higher ($P < 0.05$) than that of MC cheese at 180 days of ripening. The greenness of MC and PC cheeses was significantly lower (higher a* values) ($P < 0.05$) than that of control or SC cheese at 180 days of ripening. The yellowness (b* values) of MC cheese was significantly lower ($P < 0.05$) than that of SC cheese at 180 days of ripening. Ibáñez et al. (2015) reported that the whiteness of cheese may be related to proteolysis; lower whiteness of MC cheese may thus be attributed to higher proteolysis. The ΔE^*_{ab} values are also shown in Table 5. Sharma and Bala (2003) reported that ΔE^*_{ab} of above 2.3 leads to a just noticeable difference (JND) and, on this basis, overall, no JND was found between control cheese and the other treatments. Overall, no visible difference was found between the cheeses made from protein ingredient and control and SC cheese.

4. Conclusions

Cheddar cheeses were made from standardised cheese milk, skim milk with cream, reconstituted MCC with cream and reconstituted LHSMP with cream according to the same casein, fat and lactose content. Only minor differences in composition were found with cheese made from reconstituted MCC with added cream compared with control cheese. The cheese manufactured with MCC had significantly higher ($P < 0.05$) levels of pH 4.6-SN/TN than cheese manufactured with skim milk or LHSMP with cream. The level of intact β-and α_{S1}-CN of MC cheese was lower than rest treatments, consistent with significantly ($P < 0.05$) higher plasmin and chymosin activity. No significant difference ($P > 0.05$) was found in hardness between all treatments. Significantly higher ($P < 0.05$) springiness and cohesiveness were found in cheese manufactured from MCC and LHSMP powder, meltability and

maximum loss tangent in MC cheese were significantly ($P < 0.05$) higher than that of the other treatments. No overall differences of colour were found between all treatments. Use of novel casein-dominant dairy streams such as MCC has potential for production of Cheddar cheese with tailored functionality. The results of this study suggest that using reconstituted LHSMP with cream for the manufacture of Cheddar cheese may result in changes in functionality, while using reconstituted MCC with cream for the manufacture of Cheddar cheese may be more feasible.

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Suitability of a novel camel (*Camelus dromedarius*) chymosin as a coagulant for Cheddar cheese manufacture

Bozhao Li [a], David S. Waldron [a], MaryAnne Drake [b], John Lyne [c], Alan L. Kelly [a, *], Paul L.H. McSweeney [a]

[a] School of Food and Nutritional Sciences, University College Cork, College Road, Cork, T12 K8AF, Ireland
[b] Department of Food, Nutrition and Bioprocessing, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, NC 27695, USA
[c] Chr. Hansen A/S, DK-2970 Hørsholm, Denmark

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ABSTRACT

Cheddar-type cheese was manufactured using fermentation-produced bovine chymosin (BC), fermentation-produced camel chymosin (CC) and a modified fermentation-produced camel chymosin (mCC) and ripened for 180 days. Only minor differences were found in cheese composition and pH between the cheeses made with any of the chymosins studied. Proteolysis in cheese made with mCC was reduced compared with cheese made with BC or CC. Significantly higher instrumental and sensory hardness and significantly lower meltability were found in cheeses made using CC or mCC compared with cheese BC after 180 days of ripening. Descriptive sensory analysis results showed that cheese made with CC or mCC had less sulphur and barny flavour; the brothy flavour and bitter taste of cheese made with mCC were also lowest. In conclusion, the modified camel chymosin appears to be suitable for the manufacture of Cheddar cheese with modified functionalities.

1. Introduction

Rennet is the general name for an enzyme preparation used to coagulate milk that originates from the abomasum of ruminant mammals. Chymosin, the principal enzyme in most rennets, cleaves the Phe_{105}—Met_{106} bond of κ-casein, thereby reducing the negative charge of casein micelles and reducing steric stabilisation. Without this stabilisation, a casein micelle network is formed, leading to the separation of whey and casein curds. This property of chymosin is a key aspect of cheese manufacture, and calf chymosin is the most common coagulant used for cheese making. Other animal sources of chymosin extracted from lambs and camels are also commercially available, as well as vegetable coagulants, including enzymes from *Cynara cardunculus, Ficus carica, Arctium minus* and *Solanum dubium*, which are sometimes used in traditional cheese production (Robinson & Wilbey, 1998). Enzymes naturally produced by microorganisms (e.g., *Endothia parasitica*) can also be used, for example, for Emmental cheese manufacture (Gagnaire, Mollé, Herrouin, & Léonil, 2001).

In cheese manufacture, it is ideal for a coagulant to have low proteolytic activity, with high cleavage specificity at or near the Phe_{105}—Met_{106} bond of κ-casein, and is easy to inactivate in whey (Bansal et al., 2009). The coagulation/proteolysis (C/P) ratio, which is the ratio of milk coagulating activity to the general proteolytic activity, is related to the specificity of enzyme activity (Soodam, Ong, Powell, Kentish, & Gras, 2015). Cheese yield is an important property to consider and may be decreased as the degree of proteolysis increases or C/P ratio decreases during cheese manufacture increases (Fox & McSweeney, 1997; Soodam et al., 2015).

Camel chymosin has 85% amino acid sequence identity to calf chymosin while its clotting activity is 70% higher than that of calf chymosin, and it has 25% of the hydrolytic activity of calf chymosin (Kappeler et al., 2006; Langholm Jensen et al., 2013). Compared with calf chymosin, camel chymosin has an additional histidine residue with a positive charge on the N-terminal lobe of the enzyme, which attracts the negative charge in κ-casein, and therefore leads to a strong association of camel chymosin with the casein micelles; the higher molecular flexibility and electrostatic interaction of camel chymosin facilitate the association of enzyme and substrate (Kappeler et al., 2006; Langholm Jensen et al., 2013).

* Corresponding author.
E-mail address: a.kelly@ucc.ie (A.L. Kelly).

