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Effect of high heat treatment and β-casein-reduction on the rennet coagulation and ripening of Cheddar and Emmental cheeses manufactured from micellar casein concentrate

Thesis presented to the National University of Ireland for the degree of Doctor of Philosophy by

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

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Xiaofeng Xia

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Abstract

Microfiltration (MF) removed 53.60 – 70.29 % of native whey protein from pasteurized skim milk to permeate, and retained micellar casein concentrate (MCC) (89.64 - 93.64 % of casein expressed as percentage of total protein) in the retentate. The objective of this thesis was to formulate cheesemilk of desired composition by using MF and to study how each component affect cheese quality. In this thesis, (1) the influence of temperature, number of diafiltration (DF) steps, composition of DF media as well as type of MF membrane on the composition of MCC was examined; (2) the effect of levels of total casein, β-casein or whey protein in cheesemilk on the qualities of semi-hard cheese (Cheddar or Emmental) made therefrom were evaluated and (3) as casein micelles is more heat stable than whey protein, the heat stability of MCC and its impact on resultant cheesemilk was also analysed. Compared to MF without a DF step, DF with water increased the removal of whey protein and small molecules, such as lactose and soluble salts, from feed milk to permeate and the depletion of solutes present in the serum phase of milk increased with an increasing number of DF steps. The rennet coagulability of cheesemilk in addition to the composition, pH, texture, yield, flowability and colour in resultant cheeses were not affected by the whey protein content of the cheesemilk. However, removing whey protein from milk increased the plasmin activity of the cheesemilk formulated therefrom and also increased the level of primary proteolysis (as measured by urea-PAGE and HPLC) in the resultant Emmental cheeses. Increasing the casein content in cheesemilk led to an increased gel firming rate in milk and an increase in hardness, pH and plasmin activity as well as a decrease in moisture content and primary proteolysis in the resultant Cheddar cheese. Reducing β-casein levels from milk by 4.25 % neither affected the rennet coagulation properties of cheesemilk nor influenced the composition, pH, plasmin activity, primary proteolysis, texture profile, flowability and colour in the resultant Emmental cheese. Depletion of milk whey protein content in milk by either 53.60 % or 70.29 % largely increased milk heat stability as measured by rennet coagulation, plasmin activity and cheese quality. Subjecting MCC with 70.29 % whey protein depletion to 90 °C for 15 s neither impaired the rennet coagulation properties of cheese milk prepared therefrom nor altered the composition, texture profile, meltability and volatile profile of resultant Cheddar cheese. However for whey protein reduced-milk (53.60 %) heated at 120 °C for 15 s, the rennet coagulability and plasmin activity in the resultant cheesemilk were significantly reduced, with the flowability in the resultant Emmental cheese decreased and redness increased. Overall, the results generated from this research will help cheesemakers to formulate cheesemilk of desired composition and milk with superior heat stability by using MF and micellar casein concentrates. This research also generated new knowledge on the interactions between cheesemilk components and processes to which the milk is subjected to and how this influences the quality of semi-hard cheeses produced therefrom.

Publications

Peer-reviewed articles:

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Xia, X., Tobin, J. T., Sharma, P., Fenelon, M.A., McSweeney, P. L. H., and Sheehan, J.J. (2020). Application of a cascade membrane filtration process to standardise serum protein depleted cheese milk for Cheddar cheese manufacture. International Dairy Journal:104796.

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Xiaofeng Xia, John T. Tobin, Paul L. H. McSweeney, and Jeremiah J. Sheehan. 'Cheddar manufacture using formulations of micellar casein concentrate and Reverse Osmosis products derived from ultrafiltration permeate', The 47th Annual Food Science and Technology Conference, December 6-7, 2018, Cork, Ireland.

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Chapter 1: Production, composition and preservation of micellar casein concentrate and its application in cheesemaking: A review

1.1 Abstract

A level of 40 - 95 % of native whey proteins are removed from skim milk by microfiltration (MF) generating a whey protein-reduced or depleted micellar casein concentrate (MCC). The MCC lactose and calcium contents are influenced by the pH and temperature of the feed milk and MF process variables including the type and number of diafiltration steps used. MCC may be stabilised by freezing, refrigeration, heat treatment or spray drying and stored or transported for cheese manufacture. MCC can be combined with water and / or milk ultrafiltration permeate to prepare milk for manufacture of diverse cheese types. Manipulation of processing parameters during MF influences cheesemilk composition (casein, β-casein, whey protein, lactose and calcium contents) and thus the composition and functionality of cheeses derived therefrom.

1.2 Introduction

Microfiltration (MF) of milk at pore size 0.08 - 0.2 μm can separate casein micelles in their native form from whey proteins, with the casein micelles remaining in the MF retentate and whey proteins partitioning to MF permeate (Jost et al., 1999; Nelson and Barbano, 2005a; Govindasamy-Lucey et al., 2007; Seibel et al., 2015). Whey proteins in MF permeate sometimes referred to as 'ideal', are considered as more valuable than whey proteins derived from cheese whey because: 1, MF permeate is free from starter culture, rennet, cheese colorant, caseinomacropeptide (CMP), cheese fines, derivatives of microbial activity and fat (Nelson and Barbano, 2005a); 2, MF permeate needs less heat treatment before concentration by ultrafiltration (UF) than cheese whey (Bacher and Kønigsfeldt, 2000); 3, the process flow rate during UF of MF permeate is higher than that possible with cheese whey (Nelson and Barbano,

2005a); and 4, whey protein powder produced from MF permeate has better solubility, foaming and gel properties than powder produced from cheese whey (Bacher and Kønigsfeldt, 2000; Heino et al., 2007).

McCarthy et al. (2017) reported that microfiltration could be applied to bovine milk to produce an MF permeate of similar casein to whey protein ratio (35: 65) to that of human milk (40: 60), which could be used as protein base for infant formula production. Since removal of whey proteins from cheesemilk, as a high value by-product stream, does not significantly affect its rennet coagulation or the quality of the resultant cheese (Nelson and Barbano, 2005b), MF retentate (from here on micellar casein concentrate; MCC) can be used to prepare cheesemilk (Govindasamy-Lucey et al., 2007).

The major compositional components in MCC include casein micelles, whey proteins, lactose, milk salts and other small molecules. The casein content in MCC (as a percentage of total protein) can vary from 82.14 % to 99.34 % depending on processing factors such as the number of diafiltration steps, concentration factor and membrane type (Schreiber, 2001; Neocleous et al., 2002a; Beckman et al., 2010; Hurt and Barbano, 2010; Sauer et al., 2012; Beckman and Barbano, 2013; Zulewska and Barbano, 2013). Varying processing parameters used during the MF of milk, such as pH (Brandsma and Rizvi, 1999), temperature and the amount of water added during diafiltration (O'Mahony et al., 2014), can affect the protein profile and colloidal calcium content of MCC (Figure 1.1). The concentration of lactose and soluble calcium in MCC can vary depending on the diafiltrant used (water or UF permeate; Figure 1.1) and on the number of diafiltration steps (Nelson and Barbano, 2005a; Amelia and Barbano, 2013). Composition of cheesemilk (casein, lactose, soluble and colloidal calcium) is an important factor affecting the quality and yield of resultant

cheeses, thus a clear understanding of the factors affecting the composition of MCC, and the cheesemilk derived from the MCC is critical for cheese manufacturers.

Where milk may be surplus to current market requirements, it can be fractionated into cream or butter, MCC, whey protein concentrates or isolate, ultrafiltration (UF) permeate and/ or lactose by the use of separation technologies, and then stored for further use or transported to regions when or where there is a greater demand or market for specific dairy ingredients. The most common ways to preserve MCC for storage include spray drying, freezing and refrigeration (Davenel et al., 1997; Amelia and Barbano, 2013; Lu et al., 2015b; Gaber et al., 2020b), however the preservation method can affect the shelf life and functionality of MCC, while there are also considerations for cost and energy consumption for the various storage methods.

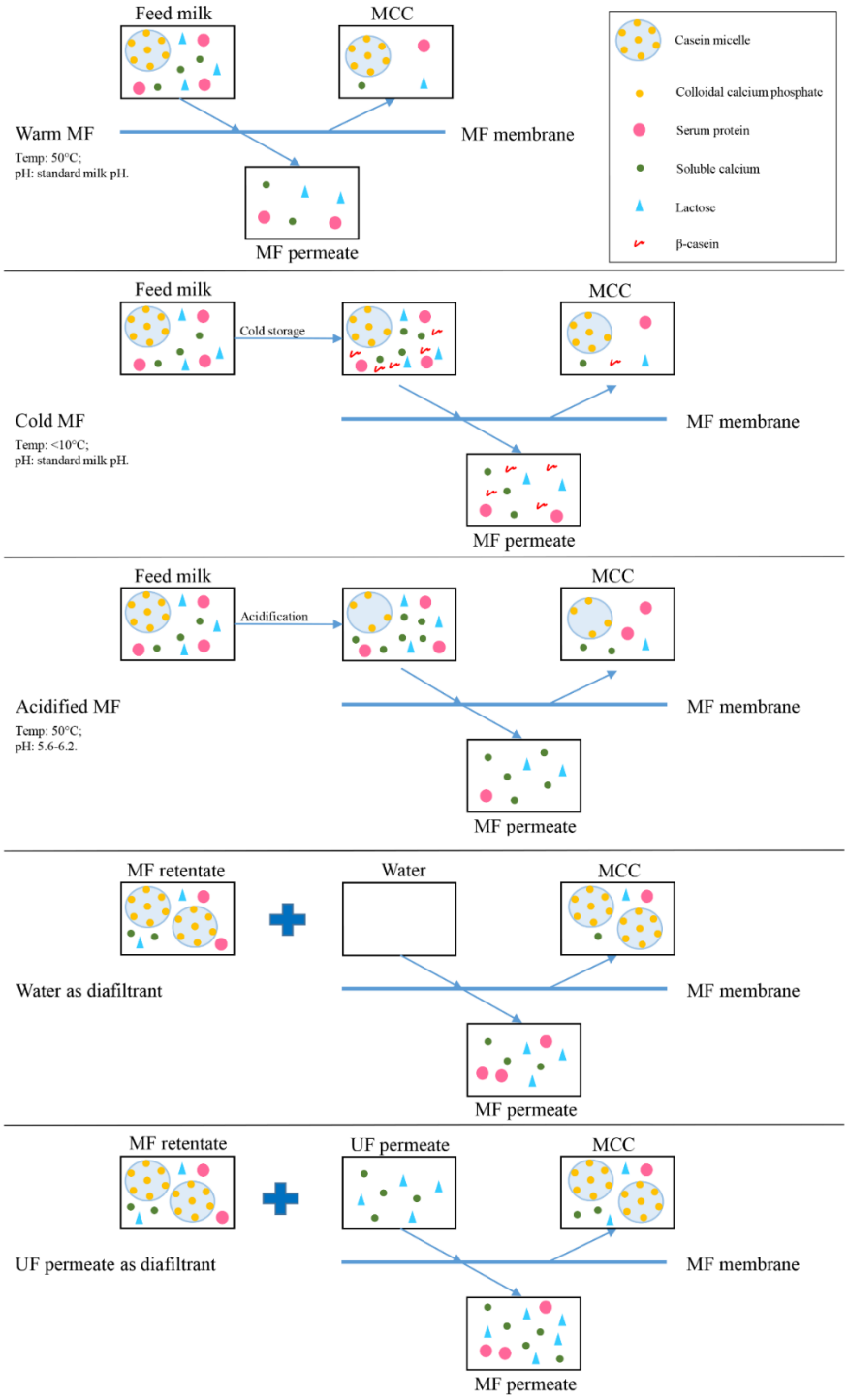

Figure 1.1. Effect of temperature, pH and type of diafiltrant on the composition of micellar casein concentrate. MF: microfiltration; MCC: micellar casein concentrate; UF: ultrafiltration.

In this review, the factors which influence MCC composition, the influence of different preservation methods on the functionality of MCC and factors influencing cheese manufacture properties of MCC are considered.

1.3 Process factors influencing MCC composition

1.3.1 *pH*

Calcium is present in milk in two forms: 69 % is present in casein micelles as colloidal calcium (colloidal calcium phosphate; CCP) and 31 % is present in the milk serum phase as soluble calcium (Gaucheron, 2005). Milk salts can be readily removed to MF permeate when dissolved in the serum phase; however it is hard to remove CCP from casein micelles unless extensive diafiltration with water is carried out (Dalgleish and Corredig, 2012; Schäfer et al., 2019a). By pre-acidifying the MF feed milk with citric acid or glucono-delta-lactone (GDL), a proportion of CCP in casein micelles can be solubilised into the milk serum phase and removed to MF permeate. This can reduce the calcium to casein ratio by 35.46-52 % in the resultant MCC compared to that in the feed milk; thus an increasing amount of colloidal calcium can be removed to the permeate by decreasing the pH of the feed milk (Figure 1.1) (Brandsma and Rizvi, 1999; Schäfer et al., 2019a). However, diafiltration with acidified water does not significantly affect the total calcium content in MCC but can result in a slight decrease in pH and increase in ionic calcium levels (Gaber et al., 2020a). Compared to MF at a standard milk pH, less whey protein is removed from the final MCC when using an acidified MF, and the permeate flux will also decrease significantly (Brandsma and Rizvi, 1999; Schäfer et al., 2019a). When carrying out acidified MF, a longer processing period is expected; in addition, an increased number of DF steps are required to increase permeate flux and improve whey protein removal. Since

demineralized MCC produced from acidified MF is mainly used to prepare cheesemilk of high casein content (8.5 %-22.87 %) and to make cheese (Ardisson-Korat and Rizvi, 2004; Schäfer et al., 2019c) (see Section 1.4.3), a greater weight of milk solids can be converted to cheese at a given time than for traditional cheesemaking. However, we suggest that future studies should determine whether the industrial scale benefit of higher conversion of milk solids to cheese during cheesemaking may outweigh the lower processing speed incurred during MF of the acidified feed. Similarly, as difficulties arise in the processing of acid whey (pH $\leq$ 6.0) during UF and spray drying, acid whey is considered un-wanted by-product (Schäfer et al., 2019c) and Schäfer et al. (2019a) suggested that the pH of feed milk should be no less than 6.2.

1.3.2 *Temperature*

MF carried out at 50 °C is called warm MF, and facilitates good permeate flux and efficient whey protein removal from MCC. It is the most common choice of the dairy industry (Samuelsson et al., 1997; O'Mahony and Tuohy, 2013; Schäfer et al., 2019a). To isolate β-casein from milk, to generate MF permeate with a protein profile close to that of human milk (casein: whey protein ratio = 40: 60), to prevent whey protein denaturation or to reduce thermophilic microbial growth in MCC, MF can be carried out at low temperature (i.e., 2 - 8.9 °C) and is called cold MF (Govindasamy-Lucey et al., 2007; O'Mahony et al., 2014; McCarthy et al., 2017). Isolation of β-casein from bovine milk is the biggest driving force for the application of cold MF in the dairy industry due to the many desirable functionalities of β-casein: (1) a good emulsion stabiliser in food matrices (Li et al., 2016); (2) a precursor for functional peptides (Post et al., 2012); (3) for enrichment for infant formula (Atamer et al., 2017);and (4) as an oral delivery system for hydrophobic drugs (Atamer et al., 2017). After cold storage (1- 4 °C) of feed milk for 12 - 48 hours, both micellar bound calcium

and β-casein can be partially dissociated from casein micelles, dissolved or dispersed in the milk serum phase (Pierre and Brule, 1981) and partitioned to MF permeate during subsequent cold MF (2 - 8.9 °C). This results in MCC with 1 - 20 % reduction in β-casein and a decreased calcium: casein ratio (Holland et al., 2011; O'Mahony et al., 2014; Seibel et al., 2015; McCarthy et al., 2017; Zulewska et al., 2018; Schäfer et al., 2019a,b) compared to MCC obtained from warm MF (Figure 1.1). The wide range in values for reduction of β-casein obtained by different researchers could be related to the different processing parameters applied, for example, varying membrane properties, thermal history of feed milk, temperature and duration of cold storage, processing temperature, number of diafiltration (DF) steps, etc.

Cold storage of feed milk before MF is the key step to maximize β-casein removal from MCC. To maximize β-casein dissociation from casein micelles and ultimately remove more β-casein from MCC, the following process steps are recommended: (1) dilution of the feed milk by 2 - 4 fold (volume or weight) with deionised water prior to cold storage; (2) an elongated storage time, i.e., 16 – 48 hours and (3) low storage temperature, i.e., 1 - 4 °C (Holland et al., 2011; O'Mahony et al., 2014; McCarthy et al., 2017; Schäfer et al., 2019b). Since operation of the membrane filtration plant will generate heat and thus will warm the milk, the β-casein may start to re-associate with casein micelles when milk temperature increase to 20 °C (Davies and Law, 1983), thus chill water is often circulated through the membrane filtration plant and sometimes also the jacket of the tanks which contains the MF retentate to maintain low processing temperature (Holland et al., 2011; O'Mahony et al., 2014; McCarthy et al., 2017; Schäfer et al., 2019b).

However, the limitations to cold MF are also evident in comparison to warm MF; the permeate flux in cold MF (McCarthy et al., 2017; Schäfer et al., 2019a) and

the levels of whey protein removed from cold MF are much lower (O'Mahony, 2014); and it is also difficult to maintain a low temperature in the feed milk and membrane system during cold MF (Zulewska et al., 2018; Schäfer et al., 2019b). However, from our own experience (Xia et al, 2021, unpublished), cold MF can remove as much whey protein from MCC as warm MF; the discrepancy between different studies might be due to the use of different membranes in various studies, although further research on this is suggested.

1.3.3 *Diafiltrant*

During MF of milk, casein micelles and bound colloidal calcium phosphate are retained in the MF retentate, allowing the milk serum phase including water, whey protein, lactose, soluble salts and other small molecules to partition to MF permeate (Neocleous et al., 2002a; Hurt and Barbano, 2015). As MF progress, both the concentration and viscosity of the MF retentate increases, resulting in a decrease in permeate flux and in the permeability of whey proteins (Smith, 2013). By adding a diafiltrant such as deionized water or UF permeate to the MF retentate, both the concentration and viscosity of MF retentate can be decreased; thus the permeate flux increases and more whey protein can be removed from the MF retentate (Nelson and Barbano, 2005a; Amelia and Barbano, 2013). Increasing the number of diafiltration steps can increase whey protein removal from MF retentate, and the composition of MCC can vary depending on the diafiltrant (i.e., deionised water or UF permeate) used and the number of steps of diafiltration applied (Nelson and Barbano, 2005a; Amelia and Barbano, 2013; Xia et al., 2020a).

Water as diafiltrant

When deionised water is added to MF retentate as a diafiltrant, the solutes (including whey protein, lactose, soluble salts, etc.) in the serum phase of MF retentate will be diluted and partially removed to MF permeate during diafiltration (Figure 1.1). An increased amount of whey protein, along with lactose and soluble salts, can be removed from the MF retentate with an increased number of diafiltration steps (Hurt et al., 2010; Schäfer et al., 2019a; Xia et al., 2020a). MF combined with a two-stage DF with water can remove 94.14 % of lactose and 22 - 29.99 % of total calcium from the feed milk (Lu et al., 2016; Xia et al., 2020a). Addition of deionised water to MF retentate, inducing partial solubilisation of colloidal calcium phosphate to the diluted serum phase in MF retentate, could also lead to the removal of a certain proportion of micellar bound salts to the MF permeate, although the exact amount is yet to be determined (Lu et al., 2016; Boiani et al., 2017, 2018; Xia et al., 2020a). Even though MCC produced in this way has very low contents of lactose and soluble salts, cheesemilk with target concentrations of casein, lactose, soluble and total salts as well as a typical milk pH (i.e., 6.60 - 6.70) can be conveniently prepared from MCC, MF permeate free of whey protein (which can be produced by UF and reverse osmosis) and water as described by Xia et al. (2020a) (Figure 1.2).

The pH of MF retentate increases after each DF step; for feed milk of typical milk pH, i.e., 6.55 - 6.76, the pH in the final MCC can reach 6.96 to 7.30 after two steps of DF. Since the pH of deionised water is 6.83 - 7.00, dissociation of colloidal calcium phosphate and a decreased concentration of buffering salts in MF retentate are suggested to be responsible for the pH increase (Hurt et al., 2010; Boiani et al., 2017; McCarthy et al., 2017; Xia et al., 2020a).

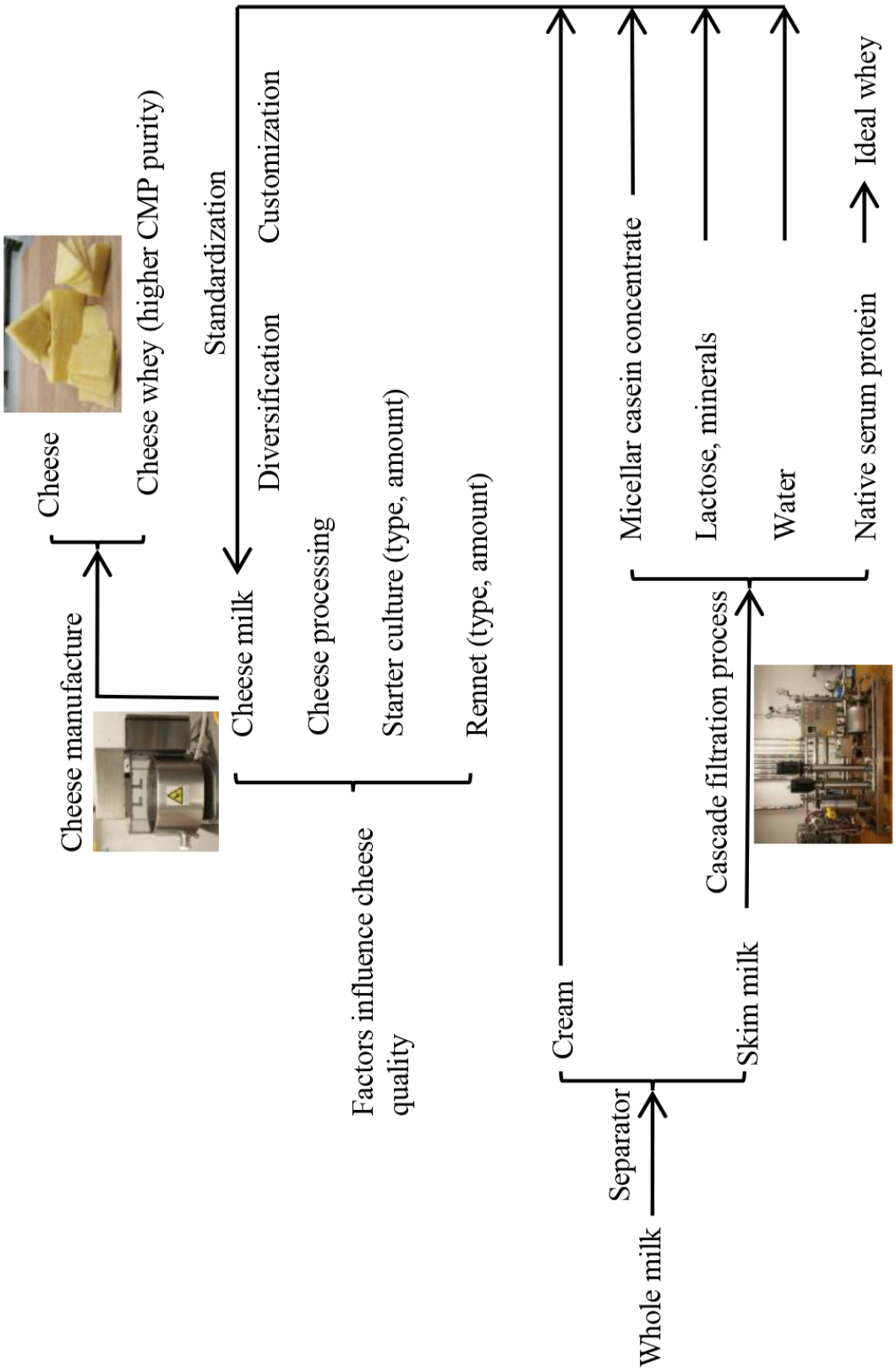

Figure 1.2. Application of microfiltration to cheese manufacture. CMP: caseinomicropeptide.

UF permeate as diafiltrant

UF permeate for use as a diafiltrant during MF can be prepared by UF of the MF permeate of the feed milk (Bulca and Kulozik, 2004; Nelson and Barbano, 2005a), UF of milk (Renhe et al., 2019) or from UF permeate powder (Aaltonen and Ollikainen, 2011). It has a similar composition to that of the serum phase of feed milk, except for being whey protein depleted (Nelson and Barbano, 2005a). After adding UF permeate to the MF retentate, the concentration of whey protein in the new serum phase is decreased, with the concentration of lactose and soluble salts being unchanged, and the calcium balance between casein micelles and the new serum phase remains unaltered as well. As a result, an increased amount of whey protein can be removed from the MF retentate to permeate with an increased number of diafiltration steps, while the pH as well as the contents of lactose and soluble salts in the MF retentate or resultant MCC remains the same as those in the feed milk (Figure 1.1) (Schreiber, 2001; Bulca and Kulozik, 2004; Nelson and Barbano, 2005a; Holland et al., 2011; O'Mahony et al., 2014; Renhe et al., 2019). A whey protein-reduced cheesemilk of target casein content can easily be prepared with such an MCC by addition of UF permeate and may be used for a standard cheese manufacture process (Figure 1.2) (Nelson and Barbano, 2005a).

General comments

Adjusting the type of diafiltrant and number of diafiltration steps used controls the composition of the serum phase in MCC, with little effect on the casein micelles. By using both water and UF permeate as diafiltrants, the lactose content in MCC cheesemilk can be reduced from 4.58 % to 3.2 - 3.9 % with MF, allowing cheese makers to make high quality Emmental cheese without the requirement of a curd wash

(Heino, 2008). The diafiltrant used, either deionised water or UF permeate, can be recovered from MF permeate by UF and / or RO and re-used to conserve water use. In a three-stage MF process where deionised water or UF permeate is used as a diafiltrant, of the total whey protein quantity removed, the first stage MF (i.e., MF without DF) and the remaining stages of MF (i.e., MF coupled with two stages of DF) account for one-half to two-thirds and one-third to one-half of total whey protein removal respectively (Nelson and Barbano, 2005a; Xia et al., 2020a). This suggests that regardless of the diafiltrant used, as the number of DF steps increase, lesser quantities of whey protein are removed from MF retentate by additional DF. Depending on the membrane type and processing conditions applied, 59.95 – 95 % of whey protein can be removed from feed milk with a 3 stage MF process, generating MCC of high casein content as percentage of total protein (i.e., 91.83 - 94.76 %) (Nelson and Barbano, 2005a; Amelia and Barbano, 2013; Xia et al., 2020a, b). Thus, dairy processors need to consider the benefits of a multi-stage MF (> 3) to remove more whey protein from MCC and produce MCC of higher casein concentration relative to the additional capital investment and operating costs including time and energy consumption required by application of additional DF steps (Nelson and Barbano, 2005a; Xia et al., 2020a).

1.3.4 *Membrane type*

Duing microfiltration, ceramic and polymeric membranes are most commonly used. In comparison to ceramic membranes, polymeric membranes are less efficient in excluding whey protein from retentate (Zulewska et al., 2009; Xia et al., 2020a,b; Chapter 5), and have less stability to chemicals and heat (Baruah et al., 2006; Karasu et al., 2010). However, the capital and running costs for polymeric membranes is lower (Lawrence et al., 2008, Beckman et al., 2010), and the permeate flux is much higher

(unpublished data in Chapter 5). Ceramic membranes with larger pore size (0.14 µm) can also produce MCC with lower casein content (91.83%) than that with smaller pore sizes (0.10 µm, 93.64 %) (Xia et al., 2020a,b). Overall, cheese manufacturers should choose membranes (ceramic vs polymetric, pore size) based on the processing conditions (pH and temperature), desired casein content in MCC and budget.

1.4 Preservation of MCC

1.4.1 *Spray drying*

Dehydration of dairy products by spray drying facilitates preservation and greater ease of transport and handling of dairy ingredients and has been widely applied across the dairy industry (Davenel et al., 1997; Schuck et al., 2007). MCC powder produced by spray drying was reported to result in cheesemilk of reduced coagulation time and faster gel firming rate as well as a higher adjusted cheese yield and composition recovery, compared to traditional cheesemilk (Pierre et al., 1992; Garem et al., 2000). However, the poor reconstitutability of fresh MCC powder and its declining rehydration ability at high storage temperature (> 20 °C) can limit its functionality (Davenel et al., 1997; Schokker et al., 2011; Burgain et al., 2016; Nasser et al., 2017b). The total rehydration time of MCC powder can remain nearly constant for 12 months of storage at 4 °C (Nasser et al., 2017a), although it would be more commercially valuable if MCC powder could be manufactured in a way to retain good solubility properties over extended storage times at higher storage temperatures (i.e., > 4°C).

Addition of sodium chloride, citrate or phosphate solutions to MCC before spray drying improves the rehydration ability of the resultant MCC powder, yet at a cost to the integral structure of casein micelles (Schuck et al., 2002). Cheesemilk

prepared from such MCC powder might have a high concentration of non-sedimentable casein and impaired rennet coagulability, as non-sedimental casein can impede aggregation of *para*-casein (Lin et al., 2016). However, more research is required to confirm if MCC powder with a disintegrated casein micelle structure is suitable for cheesemaking applications or not. The reconstitutability of MCC powder can be improved without de-structuring casein micelles by (1) adding UF permeate (serum phase of milk without whey protein), lactose, sodium caseinate, whey protein or polydextrose to MCC before spray drying; (2) producing MCC through cold microfiltration (< 10 °C); (3) acidified microfiltration (Schuck et al., 1994, 2007; Davenel et al., 1997; Gaiani et al., 2007; Schokker et al., 2011; Crowley et al., 2018; Schäfer et al., 2021) or (4) alkalization of MCC with NaOH (Panthi et al., 2021). All these methods look promising to both improve the rehydration ability of MCC powder and maintain good rennet coagulability of the corresponding cheesemilk except adding sodium caseinate, since the rennet coagulability of skim milk is impaired or even lost by fortifying with various levels of sodium caseinate (Lin et al., 2017). However, further research is required to manufacture MCC powder with good rehydration ability, rennet coagulability and cheesemaking properties at the same time.

Compared to its liquid counterpart, MCC powder has a ‘corn chip’ flavour as well as higher aroma intensity especially a cardboard flavour due to spray drying (Carter et al., 2018). While the flavour in semi-hard or hard cheeses is mainly derived from the products of proteolysis, amino acid catabolism, lipolysis and fatty acid metabolism and the metabolism of lactose, lactate and citrate (McSweeney and Sousa, 2000) during ripening, it would be interesting to determine if flavours developed during the spray drying of MCC could be detected in resultant cheeses.

1.4.2 *Freezing*

Freezing of milk or of concentrated milk at ≤ - 20 °C can provide for a long shelf life (≥ 12 months) without having a significant detrimental impact on composition and functionality (Wendorff, 2001); thus it is common practice for some cheese makers to freeze surplus milk during peak production season for use in the low production season (Voutsinas et al., 1995; Wendorff, 2001; Zhang et al., 2006). However, when the temperature of MCC is decreased from the freezing point of water (i.e., 0 °C) to the storage temperature (i.e., ≤ - 20 °C), more free water in the serum phase of MCC freezes and the concentration of serum salts increases, leading to the precipitation of calcium phosphate from the serum phase to the colloidal phase (Gaber et al., 2020b). It has been suggested that the precipitated calcium phosphate links to neighbouring casein micelles and contributes to their aggregation (Wells and Leeder, 1963; de la Fuente, 1998; Gaber et al., 2020b). By mixing partially thawed MCC with 50 °C water, MCC can be fully dispersed in water with high solubility (> 80 %) (Lu et al., 2015b). Freezing MCC at - 20 °C for 15 days had no significant impact on the pH and rennet coagualability where thawed MCC was used in cheese manufacture (Gaber et al., 2020b); however more research is required to see if prolonged storage has any effect on the rennet coagulation properties of thawed MCC.

1.4.3 *Refrigeration*

Casein number is an expression the casein content in milk (or in an equivalent formulation) as a percentage of the total protein. The casein number in bovine milk ranges from 75 to 80 % (Huppertz, 2013) and the casein number in MCC is higher due to whey protein removal; a high casein number indicates a high whey protein removal from MCC by MF. Cheesemilk is not recommended to be subjected to heat treatment

above pasteurisation (72 °C × 15 s) before cheesemaking due to impaired rennet coagulability caused by whey protein denaturation (Guinee et al., 1997). However, since casein micelles are heat stable, cheesemilk standardised from high heat treated (HHT, 90 °C × 15 s), whey protein-reduced MCC (casein number 93.64 %) or UHT treated (140 °C × 5 s) whey protein depleted MCC (casein number 99.34 %) retain good rennet coaguability, cheesemaking properties and cheese quality (Schreiber, 2001; Xia et al., 2020b).

By coupling maximum removal of lactose and non-protein nitrogen (NPN) from MCC with batch pasteurisation (69.44 °C × 30 min), MCC achieved a microbial shelf life for up to 16 weeks at 4 °C (Amelia and Barbano, 2013). Lactose and NPN can support the growth of microorganism, thus depleting lactose and NPN from MCC with multi-step DF with water facilitates preservation of MCC. MCC produced from MF where water is used as diafiltrant has a much longer heat coagulation time (HCT) at 120 °C than where UF permeate is used as diafiltrant (i.e., 49 min vs 31 min), due to its higher pH and lower contents of soluble calcium, phosphate and lactose (Renhe et al., 2019). This suggests that: (1) compared to MCC manufactured by DF with UF permeate, MCC manufactured by DF with water is more suitable to produce MCC with an extended shelf life due to higher heat stability and lack of substrate for microbial growth and; (2) heat treatment above pasteurisation (HHT and UHT) might give MCC a longer shelf life than batch pasteurisation. However, to provide more effective information to the dairy industry for extending the shelf life of MCC produced with water as a diafiltrant, further research is required. This research should focus on: (1) determination of the shelf life of HHT- or UHT treated MCC at different storage temperatures (4, 20 or 30 °C); (2) evaluation of the rennet coagulability and cheesemaking properties of cheesemilk as well as the composition, yield, texture,

proteolysis, functionalities and flavour of cheeses produced from heat treated MCC stored at different temperatures and durations.

Compared to MCC powder, refrigerated MCC is already hydrated and should reconstitute readily. Compared to frozen MCC, storing MCC at refrigeration temperatures can provide for greater energy and cost efficiencies, and there is no need to thaw and reconstitute MCC before use, even though frozen MCC may have a longer shelf life. However, to further clarify this issue, it is suggested that, a comparative study of the effect of different storage methods on (1) the shelf life and rennet coagulation properties of MCC, (2) composition, yield and flavour of cheese produced from MCC and (3) the consumption of energy and costs incurred by processing (spray drying, freezing or heat treatment), storage, transportation and reconstitution (rehydration or reconstitution after thawing) is needed.

1.5 Application of MF to cheesemilk standardisation

1.5.1 *Whey protein concentration*

Enzymes from coagulant (usually chymosin) and the principle indigenous proteinase in milk, plasmin, are the two major enzymes contributing to the initial proteolysis of caseins in cheese, with chymosin hydrolysing α_{s1}-casein most extensively and plasmin hydrolysing both β-casein and α_{s2}-casein (Aaltonen and Ollikainen, 2011). For cheese types manufactured with a high scald temperature (≥ 50 °C) like Emmental cheese, plasmin is the major enzyme responsible for its primary proteolysis (Bastian et al., 1997; Gagnaire et al., 2001). Plasmin, plasminogen and plasminogen activators are associated with casein micelles and plasminogen is converted to plasmin by plasminogen activators (Benfeldt, 2006). Because whey proteins inhibit the activities of both plasmin and plasminogen activators, an increased

level of removal of whey proteins to MF permeate by MF and DF, results in increased plasmin activity in MCC (Aaltonen and Ollikainen, 2011). At a constant casein content, cheese made from MCC derived cheesemilk has increased levels of β-casein breakdown and proteolysis during ripening than that made from raw milk (Nelson and Barbano, 2005b; Heino, 2008; Li et al., 2020). Aaltonen and Ollikainen (2011) predicted cheese yield in MCC derived cheese to decrease, due to casein degradation in the formulated milk by high plasmin activity prior to cheesemaking; however, this phenomenon was not observed by other researchers, probably because MCC was made into cheese within 24 hours of MF in their research (Nelson and Barbano, 2005b; Xia et al., 2020a). Further research is suggested to determine if longer storage times would promote greater casein breakdown and impact on resultant cheese yields when using MCC in cheese manufacture.

Gamlath et al. (2018) reported that as native whey proteins can inhibit chymosin activity as well as act as physical barriers to *para*-caseins, their reduced concentration in MCC cheesemilk results in a faster gel firming rate compared to conventional cheesemilk. Whey proteins denature and unfold after heating; with exposed free thiol groups on denatured whey proteins binding with other denatured whey protein, free κ-casein, casein micelles and plasmin, leading to decreased rennet coagulability and plasmin activity in heated cheesemilk (Anema et al., 2007; Aaltonen and Ollikainen, 2011). Due to low whey protein content in MCC, the rennet coagulation properties and plasmin activity in cheesemilk or cheese manufactured from high heat treated (90 °C × 15 s) MCC are not significantly different from those made from pasteurised (72 °C × 15 s) MCC (Bulca et al., 2004; Aaltonen and Ollikainen, 2011; Xia et al., 2020b, 2021, unpublished). By controlling the whey protein content of cheesemilk through MF, it is proposed that the rennet coagulation

and heat stability characteristics of cheesemilk as well as plasmin activity in the resultant cheese can be modified.

1.5.2 *Casein concentration*

The composition (i.e., concentrations of casein, fat, ash and lactose as well as casein: fat ratio) and pH of milk can vary with factors such as seasonality, feed, lactation stage, calving patterns and breed (Panthi et al., 2017; Soodam and Guinee, 2018). Since the temperature at setting, set to cut time, cutting and cooking procedure in industrial scale cheese manufacture are often fixed, the seasonal variance of casein content in cheesemilk can lead to the variance of cheese moisture content and cheese yield as well as a decline in protein recovered from cheesemilk to cheese (Guinee et al., 2006; Soodam and Guinee, 2018). By increasing the casein content of cheesemilk, the time needed for the gel to reach the firmness most suitable for cutting will decrease, the gel firmness at a pre-fixed set to cut time will increase and the window of time most suitable for cutting will reduce (Soodam and Guinee, 2018; Panthi et al., 2019). MF not only separates but also concentrates the casein micelles from skim milk to MCC and the casein content in MCC can be further increased by evaporation or spray drying (Davenel et al., 1997; Lu et al., 2015a; Xia et al., 2020a). The high casein content in MCC enables cheese makers to prepare high casein content cheesemilk directly from MCC. Standardisation of the casein content of cheesemilk to a concentrated level (i.e. 3.5 %) by adding MCC, not only avoids variability in cheese production and defects in cheese quality due to milk composition variance, but can also improve the yield obtained from a fixed number of cheese vat (Neocleous et al., 2002a; Soodam and Guinee, 2018), although it may create a greater volume of curd for down steam processes such as cheddaring belts and block former towers.

MCC cheesemilk of higher casein content than that of a standard milk has a lower moisture content and the resultant cheese curds are subjected to more frequent collisions during agitation in the vat, resulting in a greater pressure exerted on the curd particles. These are contributory factors to MCC cheeses having lower moisture, MNFS and higher protein contents compared to cheeses made from standard milk (Govindasamy-Lucey et al., 2007; Panthi et al., 2019a; Xia, et al., 2020). Similarly, the increased protein content (gel volume fraction) and decreased moisture content (filler volume fraction) is linked with an increased hardness in fresh MCC cheese (Neocleous et al., 2002b; Xia et al., 2020a). Lower levels of MNFS are presumed to be responsible (at least in part) for the slower proteolysis frequently observed in MCC cheese (St-Gelais et al., 1995; Neocleous et al., 2002b). Due to the higher casein content and associated bound colloidal calcium phosphate, MCC cheesemilk has higher buffering capacity than standard milk, and as a result, the pH in fresh MCC cheese is reported to be higher than in cheese made from standard milk (St-Gelais et al., 1995; Neocleous et al., 2002b; Xia et al., 2020a). By applying a lower temperature cooking regime (Neocleous et al., 2002b), cutting at a softer gel firmness, cutting the coagulum to a larger size, lowering the set temperature and wash water temperature (Govindasamy-Lucey et al., 2007), it is possible to attain moisture and MNFS contents in MCC cheeses comparable to those in cheeses made from standard cheesemilk, and similarly for cheese texture and proteolysis levels (Neocleous et al., 2002b). In addition, inoculation of starter cultures on a casein content basis rather than milk volume basis can also normalise both pH and proteolysis levels in MCC derived cheeses (Neocleous et al., 2002b; Govindasamy-Lucey et al., 2007).

1.5.3 *Milk salts*

Casein micelles and colloidal calcium phosphate contribute to 32.7 % and 20.9 % of the buffering capacity in skim milk respectively (Lucey et al., 1993). For MCC cheesemilk of high casein content, the elevated contents of casein micelles and bound colloidal calcium phosphate increases its buffering capacity. This high buffering capacity will decrease the rate of pH reduction during acidification as well as decreasing the ratio of dissociated to total CCP in the cheesemilk. Similarly it will result in increased calcium to protein ratio and cheese hardness (St-Gelais et al., 1997) as well as decreased levels of flowability and stretchability in the resultant cheeses (Ardisson-Korat and Rizvi, 2004).

A demineralised MCC produced using an acidified feed milk or MF retentate before MF (Section 1.2.1) has a decreased buffering capacity compared to typical MCC due to its reduced colloidal calcium phosphate content. Using a demineralised MCC normalises the calcium: protein ratio, as well as texture characteristics and heat induced functionalities in resultant Mozzarella cheese made from highly concentrated (casein content 15.40 to 22.87 %) MCC cheesemilk by decreasing the calcium: protein ratio in the step of MF, as well as that achieved during the cheese manufacture step (Brandsma and Rizvi, 1999, 2001a, b). Similarly, the high calcium content in cream cheese manufactured from a standard MCC derived cheesemilk with a high casein content (8.5 %) is also linked with bitterness in cheese; substitution of the standard MCC with a demineralised MCC reduced the calcium levels and associated levels of bitterness (Schäfer et al., 2019a, c). Overall, it is suggested that demineralised MCC is a suitable starting material for cheese makers to prepare cheesemilk of high casein content and low buffering capacity, as well as resultant cheeses of target calcium content.

MCC cheesemilk with a higher casein content (e.g. 3.5 %) also has faster gel firming rate and decreased set- to-cut time (time from rennet addition to achieving a gel firmness suitable for cutting; Panthi et al., 2019b), suggesting a need to modify standard operating procedures (SOPs) in industrial scale cheese processing. This problem might be avoided by fortifying or preparing cheesemilk with a demineralised MCC rather than a standard MCC, as the demineralised MCC will have slower gel firming rate due to the reduced mineral content (Caron et al., 1997), although further research is still suggested.

1.5.4 *β-Casein content*

MCC with reduced β-casein content and calcium to casein ratio can be produced by cold MF (Section 1.2.2). After restoring the calcium dissociated from the casein micelles due to cold MF, cheesemilk prepared from β-casein reduced MCC gained comparable rennet coagulation properties to that of skim milk (Holland et al., 2011). Model Cheddar cheeses made from the cheesemilk of reduced β-casein content have a better flowability, similar cheese composition and decreased hardness levels than cheeses made from standard cheesemilk (O'Mahony et al., 2008).

It is proposed that as products of β-casein hydrolysis are also linked to bitterness in cream cheese (Schäfer et al., 2019b), cream cheese manufactured from MCC of reduced β-casein content might have lower bitterness levels compared to traditional cream cheese, although further research is required to prove this. However, to further determine the commercial value of MCC of reduced β-casein content and its application in cheesemaking, further research is advised to evaluate factors such as consumer acceptance and characterisation of cheese texture and sensory properties.

1.6 Conclusions

By controlling the pH, temperature, diafiltrant type and number of DF steps used during MF, MCC with target levels of β-casein, whey protein, lactose and calcium can be produced (Figure 1.1). As a result, cheesemilk with customised contents of casein, fat, β-casein, whey protein, lactose and calcium can be formulated from MCCs together with UF permeate, water and cream, conferring bespoke composition and functionalities to the resultant cheeses (Figure 1.2). Similarly, cheese of consistent composition and quality can also be manufactured from MCC cheesemilk. By fractioning milk and stabilising the resultant MCC, whey protein products with better functionality than that manufactured from cheese whey can be produced. Further research is still required to find an economic way to preserve MCC and give it extended shelf life, high reconstitutability, good rennet coagulability, cheese make properties, high cheese yield and good cheese quality all at the same time.

Removal of native whey proteins from feed milk by MF prior to cheesemaking, can not only produce whey protein ingredients of superior functionality compared to those derived from cheese whey, but can also improve the heat stability of whey protein-reduced or depleted milk. However, further studies are needed to determine whether the benefits promoted by use of MCC for cheese manufacture, and the higher value of native whey would offset the additional capital investment required for such a manufacture process.

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Chapter 2: Application of a cascade membrane filtration process to standardise whey protein depleted cheesemilk for Cheddar cheese manufacture

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2.1 Abstract

A cascade membrane filtration process including microfiltration (MF), ultrafiltration (UF) and reverse osmosis (RO) was used to fractionate skim milk into different streams. Significant quantities of lactose and minerals were removed to permeate after MF at 0.14 μm. Cheesemilk, of similar casein content to the raw milk, was standardized simultaneously for casein, lactose, ash and total calcium from the membrane streams without requiring $CaCl_2$ and lactose addition. Whey protein depleted cheesemilk of typical casein content had similar rennet coaguability, cheese composition, texture and yield to the control; while milk of 1.5 times casein content had a faster coagulation rate and resulted in cheese of lower moisture content. On a dry matter basis, the whey protein content of MF permeate concentrated by UF was significantly higher than that in cheese whey (51.54 % vs 5.63 - 9.45 %), with significantly lower contents of ash (0.95 % vs 7.11 - 7.53 %) and lactose (9.50 % Vs 61.98 - 70.35 %) respectively.

2.2 Introduction

Microfiltration (MF) with a membrane pore size of 0.08 - 0.20 μm is commonly used to selectively partition soluble and colloidal components in milk. Dependent upon the membrane pore size for MF, casein micelles remain in the retentate, and whey proteins, lactose, minerals and other minor components permeate through the membrane (Jost et al., 1999; Nelson and Barbano, 2005; Govindasamy-Lucey et al., 2007; Seibel et al., 2015). MF retentate can be used for cheesemilk standardisation (Brandsma and Rizvi, 1999) or for the production of liquid or powdered micellar casein concentrates and isolates (Schuck et al., 1994). MF permeate often termed native, virgin or ‘ideal’ whey provides a whey protein stream free from

starter culture, cheese colorants, caseinomacropeptide (CMP), fat, cheese fines, rennet and derivatives of microbial activity compared to conventional cheese whey (Bacher and Kønigsfeldt, 2000). Process efficiencies are also achieved due to the higher purity of MF permeate, as the process speed for ultrafiltration (UF) of MF permeate is much faster than for that of cheese whey when separating and concentrating whey protein (Nelson and Barbano, 2005). Because of the negligible fat content and lower heat treatments applied to MF permeate, whey protein powders derived from MF permeate have superior functional properties compared to those manufactured from cheese whey (Bacher and Kønigsfeldt, 2000). In fact, Papadatos et al. (2003) suggested that whey protein products produced from MF permeate could be sold at a higher price than those produced from cheese whey. Furthermore, MF retentate (i.e., casein micelle concentrate) is more heat stable than skim milk as there is less whey protein present (Renhe and Corredig, 2018). Thus, optimal recovery of whey protein from skim milk to permeate during microfiltration is desired (Nelson and Barbano, 2005).

To maximise the whey protein removal from MF retentate, diafiltration (DF) with water is applied (Amelia et al., 2013), which results in a significant reduction in levels of lactose (Amelia, 2013; St-Gelais, 1995; Sauer, 2012; Outinen, 2008), calcium (Lu, 2016) and soluble milk minerals (Boiani, 2017) in MF retentate. Thus, to ensure an acceptable set to cut time during cheese manufacture, it is necessary to add $CaCl_2$ to the cheesemilk prepared from MF retentate (Heino, 2008; Zulewska et al., 2018). Similarly, low lactose content in cheesemilk caused by lactose depletion during MF and DF results in cheese with high pH (Heino, 2008). Thus, an opportunity exists to develop a membrane filtration process providing good separation of whey protein, and in parallel, facilitating the standardisation of cheesemilk to a target composition for casein, lactose and calcium contents as well as achieving a desired casein / fat ratio.

To optimise such a process, it is suggested that small molecules (whey protein, lactose and calcium) removed from the retentate after each microfiltration and diafiltration step should be quantified, so as to inform the process of standardisation of cheesemilk from MF retentate based on individual components and similarly, to optimise the membrane filtration process to produce a MF retentate which is suitable for cheesemilk standardisation.

In this study a cascade membrane filtration process was developed, where skim milk was subjected to microfiltration at 1.4 μm to remove bacterial and other cells followed by MF (pore size 0.14 μm, with 2 steps of DF with RO water, 50 °C), UF and reverse osmosis (RO) to fractionate skim milk into different streams, i.e., micellar casein concentrate (MCC; casein micelles), RO retentate (lactose and minerals), RO permeate (water) and UF retentate (whey protein). The first objective was to determine the effect of MF at 0.14 μm and DF on the composition of the MF retentates. The second objective was to develop and validate a process for the simultaneous standardisation of the casein, fat, lactose, ash and total calcium contents of cheesemilk using pasteurized cream, MCC, RO retentate and RO permeate. The third objective was to manufacture Cheddar cheese from cheesemilk standardized from membrane streams and evaluate the coagulation properties, composition, texture and yield. The composition of UF retentate and subsequent cheese wheys were also considered.

2.3 Materials and methods

2.3.1 *Cascade filtration process*

Triplicate trials were undertaken over a five month period on a cascade filtration process (Figure 2.1) with each trial conducted over three days at Moorepark Technology Limited, Co Cork, Ireland.

On day 1, raw whole milk sourced from the Teagasc Animal & Grassland Research and Innovation Centre (AGRIC), Moorepark, Co Cork, Ireland or from a local dairy company (Dairygold, Mogeely, Co. Cork, Ireland) was separated into raw cream and raw skim milk with a cream separator (GEA Westfalia, Oelde, Germany). Immediately after separation, a quantity of raw cream (20 kg, fat content 25 – 40 %) and raw skim milk (20 kg, fat content < 0.1 %) were pasteurised separately (cream, 85 °C for 20 s; skim, 72 °C for 15 s) using a pilot-scale tubular heat-exchanger (MicroThermics®, Raleigh, NC, USA), collected in sterilized containers (Thermo Scientific™ Nalgene™ Products, NY, USA) and stored at 4 °C until day 4. In parallel, 400 kg of raw skim milk was microfiltered at a membrane pore size of 1.4 μm (Tami Isoflux® ceramic membranes, Tami Industries, Nyons, France) on a pilot filtration unit (Model F, GEA Process Engineering A/S, Skanderborg, Denmark), where bacteria and spores were retained in the MF 1.4 retentate, and the bacteria-free skim milk partitioned to MF 1.4 permeate (Mistry, 2013). A quantity of 20 kg MF 1.4 permeate was transferred to two 10 L sterilized containers, cooled in an ice bath and stored at 4 °C until day 4; the remainder of the MF 1.4 permeate (350 kg) was collected in a double jacket tank and immediately cooled to 4 °C for use on day 2.