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With gene recombination technology, once the gene sequence is determined, chymosin may be produced by microbial fermentation rather than extracted from the abomasum of the animal. Calf chymosin is produced through fermentation by transferring the gene for chymosin to *Aspergillus niger* or *Kluyveromyces lactis* (Kappeler et al., 2006). Cheddar cheese manufactured using camel chymosin produced by Chr. Hansen A/S (ChyMax M™) has been shown to have higher hardness and less bitterness than cheese made from calf chymosin, due to decreased primary proteolysis (Bansal et al., 2009; Møller, Rattray, & Ardö, 2012). Also, Mozzarella cheese manufactured using camel chymosin was found to have less proteolysis, harder texture and longer shelf-life than cheese made from bovine calf chymosin (Moynihan et al., 2014).

A modified camel chymosin enzyme was developed through a structure-based design approach involving bioinformatics (amino acid diversity among chymosin sequence homologues) sequence function analysis (exchange of amino acids) and structure-based design (optimising substrate binding – κ- versus α- versus β- caseins). Selected enzymes were then expressed in a fungal model to allow production of enough protein enzyme for application in cheese model and cheese making systems to select for enzyme with optimised characteristics in terms of coagulation activity, relative coagulation versus proteolytic activity and specific casein cleavage; the resultant enzyme (CHY-MAX ® Supreme) has increased specific milk-clotting activity and greatly increased proteolytic specificity compared with the original camel chymosin (Jäckel et al., 2017; Jäckel, Lund, van den Brink, Gustafsson, & Govindarajan, 2019).

The objective of this study was to manufacture Cheddar cheese using commercial fermentation-produced bovine calf chymosin, commercially available fermentation-produced camel chymosin, and commercially available fermentation-produced camel chymosin with altered molecular structure and compare the proteolysis, texture and sensory properties of the resulting cheeses.

2. Materials and methods

2.1. Coagulants

The coagulants used for Cheddar cheese manufacture were commercially available fermentation-produced bovine calf chymosin (BC) [CHY-MAX® Extra, 600 international milk clotting units (IMCU) mL^{-1}], commercially available fermented-produced camel chymosin (CC; CHY-MAX® M 200; 200 IMCU mL^{-1}) and commercially available fermentation-produced camel chymosin with structural changes through amino acid substitution (mCC; CHY-MAX® Supreme 200, 200 IMCU mL^{-1}; Christian Hansen A/S). To determine the amount of each chymosin used for Cheddar cheese manufacture, the milk coagulation properties of each chymosin were analysed according to the method described by O'Mahony, Auty, and McSweeney (2005). Milk was coagulated by each chymosin and the gel strength (G′, storage modulus) of the sample formed at a normal cutting time (45 min) was used to determine coagulating activity (Bansal et al., 2009); G′ values were measured on an AR-G2 controlled-stress rheometer (Waters TA Instruments, Leatherhead, Surrey, UK).

2.2. Cheese manufacture

Whole milk (4.64% fat, 3.68% protein, pH 6.5) was standardised to a casein-to-fat ratio of 0.7:1.0, pasteurised at 72 °C for 15 s and cooled to 32 °C. Cheddar cheeses were manufactured according to a standard Cheddar cheese manufacturing protocol (Fox et al., 2000) from 100 kg standardised milk. An aliquot of 30 g (0.03%, w/w) of Cheddar cheese starter culture (R604Y Chr. Hansen Ltd., Little Island, Co. Cork, Ireland) was added into each sample and allowed to ripen for 30 min, followed by 60 IMCU L^{-1} of calf chymosin or camel chymosin or modified camel chymosin. The curd was cut after 45 min. Whey was drained when the pH reached 6.2 and curd was cheddared; when the pH decreased to 5.4, the curd was milled into small pieces and salted at a level of 2.5% (w/w) NaCl. The curds were then wrapped in cheesecloth and moved to moulds, which were pressed at a pressure of 2.5 kg cm^{-2} for 14 h. The cheeses were then removed, vacuum-packed (99.9% air extracted); samples were taken periodically during ripening at 8 °C for 180 days. The masses of milk, whey, starter, chymosin, salt and cheese were measured to estimate the yield of cheese.

2.3. Compositional analysis and analysis of proteolysis

The gross composition of cheeses was analysed at 14 days of ripening. Moisture was determined by the oven-drying method (IDF, 1982), protein (%N × 6.38) by the Kjeldahl method (IDF, 1986) and salt was analysed by titration with $AgNO_3$ (Fox, 1963). The pH was measured at 14 days of ripening using a calibrated pH probe placed in contact with dry grated cheeses. All results were determined in triplicate.

Urea-polyacrylamide gel electrophoresis (urea-PAGE) was applied directly on cheese samples at 14, 30, 90 and 180 days of ripening according to the method of Andrews (1983) with modifications as described by Shalabi and Fox (1987). pH 4.6-soluble extracts were prepared (Kuchroo & Fox, 1982) and their nitrogen content was determined by the Kjeldahl method (IDF, 1986) and expressed as % of the total nitrogen content of the cheese samples.

2.4. Texture profile analysis and analysis of meltability

Texture profile analysis was performed using a Texture Analyzer TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) at 30, 90, and 180 days of ripening. Cheese samples were cut into cylinders of 20-mm height and 20-mm diameter and kept at 4 °C overnight. Cheese cylinders were compressed to 25% of the initial height in two continuous compressions with a speed of 1 mm s^{-1}. Hardness, cohesiveness and springiness were also measured; five cheese samples were measured for each treatment (Li et al., 2020).

Meltability was analysed using the Schreiber meltability test as described by Altan, Turhan, and Gunasekaran (2005). Cheese samples were heated at 232 °C for 5 min, and meltability was measured as the percentage increase in diameter of the original samples; analyses were performed in triplicate at 30, 90 and 180 days of ripening.

2.5. Dynamic small-amplitude oscillatory rheology

The rheological properties of melted cheese after 30, 90 and 180 days of ripening were analysed with a controlled-stress AR-G2 rheometer (TA Instruments, Waters LLC, Leatherhead, Surrey, UK) according to the method described by Ibáñez et al. (2015). The initial temperature of cheese sample was 20 °C, and samples were heated to 80 °C at a heating rate of 2 °C per min. Storage modulus (G'), loss modulus (G"), and loss tangent (LT) were measured during heating. The maximum LT (LT_{max}) and the temperature where LT = 1, which are indicators of melting, were also recorded. Each sample was analysed in triplicate (Li et al., 2020).

2.6. Descriptive sensory analysis

Cheese samples were shipped to North Carolina State University within 72 h under refrigerated conditions after 180 days of ripening. Sensory testing was conducted on cheese samples in North Carolina State University in compliance with approval from

the North Carolina State University Institutional Review Board for Human Subjects. Cheese samples were cut into 1 × 1 × 1 cm cubes and put into 110-g lidded soufflé cups with random three-digit codes for descriptive sensory analysis. The cheeses were tempered at 10 °C for 1 h and were served at this temperature for flavour and texture profiling, with spring water and unsalted crackers for palate cleansing. Descriptive analysis of flavour used a 0 to 15-point universal intensity scale with the Spectrum™ method (Drake & Civille, 2003; Meilgaard, Carr, & Civille, 1999) and a previously established Cheddar cheese flavour sensory language (Drake, McIngvale, Cadwallader, & Civille, 2001). Texture evaluation utilised a 0 to 15-point product-specific scale and an established lexicon (Rogers et al., 2009). Flavour and texture were evaluated in separate sessions.

A descriptive sensory panel (n = 6, 6 females, ages 22–46 years), with more than 200 h collective experience with the descriptive analysis of Cheddar cheese flavour and texture, evaluated the cheeses. Paper ballots were used. Consistent with Spectrum™ descriptive analysis training, panellists were presented with reference solutions of sweet, sour, salty, and bitter tastes to learn to use consistently the universal intensity scale (Drake & Civille, 2003; Meilgaard et al., 1999). Following consistent use of the Spectrum™ scale with basic tastes, panellists learned to identify and scale flavour descriptors using the same intensity scale through presentation and discussion of flavour definitions, references and a wide array of cheeses. Discussion and evaluation of a wide array of cheeses were also conducted during training enabling panellists consistently to differentiate and replicate samples. Analysis of data collected from training sessions confirmed that panel results were consistent and that terms were not redundant, consistent with previous use of the developed language (Drake et al., 2001, 2005). Each panellist evaluated each product in duplicate.

2.7. Statistical analysis

BC, CC and mCC cheeses were made in triplicate in independent trials (i.e., 3 × 3 blocks). Statistical analysis was carried out using one-way analysis of variance (ANOVA) with R project for Windows version i386 3.4.0 to establish significant differences between samples. The probability level used for statistical significance was $P < 0.05$. The data of descriptive sensory analysis were analysed by a general linear model analysis of variance with Fisher's least significant difference (LSD) as a *post hoc* test (SAS version 9.1, Cary, NC).

3. Results and discussion

3.1. Determination of coagulant performance

To determine the amount of CC and mCC used for Cheddar cheese manufacture, a preliminary rennet coagulation rheology test was performed. At an incubation time of approximately 45 min (2700 s), the storage modulus, G', of milk samples BC, CC and mCC was almost the same (Fig. 1). With the same level of IMCU, although there was a significant difference ($P < 0.05$) in the time taken for G′ to exceed 1 Pa between BC and CC, there was no statistically significant difference ($P > 0.05$) between the gel firmness at 45 min for all of the coagulants (Table 1). Therefore, BC, CC and mCC were added to cheese-milk at a constant level of 0.06 IMCU mL^{-1} milk (i.e., 10, 30 and 30 mL 100 L^{-1} milk, respectively). Lower levels of camel chymosin were used in previous Cheddar cheese studies to obtain the same gel strength of the coagulum at the cutting time (Bansal et al., 2009; Govindasamy-Lucey, Lu, Jaeggi, Johnson, & Lucey, 2010). Moynihan et al. (2014) used identical levels of BC and CC for Mozzarella cheese manufacture at different coagulating temperatures to obtain the same gel strength at cutting.

3.2. Composition, pH, and yield

No significant differences ($P > 0.05$) were found in moisture, fat, and protein contents or pH of cheese samples (Table 2). Although the salt content and salt-in-moisture values of cheese mCC were significantly higher ($P < 0.05$) than those of the cheese BC (Table 2), the numerical differences were small, and no significant differences ($P > 0.05$) were found between samples CC and mCC. Bansal et al. (2009) and Moynihan et al. (2014) also reported that no significant differences were found in composition and pH between Cheddar or Mozzarella cheeses made with calf or camel chymosin. No significant differences ($P > 0.05$) were found in the yield of cheese (Table 2), which is in agreement with the conclusion of Bansal et al. (2009).

3.3. Proteolysis

Urea-PAGE electrophoretograms of cheese samples after 14, 30, 90 and 180 days of ripening are shown in Fig. 2. All cheese samples showed the breakdown of β-CN into β-CN (f29–209), β-CN (f106–209) and β-CN (f108–209), consistent with the action of plasmin (Eigel et al., 1984), and no differences in the breakdown of β-CN were found between samples. Bansal et al. (2009) found that the urea-PAGE electrophoretograms of cheese manufactured using calf and camel chymosin showed that hydrolysis of β-CN by calf chymosin was only linked to the hydrolysis of β-CN to β-CN (f1–189/192), which is consistent with the urea-PAGE results of our cheeses, and almost no β-CN (f1–189/192) was observed in cheese mCC (Fig. 2). The complementary peptides, β-CN (f190–209) and β-CN (f193–209) are hydrophobic and related to the bitterness of cheese (Visser, Hup, Exterkate, & Stadhouders, 1983). β-CN (f190–209) and β-CN (f193–209) were produced to a very small extent in cheese manufactured with mCC, suggesting that using camel chymosin instead of calf chymosin may decrease bitterness in cheese during maturation.