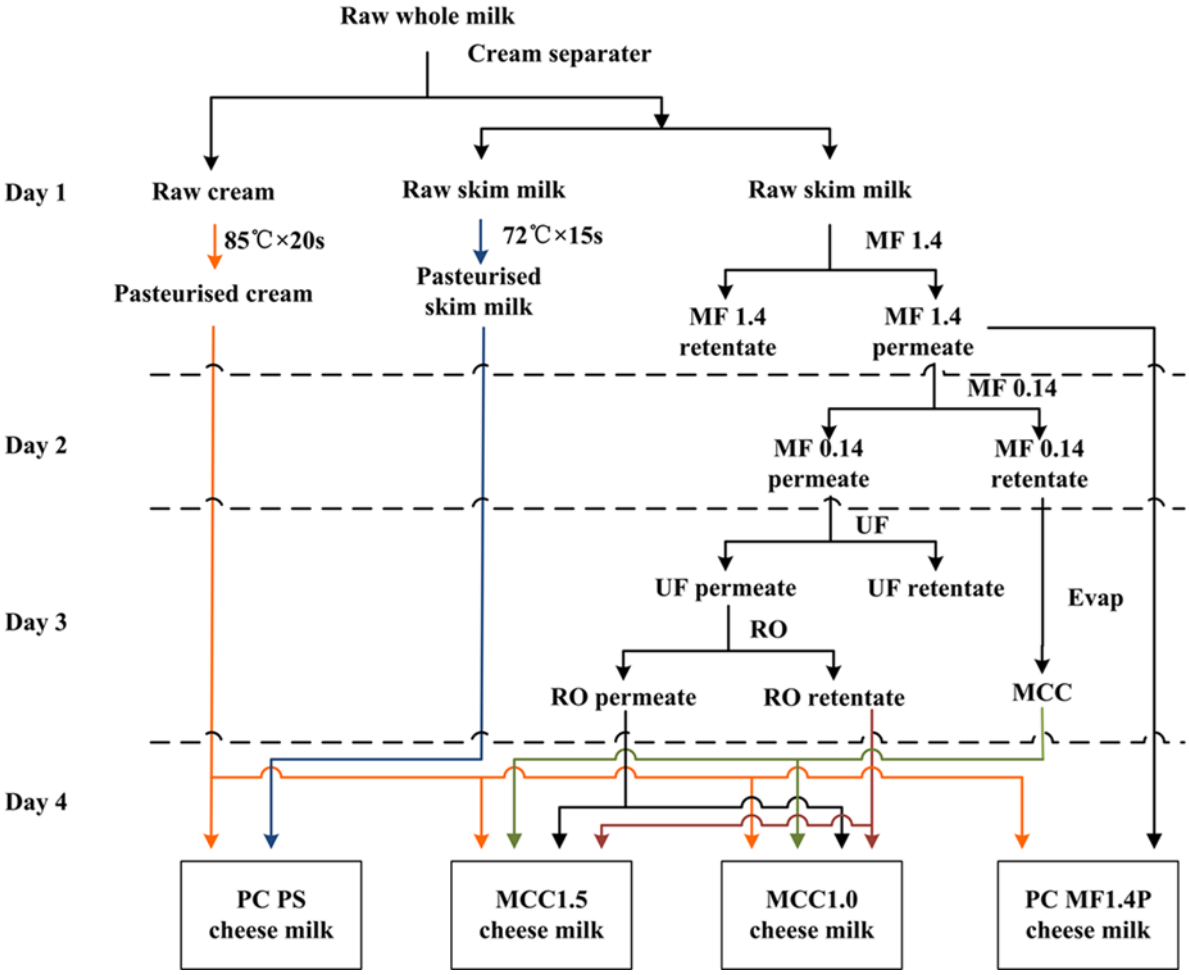

Figure 2.1. Cascade filtration process applied in preparation of milk fraction streams and in preparation of cheesemilks

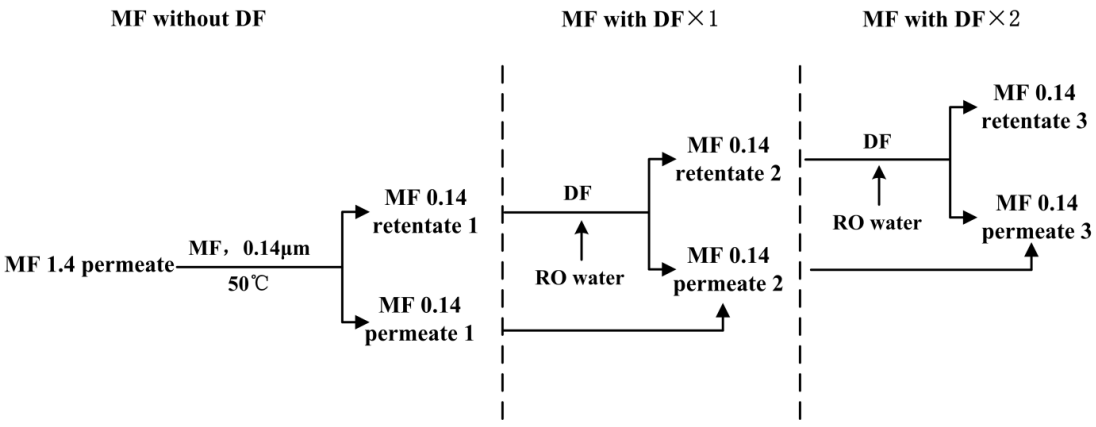

Figure 2.2. Microfiltration process with pore size 0.14 μm incorporating two diafiltration steps

On day 2 (Figure 2.2), MF 1.4 permeate was heated to 50 °C and then subjected to microfiltration using three ceramic 0.14 μm membranes in parallel, each with a surface area of 0.35 m^2 (Tami Isoflux® ceramic membranes, Tami Industries, Nyons, France). For diafiltration, when the weight of the MF 0.14 permeate reached 250 kg (for diafiltration 1) or 400 kg (for diafiltration 2) respectively, 150 kg or 100 kg of RO water (50 °C) were added to the MF 0.14 retentate immediately. The retentate and permeate obtained after each MF or DF step are referred to as MF 0.14 retentate 1, 2, 3 or MF 0.14 permeate 1, 2, 3 respectively (Figure 2.2). The temperature of MF 0.14 was maintained at 50 ± 3 °C with chilled water, both MF 0.14 permeate 3 and retentate 3 were immediately cooled to 4 °C after processing and stored until day 3.

On day 3 (Figure 2.1), the MF 0.14 retentate was evaporated at 65 °C using a single-stage falling-film evaporator (Tetra Scheffers™, Tetra Pak, Gorredijk, The Netherlands) until a brix level of 21-22 (determined by a hand held refractometer, Bellingham + Stanley Ltd, Kent, UK) was achieved in MCC. In parallel, MF 0.14 permeate was ultrafiltered using two spiral-wound membranes (Synder Filtration, Vacaville , CA, USA) with a molecular weight (MW) cut-off of 10 kDa. To partition all lactose and minerals to the UF permeate, diafiltration with RO water was carried out until the brix level of the UF permeate became 0. The UF permeate was concentrated by reverse osmosis (Hydranautics RO3840/30 membranes, Nitto, Oceanside, CA, USA) to a total solids content of 15 % in the RO retentate, containing lactose and minerals, with water removed to the RO permeate. The MCC, RO retentate and RO permeate were then transferred to sterilized containers separately, cooled in an ice bath and stored at 4 °C until day 4. All membrane filtration processes were carried out on the same filtration unit.

2.3.2 *Preparation of cheesemilk*

On day 4 (Figure 2.1), 4 cheesemilks (namely, PC PS, PC MF1.4P, MCC1.0 and MCC1.5) were prepared from the following streams: pasteurized cream, pasteurized skim milk, MF 1.4 permeate, MCC (micellar casein), RO retentate (lactose and minerals) and RO permeate (water), as described in Table 2.1. The compositional parameters (protein, fat and lactose contents) of pasteurised raw skim milk, raw cream, MCC and cheesemilks were measured by FTIR (FOSS MilkoScan™ FT+, Hillerød, Denmark). The total solids in RO retentate was analysed with a rapid moisture analyser (CEM Smart Trac, Dublin, Ireland) and the lactose content in the RO retentate was calculated as: 0.87 × total solids in RO retentate. RO permeate was considered as pure water. The casein content for PC PS, PC MF 1.4P and MCC1.0 were standardised to the same level as the raw skim milk and the casein content for MCC1.5 was standardised to 1.5 × MCC1.0. The target casein: fat ratio for all cheesemilks was 0.74, the lactose contents in MCC1.0 and MCC1.5 cheesemilks were standardised to the same level with those in PC PS and PC MF1.4P cheesemilk. Since MCC, RO retentate and RO permeate all originated from the MF 1.4 permeate, and the MF 1.4 permeate may be considered to be bacteria free (Mistry, 2013), a cheesemilk designated PC PS was prepared from pasteurized skim milk and cream, to act as control for the PC MF 1.4P, MCC1.0 and MCC1.5 cheesemilks. The purpose of PC MF 1.4P was to compare microbial removal using MF 1.4 μm to pasteurization (PC PS), a more conventional step for reduction of bacterial load and for pathogen inactivation.

Table 2.1. Component stream formulations for PC PS, PC MF1.4P, MCC1.0 and MCC1.5 cheesemilk[1, 2, and 3]

Weight of streams (kg)	PC PS	PC MF1.4P	MCC1.0	MCC1.5
Pasteurised cream	2.04	1.85	2.02	3.03
Pasteurised skim milk	10.16	0	0	0
MF 1.4 permeate	0	10.15	0	0
MCC	0	0	2.27	3.41
RO retentate	0	0	2.86	2.49
RO permeate	0	0	4.85	3.08

[1]Abbreviations: PC PS, cheesemilk prepared from pasteurized cream and pasteurized skim milk; PC MF1.4P, cheesemilk prepared from pasteurized cream and MF 1.4 permeate; MCC 1.0, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, and of the same casein content in raw skim milk; MCC 1.5, cheesemilk prepared from pasteurized cream, MCC, RO retentate and RO permeate, with casein content 1.5 times to that of the MCC1.0 (3.75 - 4.5 %);
[2]Results are means of triplicate trials;
[3]Cheesemilk formulations were calculated on a 12 kg basis.

2.3.3 *Preparation of cheese*

Each cheesemilk was formulated to 10 kg in a model cheese vat (Type CAL 10 L; Pierre Guerin Technologies, Mauze, France) and heated to 32 °C with a re-circulating water bath (Grant Y28; Grant Instrument Ltd., Cambridge, UK). The pH of the cheesemilk was standardised to 6.55 with a 4 % lactic acid solution. Starter culture (2 g per vat; R604, Chr. Hansen Ireland Ltd., Co. Cork, Ireland) was added to the cheesemilk immediately after pH standardization. After a pre-ripening period of 30 min, rennet (1.8 mL Chymax-plus (Chr. Hansen Ireland Ltd., Co. Cork, Ireland) mixed with 20 mL milli-Q water) was added to the cheesemilk. The coagulum was cut as described by Panthi et al. (2019b) at a gel firmness of 35 Pa (determined by AR-G2 rheometer; TA Instruments, New Castle, DE, USA). Subsequently the curds were cooked to 38 °C at a rate of 0.25 °C / min, drained at pH 6.15, milled at pH 5.35, salted at 2.7 % (w/w), mellowed for 25 min, moulded and then pressed at 44.23 kPa overnight. Cheeses were vacuum packed and stored in 4 °C for 7 days.

2.3.4 *Compositional analysis of membrane streams, cheesemilks and cheese wheys*

Total solids, ash, total protein, NPN, NCN, fat

Total solids and ash contents were determined as described by IDF (1964a, 2010). Total nitrogen, non-protein nitrogen (NPN) and non-casein nitrogen (NCN) were determined using the Kjeldahl method (IDF, 1964b, 1993), and a nitrogen-protein conversion factor of 6.38 was applied. MF 0.14 retentate 1, 2 and 3 and MCC were diluted with Milli-Q water to a protein concentration similar to that in skim milk during sample preparation for NCN and NPN analysis. Fat content was determined using a Gravimetric method (IDF, 1996).

Total calcium

A volume of 1 mL of sample was ashed, dissolved in 3 mL 10 % HCl, and diluted to 100 mL in volumetric flasks with milli-Q water. The solutions were further diluted (MCC: 1 in 50; MF 0.14 retentate 1, 2, and 3: 1 in 25 dilution; all the other liquid samples: 1 in 10) prior to calcium determination using an Atomic Absorption Spectrometer (AA240, Varian AA, Varian Inc., Palo Alto, CA, USA) (Gaucheron, 2005; Lin et al., 2016).

Lactose

All liquid samples were diluted 1 in 100 with Milli-Q water, filtered with a 0.2 μm nylon membrane filter (Chromacol20-SF-02(N), Thermo Scientific, Waltham, Massachusetts, United States), and analysed as described by Pirisino (1983) and Hou et al. (2014b).

2.3.5 *Rheological properties of coagulum*

The rheological properties of coagula were monitored using a rheometer (AR-G2 rheometer; TA Instruments, New Castle, DE, USA) equipped with a conical concentric cylinder geometry as described by Sandra et al. (2011). Cheesemilk was mixed for 3 min after rennet addition, and a volume of 20 mL milk was transferred to the rheometer, where a time sweep test was subsequently carried out. Conditions for the time sweep test were 32 °C with a gap distance 5920 mm, strain 0.02, and oscillation frequency 1 Hz as described by Panthi et al. (2019b), the test continued for 90 min. Rennet addition time was defined as the starting time and the following parameters was recorded or calculated from the G'/ tan δ - time curve as described by Panthi et al. (2019b): MCFR (maximum gel-firming rate), A_{40} and tan δ_{40} (the value

of G' and tan δ after 40 min of rennet addition), K_{35} and K_{70} (time for the coagulum to obtain gel firmness of 35 or 70 Pa respectively after rennet addition) and CW (cutting window, calculated from K_{35} and K_{70}).

2.3.6 *Compositional analysis of cheese*

Cheese samples were ground prior to analysis with measurements of moisture and fat contents and pH conducted on fresh samples; with the remainder frozen at - 20 °C until analysis. Frozen cheese was defrosted at 4 °C overnight prior to analysis. Moisture, protein, salt, ash and total calcium contents as well as pH in cheese were measured as described by Fenelon and Guinee (1999), fat content was determined by NMR (SMART Trac II Moisture and fat Analyzer, CEM Smart Trac, Damastown, Dublin, Ireland).

2.3.7 *Textural properties of cheese*

After storage at 4 °C for 7 days, the cheeses were sampled for texture and cheese composition analysis respectively. Cheese were prepared into 25 mm^3 cubes (six cubes per treatment), wrapped with foil paper and stored at 4 °C overnight. Texture profile analysis (TPA) was conducted on each cube with a P75 probe and 50 kg load cell (TA-XT plus, Stable Micro Systems, Godalming, Surrey, UK), the cubes were compressed to 70 % of original height at a testing speed of 1.00 mm / s. The fracture force, fracture strain and firmness were recorded and calculated as described in Hou et al. (2014a).

2.3.8 *Statistical analysis*

Triplicate trials were undertaken for the cascade filtration process, cheesemilk preparation and Cheddar cheese manufacture. The effect of MF 0.14 and diafiltration

on retentate composition, cheesemilk composition, rheological properties of coagulum as well as cheese composition, textural properties and yield were compared with least-squares difference (LSD) at 95 % significance level by one-way ANOVA using SPSS 24.0 (IBM Corp., 2016, Chicago, IL, USA).

2.4 Results and discussion

2.4.1 *Effect of MF 0.14 and diafiltration on milk composition*

As a result of MF and DF, casein micelles were separated and concentrated in MF 0.14 retentates, while small molecules including whey protein, lactose and minerals were depleted (Table 2.2). As MF and DF progressed and the casein content in MF 0.14 retentates increased, specific ratios were determined (whey protein:casein, ash:casein, total calcium:casein and lactose:casein ratios) to compare the relative loss of whey protein, ash, total calcium and lactose compared to casein in these streams during the process. After MF but without a DF step (Fig 2.2), the serum:casein, ash:casein, total calcium:casein and lactose:casein ratios in MF 0.14 retentate 1 decreased by 39.50 %, 21.40 %, 18.54 % and 67.68 % respectively compared to the MF 1.4 permeate; after two diafiltration steps (i.e., MF with DF × 1 and 2, Fig 2.2), the serum:casein, ash:casein, total calcium:casein and lactose:casein ratios in MF 0.14 retentate 3 decreased by 20.45 %, 35.32 %, 11.45 %, 26.46 % respectively when compared to MF 0.14 retentate 1. It is clear that less whey protein, minerals, total calcium and lactose were lost during MF with DF than MF without a DF step, suggesting that more small molecules were removed to the MF 0.14 permeate during MF without a DF step. It is suggested that dairy processors should consider whether the increased process costs of diafiltration would be offset by the value of increased whey protein before the application of DF or even multi-step DF with MF.

After MF together with two steps of DF, the total calcium:casein and lactose:casein ratios in MF 0.14 retentate 3 decreased by 29.99 % and 94.14 % respectively compared to MF 1.4 permeate, suggesting that calcium and lactose contents may need to be supplemented when standardising cheesemilk from MF 0.14 retentate 3. Reduced lactose content in cheesemilk can lead to increased hardness and pH in cheese (Moynihan, 2016; Hou et al. 2012, 2014a), thus it may be of benefit to apply MF to reduce or standardise lactose levels in cheesemilk as a way to control cheese pH or texture. Similarly, demineralisation of cheesemilk can decrease the buffering capacity of cheesemilk, decreasing the cheese make time (St-Gelais et al., 1997) and resulting in increased cheese moisture content (Govindasamy-Lucey et al., 2007). Thus, the demineralisation effect of MF could be beneficial to increase the moisture or moisture in non-fat substance contents in low fat cheese or in cheeses made from concentrated cheesemilk, providing sufficient milk minerals are present to ensure good rennet coaguability.

Table 2.2. Effect of microfiltration at 0.14 μm and diafiltration on the composition of resultant streams[1]

Compositional parameters	MF 1.4 permeate	MF 0.14 retentate 1	MF 0.14 retentate 2	MF 0.14 retentate 3
Total solids (% , wt/wt)	8.74^{c}	14.58^{a}	11.40^{b}	11.38^{b}
Total protein (% , wt/wt)	3.52^{b}	9.06^{a}	8.56^{a}	9.32^{a}
Casein number (%)[2]	78.95^{c}	86.98^{b}	89.76^{a}	91.83^{a}
Casein content (% , wt/wt)	2.78^{b}	7.90^{a}	7.69^{a}	8.56^{a}
Serum protein content (%, wt/wt)	0.58^{c}	0.97^{a}	0.76^{b}	0.70^{bc}
Ash content (% , wt/wt)	0.65^{b}	1.23^{a}	0.95^{ab}	0.87^{b}
Total calcium (m mol/ kg)	31.26^{b}	72.06^{a}	66.82^{a}	67.12^{a}
Lactose content (% , wt/wt)	4.51^{a}	4.07^{a}	1.61^{b}	0.77^{b}
Serum protein:casein ratio	0.21^{a}	0.12^{b}	0.10^{c}	0.08^{c}
Relative serum protein:casein ratio[3]	100.00 ^{a}	60.50 ^{b}	48.59 $^{b, c}$	40.05 ^{c}
Ash: casein ratio	0.24^{a}	0.16^{b}	0.12^{c}	0.10^{c}
Relative ash: casein ratio	100.00 ^{a}	78.60 ^{b}	52.22 ^{c}	43.28 ^{c}
Total calcium:casein ratio (m mol/g)	1.12^{a}	0.91^{b}	0.87^{b}	0.79^{c}
Relative total calcium:casein ratio	100.00 ^{a}	81.46 ^{b}	77.72 $^{b, c}$	70.01 ^{c}
Lactose:casein ratio	1.48^{a}	0.47^{b}	0.19^{c}	0.09^{c}
Relative lactose:casein ratio (%)	100.00 ^{a}	32.32 ^{b}	13.34 ^{c}	5.86 ^{d}
pH	6.76 $^{b, c}$	6.68 ^{c}	6.82 ^{b}	6.96 ^{a}

[1] Results are means of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).

[2]Casein number (%) = $\frac{\text{Casein content}}{\text{Total protein}} \times 100$.

[3]Relative serum protein:casein ratio=$\frac{\text{serum protein:casein ratio in sample}}{\text{serum protein:casein ratio in MF 1.4 permeate}}$; relative lactose:casein, ash:casein and total calcium:casein ratios were calculated in similar way.

In addition, lactose was removed from the MF 1.4 permeate at a much faster rate than whey protein and minerals (Figure 2.3), probably due to the smaller molecular size of lactose compared to that of whey proteins. Although milk salts are also small molecules, they are present in large quantities in the casein micelle in the form of colloidal calcium phosphate (Gaucheron, 2005), and thus were depleted at a slower rate than lactose. Under microfiltration, both with and without diafiltration, total calcium levels were depleted at a lower rate than for ash (Figure 2.3). This was attributed to the fact that only 31 % of total calcium is present in the serum phase, while more than 50 % of the potassium, sodium, chloride, inorganic phosphate, magnesium and citrate are present in the milk serum (Gaucheron, 2005); thus minerals dissolved in the serum phase are more likely to partition in the permeate during MF and DF.

Gaucheron (2005) reported that soluble calcium amounts to 31 % of total calcium, and in the current study the total Ca: casein ratio in MF 0.14 retentate 3 was 70 % of that in MF 1.4 permeate (Table 2.2), suggesting that all the soluble calcium originally present in MF 1.4 permeate partitioned to MF 0.14 permeate 3 during MF and DF. Thus, to maintain the calcium equilibrium, we presume that a certain amount of colloidal calcium phosphate (CCP) dissociated and dissolved in the serum phase of MF 0.14 retentate 3, leading to a lower colloidal calcium:casein ratio in MF 0.14 retentate 3 compared to the original skim milk, although further research is required to prove this assumption. During diafiltration, the addition of RO water will dilute the serum phase of the MF 0.14 retentate, which may disrupt the calcium equilibrium between casein micelles (CM) and the serum phase. As a result, part of the colloidal calcium phosphate (CCP) within the CM may be dissolved in the diluted serum phase and ultimately removed to MF 0.14 permeate during diafiltration. Alexander et al.

(2011) and Li et al. (2014) reported that part of the CCP inside CM was washed away during ultrafiltration (UF) and DF (with RO water) of milk. Both Boiani (2017, 2018) and Lu et al. (2016) suggested that part of the CCP might be removed during MF and DF with water, although this assumption was not proven in their research. CCP is very important for rennet induced gelation of milk in cheese manufacture; when the colloidal calcium: casein ratio is lower than 70 % of the original level, a rennet induced gel cannot be formed (Shalabi and Fox, 1982; Choi et al., 2007). CCP loss from CM can also cause weak gels (Udabage et al., 2001) and it becomes difficult to reverse or fortify CCP loss when a large amount of CCP is lost through membrane filtration (Ferrer et al., 2014). Thus, when water is used as diafiltrant during microfiltration, and especially when multiple DF steps are carried out, the colloidal calcium: casein ratio in MF retentate should be monitored when the retentate is used to prepare cheesemilk directly.

A significant increase in pH was observed between MF 1.4 permeate and MF 0.14 retentate 3, and the pH of MF 0.14 retentate 1, 2 and 3 increased significantly after each diafiltration step (Table 2.2). Boiani (2017) also observed a pH increase in MF retentate after microfiltration and diafiltration with water, i.e., from 6.55 in skim milk to 7.02 in MF retentate. We suggest that partial solubilization of CCP from casein micelles might have led to the increased retentate pH (Fox et al., 2015).

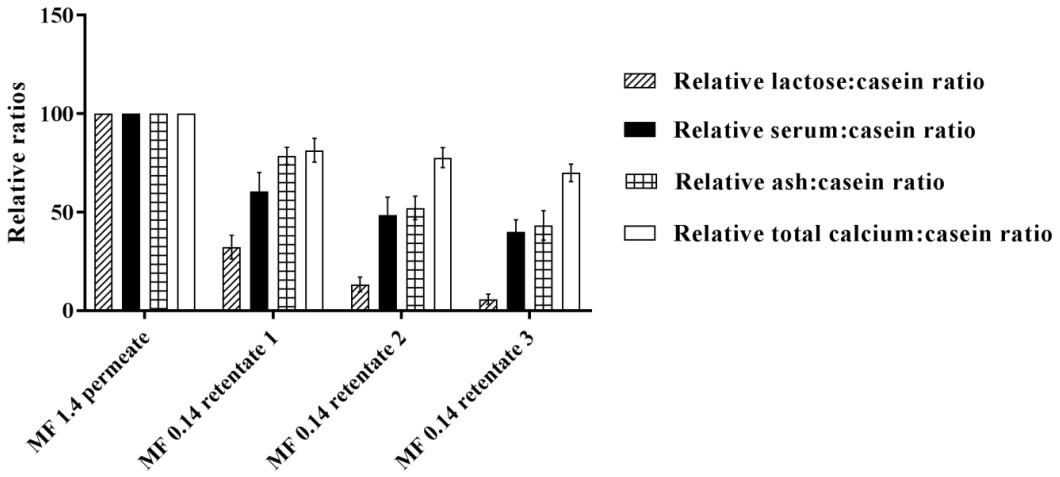

Figure 2.3. Relative lactose:casein, serum:casein, ash:casein, and total calcium:casein ratios in MF 1.4 permeate and MF 0.14 retentate 1, 2, and 3 streams respectively. Relative lactose:casein ratio was determined as: $\frac{\text{lactose:casein ratio in sample}}{\text{lactose:casein ratio in MF 1.4 permeate}}$; relative lactose:casein, ash:casein and total calcium:casein ratios were calculated in similar way; Figure 2.3 is derived from data in Table 2.2.

2.4.2 *Cheesemilk composition*

The streams generated (pasteurised cream, pasteurised skim milk, MF 1.4 permeate, MCC, RO retentate and RO permeate) were combined to formulate four cheesemilks (Table 2.1). For cheesemilks of the same casein content, i.e., PC PS, PC MF1.4P, and MCC1.0, there was no significant difference between their contents of total solids, total protein, casein, total calcium and lactose (Table 2.3). Similarly no significant difference between PC MF 1.4P and MCC1.0 was observed for ash content. The lactose content in MCC 1.5 cheesemilk was similar to those of the other three cheesemilks as a result of lactose standardisation. The ash and total calcium contents in MCC1.5 cheesemilk were significantly higher ($P < 0.05$) than those in the other cheesemilk samples, and was attributed to the significantly higher casein content in the former. The ash: casein ratio and total calcium: casein ratio in the MCC1.5 cheesemilk were also significantly lower, although similar in magnitude, to the other three cheesemilks (Table 2.3).