All cheese samples showed a breakdown of α_{S1}-CN, and the rate of hydrolysis was fastest for the cheese BC, followed by cheese CC and then mCC. After 180 days of ripening, cheese mCC retained some intact α_{S1}-CN while that in cheeses BC and CC was broken down completely. The level of hydrolysis of α_{S1}-CN in cheese mCC at 180 days of ripening was similar to that in cheese BC at 14 days of ripening. McSweeney, Olson, Fox, Healy, and Hojrup (1993) reported that, during primary proteolysis, intact α_{S1}-CN is broken into α_{S1}-CN (f1–23) and α_{S1}-CN (f24–199). Overall, primary proteolysis of α_{S1}-CN in cheese by the action of mCC was slower than in cheeses BC and CC.

The levels of pH 4.6-soluble nitrogen as a percentage of total nitrogen (pH 4.6-SN/TN) in cheese made using different chymosins at 90 and 180 days of ripening were significantly different ($P < 0.05$) (Table 2). At each time point, the level of pH 4.6-SN/TN in cheese BC was significantly higher ($P < 0.05$) than that in cheese CC, followed by that in mCC cheese. After 180 days of ripening, the level of pH 4.6-SN/TN value in mCC cheese (9.86%) was approximately half of that in cheese BC (18.68%) and significantly lower ($P < 0.05$) than that in cheese CC (13.64%).

Moynihan et al. (2014) reported that the levels of pH 4.6-SN/TN for Mozzarella cheeses were made with the same levels of calf and camel chymosins after 84 days of ripening were 12% and 7%, respectively. Bansal et al. (2009) reported that the pH 4.6-SN/TN

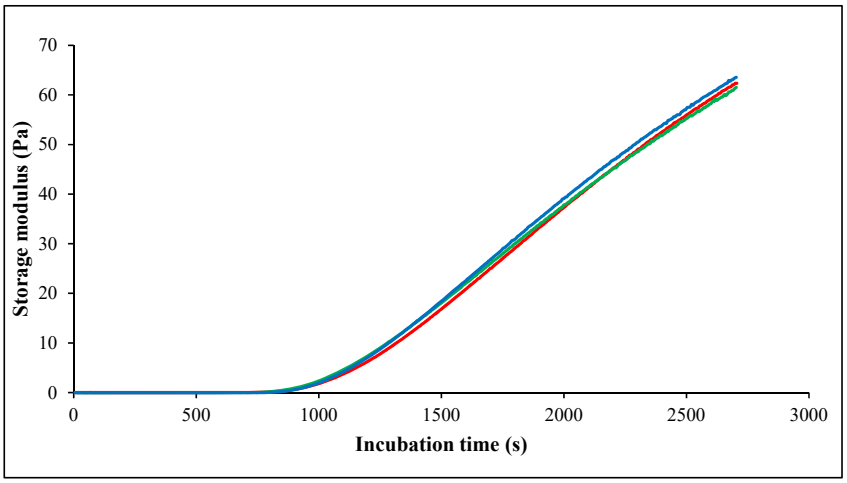

Fig. 1. Storage modulus (G′) as a function of incubation time of milk renneted by bovine calf chymosin (red), camel chymosin (green) and modified camel chymosin (blue). Data shown are one of the three replicates; all replicates were similar.

Table 1
Rheological properties of milk coagula produced by bovine calf chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC).[a]

Chymosin	Time G′> 1 Pa (s)	Gel firmness (G′ at 45 min, Pa)
BC	1031 ± 80[b]	57.7 ± 4.3[a]
CC	858 ± 50[a]	66.0 ± 4.2[a]
mCC	959 ± 26[ab]	61.1 ± 2.7[a]

[a] All enzymes were at a concentration of 60 ICMU L^{-1}. Data are means ± standard deviation, from three independent trials; means within the same column not sharing a similar superscript letter differ ($P < 0.05$).

values for Cheddar cheeses manufactured with calf chymosin and camel chymosin after 180 days of ripening were 19.93% and 14.99%, respectively, which is close to the values found in this study (18.68% and 13.64%), although lower levels of camel chymosin were used in this study. O'Keeffe, Fox, and Daly (1978) reported that the main factor influencing pH 4.6-soluble nitrogen levels in cheese is the coagulant; therefore, these results indicate that the level of primary proteolysis in cheese mCC was lower than that in cheeses BC and CC, which suggests that, at the same level of usage, coagulant mCC has lower proteolytic activity than calf chymosin and camel chymosin.

3.4. Texture profile analysis

Cheeses CC and mCC were significantly harder ($P < 0.05$) than cheese BC after 30, 90 and 180 days of ripening (Fig. 3A). Bansal et al. (2009) reported that the hardness of cheese made with camel chymosin was significantly higher ($P < 0.05$) than that of cheese made with calf chymosin after 150 days of ripening which is consistent with these results. No significant differences ($P > 0.05$) were found in springiness between all treatments at 30 and 90 days of ripening, although the springiness of cheese mCC was significantly higher ($P < 0.05$) than that of cheese BC after 180 days of ripening (Fig. 3B). The cohesiveness of cheese mCC was significantly higher ($P < 0.05$) than that of cheese BC and CC after 30 or 180 days of ripening (Fig. 3C). The results of springiness and cohesiveness of cheese made with bovine or camel chymosins are in agreement with previous research that no significant differences were found between the springiness and cohesiveness of cheese made with calf or camel

Table 2
Composition, pH, yield, and levels of soluble N in Cheddar cheeses made from milk coagulated with bovine calf chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC).[a]

Parameter	BC	CC	mCC
Moisture (%)	37.8 ± 0.9[a]	37.6 ± 0.7[a]	37.27 ± 0.40[a]
Protein (%)	26.1 ± 0.3[a]	26.0 ± 0.5[a]	25.99 ± 0.53[a]
Salt (%)	1.23 ± 0.10[a]	1.26 ± 0.06[ab]	1.37 ± 0.15[b]
Fat (%)	32.6 ± 0.7[a]	32.1 ± 0.8[a]	31.90 ± 0.72[a]
pH	5.22 ± 0.03[a]	5.22 ± 0.05[a]	5.22 ± 0.03[a]
MNFS (%)	57.8 ± 2.0[a]	58.6 ± 1.1[a]	57.78 ± 0.63[a]
FDM (%)	52.3 ± 1.1[a]	51.5 ± 1.3[a]	50.85 ± 1.15[a]
Salt in moisture (%)	3.26 ± 0.28[a]	3.35 ± 0.14[ab]	3.67 ± 0.43[b]
Yield (kg cheese 100 kg^{-1} milk)	9.92 ± 0.45[a]	9.89 ± 0.32[a]	9.76 ± 0.23[a]
Moisture adjusted yield (kg cheese 100 kg^{-1} milk)	9.83 ± 0.28[a]	9.89 ± 0.27[a]	9.89 ± 0.33[a]
pH 4.6-SN/TN at 90 days (%)	13.8 ± 1.2[c]	9.72 ± 0.83[b]	6.58 ± 0.50[a]
pH 4.6-SN/TN at 180 days (%)	18.7 ± 0.5[c]	13.6 ± 0.8[b]	9.86 ± 0.60[a]

[a] Abbreviations are: MNFS, moisture in non-fat substance. FDM, fat in dry matter. Analysis for moisture, fat, protein, salt, moisture in non-fat substance (MNFS), fat in dry matter (FDM), salt in moisture and pH value were performed at 14 days of ripening and pH 4.6-SN/TN levels at 90 and 180 days of ripening. Yield of cheese was measured at 0 day of ripening. Moisture adjusted yield was adjusted to 37.5% moisture. Values are means ± standard deviations, from three independent trials; means within the same row not sharing a common superscript differ significantly ($P < 0.05$).

Table 3
Rheological properties of cheeses made from bovine calf chymosin (BC), camel chymosin (CC) and modified camel chymosin (mCC) at 30, 60 and 180 days of ripening, as assessed by dynamic small amplitude oscillatory rheology.[a]

Storage time	LT_{max}	Temperature at LT_{max} (°C)	Temperature at LT = 1 (°C)
30 d			
BC	2.32 ± 0.22[a]	71.6 ± 2.0[a]	55.6 ± 1.7[a]
CC	2.36 ± 0.23[a]	73.4 ± 1.3[ab]	54.5 ± 1.9[a]
mCC	2.55 ± 0.23[a]	74.9 ± 1.7[b]	54.7 ± 1.1[a]
90 d			
BC	1.67 ± 0.15[a]	64.7 ± 2.0[a]	56.6 ± 1.5[a]
CC	1.80 ± 0.20[a]	67.6 ± 1.3[b]	57.9 ± 1.2[a]
mCC	2.37 ± 0.34[b]	70.6 ± 3.1[c]	57.2 ± 1.4[a]
120 d			
BC	1.66 ± 0.18[a]	66.6 ± 1.3[a]	58.2 ± 2.1[a]
CC	1.79 ± 0.28[a]	67.5 ± 1.3[ab]	58.7 ± 0.9[a]
mCC	2.29 ± 0.37[b]	70.2 ± 3.8[b]	58.7 ± 1.3[a]

[a] Data are means ± standard deviation, from three independent trials; means within the same column not sharing a similar superscript differ significantly ($P < 0.05$).

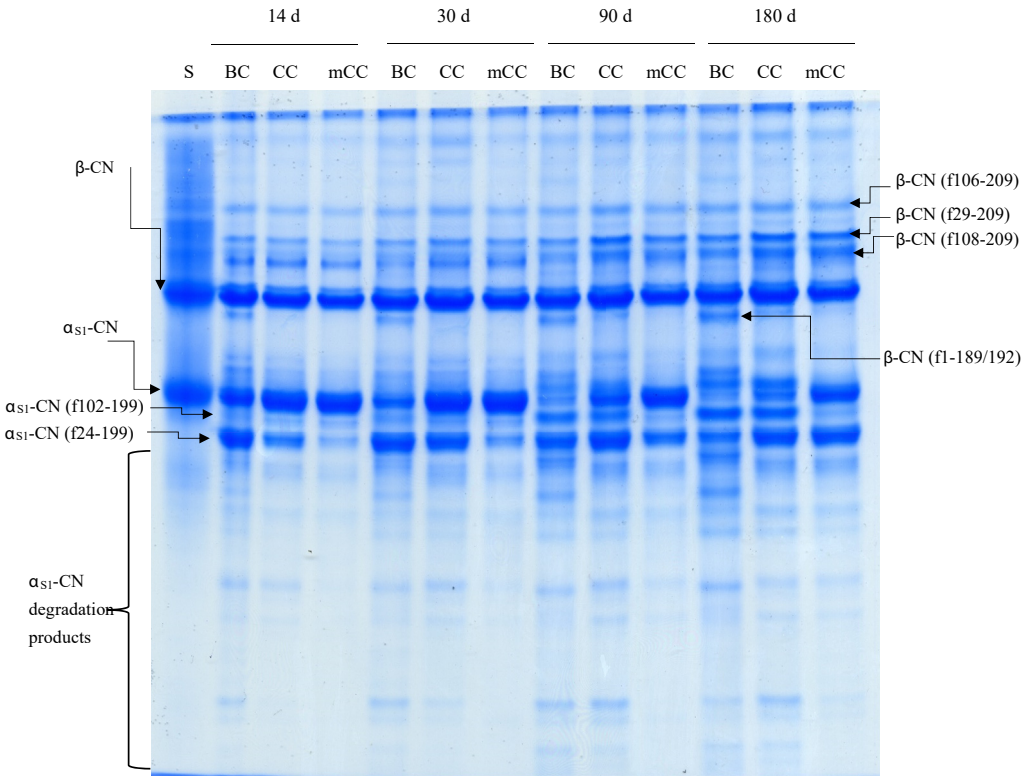

Fig. 2. Urea-polyacrylamide gel electrophoretograms of Cheddar cheeses made from milk coagulated with bovine calf chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC) at 14, 30, 90 and 180 days of ripening. Sodium caseinate (S) was used as standard on both sides of the gel. The figure corresponds to data for one of the three cheese replicates; all replicates were similar.

chymosins (Bansal et al., 2009). The higher hardness of cheeses made with both camel chymosins may be attributed to the lower level of primary proteolysis caused by the camel chymosins.