Although only the casein and lactose contents as well as casein: fat ratio in MCC 1.0 and MCC 1.5 cheesemilks were deliberately standardised during cheesemilk preparation, it was observed that the ash and total calcium contents in the MCC1.0 cheesemilk also achieved standardisation, while the ash: casein, total calcium: casein ratios in MCC1.5 cheesemilk were lower, although similar in magnitude. This was attributed to the fact that the cascade membrane filtration process resulted in all casein micelles originally present in skim milk being separated and concentrated in the MCC, while the lactose and minerals were either retained in the MCC or concentrated in the RO retentate.

The pH of the four cheesemilks were approximately 6.63 (Table 2.3) which were in the range of natural milk pH as suggested by Fox et al. (2017). The PC PS and PC MF1.4P cheesemilks were prepared from pasteurised cream (pH 6.61 - 6.65), pasteurised skim milk (pH 6.72 - 6.74) and MF 1.4 permeate (pH 6.76). The MCC1.0 and MCC1.5 cheesemilks were prepared from pasteurised cream, MCC (pH 6.85), RO retentate (pH 6.19) and RO permeate (6.43). Although the pH of the RO retentate and RO permeate were low, this was offset by the high pH and high buffering capacity of MCC (casein micelles and milk serum) resulting in a cheesemilk pH of 6.63.

Table 2.3. Compositional ratios of cheesemilks formulated from streams produced by the cascade filtration process[1, 2]

Compositional parameters	PC PS	PC MF1.4P	MCC1.0	MCC1.5
Total solids (% , wt/wt)	12.53[b]	12.53[b]	12.09[b]	15.72[a]
Total protein (% , wt/wt)	3.55[b]	3.40[b]	3.34[b]	4.96[b]
Casein number[3]	80.79[b]	79.55[b]	85.90[a]	87.03[a]
Casein content (% , wt/wt)	2.87[b]	2.71[b]	2.87[b]	4.32[a]
Serum protein content (%, wt/wt)	0.49[a]	0.51[a]	0.30[b]	0.45[a]
Fat content (%)	4.05 [b]	3.99 [b]	4.18 [b]	6.02 [a]
Casein: fat ratio	0.71 [a]	0.68 [a]	0.69 [a]	0.73 [a]
Ash content (% , wt/wt)	0.72[b]	0.65[c]	0.66[c]	0.83[a]
Total calcium (m mol/ kg)	29.17[b]	28.19[b]	29.04[b]	40.79[a]
Lactose content (% , wt/wt)	4.32[a]	4.14[a]	4.11[a]	4.45[a]
Ash:casein ratio	0.25[a]	0.24[a]	0.23[a]	0.19[b]
Total calcium:casein ratio	1.02[a]	1.03[a]	1.07[a]	0.96[b]
Lactose:casein ratio	1.56[a]	1.61[a]	1.45[a]	0.95[b]
pH	6.62[a]	6.63[a]	6.63[a]	6.63[a]

[1]Abbreviations: PC PS, cheesemilk prepared from pasteurized cream and pasteurized skim milk; PC MF1.4P, cheesemilk prepared from pasteurized cream and MF 1.4 permeate; MCC 1.0, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, and of the same casein content in raw skim milk; MCC 1.5, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, casein content 1.5 times that of the MCC1.0 (3.75 - 4.5 %);

[2] Results are means of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).

[3]Casein number (%) = $\frac{\text{Casein content}}{\text{Total protein}} \times 100$.

2.4.3 *Coagulum rheology*

The Maximum Gel Firming Rate (MGFR) during coagulation of the MCC1.5 cheese was significantly higher than for the other cheeses, corresponding with a significantly higher gel firmness at 40 min (A_{40}) and significantly reduced time to obtain gel firmness of 35 and 70 Pa (K_{35} and K_{70}) (Table 2.4). Cheesemilk pH in all vats was standardized to 6.55, however the rennet was added on a volume basis, and in milk of a higher casein content (MCC1.5), the para-caseins had a greater chance of collision, thus forming a more dense 3-D network, resulting in a higher gel firming rate and gel firmness at any given time (Guinee et al., 1996; Sandra et al., 2011; Panthi et al., 2019b). Due to the faster gel firming rate for the MCC1.5, the time for the gel's elastic modulus (G') to reach 35 Pa (K_{35}) and 70 Pa (K_{70}) (used to calculate cutting window; Panthi et al; 2019b) were significantly lower than the other coagulum, and as a result, the cutting window (CW) in MCC1.5 was significantly narrower than for the other cheeses. The reduced cutting window would result in problems for cheese makers during cutting, e.g., curd tearing and shattering and increased fat loss in cheese whey (Guinee et al., 1994). This may be avoided by application of a lower set temperature to reduce gel firming rate (Guinee et al., 1996; Panthi et al., 2019b), cutting of the coagulum when softer (a lower G') (Govindasamy-Lucey et al., 2007) or overlay of the curds with UF permeate before and after cutting (Panthi et al., 2019a). The tendency for all gels to synerese was not influenced by their differing casein contents, as suggested by their similar tan δ value at 40 min in agreement with Panthi et al. (2019b).

Table 2.4 Coagulation properties, cheese yield and texture of cheese manufactured from PC PS, PC MF1.4P, MCC1.0 and MCC1.5 cheesemilks[1, 2]

Parameters	PC PS	PC MF1.4P	MCC1.0	MCC1.5
Gel coagulation				
MGFR (Pa/min)[3]	2.69[b]	2.45[b]	3.88[b]	18.49[a]
A_{40} (Pa)[4]	36.76[b]	39.02[b]	70.20[b]	310.95[a]
Tan δ_{40}[4]	0.28[a]	0.26[a]	0.28[a]	0.28[a]
K_{35} (min)[5]	40.67[a]	38.28[a]	31.16[a]	18.00[b]
K_{70} (min)[5]	56.00[a]	58.54[a]	41.89[a]	20.49[b]
CW (min)[6]	15.33[a,b]	20.26[a]	10.46[b]	2.50[c]
Cheese yield[7]				
Y_a (kg/100 kg)	10.89[b]	10.55[b]	11.33[b]	16.01[a]
Y_{ma}	11.22[b]	11.21[b]	11.98[b]	17.31[a]
Y_{afcam}	9.36[a]	9.38[a]	9.53[a]	9.21[a]
Y_{mafcam}	9.62[a]	9.96[a]	10.07[a]	9.96[a]
Texture				
Fracture stress (kPa)	501.35[a,b]	447.58[b]	516.05[a,b]	627.34[a]
Fracture strain	0.69[a]	0.72[a]	0.71[a]	0.70[a]
Firmness (N)	306.24[a,b]	266.69[b]	310.49[a,b]	380.27[a]

[1] Results are means of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2] Abbreviations: PC PS, cheesemilk prepared from pasteurized cream and pasteurized skim milk; PC MF1.4P, cheesemilk prepared from pasteurized cream and MF 1.4 permeate; MCC 1.0, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, and of the same casein content in raw skim milk; MCC 1.5, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, casein content 1.5 times that of the MCC1.0 (3.75 - 4.5 %);. .
[3] MGFR: maximum gel firming rate, calculated from $\Delta G'/\Delta t$ curve.
[4] A_{40} and tan δ_{40}: the value of G' or tan δ after 40 min of rennet addition in respective.
[5] K_{35} and K_{70}: the value of G' after 35 or 70 min of rennet addition separately.
[6] CW: cutting window, K_{70}-K_{35}.
[7] Y_a= actual yield (kg/ 100 kg milk); Y_{ma}= moisture-adjusted yield; Y_{afcam}= yield per 100 kg of milk normalized to reference fat (3.4 %, w/w) and casein (2.53 %, w/w) levels; Y_{mafcam}= moisture-adjusted yield per 100 kg of milk normalized to reference fat (3.4 %, w/w) and casein (2.53 %, w/w) levels.

For cheesemilk of similar casein and total calcium contents, no significant difference was observed for gel firming rates, suggesting that methods to decrease the bacteria load (pasteurization vs MF1.4) as well as milk whey protein content did not have a significant impact on their rennet induced gelation properties.

2.4.4 *Cheese composition*

The moisture and MNFS contents in the MCC1.5 cheese were significantly lower than those in the PC PS cheese and were lower in magnitude (although not significantly) than the PC MF1.4P and MCC1.0 cheeses (Table 2.5). It has previously been reported that cheese coagulum formed from milk of higher casein content have lower moisture contents than those originated from milks of lower casein content, due to the lower moisture content in cheesemilk of higher casein content (Panthi et al., 2019a); in addition, such gels are more prone to syneresis due to the higher casein concentration and higher pressure created by more frequent curd particles collisions (Guinee et al., 2006). Since the casein content and ash content in MCC1.5 cheese were significantly higher than the other cheeses (Table 2.5), it is expected that the buffering capacity in this cheese would be higher thus resulting in the significantly higher pH (Table 2.5).

There was no significant difference in all other compositional parameters between PC PS, PC MF1.4P and MCC1.0 cheeses (Table 2.5). It was concluded that use of MF to remove bacteria and whey protein content in cheesemilk had no significant impact on the cheese composition.

Table 2.5. Composition at 7 days of cheeses manufactured from PC PS, PC MF1.4P, MCC1.0 and MCC1.5 cheesemilks[1, 2]

Compositional parameters	PC PS	PC MF1.4P	MCC1.0	MCC1.5
Protein content (%)	24.61[a]	24.11[a]	24.42[a]	25.96[a]
Fat content (%)	32.27[a]	34.07[a]	33.91[a]	33.65[a]
Pro: fat ratio	0.76[a]	0.71[a]	0.73[a]	0.78[a]
Moisture content (%)	36.71[a]	34.69[a,b]	34.98[a,b]	33.50[b]
FDM (%)[3]	50.96[a]	52.14[a]	52.12[a]	50.59[a]
MNFS (%)[4]	54.18[a]	52.6[a,b]	52.92[a,b]	50.53[b]
Salt content (%)	1.39[a]	1.34[a]	1.32[a]	1.53[a]
S/M (%)[5]	3.82[a]	3.86[a]	3.79[a]	4.57[a]
Ash content (%)	3.28[b]	3.30[b]	3.34[b]	3.89[a]
Total calcium (mg/ 100 g cheese)	711.21[b]	716.37[b]	732.87[b]	809.50[a]
Calcium to protein (mg/ g)	28.92[a]	29.73[a]	29.99[a]	31.16[a]
pH	5.09[b]	5.08[b]	5.15[b]	5.33[a]

[1]Results are means of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2] Abbreviations: PC PS, cheesemilk prepared from pasteurized cream and pasteurized skim milk; PC MF1.4P, cheesemilk prepared from pasteurized cream and MF 1.4 permeate; MCC 1.0, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, and of the same casein content in raw skim milk; MCC 1.5, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, casein content 1.5 times that of the MCC1.0 (3.75 - 4.5 %);
[3] FDM: fat in dry matter.
[4] MNFS: moisture in non-fat substance.
[5] S/M: salt in moisture.

2.4.5 *Cheese texture*

The fracture stress and firmness of the MCC1.5 cheese were significantly higher than those of PC MF1.4P cheese and were higher in magnitude, although not significantly so than PC PS and MCC1.0 cheeses at day 7 of ripening (Table 2.4). The firmer texture obtained by MCC1.5 cheese is attributed to the combined effect of its higher curd-forming protein content (Guinee, 2016) and its lower curd-filler moisture content (Neocleous et al., 2002). Similarly, higher (although not significantly so)

levels of S/M in MCC1.5 cheese could also enhance the hydration and swelling of para-casein strands in cheese network, making the curd more resistant to deformation (Pastorino et al., 2003; McCarthy et al., 2016). Neocleous et al. (2002) also reported that fresh cheese produced from concentrated cheesemilk had increased hardness due to higher protein and lower moisture contents compared to control cheeses (made from typical cheesemilk); however increasing the moisture content in cheese manufactured from concentrated milk through adjustment of cheesemaking procedures can result in cheese with a comparable texture to the control. No significant difference was observed for fracture strain between the four cheeses (Table 2.4).

2.4.6 *Cheese yield*

The actual yield (Ya) and moisture adjusted cheese yield (Yma; target moisture content: 38.5 %), as defined by Guinee et al (2006) were significantly higher for the MCC1.5 cheese compared to the other cheeses (Table 2.4). This was attributed to significantly higher casein content in the MCC1.5 cheesemilk. It reflects the ability to produce more curd per vat when utilizing concentrated cheesemilk as reported by Neocleous et al. (2002b) and St-Gelais et al. (1995). The difference for Yma between MCC1.5 cheese and the other cheeses was more pronounced than for Ya, reflected by the significantly lower moisture content in MCC1.5 cheese (Neocleous et al., 2002; Guinee et al., 2006). To eliminate the effect of different fat and casein concentrations in the cheesemilks between the vats, both Ya and Yma per 100 kg of cheesemilk were adjusted to arbitrary levels of fat (3.4 %, wt/wt) and casein (2.53 %, wt/wt) contents as described by Guinee et al (2006), i.e., yield of cheese per 100 kg fat- and casein-adjusted milk (Yafcam) and moisture adjusted yield of cheese per 100 kg fat- and casein- adjusted milk (Ymafcam). No significant difference was found for Yafcam and Ymafcam between four cheeses, supporting the conclusion that the significantly

higher Ya and Yma for the MCC1.5 cheese was due only to the significantly higher casein content in the cheesemilk (Guinee et al., 2006).

2.4.7 *Composition of cheese whey and UF retentate*

The weight of MCC1.5 cheese whey was significantly lower than the other three cheese wheys (Table 2.6), in accordance with the findings of Outinen et al. (2010) and Daviau et al. (2000), which could be due to the lower moisture content (reflected by higher total solids content) in MCC1.5 cheesemilk than the other cheesemilks (Table 2.3) (Daviau et al., 2000).

The UF retentate produced in the cascade filtration process has a much higher purity of whey protein compared to cheese whey. Even though the total solids in the UF retentate (3.78 %) was much lower than those in cheese whey (6.03 - 6.76 %, Table 2.6), the whey protein content and whey protein as a percentage of total solids in the UF retentate (1.94 %, 51.54 %) were significantly higher than those in cheese whey (0.34 - 0.62 %, 5.63 - 9.45) respectively (Table 2.6). The high purity of whey protein in UF retentate is mainly attributed to the low or negligible amount of lactose and minerals as well as the absence of curd fines in this stream (Table 2.6). Similarly, starter bacteria, enzymes and colorants added during cheese manufacture will also be absent. The high purity and concentration of whey protein and the absence of thermal history confers better functionality (gelation and foaming properties, solubility, Bacher, 2000; Heino et al, 2007) to the UF retentate, making it a source of whey protein of higher value compared to cheese whey. Furthermore, the significantly lower ash content (0.95 %) calculated on dry matter basis in UF retentate than that in cheese whey (7.11 - 7.53 %) makes the whey protein products produced from UF retentate significantly more valuable particularly for applications in infant milk formula

(Bylund, 2015) (Table 2.6), where it is necessary to undertake demineralisation of standard cheese whey, as well as applications in ice cream and bakery products.

Table 2.6. Composition of UF retentate and cheese whey manufactured from PC PS, PC MF1.4P, MCC1.0 and MCC1.5 cheesemilks[1,2]

Compositional parameters	UF retentate	Cheese whey			
		PC PS	PC MF1.4P	MCC1.0	MCC1.5
Weight (kg/10 kg of cheese milk)	N/A[3]	8.61[a]	8.56[a]	8.47[a]	7.96[b]
Total solids (% , wt/wt)	3.78[c]	6.75[a]	6.60[a]	6.03[b]	6.76[a]
Fat (%, wt/wt)	N/A[4]	0.39[b]	0.42[b]	0.34[b]	0.63[a]
Protein (% , wt/wt)	3.13[a]	0.93[b]	0.95[b]	0.62[b]	0.86[b]
Serum protein content (%, wt/wt)	1.94[a]	0.60[b]	0.62[b]	0.34[c]	0.48[b,c]
Serum protein (% of total solids)	51.54[a]	8.85[b]	9.45[b]	5.63[b]	7.13[b]
Ash content (% , wt/wt)	0.04[b]	0.51[a]	0.50[a]	0.45[a]	0.48[a]
Ash content (% of total solids)	0.95[b]	7.51[a]	7.53[a]	7.47[a]	7.11[a]
Lactose content (% , wt/wt)	0.35[b]	4.37[a]	4.24[a]	4.24[a]	4.19[a]
Lactose content (% of total solids)	9.50[b]	64.77[a]	64.21[a]	70.35[a]	61.98[a]
pH	6.75[a]	5.78[b]	5.68[b]	5.69[b]	5.79[b]

[1] Results are means of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2] Abbreviations: PC PS, cheesemilk prepared from pasteurized cream and pasteurized skim milk; PC MF1.4P, cheesemilk prepared from pasteurized cream and MF 1.4 permeate; MCC 1.0, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, and of the same casein content in raw skim milk; MCC 1.5, cheesemilk prepared from pasteurized cream, MCC, RO retentate, RO permeate, casein content 1.5 times that of the MCC1.0 (3.75 - 4.5 %);
[3]N/A: Not applicable;
[4]N/A: Not available.

2.5 Conclusions

Large amounts of whey protein, lactose and minerals were depleted from the retentate by microfiltration at pore size 0.14 μm without diafiltration; while lower amounts of whey proteins, lactose and minerals were removed during MF0.14 with diafiltration when RO water was used as a diafiltrant. The comparable depletion level for small molecules during MF and DF was: lactose> whey protein> ash> total calcium.

It was shown that whey protein depleted cheesemilk can be accurately standardised from pasteurized cream, MCC, RO retentate and RO permeate as, in particular when standardising the lactose content in cheesemilk with RO retentate, the mineral content and total calcium content were also standardised simultaneously. The whey protein depleted cheesemilk also had a comparable pH to the control.

Cheesemilk standardised from membrane streams of typical casein content had comparable rennet coagulation properties, cheese composition, yield and texture to the control. Cheesemilk with an elevated casein content had a faster gel firming rate, narrower cutting window, decreased cheese moisture as well as increased pH, hardness and actual cheese yield.

The whey protein stream removed from milk by MF and concentrated by UF retaining its globular structure had significantly higher whey protein purity, lower ash and lactose contents as well as an absence of starter culture, cheese fines, fat and rennet in comparison to cheese whey.

In this cascade filtration process, all streams originating from the whole milk can be utilized: cream, MCC, RO retentate and RO permeate for cheese production; UF retentate and cheese wheys can be used to produce whey protein products. Overall, this research showed that the cascade membrane filtration process utilised in this

research can produce whey protein depleted cheesemilk of target composition, resulting in Cheddar cheese of standard quality and a native whey protein stream of high purity.

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Chapter 3: Effect of thermal treatment on whey protein-reduced micellar casein concentrate: An evaluation of rennet coagulability, cheese composition and yield

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3.1 Abstract

Microfiltration at 0.10 μm removed ~70.29 % of whey proteins from milk and the resultant micellar casein concentrates (MCC) were subjected to: no heat treatment (control), pasteurization (72 °C × 15 s) and high heat treatment (HHT, 90 °C × 15 s) before formulation of cheesemilk for Cheddar cheese manufacture. MCC showed good heat stability due to low whey protein content. For cheesemilk of typical casein content, both pasteurization and HHT did not significantly influence pH, calcium distribution and rennet coagulability, or subsequent cheese composition and yield; although HHT elongated cheese make time significantly. On increasing casein content from 3.09 % to 4.31 %, there was no significant difference for rennet to cut time between cheeses made from milk with different thermal histories and casein contents. Overall, HHT of MCC had no significant impact on cheese make properties, cheese composition and yield of Cheddar cheese.

3.2 Introduction

Heating of milk at temperatures ≥ 70 °C can cause whey protein denaturation; such denatured whey proteins can form complexes with other denatured whey proteins or with κ-casein (both on the surface of casein micelles or in milk serum phase) through thiol-disulphide bond exchange reactions (Bulca et al., 2004). Since disulphide bonds formed between whey proteins and casein micelles are located in the *para*- κ-casein region; the denatured whey proteins will be attached to the *para*-casein micelles after rennet addition and thus, incorporated into cheese curd (Anema et al., 2007). Partition of denatured whey proteins from cheesemilk to cheese provides a way to increase cheese yield (Banks et al., 1987; Singh and Waungana, 2001). However, both whey protein/ *para*-casein micelle complexes and whey protein/ soluble κ-casein

complexes can impair the rennet coagulability of cheesemilk (Kethireddipalli et al., 2010). As a result, Guinee et al. (1997) and Fox et al. (2017d) suggested that heat treatment above HTST pasteurization (72 °C × 15 s) conditions should not be applied to cheesemilk prior to cheese manufacture.

Native whey proteins can be separated from milk using microfiltration (MF) (Garem et al., 2000) and are considered as 'ideal whey protein' due to their superior functional and nutritional value over whey proteins recovered from cheese whey (Bacher and Kønigsfeldt, 2000; Heino et al., 2007), as well as having an absence of caseinomacropeptide, starter bacteria, colorants, coagulant enzymes, cheese fines and fat in this stream. Whey protein-reduced or depleted milk, called micellar casein concentrate (MCC), can be used to formulate cheesemilk (Neocleous et al., 2002; Heino, 2008). MCC produced by MF has an enhanced heat stability compared to milk of typical casein and whey protein contents (Renhe and Corredig, 2018). Milk which is partially or completely reduced of whey protein content can be subjected to high heat treatment (above pasteurization conditions) with little or no impairment of rennet coagulation properties, and the lower the whey protein content, the higher the heat stability (Bulca et al., 2004). Thus it could be hypothesized that high heat treatment (more intensive than HTST, 72 °C × 15 s) could be applied to denature and recover residual whey proteins in MCC to cheese curd leading to increased cheese yield without compromising rennet gelation and cheese making properties.

Previous research by this group (Xia et al., 2020) produced MCC of high casein number (casein content as a percentage of total protein, ～91 %) using a cascade membrane filtration process, this MCC had a high pH (～7.0) as a result of diafiltration (DF) with RO water during the MF. Beliciu et al. (2012) suggested that aggregation

or gelation in sterilised MCC can be prevented when the pH in unheated MCC is > 6.7 and thus it is postulated that MCC produced by MF and DF with RO water might have a good heat stability. The objective of this study was to: 1) characterise the heat stability of MCC manufactured by MF and DF with RO water and; 2) evaluate the rennet coagulability, cheese making properties and cheese yield of cheesemilks standardised from MCC of different thermal histories.

3.2 Materials and methods

3.2.1 *Cascade filtration process*

A pilot scale cascade membrane filtration process was carried out in triplicate (Figure 3.1) at Moorepark Technology Limited, Fermoy, Co Cork, Ireland:

Pasteurized skim milk was sourced from a local dairy company (Dairygold, Mitchelstown, Co Cork), stored at 4 °C overnight, pre-heated to 50 °C for 30 min and then microfiltered at a membrane pore size of 0.1 μm (Pall Corporation, New York, USA, model no. EP 3730, surface area 0.35 m^2, length 1020 mm) on a GEA Model F filtration unit (GEA Process Engineering A/S, Skanderborg, Denmark) at 50 °C. The volume concentration factor (VCF) was 3. Two steps of diafiltration with RO water (50 °C) were also undertaken during MF, with a dilution factor of 2. MF retentate was immediately chilled and stored at 4 °C until day 2. MF permeate was firstly subjected to reverse osmosis (VCF = 5) and then ultrafiltration, where RO permeate (water) and UF permeate (containing lactose and minerals) were collected in sterilized containers, chilled in an ice bath and stored at 4 °C until day 30.

On day 2 (Figure 3.1), MF retentate was divided into three portions and subjected to the following treatments using a pilot-scale tubular heat-exchanger (MicroThermics®, Raleigh, NC, USA): portion 1, unheated (control), denoted as CON

MCC; portion 2, pasteurized at 72 °C × 15 s, denoted as PS MCC; portion 3, high heat treatment: 90 °C for 15 s, denoted as HHT MCC. The MCCs were stored separately in sterilized containers, cooled in an ice bath and stored at 4 °C until day 3.

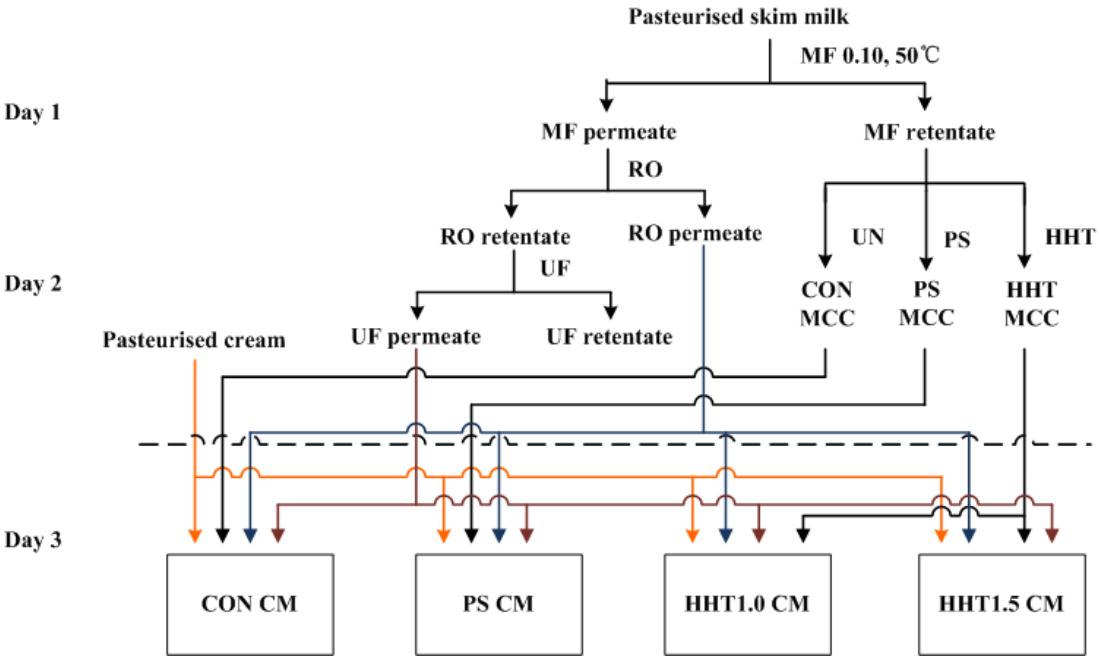

Figure 3.1. Cascade filtration process applied in Cheddar cheesemaking. Abbreviation: MF, microfiltration; RO, reverse osmosis; UF, ultrafiltration; UN, unheated; PS, pasteurisation (72°C×15s); HHT, high heat treatment (90°C×15s); MCC, micellar casein concentrate; CON, control; CM, cheesemilk.