3.5. Descriptive sensory analysis

After 180 days of ripening, no significant differences ($P > 0.05$) were found in milky, whey, milk fat or mothball or sour taste, salty taste, sweet or umami tastes among the cheeses. The fruity flavour of cheese CC was significantly lower ($P < 0.05$) than that of cheese BC and mCC. The sulphur and barny flavours of cheeses CC and mCC were significantly lower ($P < 0.05$) than that of cheese BC. The brothy flavour and bitter taste of cheese mCC were significantly lower ($P < 0.05$) than that of cheese CC, followed by cheese BC (Table 4). Bansal et al. (2009) found that cheese made with camel chymosin has less sulphur, brothy flavours and less bitterness than cheese made with bovine calf chymosin.

Significant differences were also found in sensory texture attributes. The hand firmness of cheese mCC was significantly higher ($P < 0.05$) than that of cheese BC (Table 5). This is in agreement with instrumental texture analysis, which showed that the hardness of cheeses CC and mCC was significantly higher than that of cheese BC after 180 days of ripening (Fig. 3A). The rate of recovery measured by hand and springiness were not detected in cheese BC or CC, and only the cheese made with modified camel chymosin (mCC) showed the rate of recovery and springiness

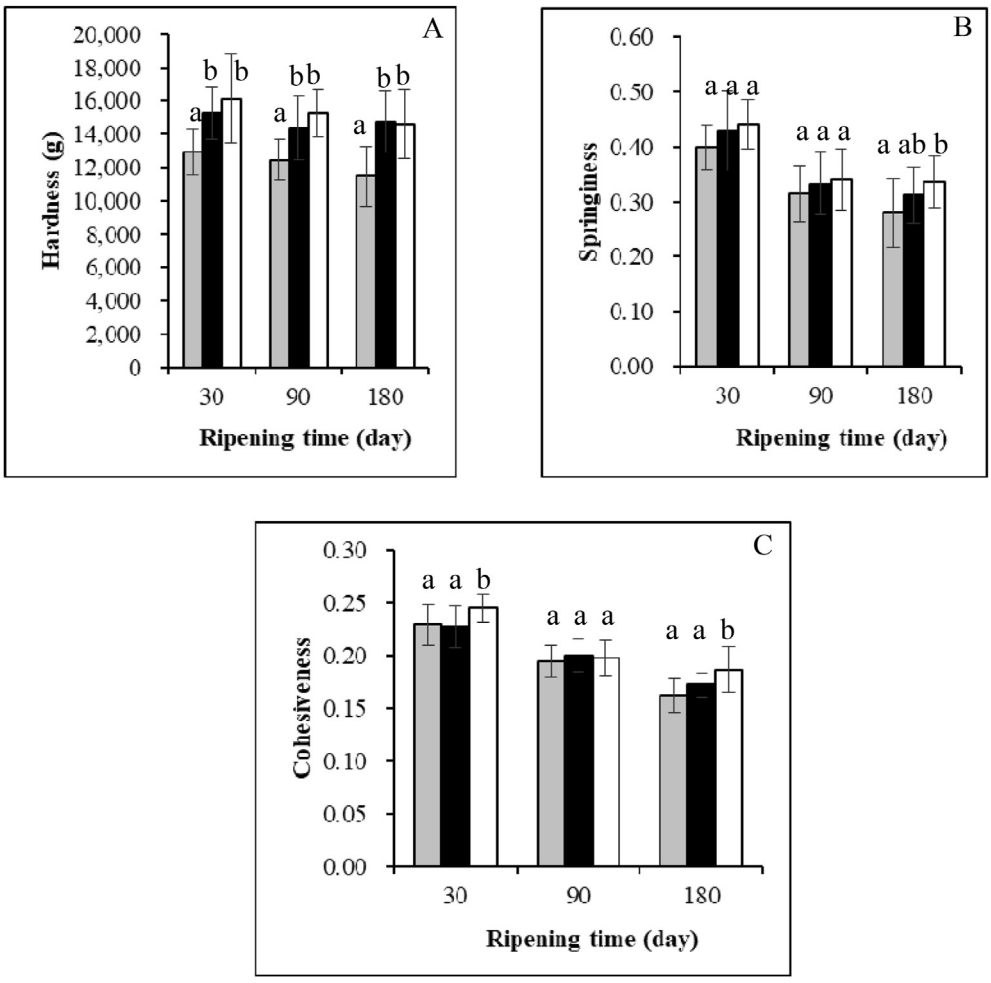

Fig. 3. Changes in TPA hardness (A), springiness (B) and cohesiveness (C) of Cheddar cheeses made from milk coagulated with bovine calf chymosin (BC, *grey bars*), camel chymosin (CC, *black bars*) or modified camel chymosin (mCC, *white bars*) at 30, 90 and 180 days of ripening. [a,b] Means at the same time point not sharing a common superscript differ ($P < 0.05$). Values are means of three independent trials. Error bars indicate standard deviation.