Table 3.1. Formulations on a weight basis for serum protein reduced cheesemilk of different thermal history and casein content[1, 2 and 3]

Weight of streams (kg)	CON CM	PS CM	HHT1.0 CM	HHT1.5 CM
Pasteurised cream	1.11	1.11	1.11	1.67
CON MCC[4]	4.27	0	0	0
PS MCC[4]	0	4.27	0	0
HHT MCC[4]	0	0	4.27	6.41
UF permeate[5]	5.06	5.06	5.06	4.72
RO permeate[5]	1.57	1.57	1.57	0

[1]Results are means of triplicate trials;
[2]Cheesemilk formulations were calculated on a 12kg basis.
[3]Cheesemilk were standardised from control micellar casein concentrate (CON CM), pasteurised micellar casein concentrate (PS CM) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 CM, HHT1.5 CM).
[4]CON MCC, control micellar casein concentrate; PS MCC, pasteurised micellar casein concentrate, 72°C×15s; HHT MCC, high heat treated micellar casein concentrate, 90°C×15s.
[5]UF permeate or RO permeate refer to permeate from ultrafiltration or reverse osmosis respectively.

3.2.2 *Preparation of cheesemilk*

On day 3 (Figure 3.1), 4 cheesemilks, namely CON- (control), PS-, HHT1.0-, and HHT1.5 cheesemilk (CM) were prepared from the following streams: pasteurized cream (fat), CON-, PS-, and HHT MCC (casein), UF permeate (lactose and minerals) and RO permeate (water) as described in Table 1. The protein, fat and lactose contents in the pasteurised cream, MCCs and cheesemilks were measured by FTIR (FOSS MilkoScan™ FT+, Hillerød, Denmark), the total solids in UF permeate was measured by microwave (CEM Smart Trac moisture analyser, Damastown, Dublin, Ireland), while RO permeate is essentially water and its composition was not determined. The lactose content, obtained by multiplying total solids by 0.87 and expressed as a percentage of the total solids in UF permeate, was estimated to be ~ 87 %, in keeping

with levels observed by previous studies (unpublished data) undertaken by this research group. The casein contents for CON-, PS-, and HHT1.0 CM were standardised to 2.72 - 2.74 % and the casein content in HHT1.5 CM was standardised to 1.5 times the casein content in HHT1.0 CM. The casein: fat ratio and lactose content in the four cheesemilks were standardised to 0.74 and 4.45 - 4.52 % respectively. HHT1.5 CM was formulated to mitigate any potential negative influence of high heat treatment (90 °C for 15 s) on the rennet coagulability of cheesemilk, by increasing casein concentration.

3.2.3 *Preparation of cheese*

On the same day of cheesemilk formulation, Cheddar cheese was manufactured as described by Xia et al. (2020).

3.2.4 *Calcium in MCC and cheese milk*

Total calcium in MCCs, cheesemilk and cheeses as well as colloidal- and soluble calcium in cheesemilk were determined with atomic absorption spectrometry (AA240, VarianAA, Varian Inc., CA, USA) as described by Guinee et al. (2000), Gaucheron (2005) and Lin et al. (2016). Colloidal- and soluble calcium were measured in fresh milk.

3.2.5 *Composition of liquid samples and cheese at 14 days*

Total solids and ash contents in the liquid samples (including MCCs, cheesemilk and cheese whey) and cheeses were determined by the methods described in IDF (2010) and IDF (1964a) respectively. The fat content in liquid samples was measured by a gravimetric method (IDF, 1996) and in cheese by NMR (CEM SMART Trac II, Damastown, Dublin, Ireland). Total nitrogen, non-protein nitrogen and non-

casein nitrogen contents were determined using the Kjeldahl (IDF, 1964b, 1993) with a nitrogen-protein conversion factor of 6.38. The casein number and native whey protein content (NWP, expressed as a percentage of total protein) were calculated as described by Lin et al. (2018). The percentage of whey protein denaturation (%WPD, as a percentage of total whey protein) in MCCs and cheesemilk was calculated with equations adapted from Lin et al. (2018):

$$\%WPD=\frac{100\times(NWP_{CON}-NWP_h)}{NWP_{CON}},$$

where NWP_{CON} represented the level of native whey protein in CON MCC or CON CM and NWP_h represented the level of native whey protein in heated MCCs (PS MCC and HHT MCC) or cheesemilk prepared from heated MCCs (PS CM and HHT1.0 CM). The level of whey protein denaturation arising due to pasteurisation of the feed milk was not considered during the calculation of %WPD in MCCs to enable a comparison of the effects of the various heat treatments on the MCCs.

3.2.6 *Rennet coagulation characterisation*

A volume of 20 mL of cheesemilk was transferred from the cheese vat to a rheometer (AR-G2 rheometer; TA Instruments, New Castle, DE, USA) 3 min after rennet addition, and a time sweep and frequency sweep were carried out as described by Xia et al. (2020). Rennet coagulation time (RCT) (Sandra et al., 2011), storage modulus and tan δ at 40 min after rennet addition (A_{40} and tan δ_{40} respectively) and time to achieve storage modulus 35 Pa (K_{35}) or 70 Pa (K_{70}) (Panthi et al., 2019b) were recorded from the storage modulus-time curve. Since the coagulum was cut on achieving gel firmness of 35 Pa, K_{35} was used to represent set to cut time (time from rennet addition to cutting). After a frequency sweep, the following equation can be derived from the frequency (ω)-storage modulus (G') curve:

$$\text{Log } G' = n^* \log \omega + K;$$

Where n was defined as degree of frequency dependence (Tunick, 2010).

3.2.7 *Cheese yield*

Actual and compositional adjusted cheese yield was calculated as described by Guinee et al. (2006):

1. Y_a, actual cheese yield per 100 kg of cheesemilk (kg/ 100 kg of cheesemilk);
2. Y_{ma}, moisture adjusted (to 38.5 %) cheese yield (kg/ 100 kg of cheesemilk):

   $Y_{ma}=Y_a\times(\frac{100-Ma}{100-Mr})$

Where M_a and M_r refer to the actual and reference (38.5 %) cheese moisture content respectively.

3. Y_{afcam}, actual cheese yield per 100 kg of fat and casein adjusted milk:

   $Y_{afcam}=Y_a\times(\frac{Frm+Crm}{Fcm+Ccm})$

Where F_{cm} and C_{cm} refer to the actual fat and casein concentrations in cheesemilk, and F_{rm} (3.4 %) and C_{rm} (2.53 %) the concentrations in the reference milk.

4. Y_{mafcam}, moisture adjusted cheese yield per 100 kg of fat and casein adjusted milk, which was calculated from Yma with a similar formula to that described in formula 3.

3.2.8 *Statistical analysis*

The cascade filtration process and cheese manufacture trials were carried out in triplicate. The effect of heat treatment on the composition of MCC, cheesemilk, cheese composition, yield, texture and gel properties were compared with least-squares difference (LSD) at a 95 % significance level in a one-way ANOVA using IBM SPSS statistics 24.0 (IBM Corp., 2016, Chicago, IL, USA).

3.3 Results and discussion

3.3.1 *Composition of MCC*

A level of 70.29 % of whey protein originally present in pasteurised skim milk was removed to permeate after microfiltration at 0.1 μm, giving a whey protein-reduced MCC (casein number: 93.64 %, Table 3.2). The total solids, total protein, ash and total calcium contents in MCC were not affected by heat treatment (Table 3.2). As the intensity of heat treatment increased, the native whey protein (NWP, as a percentage of total protein) content in MCC decreased and %WPD increased (Table 3.2). There was no significant difference in the NWP content and %WPD between CON MCC and PS MCC, which was not surprising, since pasteurization only leads to 1 % whey protein denaturation in skim milk (casein number: 75 %) as reported by Guinee et al. (1996b). The %WPD in HHT MCC was significantly higher than the CON- and PS MCC, corresponding to the significantly lower NWP content in this stream (Table 3.2). The %WPD (15.97 %, Table 3.2) in HHT MCC (90 °C × 15 s) observed in the current research was substantially lower than the %WPD (36.1 %) reported in skim milk (casein number: 74.2 %) after high heat treatment (88 °C × 15 s) by Guinee at al. (1997). The enhanced heat stability in MCC (manifest by the lower %WPD in HHT MCC) was attributed to its low whey protein content due to whey protein reduction (Bulca et al., 2004).

No significant difference was observed in pH levels between MCCs with different thermal histories although the heated MCCs were lower in magnitude. It has been shown previously that during heat treatment, soluble calcium content decreased, resulting in increased colloidal calcium content and a pH drop in milk (Pouliot et al., 1989 a,b, c; On-Nom et al., 2010). Both the soluble calcium content and pH levels in

heated milk can be almost or fully restored to their original level after cooling when the heating temperature is less than 95 °C (Kannan and Jenness, 1961; Pouliot et al., 1989d; Beliciu et al., 2012). As the pH values in heated MCC were similar to the control MCC, it is assumed that the soluble calcium content and pH in heated MCC almost or totally returned to original levels after cooling.

Table 3.2. Composition and pH of control-, pasteurised-, and high heat treated micellar casein concentrate[1, 2]

Compositional parameters	CON MCC	PS MCC	HHT MCC
Total solids (% , wt/wt)	11.09±1.30[a]	10.96±1.16[a]	10.99±1.16[a]
Total protein (% , wt/wt)	8.80±1.05[a]	8.79±1.06[a]	8.76±1.08[a]
Casein number[3]	93.64±0.53[b]	93.88±0.39[ab]	94.59±0.35[a]
Serum protein (%, wt/ wt)	0.52±0.08[a]	0.52±0.08[a]	0.52±0.08[a]
Serum protein denaturation[4]			
NWP (% of TP)	5.91±0.30[a]	5.75±0.28[a]	4.96±0.22[b]
%WPD	0.00±0.00[b]	2.69±0.64[b]	15.97±2.96[a]
Ash (%, wt/ wt)	0.94±0.11[a]	0.92±0.11[a]	0.93±0.10[a]
Total Ca (m mol kg^{-1})	73.0±7.2[a]	72.5±6.6[a]	71.9±6.9[a]
pH	6.90±0.10[a]	6.82±0.12[a]	6.83±0.12[a]

[1]Results are means minus and plus the standard deviation of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2]CON MCC, control micellar casein concentrate; PS MCC, pasteurised micellar casein concentrate, 72°C×15s; HHT MCC, high heat treated micellar casein concentrate, 90°C×15s.
[3]Casein number (%) = $\frac{\text{Casein content}}{\text{True protein content}} \times 100$.
[4]NWP = native whey protein, expressed as a percentage of total protein; %WPD = percentage of whey protein denaturation, expressed as a percentage of total whey protein.

3.3.2 *Composition of cheesemilk*

Similar contents of total solids, total protein, casein, fat, ash, total calcium as well as casein: fat ratio (Table 3.3) were achieved in CON-, PS- and HHT1.0 cheesemilks as a result of cheesemilk standardization. There was no significant difference in the NWP content and %WPD between CON- and PS cheesemilk. The %WPD in HHT1.0 cheesemilk was significantly higher than the other cheesemilks (Table 3.3), in line with the findings for MCC (Table 3.2). The colloidal- and soluble calcium contents, %soluble calcium, colloidal calcium per gram casein and the pH of cheesemilk with different thermal histories were also similar (Table 3.3), and comparable to the values reported by Gaucheron (2005). This suggests that a similar calcium distribution between the colloidal and soluble phases in CON-, PS- and HHT cheesemilks was achieved as was a complete restoration of soluble calcium in the heated MCC.

The total solids, total protein, casein, ash and total-, soluble-, colloidal calcium contents in HHT1.5 cheesemilk were significantly higher than those in HHT1.0 cheesemilk, due to the higher casein content in this HHT1.5 cheesemilk. Similarly, the fat content was higher as the milk had been standardised on a fat to casein basis. The casein: fat ratio, soluble calcium as percentage of total calcium, colloidal calcium per gram casein and pH in the HHT1.5 cheesemilk were similar to the other three milks (Table 3.3), reflecting an accurate standardization of the HHT1.5 cheesemilk.

Table 3.3. Composition and pH of serum protein reduced cheesemilk of different thermal histories and casein contents [1, 2]

Compositional parameters	CON CM	PS CM	HHT1.0 CM	HHT1.5 CM
Total solids (% , wt/wt)	12.50±0.49[b]	12.62±0.53[b]	12.81±0.81[b]	15.51±0.33[a]
Total protein (% , wt/wt)	3.45±0.39[b]	3.44±0.41[b]	3.44±0.40[b]	4.74±0.32[a]
Casein number	88.65±1.74[a]	88.66±1.73[a]	89.59±1.42[a]	90.86±1.18[a]
Serum protein denaturation[3]				
NWP (% of TP)	7.49±2.51[a]	7.23±2.80[a]	6.75±2.19[a]	6.42±2.53[a]
%WPD	0.00±0.00[b]	0.00±0.00[b]	9.99±1.19[a]	N/A[4]
Casein content (% , wt/wt)	3.06±0.39[b]	3.05±0.40[b]	3.09±0.39[b]	4.31±0.33[a]
Fat content (% , wt/wt)	3.93±0.29[b]	3.86±0.29[b]	3.91±0.37[b]	5.58±0.29[a]
Casein: fat ratio	0.78±0.06[a]	0.79±0.05[a]	0.79±0.04[a]	0.77±0.02[a]
Ash (%, wt/ wt)	0.73±0.05[a]	0.75±0.06[a]	0.76±0.09[a]	0.86±0.06[a]
Calcium				
Total Ca (m mol kg^{-1})	32.34±1.78[b]	32.22±1.32[b]	31.28±3.46[b]	41.42±1.51[a]
Colloidal Ca (m mol kg^{-1})	21.90±1.30[b]	21.63±1.87[b]	20.44±0.51[b]	28.67±2.23[a]
Colloidal Ca /casein (m mol/g casein)	0.73±0.13[a]	0.72±0.14[a]	0.68±0.07[a]	0.68±0.12[a]
Soluble Ca (m mol kg^{-1})	10.44±3.03[a]	10.59±2.92[a]	10.84±2.95[a]	12.75±3.66[a]
%soluble Ca	32.01±7.56[a]	32.69±7.85[a]	34.22±5.94[a]	30.60±7.90[a]
pH	6.49±0.04[a]	6.49±0.06[a]	6.52±0.07[a]	6.53±0.07[a]

[1]Results are means minus and plus the standard deviation of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).

[2]Cheesemilk were standardised from control micellar casein concentrate (CON CM), pasteurised micellar casein concentrate (PS CM) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 CM, HHT1.5 CM).
[3]NWP = native whey protein, expressed as a percentage of total protein; %WPD = percentage of whey protein denaturation, expressed as a percentage of total whey protein.
[4]N/A: not available.

3.3.3 *Rennet coagulation property*

For cheesemilks of typical casein content, the rennet coagulation properties were not significantly affected by pasteurisation, with similar RCT, A_{40}, K_{35} and K_{70} between CON- and PS cheesemilks (Table 3.4). This was in keeping with the similar levels of %WPD in CON- and PS cheesemilks (Table 3.3) and reports of negligible effects of pasteurisation on the coagulability of skim milk (Fox et al., 2017a). Even though the set to cut time (K_{35}) in HHT1.0 CM increased by 21.84 % compared to that in CON CM, it (22.42 min) still ranged between the value cheese makers usually use in cheese manufacture: 20 to 30 min (Govindasamy-Lucey et al., 2004; Heino, 2008; Panthi et al., 2019b). Guinee et al., (1997) reported that the set to cut time at 20 Pa in high heat treated (88 °C × 15 s) cheesemilk (around 70 min) is nearly twice to that in raw cheesemilk (around 33.33 min), leading to the suggestion that cheesemilk of typical casein number should not undergo high heat treatment (i.e., > 72 °C × 15 s) due to high levels of whey protein denaturation (Guinee et al., 1997; Fox et al., 2017c). The lower level of whey protein denaturation as a result of whey protein reduction was shown to mitigate this issue (Bulca et al., 2004; Renhe and Corredig, 2018).

Gel firming rate is improved by increasing the casein content in cheesemilk (Guinee et al., 1997; Panthi et al., 2019b), as a result, K_{35} and K_{70} decrease and A_{40} increases when the casein content in cheesemilk increase (Panthi et al., 2019b). For the cheesemilks prepared from high heat treated MCC, the significantly higher A_{40}

value as well as lower K_{35} and K_{70} value in HHT1.5 cheesemilk suggested that the gel firming rate increased when the casein content increased from 3.09 % to 4.31 % (Figure 3.2, Table 3.4). Interestingly, the set to cut time (K_{35}) in HHT1.5 CM was similar to that of the control milk (CON CM) (Table 3.4). Suggesting that there is no need to change the set to cut time when manufacturing Cheddar cheese from HHT cheesemilk with casein concentration as high as 4.31 %, manufacture cheese from cheesemilk with high casein concentration can allow cheese makers to produce more cheese with fixed facility and labour (Neocleous et al., 2002).

The degree of frequency dependence for storage modulus (n) is an indication of gel structure (Chen et al., 1999), with n = 0 for ideal covalent cross-linked gels and n > 0 for non-covalent cross-linked gels; an increased n value indicates an increased viscoelasticity (Zhou and Mulvaney, 1998; Rosalina and Bhattacharya, 2002; Tunick, 2010). There was no significant difference between the n value of the gels in this research (Table 3.4) arising from the varying heat treatments and casein contents.

Table 3.4. Gel forming properties of serum protein reduced cheesemilk of different thermal histories and casein contents [1, 2]

Parameters	CON CM	PS CM	HHT1.0 CM	HHT1.5 CM
Degree of frequency dependence, n	0.16±0.01[a]	0.16±0.00[a]	0.16±0.00[a]	0.17±0.00[a]
RCT (min)[3]	11.83±2.59[a]	13.63±3.71[a]	13.79±3.23[a]	15.07±1.77[a]
A_{40} (Pa)[4]	164.13±44.43[b]	162.10±58.97[b]	132.15±16.05[b]	288.63±78.90[a]
K_{35} (min)[5]	18.40±1.44[a]	20.46±6.43[a]	22.42±3.66[a]	19.17±0.76[a]
K_{70} (min)[5]	22.83±1.63[a]	24.87±7.99[a]	27.54±4.23[a]	22.44±2.01[a]

[1]Results are means minus and plus the standard deviation of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2]Cheesemilk were standardised from control micellar casein concentrate (CON CM), pasteurised micellar casein concentrate (PS CM) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 CM, HHT1.5 CM).
[3]RCT: the time required for the G' to reach the value of 0.1 Pa after rennet addition.
[4]A_{40}: the storage modulus (G') of gel 40min after rennet addition.
[5]K_{35} or K_{70}: the time it take for the G' to reach the value of 35 or 70Pa respectively after rennet addition.

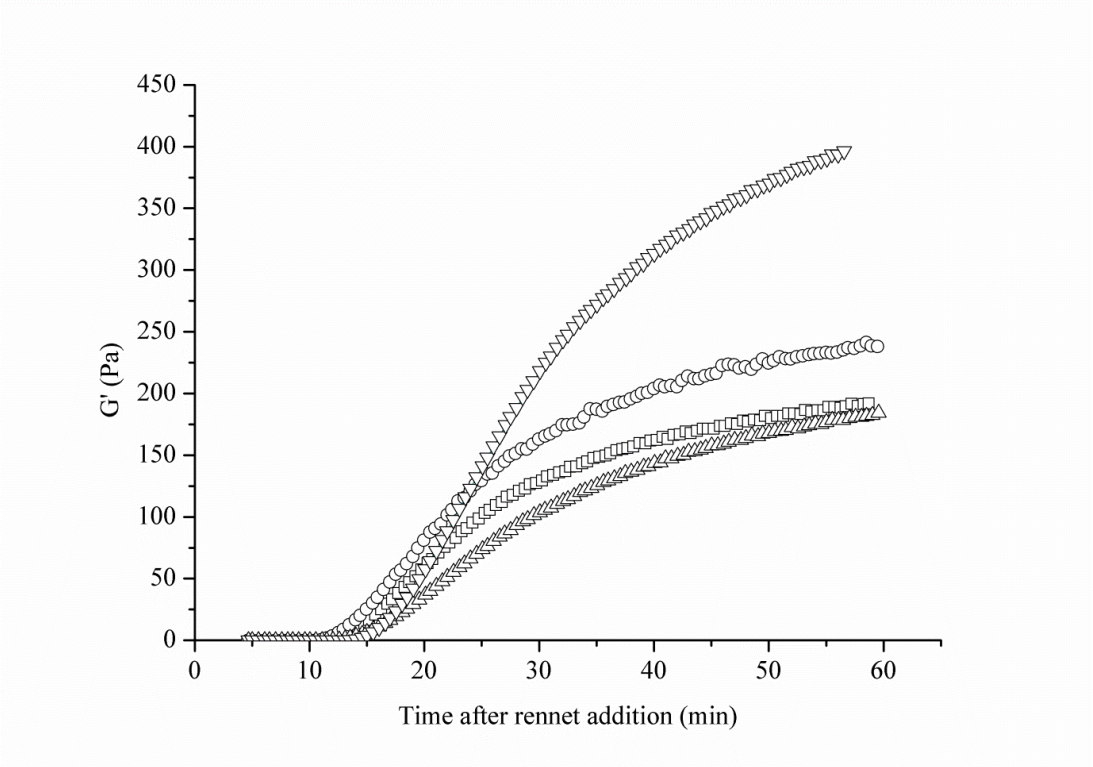

Figure 3.2. Storage modulus (G') of rennet coagulations formed from cheese milks standardised from control- ( ), pasteurised- (○), and high heat treated micellar casein concentrate of typical (Δ) or 1.5 times typical casein content ( ).

3.3.4 *Cheese manufacture time*

The rennet addition to drain time in HHT1.0 cheese was significantly higher than those of CON- and PS cheeses, with no significant difference in drain to mill time between the three cheeses (Table 3.5), as a result, the total make time for the HHT1.0 cheese was longer than for the CON- ($P < 0.05$) and PS cheeses ($P = 0.055$) (Table 3.5). The pH and lactose content in cheesemilk, starter culture inoculum levels and cheese making procedures for the CON-, PS-, and HHT1.0 cheeses were the same, as were the concentrations of casein, total protein, ash, total-, soluble- and colloidal calcium contributing to the buffering capacity (Lucey et al., 1993a, b) in milk (Table 3.3). It has been reported that heat treatment of milk can influence the acid production capacity of lactic acid bacteria inoculated into the milk (Greene and Jezeski, 1957a, b; Singh et al., 1980; Stulova et al., 2011), and this capacity could be inhibited under certain temperature-time heat combinations depending on levels of denatured whey protein and the availability of –SH groups. Given the significantly higher levels of whey protein denaturation prior to HHT cheese manufacture, it is proposed that the acid production capacity of the starter culture (*Lactococcus lactis*) in HHT 1.0 cheesemilk may have been reduced (Figure 3.3), leading to the extended cheese make time. However, further research is required to definitively prove this.

A significant increase in the time from rennet addition to drain as well as for total make time in HHT1.5 cheese compared to the CON- and PS cheeses (Table 3.5) may be due to a greater buffering capacity resulting from higher casein and ash contents (Table 3.3), as reported by St-Gelais et al. (1998). Extended manufacture times can result in lower cheese moisture content (St-Gelais et al., 1997). Xia et al. (2020) reported loss of a large proportion of minerals from MCC during microfiltration and diafiltration with water requiring addition of milk salts to MCC to

fortify the ash content in cheesemilk. Future studies should determine the possibility of decreasing the buffering capacity of cheesemilk of high casein concentration by adding less UF permeate (which was used to fortify lactose and milk salts in cheesemilk) to MCC during cheesemilk preparation. Similarly, increasing the starter culture addition in casein concentrated cheesemilk to improve acid production during cheese processing would also be an option (O'Keeffe et al., 1975; Guinee et al., 1996a).

Table 3.5. Manufacture times for Cheddar cheeses made from serum protein reduced cheesemilk of different thermal histories and casein contents [1, 2]

Manufacture time (min)	CON CM	PS CM	HHT1.0 CM	HHT1.5 CM
Rennet addition to drain	97.73±37.02^{b}	104.64±17.01^{b}	151.28±21.44^{a}	165.83±7.78^{a}
Drain to mill	65.67±21.13^{a}	72.00±25.94^{a}	78.33±19.86^{a}	97.33±6.43^{a}
Total make time[3]	163.40±35.17^{c}	176.64±42.54^{bc}	229.61±16.99^{ab}	263.17±4.31^{a}

[1]Results are means minus and plus the standard deviation of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2]Cheesemilk were standardised from control micellar casein concentrate (CON CM), pasteurised micellar casein concentrate (PS CM) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 CM, HHT1.5 CM).
[3]Total make time: from rennet addition to drain.

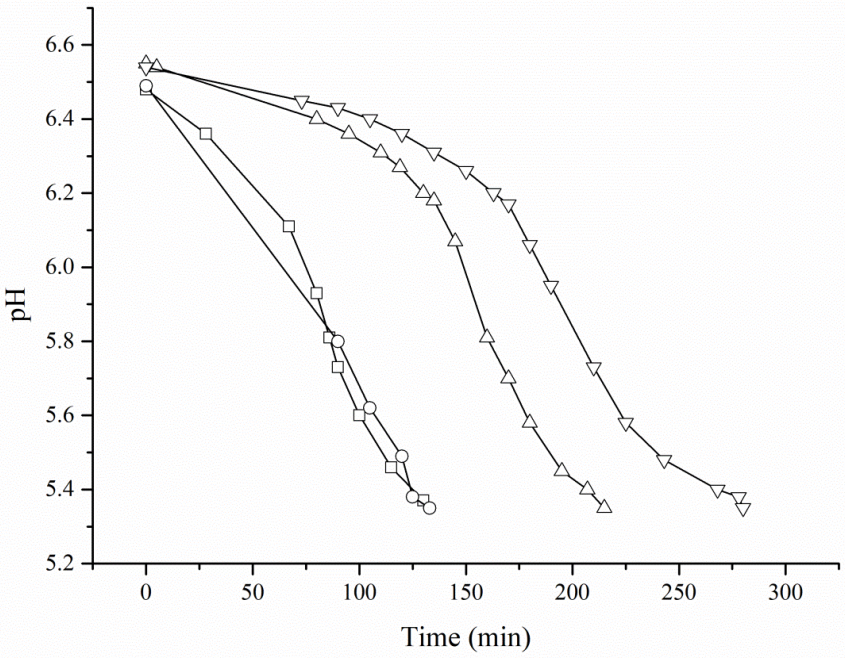

Figure 3.3. Change of pH as a function of cheese manufacture time from cheesemilks standardised from control- ( ), pasteurised (□) (○), and high heat treated micellar casein concentrate of typical (Δ) or 1.5 times typical casein content ( ). ▽

3.3.5 *Composition of 14 days cheese*

No significant difference was observed between CON-, PS and HHT1.0 cheeses (Table 3.6), for contents of protein, fat, moisture, salt, ash and total calcium as well as protein: fat ratio, FDM, MNFS, S/M, Calcium/protein and pH, showing that the thermal treatments applied and any subsequent whey protein denaturation did not affect the Cheddar cheese composition. Guinee et al. (1995) and Rynne et al. (2004) reported that cheese made from high heat treated (88 °C × 15 s or 87 °C × 26 s) cheesemilk of typical whey protein content had increased moisture contents due to reduced syneresis; it was suggested that the retarded aggregation and fusion of denatured whey protein-para-casein complexes should be responsible for the impaired syneresis in severely heat treated cheesemilk (Rynne et al., 2004). A higher tan δ value

corresponded to improved syneresis in rennet induced gels (Van Vliet et al., 1991), similar tan δ40 values (data not shown) for all coagula observed in the current research suggests that the heat treatment applied (72 °C × 15 s or 90 °C × 15 s) did not affect syneresis during rennet induced coagulation of whey protein depleted cheesemilk, with similar cheese moisture contents in CON-, PS- and HHT1.0 cheeses further supporting this.

For each independent trial, the contents of moisture and MNFS in HHT1.5 cheese were lower than those in the other three cheeses (data not shown). Due to the large deviation between trials, these difference were not significant (Table 3.6). Panthi et al. (2019a) reported that for concentrated cheesemilk, lower quantities of cheese whey can lead to curd tearing and small curd particles during cutting and stirring.

Table 3.6. Composition and pH of Cheddar cheeses manufactured from serum protein reduced cheesemilk of different thermal histories and casein contents at 14 days[1, 2]

Compositional parameters	CON cheese	PS cheese	HHT1.0 cheese	HHT1.5 cheese
Protein content (%)	26.23±1.50[a]	25.90±2.60[a]	26.63±1.69[a]	27.56±1.35[a]
Fat content (%)	30.42±0.81[a]	30.44±1.11[a]	30.47±0.97[a]	31.40±0.62[a]
Pro: fat ratio	0.86±0.07[a]	0.85±0.07[a]	0.88±0.07[a]	0.88±0.06[a]
Moisture content (%)	36.27±1.59[a]	36.33±4.22[a]	35.62±1.91[a]	33.43±0.82[a]
FDM (%)[3]	47.75±1.77[a]	47.90±2.15[a]	47.36±1.74[a]	47.21±1.40[a]
MNFS (%)[4]	52.13±2.37[a]	52.19±5.40[a]	51.24±2.62[a]	48.74±1.54[a]
Salt content (%)	1.76±0.10[a]	1.73±0.09[a]	1.82±0.07[a]	1.80±0.18[a]
S/M (%)[5]	4.87±0.47[a]	4.79±0.45[a]	5.12±0.44[a]	5.40±0.57[a]
Ash content (%)	3.99±0.24[a]	3.88±0.32[a]	4.11±0.18[a]	4.22±0.13[a]
Ca (mg/100g)	775.45±25.03	713.02±108.6	756.40±47.	782.81±64.6
Calcium/protein (mg/g of protein)	29.66±2.64[a]	27.43±1.59[a]	28.41±0.14[a]	28.37±1.09[a]
pH	5.29±0.10[a]	5.23±0.09[a]	5.26±0.06[a]	5.33±0.09[a]

[1]Results are means plus and minus the standard deviation of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2]Cheddar cheeses were manufactured from cheesemilk standardised from control micellar casein concentrate (CON cheese), pasteurised micellar casein concentrate (PS cheese) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 cheese, HHT1.5 cheese).
[3]FDM= fat in dry matter.
[4]MNFS=moisture in non-fat substance.
[5]S/M=salt in moisture.

3.3.6 *Cheese yield*

There was no significant difference in cheese yield between CON- and PS cheese (Table 3.7), which was expected due to the negligible levels of whey protein denaturation in the PS cheesemilk. Similarly, high heat treatment of cheesemilk did not affect the actual- and moisture adjusted cheese yield (Table 3.7), contrary to earlier expectation. The moisture adjusted actual yield (Y_{ma}) of HHT1.0 cheese, predicted to be 12.36 kg if all the denatured whey protein in HHT1.0 cheesemilk was recovered to the resultant cheese, was achieved (Table 3.7), suggesting that the low levels of whey protein present and in a denatured state due to HHT had a negligible impact on the yield of Cheddar cheese.

The actual (Y_a) and moisture adjusted (Y_{ma}) cheese yield of cheeses made from the high heat treated concentrated cheesemilk (HHT1.5) was significantly higher than for cheeses made from un-concentrated cheesemilks in accordance with Xia et al. (2020) and was attributed to the higher solids content in the HHT1.5 cheesemilk as the casein and fat adjusted cheese yields (Y_{afcam} and Y_{mafcam}) were similar between all four cheese types (Table 3.7).

Table 3.7. Recoveries of components and yields of Cheddar cheese made from serum protein reduced cheesemilk with different thermal histories and casein contents [1, 2]

Parameters	CON cheese	PS cheese	HHT1.0 cheese	HHT1.5 cheese
Recovery to cheese				
Fat (% total milk fat)[3]	91.90±1.89[a]	94.83±1.56[a]	92.21±3.25[a]	90.58±4.94[a]
Protein (% total milk protein)[4]	90.50±1.32[a]	90.51±1.24[a]	91.52±0.49[a]	93.73±0.79[b]
Cheese yield[5]				
Y_a (kg/100kg)	11.87±0.61[b]	12.00±0.35[b]	11.80±0.69[b]	16.07±0.46[a]
Y_{ma}(kg/100kg)	12.31±0.94[b]	12.44±1.17[b]	12.36±1.06[b]	17.39±0.48[a]
Y_{afcam}(kg/100kg)	10.10±0.45[a]	10.36±0.78[a]	10.05±0.53[a]	9.65±0.46[a]
Y_{mafcam}(kg/100kg)	10.45±0.21[a]	10.68±0.17[a]	10.50±0.24[a]	10.44±0.40[a]

[1]Results are means minus and plus the standard deviation of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2]Cheddar cheeses were manufactured from cheesemilk standardised from control micellar casein concentrate (CON cheese), pasteurised micellar casein concentrate (PS cheese) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 cheese, HHT1.5 cheese).
[3]Fat (% of total milk fat) $= \frac{\text{fat content in cheese} \times \text{weight of cheese}}{\text{fat content in cheese milk} \times \text{weight of cheese milk}} \times 100$
[4]Protein (% of milk protein) $= \frac{\text{protein content in cheese} \times \text{weight of cheese}}{\text{protein content in cheese milk} \times \text{weight of cheese milk}} \times 100$
[5]Y_a= actual yield (kg/100kg milk); Y_{ma}= moistrure-adjusted yield; Y_{afcam}= yield per 100kg of milk normalized to reference fat (3.4%, w/w) and casein (2.53%, w/w) levels; Y_{mafcam}= moisture-adjusted yield per 100kg of milk normalized to reference fat (3.4%, w/w) and casein (2.53%, w/w) levels.

3.4 Conclusions

Pasteurization (72 °C × 15 s) of MCC had no significant influence on the %WPD of pasteurised MCC or on the %WPD, calcium distribution and pH in subsequent cheesemilk. Similarly, the rennet coagulation properties of cheesemilk, cheese make time, cheese composition and yield were not influenced by pasteurization of MCC compared to the control. High heat treatment (90 °C × 15 s) of the MCC resulted in increased %WPD in both MCC (15.97 %) and the resultant cheesemilk,

although lower than that reported in studies on milk and was attributed to the low whey protein content in MCC prior to heat treatment. The calcium distribution and pH of whey protein-reduced cheesemilk were not affected by HHT, nor were the cheese composition, pH and yield. HHT also elongated the cheese make time significantly during Cheddar cheese manufacture compared to CON cheese, although it did not result in a significant reduction in cheese moisture content.

After partial removal of whey protein, the gel firming rate for HHT cheesemilk of typical casein concentration (3.09 %) was not significantly affected by denaturation of the whey protein. The gel firming rate increased by increasing the casein concentration in cheesemilk from 3.09 % to 4.31 % with set to cut time decreased insignificantly.

Despite prior expectation, cheese yield was not significantly improved by HHT of MCC, and similarly HHT did not significantly impact on the rennet coagulability, cheese making properties and cheese composition from whey protein depleted cheesemilk. As Amelia and Barbano (2013) reported that pasteurised MCC had a long shelf life (> 16 weeks) at 4°C, future research should determine whether the heat treatment of MCC (90 °C × 15 s) would result in an extended shelf life of MCC providing a commercial means of protein fortification of cheesemilk to mitigate seasonal variations in milk protein content. Overall, HHT of MCC prior to cheese manufacture did not negatively influence the cheese manufacture process, or composition and yield of resultant Cheddar cheese.

3.5 References

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Chapter 4: Effect of heat treatment on whey protein-reduced micellar casein concentrate: A study of texture, proteolysis levels and volatile profiles of Cheddar cheeses produced therefrom

4.1 Abstract

Micellar casein concentrate (MCC) of high casein content, (93.64 % of total protein), was produced by microfiltration of pasteurised skim milk. Cheesemilk of typical (1×) or high (1.5×) casein content were formulated from MCC which received either: no further heat treatment; pasteurisation (72 °C×15 s) or high heat treatment (90 °C×15 s), prior to combination with other membrane streams and cream. Cheddar cheeses were manufactured in triplicate and ripened for 180 days. Cheeses made from pasteurised or high heat treated MCC had similar pH as well as comparable flowability, hardness and volatile profiles over 180 days of ripening. On increasing cheesemilk casein content, levels of primary proteolysis in resultant cheeses decreased, and pH and hardness levels increased. Overall, increasing the heat treatment temperature of MCC from 72 to 90 °C did not impair the texture, functionality and volatile profile of resultant Cheddar cheeses.

4.2 Introduction

Micellar casein concentrate (MCC) is produced by microfiltration (MF) of skim milk removing native whey proteins in the MF permeate and retaining native casein micelles in the MF retentate (Neocleous et al., 2002b). MF permeate is free from rennet, starter culture, caseinomacropeptide, cheese fines, fat, colorant and lactic acid, and is considered as an ideal stream for native whey protein production compared to cheese whey (Bacher and Kønigsfeldt, 2000). Increasing the casein content of cheesemilk through formulation of cheesemilk directly from MCC and permeate streams, cheese of consistent quality and yield can be obtained where seasonal milk supplies are an issue. Similarly, higher cheese yields can be achieved from fixed equipment and labor inputs (Neocleous et al., 2002a; Soodam and Guinee, 2018).

On heating cheesemilk of typical whey protein content, the exposed thiol group in denatured whey protein, especially β-lactoglobulin (β-LG) can form aggregates with itself, soluble κ-casein or casein micelles. These interactions can impede the aggregation and rearrangement of para-casein micelles and ultimately impair the rennet coagulation properties of cheesemilk as well as syneresis of the resultant gel (Bulca et al., 2004; Rynne et al., 2004; Anema et al., 2007). For this reason, by partially removing whey protein in its native state from skim milk with MF, the resultant MCC has a better heat stability than skim milk (Bulca and Kulozik, 2004; Renhe and Corredig, 2018). Cheesemilk prepared from high heat treated MCC (HHT, 90 °C×15 s) has good rennet coagulation and cheesemaking properties and the resultant cheeses have comparable cheese moisture contents and yield to cheeses made from a control unheated MCC cheesemilk (Xia et al., 2020b). However, it is not yet known whether manufacture of cheeses from HHT MCC will influence ripening, structural and quality indices such as pH, levels of proteolysis, texture, flowability and also the volatile profile.

Metabolism of residual lactose and of lactate and citrate, lipolysis and proteolysis are the three major biochemical events which take place during cheese ripening. Amongst these, proteolysis, which influences the evolution of pH, texture, flowability and flavor, has traditionally been considered as the most important (Fox, 1989; McSweeney and Sousa, 2000). In primary proteolysis, intact casein, which provides structural integrity to cheese, is partially hydrolysed into peptides through the activity of enzymes, i.e., residual chymosin hydrolysing αs1-casein and plasmin induced hydrolysis of β-casein. In secondary proteolysis, the peptides produced from primary proteolysis are further hydrolysed into smaller peptides and free amino acids

by proteinases and peptidases released through lysis of starter culture or non-starter lactic acid bacteria (NSLAB), forming precursors for cheese flavour development.

Higher levels of primary proteolysis result in a softer cheese body and more substrate for secondary proteolysis, and thus more precursors for flavor and taste (Ardö et al., 2017). For milk of typical whey protein content, free thiol groups present on denatured β-LG can interact with plasmin associated with casein micelles after heat treatments that are more severe than pasteurization (i.e., 80-95 °C × 180-30 s), leading to reduced plasmin activity (Somers and Kelly, 2002; Crudden et al., 2005; Benfeldt, 2006; Stoeckel et al., 2016). Increasing the temperature from 72 to 90 °C and holding time from 15 to 60 s during heat treatment can result in a greater reduction in plasmin activity in the resultant cheese during ripening (Benfeldt et al., 1997). The plasmin activity in milk of typical whey protein content is reduced by around 50 % after HHT (90 °C × 15 s) as well as within the resultant cheese; as a result, the intact β-casein level in HHT cheese is about three times higher than that in cheese made from pasteurized milk (Benfeldt et al., 1997).

The reduction of β-casein hydrolysis can lead to higher fracture strain (Lamichhane et al., 2019) and lower flavor levels in cheese (Jameson and Lelievre, 1996). However, the plasmin activity in whey protein-reduced MCC (casein accounting for 94.17 % of total protein) was only slightly decreased after heat treatment of 95 °C for up to 15 s due to less available denatured β-LG (Aaltonen and Ollikainen, 2011). Previously Xia et al. (2020b) reported a reduction in whey protein levels of 70.29 % in MCC (casein accounting for 93.64 % of total protein) which is very similar to that reported by Aaltonen and Ollikainen (2011). As a continuation of the study of Xia et al. (2020b), this study aims to determine if the plasmin activity in cheese made from HHT MCC (90 °C × 15 s), as described in Xia et al. (2020b), is

significantly different than that in cheeses made from pasteurised MCC (72 °C × 15 s), and similarly if levels of β-casein degradation during cheese maturation were affected.

Xia et al. (2020b) estimated that whey protein accounted for 0.74 % of the total protein in Cheddar cheese made from HHT MCC. Some researchers have suggested that whey protein in its native or denatured format incorporated into the cheese matrix could impede the activity of residual rennet and perhaps other enzymes, leading to decreased degradation of α_{s1}-casein (Creamer et al., 1987; Harper et al., 1989; Bech, 1993; Miloradovic et al., 2017), while others report the opposite (Aydemir, 2018) or no effect (Benfeldt et al., 1997). Further research should also determine if low levels of denatured whey protein affect degradation of α -casein in Cheddar cheese produced from HHT MCC.

The effect of high heat treatment of MCC on the rennet coagulation properties of cheese milk, as well as the composition and yield of resultant Cheddar cheese was studied previously (Xia et al., 2020b). As a follow on study, the objective of the current research is to determine whether the pH and plasmin activity of experimental cheeses, and levels of primary proteolysis, texture, flowability and cheese volatile profiles during ripening are affected by the application of high heat treatment. Such high heat treatment may be applied to prolong the shelf life of MCC as described by Amelia and Barbano (2013). Similarly, the effects of high heat treatment were also determined on the characteristics of cheese manufactured with a higher casein content.

4.3 Materials and methods

4.3.1 *Cheddar cheese manufacture*

Membrane streams, whey protein-reduced cheesemilk and Cheddar cheese were prepared as described by Xia et al. (2020b). In summary, pasteurised skim milk was fractionated into micellar casein concentrate (MCC), UF permeate (contains lactose and minerals) and RO permeate (water) by microfiltration at 0.10 μm, and concentrated by ultrafiltration and reverse osmosis, all within one day. On the following day, MCC aliquots were divided into portions that received no further heat treatment, or were pasteurised (72 °C × 15 s) or high heat treated (HHT, 90 °C × 15 s); these are referred to as control MCC (CON MCC), pasteurised MCC (PS MCC) or high heat treated MCC (HHT MCC) respectively. On the third day, four individual cheesemilks (CMs) were formulated and standardised from the MCCs, UF permeate, RO permeate and pasteurised cream and were termed CON CM, PS CM, HHT1.0 CM and HHT1.5 CM. The casein contents in CON-, PS- and HHT1.0 CM were standardised to 2.72- 2.74 % and the casein content in HHT1.5 CM was standardised to 1.5 times the casein content in HHT1.0 CM. The casein: fat ratio and lactose contents in all cheesemilks were standardised to 0.74 and 4.45- 4.52 %, respectively. Subsequent to cheesemilk standardisation, Cheddar cheeses were manufactured on the same day as described by Xia et al. (2020a).

After removal from pressing, the cheeses were vacuum packed and stored at 4 °C for 14 days and then transferred to 8 °C for ripening for the remainder of the 180 d period.

4.3.2 *Plasmin activity*

MCCs or cheesemilks (30 mL) was added to 10 mL of 40 mM trisodium citrate and mixed gently. Subsequently, the mixture was centrifuged at 29,000 g for 20 min at 4 °C, and the resultant supernatant was then filtered through 0.45 μm filters. Finely grated cheese (10 g) sampled from day 180 of ripening was dispersed in 90 ml 40 mM tri-sodium citrate solution and equilibrated at 37 °C for 15 min. The mixture was then homogenized using a stomacher. Plasmin activity in both liquid and cheese samples was measured using a modified method of Richardson and Pearce (1981) as described by Li et al. (2020). The results were expressed as nmol AMC mL-1 min-1, which equated to one unit of plasmin activity.

4.3.3 *Urea-polyacrylamide gel electrophoresis*

Urea-polyacrylamide gel electrophoresis (PAGE) analysis of the Cheddar cheeses was conducted with Protean II xi vertical slab gel unit (Bio-rad Laboratories Ltd., Watford, Herts, UK) as described by Lamichhane et al. (2019). Ground cheese samples with fixed weight (i.e., 20 mg) after 3, 30, 90 and 180 days of ripening were mixed with 1 mL of sample buffer, incubated at 55 °C for 10 min in a water bath and then loaded onto the gels (20 μL per lane). After electrophoresis, the gel was stained with Coomassie based staining solution (InstantBlueTM, ISB1L, Expedeon) for 24 hours, de-stained with water for 2 hours and scanned.

4.3.4 *pH, pH4.6-SN, flowability and texture*

Cheeses were sampled at day 3, 30, 90 and 180, and levels of pH and pH 4.6-soluble nitrogen as a percentage by weight of cheese (pH4.6SN) were monitored as per Fenelon and Guinee (2000). Flowability and the texture profile were measured as described by McCarthy et al. (2016) and Hou et al. (2012) respectively. Cheese

firmness, fracture stress and fracture strain were recorded and calculated by the methods described by Hou et al. (2012).

4.3.5 *Analysis of volatiles*

Cheese samples from each of triplicate trials were analysed, in triplicate, for volatile compounds by headspace solid phase microextraction (HS-SPME) gas chromatography mass spectrometry (GC-MS) as per Lamichhane et al. (2018).

4.3.6 *Statistical analysis*

Trials for the cascade membrane filtration process and Cheddar cheese manufacture were carried out in triplicate. A split-plot design was applied to study the effect of heat treatment, casein concentration and ripening time as well as their interactive effects on the pH, flowability, fracture stress, fracture strain and firmness of Cheddar cheese. Analysis of variance was carried out with general linear model using SAS 9.4 (SAS Institute, 2012), the differences between means were evaluated with the Turkey test at 95 % significance level.

4.4 Results and discussion

4.4.1 *Cheese composition at 14 days of ripening*

The composition and pH of Cheddar cheeses at day 14 of maturation was measured and reported, previously, by Xia et al. (2020b), the results are provided in this research as supplumentary material (Table S 4.1). In general, no significant difference was observed on the pH and contents of protein, fat, moisture, salt and ash between experiemntal cheeses (Table S 4.1).

4.4.2 *Changes in pH during ripening*

From day 30 of ripening onwards, the pH in cheese increased significantly with increasing casein concentration in the cheesemilk ($P < 0.05$), and with advancing cheese ripening time ($P < 0.001$), as was also reported previously (Rynne et al., 2007; Hou et al., 2012; Soodam et al., 2014) (Table 4.1, Figure 4.1). Although not significantly different, the moisture and MNFS contents were lower in magnitude with higher protein and ash contents in cheese manufactured from cheesemilk with higher casein concentration (Table S 4.1), which may have resulted in a lower lactose and lactic acid content (Rynne et al., 2007), a higher buffering capacity (Lucey and Fox, 1993) and a significantly lower pH. Different thermal treatments (control / no heat, PS and HHT) received by MCCs had no significant influence on the pH of cheese over maturation, which might be explained by the similar contents of protein, moisture and ash in CON-, PS- and HHT1.0 cheese (Table S 4.1).

Table 4.1. Statistical significances (P-values) for mean changes in pH, flowability and % pH4.6SN/ 100 g cheese in Cheddar cheeses manufactured from cheesemilk with different thermal history and casein concentration[1]

Parameters	Treatment	Time	Interactive effect (treatment × time)
pH	**	***	*
% pH4.6SN/ 100 g cheese	***	***	***
Firmness (N)	**	***	NS
Fracture stress (N)	***	***	**
Fracture strain	NS	***	NS
Flowability (%)	NS	NS	NS

[1] Significant levels: NS, P > 0.05; *P < 0.05; ** P < 0.01; *** P < 0.001.

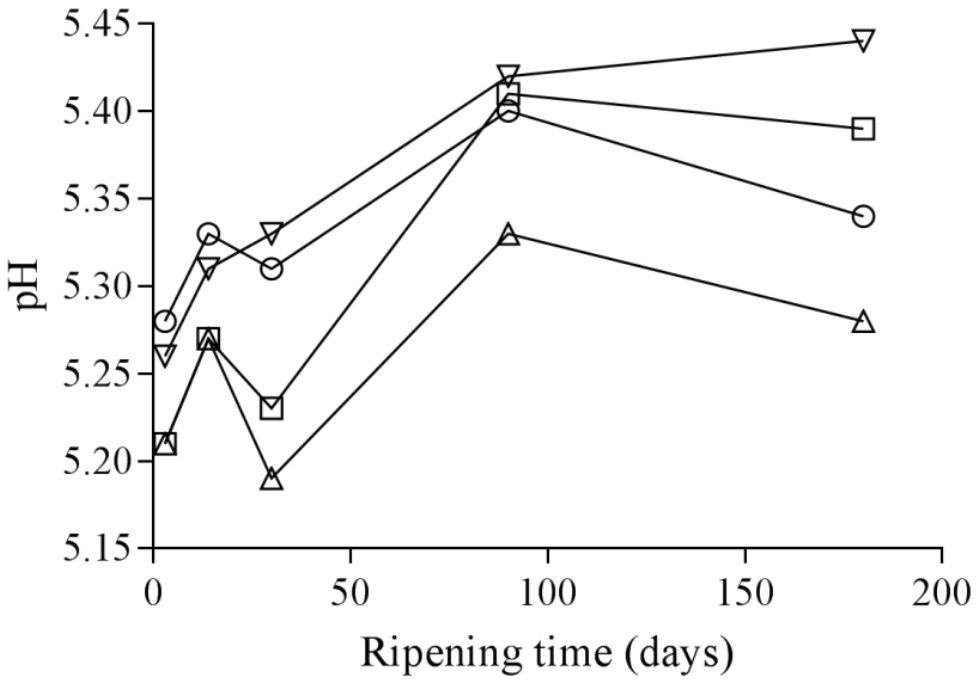

Figure 4.1. Mean pH values during ripening of Cheddar cheeses made from MCCs of different thermal histories: control (○), pasteurised (□), high heat treated (Δ) and casein concentration: of 1.5 times typical casein concentration (▽).

4.4.3 *Plasmin activity*

Even though whey proteins were partially removed from the MCC (70.29 % of whey protein was removed from the feed milk), the plasmin activity in MCC decreased significantly on increasing heat temperature from unheated to 90 °C (Figure 3.2A). Plasmin activity in milk is reduced after heat treatment due to disulphide bond formation between plasmin and denatured β-LG (Aaltonen and Ollikainen, 2011). The low levels of denatured whey proteins observed in both PS- and HHT MCCs (Xia et al., 2020b) might have led to a partial inactivation of plasmin in the heat treated MCCs. The difference in plasmin activity between PS- and HHT MCC was carried forward to their corresponding cheesemilk (Figure 3.2B) and cheeses (Figure 3.2C), with no significant difference between PS- and HHT cheeses. The plasmin activity in cheese was determined and compared on a cheese weight basis. At day 180 of ripening, the plasmin activity in HHT1.5 cheese was significantly higher than that in HHT1.0 cheese (Figure 3.2C), possibly relating to a significantly higher casein content in the cheesemilk (Xia et al., 2020b) since both plasmin and plasminogen are associated with casein micelles in milk (Neocleous et al., 2002b).