Table 4
Flavour profiles for Cheddar cheeses made with bovine calf chymosin (BC), camel chymosin (CC) or modified camel chymosin (mCC) after 180 days of ripening.[a]

Flavour	Treatments		
	BC	CC	mCC
Cooked/milky	3.1 ± 0.3[a]	3.1 ± 0.4[a]	3.3 ± 0.3[a]
Whey	1.0 ± 0.3[a]	1.0 ± 0.4[a]	1.2 ± 0.4[a]
Milkfat	3.0 ± 0.4[a]	3.2 ± 0.4[a]	3.0 ± 0.4[a]
Fruity	1.5 ± 0.4[a]	0.7 ± 0.3[b]	1.2 ± 0.5[a]
Sulphur	2.3 ± 0.4[a]	1.5 ± 0.3[b]	1.6 ± 0.3[b]
Brothy	2.1 ± 0.4[a]	1.8 ± 0.5[b]	1.5 ± 0.4[c]
Barny	2.4 ± 0.4[a]	2.0 ± 0.5[b]	1.9 ± 0.4[b]
Mothball/faecal	0.5 ± 0.4[a]	0.5 ± 0.5[a]	0.5 ± 0.4[a]
Sour taste	3.1 ± 0.3[a]	3.2 ± 0.3[a]	3.2 ± 0.4[a]
Bitter taste	2.0 ± 0.5[a]	1.3 ± 0.4[b]	0.9 ± 0.3[c]
Salty taste	3.0 ± 0.4[a]	3.0 ± 0.4[a]	3.1 ± 0.3[a]
Sweet taste	1.9 ± 0.3[a]	1.9 ± 0.4[a]	1.9 ± 0.4[a]
Umami taste	3.2 ± 0.5[a]	3.2 ± 0.4[a]	3.1 ± 0.5[a]

[a] Data are means from duplicate analyses by 6 trained panellists of three independent trials; means within the same row not sharing a similar superscript differ significantly ($P < 0.05$). Attributes not listed were not detected in cheeses. Cheese flavours (Drake et al., 2001) were scored on a 0 to 15-point universal intensity scale (Spectrum method, Meilgaard et al., 1999). Most cheese flavours fall between 0 and 5 (Drake, 2007).

(Table 5). The TPA results also showed significantly higher springiness ($P < 0.05$) in cheese mCC compared with cheese BC after 180 days of ripening (Fig. 3B).

At first bite, cheeses made with both camel chymosin types showed significantly higher ($P < 0.05$) firmness than that of cheese made with calf chymosin; the fracturability of cheese made with modified camel chymosin was significantly higher ($P < 0.05$) than that of cheese made with calf and camel chymosin. During mastication, mCC cheese showed a significantly lower ($P < 0.05$) degree of breakdown, cohesiveness and adhesiveness than that of cheeses BC and CC. In contrast, TPA results showed that the cohesiveness of cheese mCC was significantly higher ($P < 0.05$) than that of the other batches of cheese after 180 days of ripening, the reason for which is unclear. The different results of cohesiveness between the TPA and the sensory test were observed before: Bansal et al. (2009) found no significant differences ($P > 0.05$) in cohesiveness between the cheeses made from bovine or camel chymosins; however, the sensory cohesiveness of the cheese made from camel chymosin in that study was lower than that of the cheese made from bovine chymosin.

After expectoration, the smoothness of mouthcoating of cheese made with modified camel chymosin (mCC) was

Table 5
Sensory texture profiles for Cheddar cheeses made with bovine calf chymosin (BC), camel chymosin (CC) or highly specific camel chymosin (mCC) after 180 days of ripening.[a]

Attributes	Treatments		
	BC	CC	mCC
Hand firmness	13.3 ± 0.7[b]	13.9 ± 1.0[ab]	14.3 ± 0.9[a]
Hand rate of recovery	ND	ND	7.8 ± 1.2
Springiness	ND	ND	7.8 ± 1.3
First bite			
Firmness	9.0 ± 0.4[b]	9.7 ± 0.8[a]	10.1 ± 1.1[a]
Fracturability	7.4 ± 1.0[b]	7.5 ± 1.2[b]	8.4 ± 1.1[a]
Mastication			
Degree of breakdown	10.3 ± 1.2[a]	9.6 ± 1.1[b]	8.8 ± 1.2[c]
Cohesiveness	9.8 ± 1.0[a]	9.6 ± 1.0[a]	8.6 ± 1.2[b]
Adhesiveness	9.7 ± 1.2[a]	9.6 ± 0.9[a]	8.7 ± 1.3[b]
Smoothness of mass	9.8 ± 0.9[a]	9.8 ± 1.2[a]	9.7 ± 1.0[a]
After expectoration			
Smoothness mouthcoat	10.4 ± 1.3[a]	9.9 ± 1.0[a]	8.8 ± 0.9[b]

[a] Data are means from duplicate analyses by 6 trained panellists of three independent trials (ND, not detected); means within the same row not sharing a similar superscript differ significantly ($P < 0.05$). Attributes not listed were not detected in cheeses. Cheese texture attributes (Rogers et al., 2009) were scored on a 0 to 15-point product-specific intensity scale (Spectrum method, Meilgaard et al., 1999).

significantly lower ($P < 0.05$) than that of cheeses BC and CC. Bansal et al. (2009) found that cheeses made with calf chymosin showed higher smoothness and mouthcoating and higher cohesiveness than cheeses made with camel chymosin, which is consistent with these results. Differences in sensory flavour and texture between treatments may be related to different extents of proteolysis in cheeses (Bansal et al., 2009).

3.6. Meltability

At 30 days of ripening, no significant differences ($P > 0.05$) in meltability were found between treatments, but the meltability of both cheeses CC and mCC were significantly lower ($P < 0.05$) than that of cheese BC at 90 and 180 days of ripening. No significant differences were found between the meltability of cheeses CC and mCC at 30, 90 and 180 days of ripening (Fig. 4). The meltability of cheese increases with the breakdown of α_{S1}-CN and β-CN (Bogenrief & Olson, 1995; Dave, McMahon, Oberg, & Broadbent, 2003). No differences in breakdown of β-CN were found between samples, while lower extents of breakdown of α_{S1}-CN were found in cheeses CC and mCC compared with cheese BC. The lower meltability of cheeses CC and mCC may thus be linked to their lower levels of proteolysis.