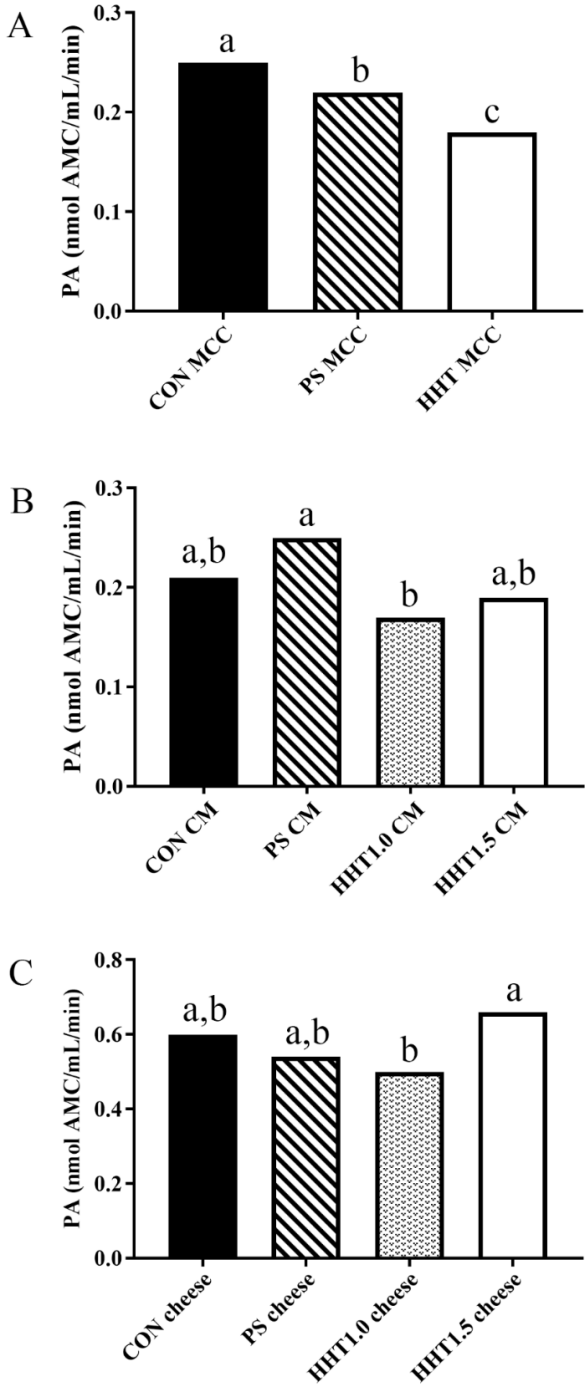

Figure 4.2. Plasmin activity in micellar casein concentrates (A), whey protein-reduced cheesemilks (B) and Cheddar cheeses made there from at day 180 of ripening (C). Results are means of triplicate trials, values within a figure not sharing the same superscript differ significantly ($P < 0.05$). Abbreviations: micellar casein concentrate were not heated (CON MCC), pasteurised (PS MCC) or high heat treated (HHT MCC); cheesemilks were standardised from control MCC (CON CM), pasteurised MCC (PS CM) or high heat treated MCC with typical or 1.5 times typical casein content (HHT1.0 CM, HHT1.5 CM), the Cheddar cheeses made therefrom were called CON-, PS-, HHT1.0- or HHT 1.5 cheese respectively.

4.4.4 *Urea-PAGE*

There was no significant difference in the protein contents between Cheddar cheeses (Table S 4.1), and the pattern of casein hydrolysis during ripening was monitored by urea-PAGE on a cheese weight basis. Less hydrolysis of α_{s1}- and β-casein in the PS-, HHT1.0- and HHT1.5 cheeses than those in the CON cheese along with similar levels of intact β-casein in cheeses PS-, HHT1.0- and HHT1.5 were observed over maturation (Fig. 4.3). However, no significant difference in plasmin activity was observed between CON, PS and HHT1.5 cheeses (Figure 4.2C). Similarly, the retention and activity of residual chymosin in these cheeses is expected to be similar due to their comparable contents of moisture, protein and pH (Table S 4.1). Thus it is proposed that one or more enzymes, other than chymosin and plasmin might be present in the CON cheese which might be responsible for the higher levels of hydrolysis of α_{s1}- and/ or β-casein. Similarly, the increasing intensity of an unknown band located on the top of the CON cheese lane, as a function of ripening time (Figure 4.3) may also be related to this enzyme(s), though more research is required to support this hypothesis. This hypothesis is further inked to the higher levels of volatile compounds in the CON cheeses, as discussed later

For all cheeses, the degradation of α_{s1}-casein was much more intensive than β-casein particularly at day 90 and 180, in agreement with previous studies on Cheddar cheese (Lawrence et al., 1987, Rynne et al., 2004, McMahon et al., 2014, McCarthy et al., 2017b). Milk heat treatment intensity did not have a significant impact on the breakdown patterns of α_{s1}-casein on comparing cheeses PS- and HHT1.0 (Fig. 4.3). Residual coagulant within the cheese moisture phase hydrolyses α_{s1}-casein during cheese ripening, and results suggest that the levels of denatured whey protein

recovered in cheese HHT1.0 did not impede the hydrolysis of α_{s1}-casein, which is in agreement with the studies of Benfeldt et al. (1997).

Urea PAGE showed lower levels of hydrolysis of α_{s1}-casein in the HHT1.5 cheese compared to the other cheeses at all sampling dates (Fig 4.3). This was attributed to the addition of chymosin to milk on a weight basis during cheese manufacture and thus a lower chymosin to casein ratio in the resultant cheese (Neocleous et al., 2002b). The significantly higher pH in HHT1.5 cheese ($P < 0.05$) than that in HHT1.0 cheese from day 30 to day 180 of maturation might account for its lower levels of α_{s1}-casein degradation as well, since the activity of residual chymosin decreases at higher pH (Hickey et al., 2017).

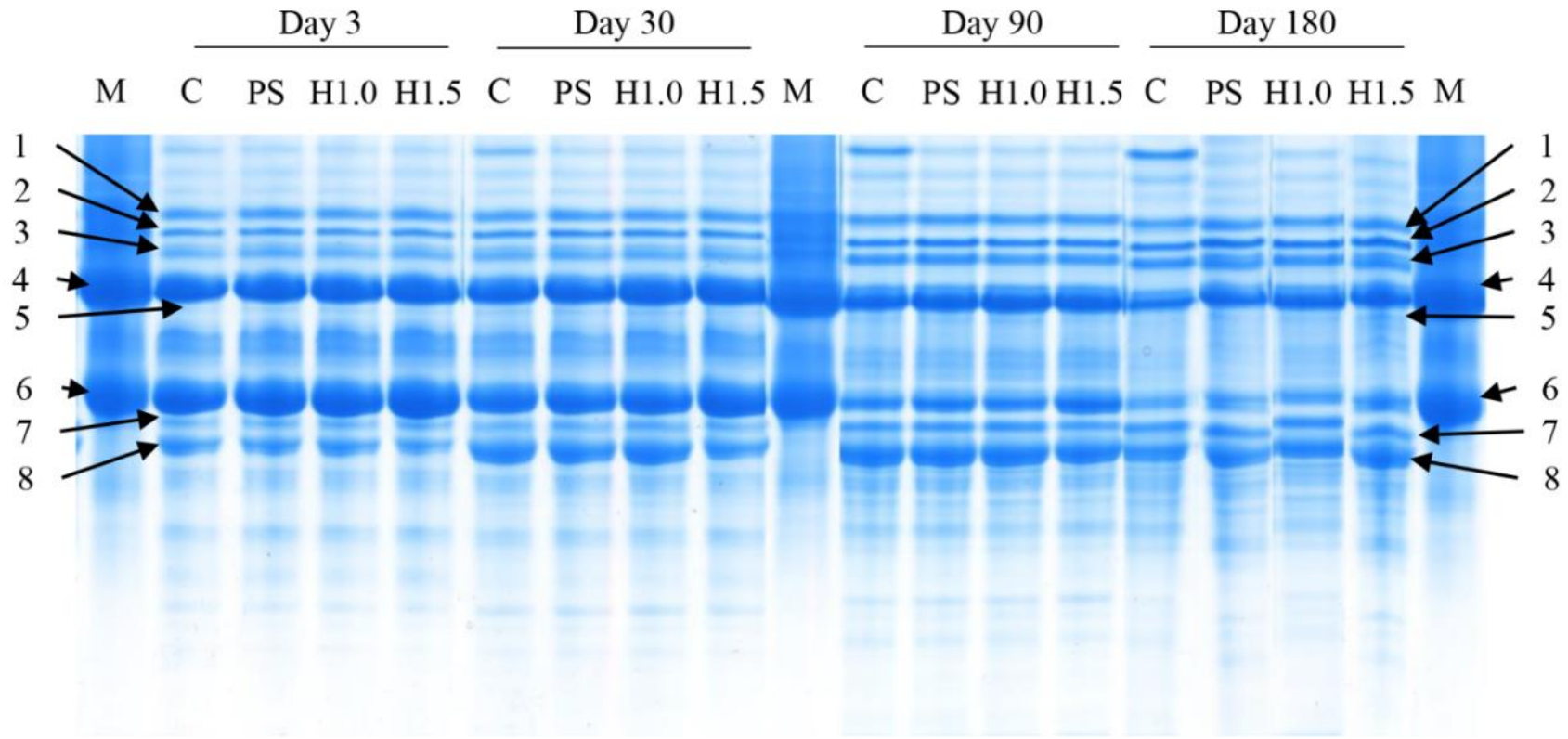

Figure 4.3. Urea-polyacrylamide gel electrophoretograms of Cheddar cheeses made from MCCs with different thermal histories: control (CON), pasteurised (PS), high heat treated (HHT1.0) and casein concentration of 1.5 times typical casein concentration (HHT1.5) at day 3, 30, 90 and 180 of ripening. Abbreviation: M, Marker (sodium caseinate); C, control cheese; PS, cheese made from pasteurised MCC; H1.0, cheese made from high heat treated MCC with typical casein concentration; H1.5, cheese made from high heat treated MCC with 1.5 times typical casein concentrate. Number: 1, β-casein (f106-209); 2, β-casein (f29-209); 3, β-casein (f108-209); 4, β-casein; 5, β-casein (f1-192); 6, α_{s1}-casein; 7, α_{s1}-casein (f102-199); 8, α_{s1}-casein (f24-199).

4.4.5 *Primary proteolysis: pH4.6-SN/100 g cheese*

Levels of pH4.6SN per 100 g cheese were determined to compare the influence of treatments applied to the MCC on primary proteolysis. These levels increased significantly during ripening time for all cheeses ($P < 0.0001$, Table 4.1, Figure 4.4) indicating progression of proteolysis (Bertolino et al., 2011; Hou et al., 2014). The levels of pH4.6SN/ 100 g cheese in the PS cheeses was comparable to those in full fat Cheddar cheese as reported by McCarthy et al. (2017b). There was also a significant interaction between treatment and time where, from day 30 of ripening onwards, the levels of pH4.6SN/100g cheese were significantly ($P < 0.001$) higher in the CON than in the PS- and HHT1.0 cheeses, in agreement with the urea-PAGE results, and this is attributed to increased levels of α_{s1}- and β-casein degradation in CON cheese. No significant difference was observed for levels of pH4.6-SN per 100 g cheese between PS- and HHT1.0 cheese during maturation except on day 180 of ripening ($P < 0.01$). For cheeses sampled at day 30, 90 and 180, cheese made from cheesemilk with higher casein content also had significantly lower level of pH4.6SN/100 g cheese ($P < 0.0001$) in agreement with the results of Neocleous et al. (2002b), which is probably owe to a decreased chymosin to casein ratio on increasing casein content. Similar trends were also observed for levels of pH4.6SN expressed as % of TN in cheese (results not shown) given the similar protein levels in all cheeses (Table S 4.1).

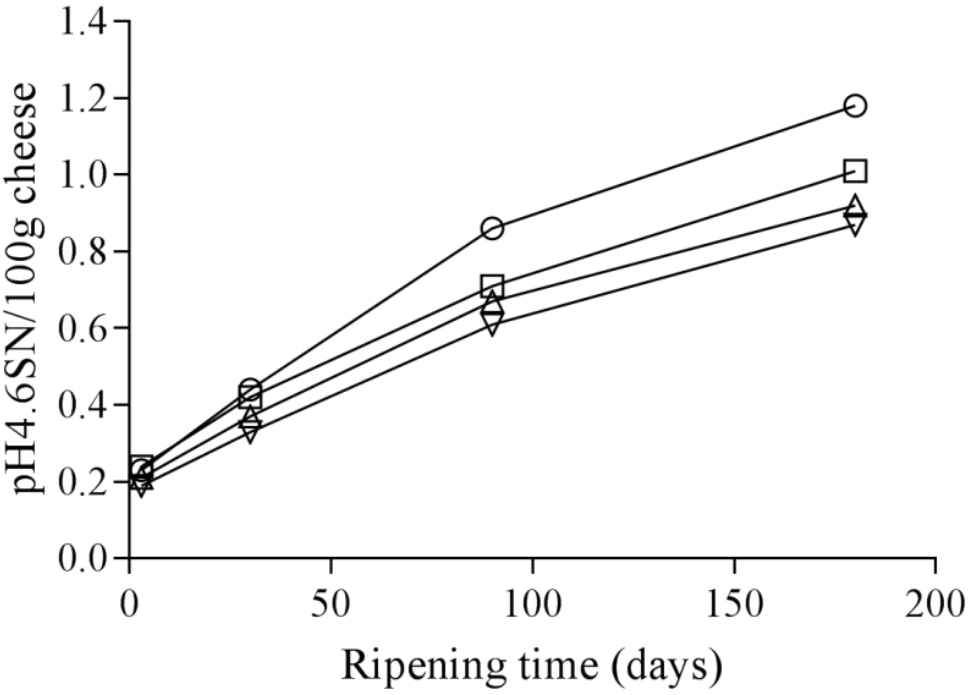

Figure 4.4. Mean levels of pH4.6SN/ 100 g cheese in Cheddar cheese made from MCC with different thermal history: control (○), pasteurised ( ), high heat treated (Δ) and casein concentration: 1.5 times typical casein concentration ( ) during ripening.

4.4.6 *Texture analysis*

Apart from a slight increase at day 30 of ripening, the fracture stress, fracture strain and firmness of the Cheddar cheeses all decreased significantly ($P < 0.0001$) as ripening progressed, suggesting that the texture of cheese become softer and shorter as a result of age-related proteolysis of the para-casein network, as well as solubilisation of colloidal calcium, as reported by other studies (Soodam et al., 2015; Lamichhane et al., 2019). A significant interaction between treatment and time was observed for fracture stress (Table 4.1), and PS- and HHT1.0 cheeses had similar ($P>0.05$, Fig. 4.5) but significantly higher fracture stress values than that of the control cheese between day 30 and day 180 of maturation ($P < 0.05$), which was associated with higher levels of intact casein and significantly lower levels of pH4.6SN/ 100 g cheese in the PS- and HHT1.0 cheeses (Fig 4.2, 4.4, Table 4.1). Between day 90 and

180 of maturation, HHT1.5 cheese had a significantly higher fracture stress ($P < 0.05$) than HHT1.0 cheese, attributed to lower levels of casein hydrolysis, as indicated by the significantly lower levels of pH4.6SN/ 100 g cheese of the HHT1.5 cheese (Fig 4.4, Table 4.1). St-Gelais et al. (1995) and Neocleous et al. (2002b) also reported that cheeses made from high casein content cheesemilk of lower levels of proteolysis and a harder texture; however, this change could be mitigated by increasing the MNFS levels in cheese through adjusting processing parameters, as well as increasing levels of proteolysis through the use of higher chymosin levels (Neocleous et al., 2002b).

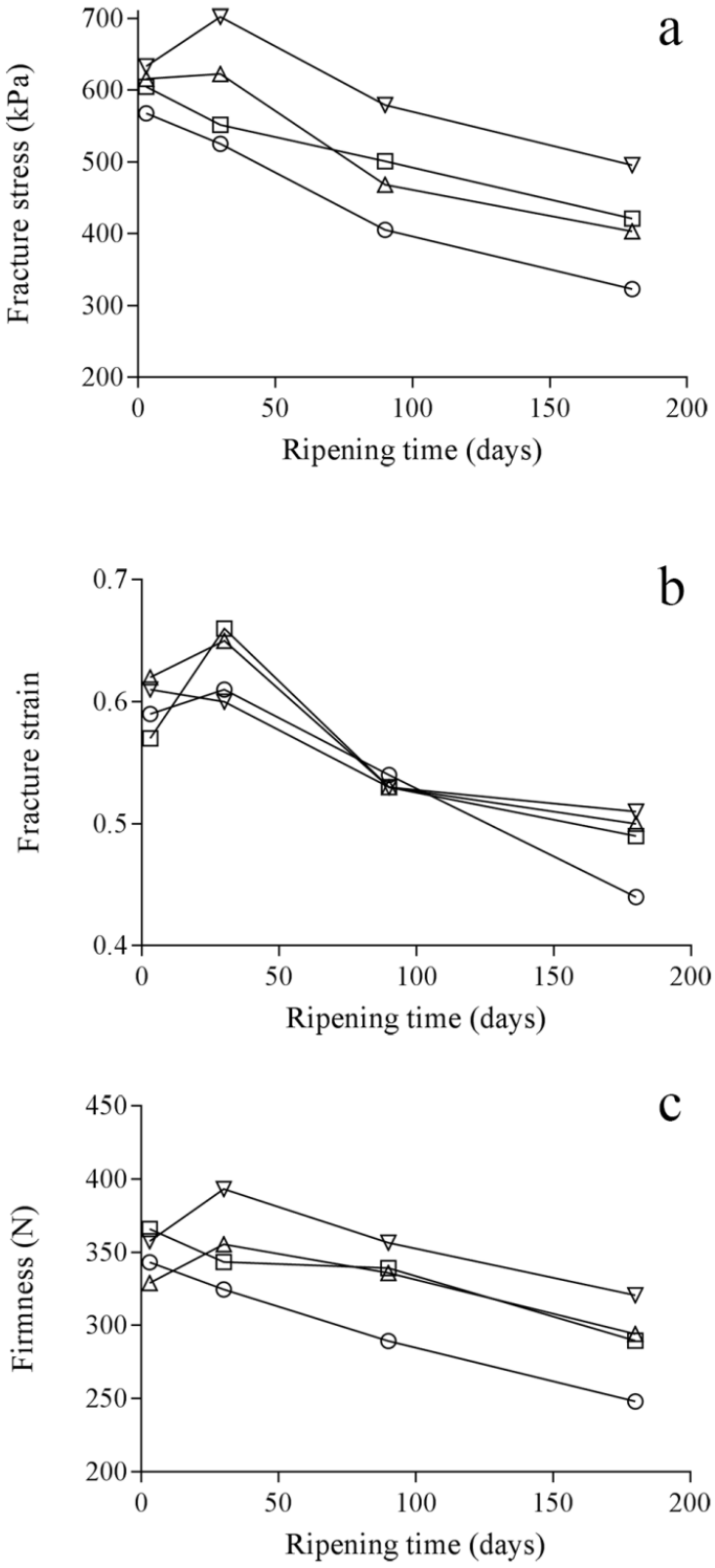

Figure 4.5. Levels of Fracture stress (a), Fracture strain (b) and Firmness (c) of Cheddar cheeses with different thermal history: control (○), pasteurised (□), high heat treated (Δ) and casein concentration: 1.5 times typical casein concentration (▽) during ripening.

4.4.7 *Flowability*

For cheese manufactured from cheesemilk of typical whey protein content, increasing temperature of heat treatment significantly reduces the flowability of Cheddar cheese throughout cheese ripening, probably due to the inclusion of denatured whey protein (Rynne et al., 2004). However, in this study, heat treatment of the MCC used for cheese manufacture did not have a significant impact on the flowability of cheeses manufactured from cheesemilk depleted with respect to whey protein (Table 4.1, Figure 4.6), possibly due to the low amount of denatured whey protein retained in the resultant cheeses. The flowability of Cheddar cheeses increased with ripening time as a result of proteolysis in line with other reports (Rynne et al., 2007; Rynne et al., 2008; McCarthy et al., 2017a) and decreased with increased casein content in cheesemilk (Figure 4.6) due to lower levels of primary proteolysis; however, neither effects as significant (Table 4.1).

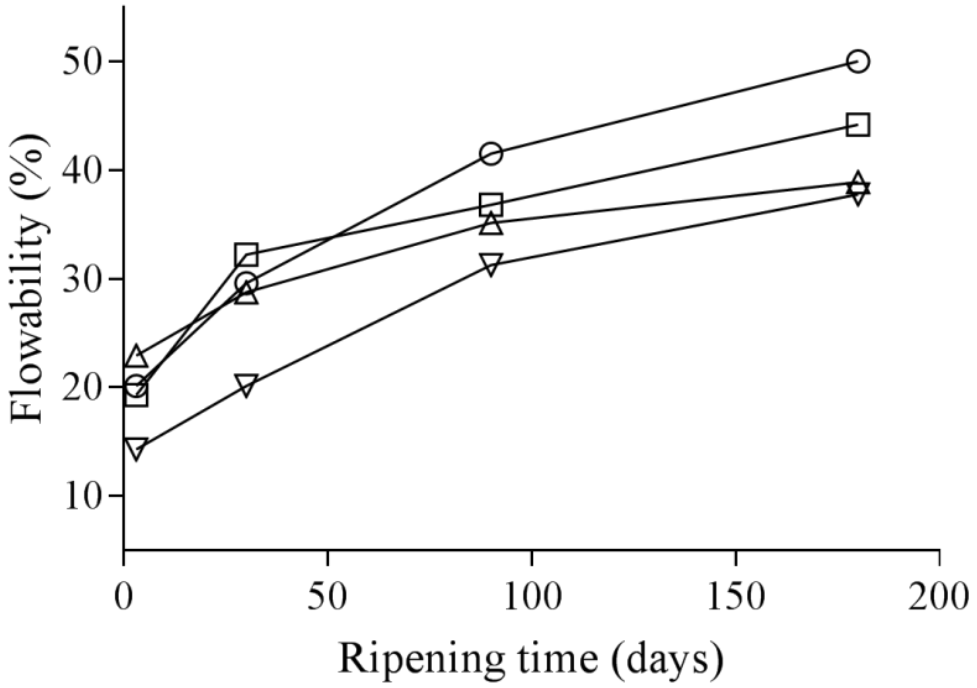

Figure 4.6. Levels of flowability of Cheddar cheese with different thermal history: control (○), pasteurised (□), high heat treated (Δ) and casein concentration: 1.5 times typical casein concentration (▽) during ripening.

4.4.8 *Volatile compounds*

Thirty nine volatiles, incorporating 8 alcohols, 5 aldehydes, 6 ketones, 6 esters, 5 sulphur compounds, 5 acids, 1 terpene, 1 furan,1 lactone and benzene, were identified (Table 4.2). Principal component analysis (PCA) of volatile profiles discriminated the Cheddar cheeses on the basis of different thermal treatments of the MCC and on the basis of casein content in the cheesemilk. The total variance was 93.4 % with the PC-1 axis accounting for 70.4 % of difference while PC-2 accounted for 23 % (Fig 4.7). This value shows a large discrimination based on volatile profiles.

PC-1 separated the control cheese from the rest of the cheeses (i.e., PS-, HHT1.0- and HHT 1.5 cheese) (Fig 4.7). Compared to cheeses manufactured from pasteurised or high-heat-treated MCC, the control cheese was characterised by higher levels of alcohols (7 out of 8), aldehydes (5 out of 5), esters (6 out of 6), acids (4 out of 5), terpenes (1 out of 1), benzene and lactone (1 out of 1) compounds as well as lower levels of sulphur (4 out of 5) and furan (1 out of 1) compounds. The higher levels of ethyl esters in CON cheese compared to other experimental cheeses is related to its higher contents of ethanol and short chain acids, which are the substrates for these ethyl esters. Typically ethanol is a limiting factor in the synthesis of ethyl ester, and acids also play an essential role during this process (Thierry et al., 2006). The lower levels of 2, 3-butanedione and acetoin as well as the higher levels of their metabolism products, i.e., 2-butanone and 2-butanol, suggests substantially more pyruvate metabolism and glycolysis overall in the CON cheese also evident by higher levels of ethanol, acetaldehyde and acetic acid (Eugster et al., 2019). CON cheese also had enhanced lipid oxidation, lipolysis and metabolism of certain amino acids (phenylalanine, leucine and methionine) in comparison to the cheeses MCC-, HHT1.0- and HHT1.5, given to the higher levels of corresponding metabolites: 1-

propanol, 1-octanol, nonanal, 2-pentanone and 2-heptanone from lipid oxidation; butanoic, hexanaoic and octanoic acid from lipolysis; phenylethyl alcohol, benzaldehyde and benzeneacetaldehyde from phenylalanine metabolism; 2-methyl-1-propanol, 3-methyl-1-butanol and 3-methyl butanal from leucine metabolism and 3-(methylthio)-1- propanol from methionine (McSweeney and Sousa, 2000, Ganesan and Weimer, 2017, Kilcawley, 2017). It is well known that ethyl ester and acid contribute to the fruity or cheesy, sour, pungent flavour, while ethanol and 3-(methylthio)-1- propanol are related to the alcoholic or baked potato aroma in cheese separately (Kilcawley, 2017). Due to their higher levels of volatile compounds, it was postulated that CON cheeses might be more aromatic or have a more intense flavour profile compared to the other cheeses.

Although no descriptive sensory analysis was undertaken in this study, 2 experienced cheese graders did evaluate the cheeses to provide supplementary information to the volatile profiling. Although levels of bitterness and sourness appeared higher in one of the trial replicates, overall and across the trial replicates the PS, HHT1.0 and HHT1.5 had a typical Cheddar cheese flavour, while the CON cheeses was considered to taste more similar to raw milk cheeses, in line with the volatile profiles as measured by GC-MS and as described above. In this study, the initial milk was skimmed and pasteurised prior to MF and MCC manufacture. Subsequently the CON cheesemilk formulated from the MCC did not receive further heat treatment while the remainder of the cheesemilks manufactured from heated MCC received a heat treatment (pasteurisation or high heat treatment). Even with careful CIP of equipment and strict good manufacturing practice during membrane filtration processing during pilot plant trials, higher levels and increased diversity of microbiota and bioactive compounds are likely to have developed in the unheated

MCC compared to those in heated MCCs, which were subsequently carried forward to the CON cheeses as reflected in their volatile profile.