3.7. Dynamic small-amplitude oscillatory rheology

No significant differences ($P > 0.05$) were found in the temperature at LT = 1 between all treatments after 30, 90 and 180 days of ripening (Table 3). The temperature at LT = 1 is the temperature at which the cheese started to melt (Gunasekaran & Ak, 2002). After 30 days of ripening, no significant differences ($P > 0.05$) were found in LT_{max} values between all the treatments; the temperature at LT_{max} of sample mCC was significantly higher ($P < 0.05$) than that of the sample BC. After 90 and 180 days of ripening, the LT_{max} of cheese mCC was significantly higher ($P < 0.05$) than that of cheeses BC and CC and no significant differences ($P > 0.05$) were found between cheeses BC and CC; the temperature at LT_{max} of cheese mCC was significantly higher ($P < 0.05$) than that of cheese BC. Moynihan et al. (2014) did not find any significant difference in the LT_{max} values between Mozzarella cheese made with BC and CC, which is consistent with the results of this study. The higher LT_{max} relates to higher meltability (Lucey, Johnson, & Horne, 2003), which indicates that the meltability of cheese mCC was significantly higher ($P < 0.05$) than that of cheeses BC and CC at the temperature between 20 and 80 °C.

Proteolysis affects the rheological properties of cheese during ripening (Lucey et al., 2003). Cheese mCC showed the lowest proteolysis, while the meltability of cheese mCC at the temperature between 20 and 80 °C was not the lowest, the reason of which is unknown. In contrast, the results of the Schreiber test showed that cheeses made with bovine calf chymosin had the highest meltability at high temperatures (232 °C). Cooke, Khosrowshahi, and McSweeney (2013) found that the differences between the results of the Schreiber test and dynamic small-amplitude rheology may be linked to the different extent of temperature exposure and different speed of moisture, fat, and protein separation at different temperatures. At lower temperatures (<80 °C), the cheeses made with mCC showed higher meltability, while cheeses made with BC showed greater meltability at high temperatures (232 °C).

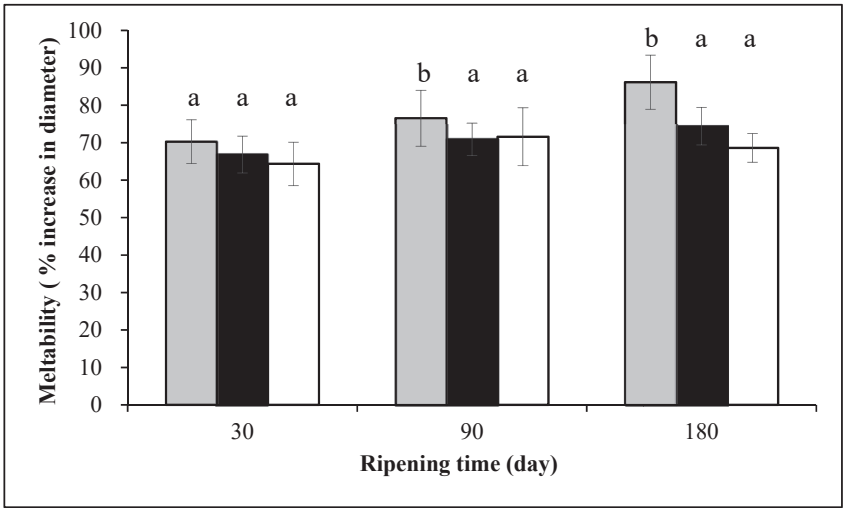

Fig. 4. Meltability (diameter increase %) of Cheddar cheeses made from milk coagulated with bovine calf chymosin (BC, *grey bars*), camel chymosin (CC, *black bars*) or modified camel chymosin (mCC, *white bars*) at 30, 90 and 180 days of ripening. [a,b] Means at the same time point not sharing a common superscript differ ($P < 0.05$). Values are means of three independent trials. Error bars indicate standard deviation.

4. Conclusion

Cheddar cheeses were manufactured using the same IMCU level of fermentation-produced bovine or fermentation-produced camel or fermentation-produced camel chymosin with structural changes as a coagulant. The same curd strength was obtained at the same time from three different chymosins during manufacture. Minor differences were found in the composition and pH of the 14 days old cheeses made with fermentation produced calf chymosin, camel chymosin or modified camel chymosin. Levels of pH 4.6 SN/TN and urea-PAGE analysis showed that the proteolysis of cheese mCC may be lower than that of cheeses BC and CC, especially lower in the hydrolysis of α_{S1}-CN and β-CN. Cheeses manufactured using modified camel chymosin (mCC) showed significantly higher hardness, springiness and cohesiveness and significantly lower meltability compared with the cheese manufactured using calf chymosin (BC). Descriptive sensory analysis suggested that cheese made with both camel chymosins had lower ($P < 0.05$) sulphur, brothy, barny flavours and bitter taste than cheese made with calf chymosin. The sensory firmness of cheese made with both camel chymosins was significantly lower ($P < 0.05$) than that of cheese BC which is consistent with the texture profile analysis results. The springiness, cohesiveness and smoothness of mouthcoating of cheese mCC were significantly lower ($P < 0.05$) than that of cheeses BC and CC. At low temperature (80 °C), cheese mCC showed significantly higher meltability than cheeses BC and CC, while at high temperature (232 °C) cheese BC showed greater meltability than that of cheeses CC and mCC. These results suggest that using the modified camel chymosin for Cheddar cheese manufacture is feasible and may result in lower levels of primary proteolysis, modified texture and different flavour compared with the use of calf and camel chymosin.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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