Both PS- and HHT1.0- cheeses were grouped on the positive side of PC-2 and the negative side of PC-1 axis (Figure 4.7), suggesting a similar volatile profile between these two treatments.

By increasing the casein content in cheesemilk from 3.09 % to 4.31 %, the contents of esters (5 out of 6), sulphur compounds (3 out of 6), acids (4 out of 5), furan (1 out of 1) and lactone (1 out of 1) compounds decreased and benzene increased (Table 4.2). Both lactone and sulphur compounds have a positive contribution to Cheddar cheese flavour (Singh et al., 2003; Burbank and Qian, 2008) and it was previously reported that cheeses made from concentrated cheesemilk may have reduced flavour intensity (Neocleous et al., 2002b). The PC-2 axis also differentiated HHT1.5 cheese from PS- and HHT1.0 cheese (Fig 4.7). The lower levels of volatile compounds in HHT1.5 cheese might be attributed to the lower levels of primary proteolysis, as shown by the pH4.6SN/100 g cheese results.

Table 4.2. Mean volatile compound peak areas in Cheddar cheeses manufactured from cheesemilk with difference thermal history and casein concentration at day 180 of ripening[1, 2]

Volatile compounds	CON cheese	PS cheese	HHT1.0 cheese	HHT1.5 cheese
Alcohol				
Ethanol	1.21E+07^{a}	7.10E+06^{a}	7.45E+06^{a}	7.84E+06^{a}
Isopropyl Alcohol	1.21E+06^{a}	2.15E+05^{b}	1.09E+06^{a}	1.99E+06^{a}
1-Propanol	4.37E+04^{a}	2.00E+03^{b}	2.48E+03^{b}	0^{c}
2-Butanol	2.21E+05^{a}	0^{b}	0^{b}	0^{b}
2-Methyl-1-propanol	8.38E+04^{a}	1.64E+04^{a}	1.83E+04^{a}	7.89E+03^{a}
3-Methyl-1-butanol	2.26E+06^{a}	6.97E+05^{b}	4.53E+05^{b}	7.71E+05^{b}
1-Octanol	2.52E+05^{a}	2.18E+05^{a}	1.80E+05^{a,b}	8.85E+04^{b}
Phenylethyl Alcohol	6.71E+04^{a}	9.84E+03^{b}	7.96E+03^{b}	6.50E+03^{b}
Aldehyde				
Acetaldehyde	2.74E+05^{a}	7.99E+04^{a,b}	2.71E+04^{b}	4.23E+04^{b}
3-Methylbutanal	2.26E+05^{a}	9.58E+04^{a}	4.84E+04^{a}	1.51E+05^{a}
Benzeneacetaldehyde	2.85E+04^{a}	1.99E+04^{a}	1.45E+04^{a}	1.43E+04^{a}
Benzaldehyde	3.08E+04^{a}	1.93E+04^{b}	1.23E+04^{b}	1.48E+04^{b}
Nonanal	1.64E+04^{a}	1.32E+04^{a,b}	1.11E+04^{b}	1.10E+04^{b}
Ketone				
Acetone	1.02E+05^{a}	1.37E+05^{a}	1.39E+05^{a}	1.21E+05^{a}
2,3-Butanedione	3.30E+05^{a}	5.01E+05^{a}	4.55E+05^{a}	6.74E+05^{a}
2-Butanone	2.83E+06^{a}	4.62E+05^{a}	2.88E+05^{a}	3.18E+05^{a}

2-Pentanone	2.71E+05[a]	2.37E+05[a]	2.45E+05[a]	1.45E+05[a]
Acetoin	4.86E+05[a]	8.12E+05[a]	6.58E+05[a]	2.01E+06[a]
2-Heptanone	2.69E+05[a]	2.04E+05[a,b]	1.90E+05[a,b]	1.39E+05[b]
Ester				
Ethyl acetate	2.07E+06[a]	3.92E+05[a]	6.08E+05[a]	4.77E+05[a]
Ethyl butanoate	5.85E+05[a]	5.25E+05[a,b]	1.72E+05[b]	1.91E+05[b]
Ethyl hexanoate	9.15E+04[a]	3.04E+04[b]	3.17E+04[b]	3.02E+04[b]
Ethyl octanoate	4.18E+04[a]	1.12E+04[b]	9.89E+03[b]	7.87E+03[b]
Ethyl decanoate	4.23E+04[a]	1.53E+04[b]	9.91E+03[b]	9.18E+03[b]
Ethyl dodecanoate	1.50E+04[a]	4.96E+03[b]	2.64E+03[b]	2.51E+03[b]
Sulphur				
Methanethiol	1.07E+04[a]	1.03E+04[a]	1.28E+04[a]	1.42E+04[a]
Dimethyl sulphide	6.56E+03[a]	9.67E+03[a]	9.57E+03[a]	6.43E+03[a]
Dimethyl disulphide	2.26E+03[a]	4.63E+03[a]	5.12E+03[a]	4.51E+03[a]
Dimethyl sulfone	1.36E+04[a]	2.26E+04[a]	1.67E+04[a]	1.47E+04[a]
3-(Methylthio)-1-propanol	1.01E+05[a]	8.28E+03[a]	0[b]	7.56E+03[a]
Acid				
Butanoic acid	3.05E+06[a]	2.97E+06[a]	2.24E+06[a,b]	1.81E+06[b]
Hexanoic acid	9.55E+05[a]	7.43E+05[a,b]	5.05E+05[a,b]	2.67E+05[b]
3-Methylbutanoic acid	9.03E+04[a]	1.56E+05[a]	2.32E+05[a]	3.06E+05[a]
Acetic acid	3.08E+06[a]	2.23E+06[a]	2.76E+06[a]	1.83E+06[a]
Octanoic acid	5.20E+04[a]	4.17E+04[a,b]	2.51E+04[a,b]	1.04E+04[b]

Benzene				
Toluene	2.14E+05[a]	2.01E+05[a]	2.05E+05[a]	2.19E+05[a]
Terpene				
D-Limonene	1.29E+04[a]	1.02E+04[a,b]	8.05E+03[b]	7.43E+03[b]
Furan				
2-Methylfuran	3.53E+03[a]	5.79E+03[a]	4.66E+03[a]	4.22E+03[a]
Lactone				
γ-Decalactone	1.60E+04[a]	1.40E+04[a,b]	1.10E+04[b,c]	9.06E+03[c]

[1] Results are means of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2] Cheddar cheeses were manufactured from cheesemilk standardised from control micellar casein concentrate (CON cheese), pasteurised micellar casein concentrate (PS cheese) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 cheese, HHT1.5 cheese).

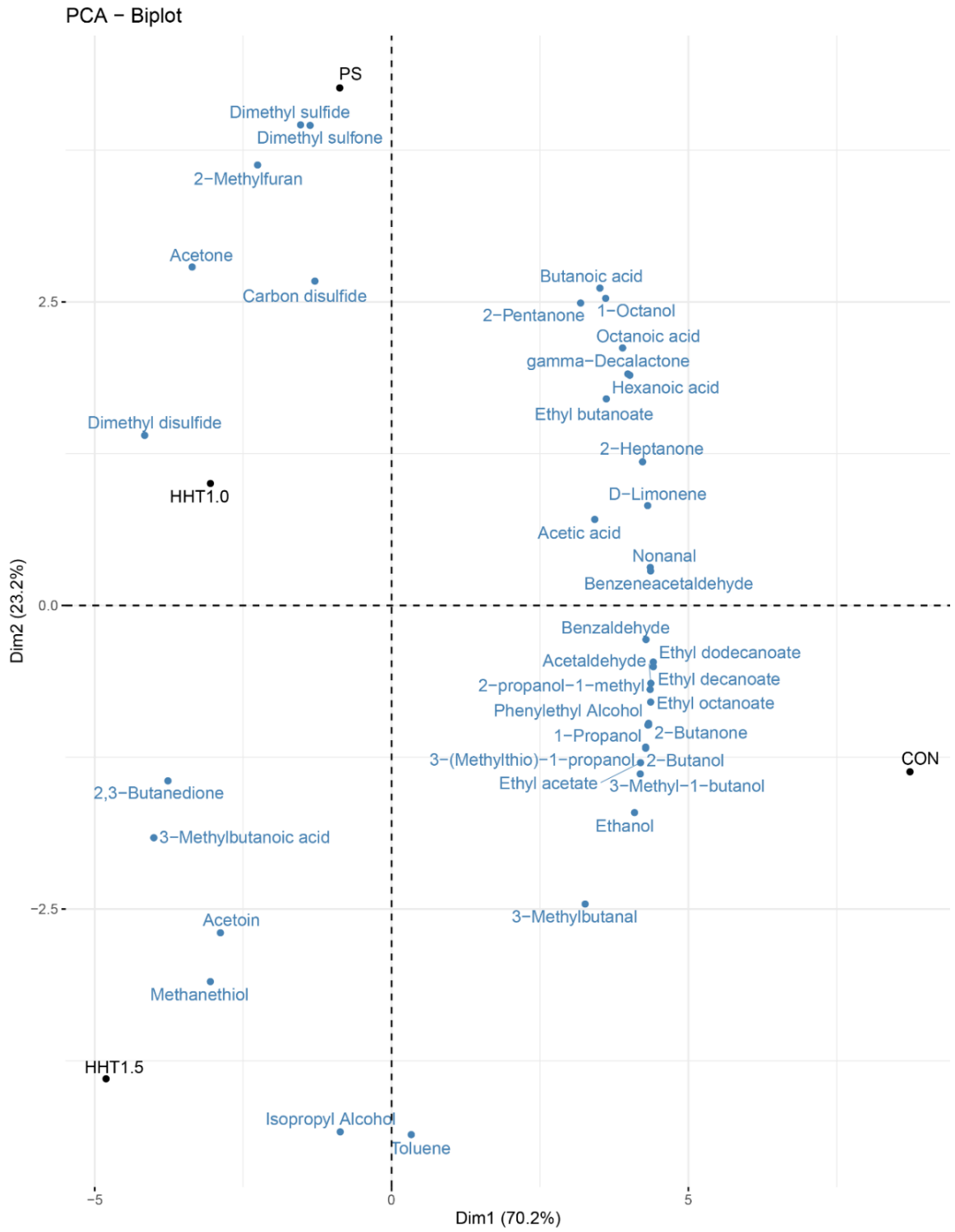

Figure 4.7. PCA Bi-plot of separation based on volatile profile of Cheddar cheese at day 180 of ripening. Results are means of triplicate trials. Cheddar cheeses were manufactured from cheesemilk standardised from control micellar casein concentrate (CON cheese), pasteurised micellar casein concentrate (PS cheese) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 cheese, HHT1.5 cheese).

4.5 Conclusions

The pH during maturation of the Cheddar cheeses prepared from MCC was not significantly affected by heat treatment (i.e., no further heat treatment, 72 °C × 15 s or 90 °C × 15 s) applied to the MCC, however, it was influenced by the casein content of the cheesemilk.

Due to prior whey protein reduction of the MCC, levels of plasmin activity and flowability in cheese were not significantly affected by increasing heating temperature on MCC from 72 to 90 °C. As a result of lower levels of primary proteolysis, the hardness in cheese made from heated MCC was significantly higher than in cheese made from unheated MCC, and the level of volatile compounds was also lower. However, increasing heating temperature from 72 to 90 °C had no significant impact on the primary proteolysis levels in cheese, explaining the similar hardness and volatile profile in PS- and HHT1.0 cheese. Overall, high heat treatment (90 °C × 15 s) can be applied to MCC before cheesemaking without generating any negative impact on the functionality, texture and volatile profile of resultant Cheddar cheeses. Since plasmin associates with casein micelles, and can hydrolyse casein leading to impaired gel strength and cheese yield (Aaltonen and Ollikainen, 2011), the rennet coagulation and cheese yield of HHT MCC also needs to be studied as a function of storage time. By holding the heating time unchanged (i.e., 15 s), it would be interesting to explore the maximum heat temperature that could be applied to MCC while maintaining good rennet coagulability and cheese quality. In conclusion, high heat treatment of MCC does not restrict its application for cheese manufacture and for maintaining key quality indices during cheese maturation.

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Supplemental Table 4.1. Composition and pH of Cheddar cheeses manufactured from whey protein depleted cheesemilk of different thermal histories and casein contents at 14 days of ripening[1, 2 and 3]

Compositional parameters	CON cheese	PS cheese	HHT1.0 cheese	HHT1.5 cheese
Protein content (%)	26.23[a]	25.90[a]	26.63[a]	27.56[a]
Fat content (%)	30.42[a]	30.44[a]	30.47[a]	31.40[a]
Pro: fat ratio	0.86[a]	0.85[a]	0.88[a]	0.88[a]
Moisture content (%)	36.27[a]	36.33[a]	35.62[a]	33.43[a]
FDM (%)[4]	47.75[a]	47.90[a]	47.36[a]	47.21[a]
MNFS (%)[5]	52.13[a]	52.19[a]	51.24[a]	48.74[a]
Salt content (%)	1.76[a]	1.73[a]	1.82[a]	1.80[a]
S/M (%)[6]	4.87[a]	4.79[a]	5.12[a]	5.40[a]
Ash content (%)	3.99[a]	3.88[a]	4.11[a]	4.22[a]
Ca (mg/ 100 g)	775.45[a]	713.02[a]	756.40[a]	782.81[a]
Calcium/ protein (mg/ g of protein)	29.66[a]	27.43[a]	28.41[a]	28.37[a]
pH	5.29[a]	5.23[a]	5.26[a]	5.33[a]

[1]These results were published in Xia et al. (2020b) previously.
[2]Results are means of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[3]Cheddar cheeses were manufactured from cheesemilk standardised from control micellar casein concentrate (CON cheese), pasteurised micellar casein concentrate (PS cheese) or high heat treated micellar casein concentrate with typical or 1.5 times typical casein content (HHT1.0 cheese, HHT1.5 cheese).
[4] FDM= fat in dry matter.
[5] MNFS=moisture in non-fat substance.
[6] S/M=salt in moisture.

Chapter 5: Effect of β-casein reduction and high heat treatment of micellar casein concentrate on the rennet coagulation properties, composition and yield of Emmental cheese made therefrom

5.1 Abstract

Cheesemilks were formulated from low heat skim milk powder without β-casein reduction (LH CM), micellar casein concentrate with 1.83 % β-casein reduction (MCC CM) and β-casein reduced micellar casein concentrate with 4.25 % β-casein reduction (LBCM or HHT LB CM which was subjected to high heat treatment, i.e., 120 °C × 15 s;) and used to manufacture Emmental cheeses. MCC- and LB CM had similar rennet coagulation properties and similar compositions and yield when Emmental cheeses were made therefrom. Compared to LB CM, HHT LB CM had an increased level of denatured whey protein and a reduced, yet acceptable, gel firming rate. Higher fat losses, but lower protein losses, from cheesemilk to whey in HHT LB cheeses resulted in a similar yield compared to LB CM cheese. Overall, this research demonstrated the possibility of removing β-casein from micellar casein concentrate or applying high heat treatment to whey protein-reduced milk without adversely affecting cheese composition and yield.

5.2 Introduction

Casein and whey proteins are the two major protein types in bovine milk, where casein constitutes around 80 % of total protein (Fox et al., 2015). Bovine casein includes α_{s1}- (45.0 %), α_{s2}- (12.0 %), β- (35.0 %) and κ casein (8.0 %) (Farrell et al., 2004). Several methods have been developed to isolate and purify β-casein from bovine milk due to its multiple functionalities: β-casein can act (1) as an efficient emulsifier and foam stabiliser in food (Li et al., 2016); (2) as a precursor for bioactive peptides with antihypertensive, opioid or mineral-binding properties (Post et al., 2012); (3) for enrichment of infant milk formulae (Atamer et al., 2017) and (4) as an oral delivery system for hydrophobic drugs (Atamer et al., 2017). When the temperature of milk drops below 5 °C, β-casein partially dissociates from casein micelles and disperses in the serum phase as monomers (Downey and Murphy, 1970; Li et al., 2019).

Thus, cold microfiltration (1- 8.9 °C) of skim milk or micellar casein concentrate (MCC) is used to produce β-casein of high purity (O'Mahony et al., 2014; Schäfer et al., 2019) or β-casein-enriched whey protein with a similar protein profile to human milk (McCarthy et al., 2017), both resulting in β-casein-reduced MCC as a co-product. MF at low temperature (< 15 °C) also reduces microbial growth and avoids whey protein denaturation during extended processing runs compared to warm MF (MF at 50 °C) (Govindasamy-Lucey et al., 2011).

However, O'Mahony et al. (2009) suggested that by altering the β-casein content in cheesemilk by cold MF, the rennet coagulation properties may be altered. Seibel et al. (2015) also reported that cheesemilk with a reduced level of β-casein had a weaker rennet-induced gel compared to that of control milk; however in contrast, Holland et al. (2011) found no effect. In both studies, the contents of casein and total calcium and pH levels in the β-casein reduced and control cheesemilk were similar. Thus, further research is required to determine if rennet coagulation properties of cheesemilk differ when formulated from β-casein reduced MCC or from typical MCC.

Applying a more intensive heat treatment on MCC can extend the microbial shelf life of MCC (Amelia and Barbano, 2013). MCC has a better heat stability than skim milk due to a lower whey protein content; cheesemilk standardised from high heat treated (HHT, 90 °C × 15 s) MCC was shown to have good rennet coagulability and cheesemaking properties, and a comparable cheese compositions to that made from unheated MCC (Xia et al., 2020b). However it is of interest to determine if a higher thermal temperature (i.e.> 90 °C) can be applied to MCC (and maintaining heating time of 15 s) without significantly impacting on the rennet coagulability and cheesemaking properties of cheesemilk formulated from MCC.

To investigate the effect of β-casein content in cheesemilk on rennet coagulability and cheese quality, cold microfiltration was used to reduce the β-casein and whey protein levels in

skim milk. The rennet coagulation and cheesemaking properties of the β-casein-reduced cheesemilk as well as the cheese composition, pH and yield of Emmental cheeses made therefrom were evaluated and compared to those made from a typical cheesemilk. Similarly, milk reconstituted from β-casein reduced MCC was subjected to heat treatments of 72 °C × 15 s or 120 °C × 15 s and its influence on rennet coagulability and cheese composition and yield were also characterised.

5.3 Materials and methods

5.3.1 *Cascade filtration process*

Membrane filtration trials were carried out on pilot plant scale at Moorepark Technology Limited, Fermoy, Co. Cork, Ireland. One day before each trial, 1000 kg pasteurised skim milk was sourced from a local dairy company (Dairygold, Mitchestown, Co Cork). An initial, 300 kg quantity of milk was chilled to 1 °C for use on day 1 and the remainder of the milk was stored at 4 °C for use on day 2 and day 3 respectively. On day 1, pasteurised skim milk was microfiltered with an 800 kDa polyvinyldiene fluoride (PVDF) membrane (Model: FR-2B-3838, Synder Filtration, Vacaville, CA, USA) and diafiltered twice with chilled reverse osmosis (RO) water (3 °C). The processing temperature was maintained at 8.5 °C and the volumetric concentration factors (VCF) for both cold MF and DF were 3. During cold MF, both whey proteins and some β-casein partitioned to the MF permeate, while β-casein reduced casein micelles were retained in the MF retenate, chilled at 1 °C until day 2 and was denoted β-casein reduced micellar casein concentrate (LB MCC).

On day 2, a portion of the pasteurised skim milk (300 kg) was heated to 47 °C, and microfiltered with the same polymeric membrane as used previously for the cold MF, followed by two steps of DF with warm RO water (47 °C). The processing temperature and VCF for warm MF and DF were 47 °C and 3 separately. The retentate produced by warm MF was called MCC and was chilled immediately after processing for use on day 3. Also on day 2, the LB

MCC was spray dried on a pilot scale single stage spray dryer (Anhydro Laboratory Spray Dryer, SPX Flow Technology, Kolding, Denmark) equipped with nozzle atomisation; the inlet and outlet temperatures were 185 and 85 °C respectively.

One day 3, pasteurised skim milk was evaporated at 65 °C to 42 % total solids with a single-effect falling-film evaporator (Anhydro F1 Laboratory; Copenhagen, Denmark). Both the evaporated skim milk and MCC were spray dried using the same method as that for the LB MCC. The spray dried pasteurised skim milk was denoted as low heat skim milk powder (LHSMP). All powders were packaged, sealed and stored at 4 °C immediately after manufacture to ensure good rehydration properties during storage (Nasser et al., 2017).

5.3.2 *Preparation of cheesemilk*

Recombined milks were formulated from LHSMP, MCC, LB MCC and milk permeate powder (Profile^{TM} P8 permeate powder W463-25 kg, Kerry Group, Listowel, Ireland), $CaCl_2$ (calcium chloride anhydrous powder Reag. Ph Eur, Merck Chemicals Ltd, Nottingham, United Kingdom) and RO water two days before cheesemaking (Table 5.1) and were denoted as: LH-, MCC-, LB- and HHT LB milk separately. The casein, lactose and ash contents in these milks were standardised to 2.89 %, 5.07 % and 0.77 % respectively. The ingredients were rehydrated with RO water at 55 °C, mixed (Silverson model L4RT, Silverson, Chesham, UK) at high shear (6300 rpm) for 20 min and then stirred (Model RW16; IKA-Werke GmbH, Staufen im Breisgau, Germany) at 250 rpm for 20 h (with the first two hours at room temperature and the remaining 18 hours at 4 °C) in a 15 L sterile container.

One day before cheesemaking, the LH-, MCC- and LB milks were pasteurised at 72 °C for 15 s and the HHT LB milk heat treated at 120 °C for 15 s with a pilot scale heat exchanger (MicroThermics®, Raleigh, NC, USA). On the day of cheesemaking, pasteurised cream (Dairygold, Mitchelstown, Co Cork) was added to the recombined milks to achieve a

standardised casein content of 2.79 % and a casein to fat ratio of 0.79: 1 to generate LH, MCC-, LB- and HHT LB cheesemilk (CM).

Table 5.1. Formulations by weight for recombined milk[1, 2 and 3]

Weight of ingredients or water (kg)	LH milk	MCC milk	LB milk	HHT LB milk
LHSMP	1.26	0	0	0
MCC powder	0	0.56	0	0
LB MCC powder	0	0	0.55	0.55
$CaCl_2$	0	0.003	0.003	0.003
Milk permeate powder	0	0.65	0.64	0.64
RO water	11.74	11.80	11.82	11.82

[1]Trials undertaken in quadruplicate all with the same formulation.
[2]Milk formulations were prepared to a total weight of 13 kg.
[3]Reconbined milk were prepared from low heat skim milk powder (LH milk), micellar casein concentrate powder (MCC milk) or β-casein reduced micellar casein concentrate powder of different thermal history (LB milk or HHT LB milk).

5.3.3 *Cheese manufacture*

Standardised cheesemilks (10 kg) were poured into mini-cheese vats (Type CAL 10 L; Pierre Guerin Technologies, Mauze, France), warmed to 32 °C and adjusted to pH 6.60 with 4 % food grade lactic acid (Sigma-Aldrich). Starter cultures were premixed with 400 mL of cold cheesemilk and an aliquot of this milk was inoculated to each cheesemilk to achieve a final inoculum of 0.05 g L^{-1} LH-B03 (*Lactobacillus helveticus*); 0.1 g L^{-1} TH-4 (*Streptococcus thermophilus*) and 0.025 g L^{-1} PS-40 (*Propionibacterium freudenreichii subsp. shermanii*), all from Chr. Hansen Ireland Ltd., Co. Cork, Ireland.

To obtain an acceptable set to cut time (from rennet addition to cut), as deduced from preliminary trials, 0.1 % (v/w) of 1 m mol kg^{-1} $CaCl_2$ (Li et al., 2020) was added to LH CM and 0.6 % (v/w) to the other cheesemilks 3 min before rennet addition. Heat labile coagulant

(*Cryphonectria parasitica proteinase*; 0.55 mL Thermolase® 625, Chr. Hansen Ireland Ltd., Co. Cork, Ireland, diluted with 10 mL milli-Q water) was added to each cheese vat after 60 min of pre-ripening with the starter cultures. Ninety seconds after rennet addition, a 20 mL aliquot of renneted cheesemilk was transferred to a rheometer (AR-G2 rheometer; TA Instruments, New Castle, DE, USA). Cheese coagula were cut after the gel firmness reached 35 Pa (Panthi et al., 2017) with a cut program of 5 min duration. Thereafter, the curd-whey mixture was cooked over 31 min by increasing the temperature initially at a rate of 0.5 °C/ min from 32 °C to 45 °C and subsequently from 45 °C to 50 °C at a rate of 1 °C/ min. The bulk of whey was drained immediately after max scald, subsequently curds were pre-pressed under warm cheese whey at 6.12 kPa for 25 min, then moulded and pressed at 29.49 kPa for 3.5 h. On achieving a pH of 5.30, the cheeses were brined for 7.5 h as described by Lamichhane et al. (2019), vacuum packed and ripened for 120 days (8 °C from day 0 to day 14, 22 °C from day 15 to 70 and 4 °C from day 71 to 120).

5.3.4 *Calcium in liquid samples*

Concentrations of total-, soluble- and ionic calcium in cheesemilks were determined before and after $CaCl_2$ addition as described by Lin et al. (2016). Colloidal calcium content was calculated as the difference between total- and soluble calcium contents in each milk, fresh samples were used for all measurements.

5.3.5 *Protein profile in liquid samples*

The protein profile of the pasteurised skim milk, MCC and β-casein reduced MCC were measured in duplicate by reverse phase-HPLC as described by McCarthy et al. (2017), where pasteurised skim milk was diluted 1 in 20 with urea buffer while MCC and β-casein reduced MCC were diluted 1 in 40 before measurement.

5.3.6 *Composition of powders and liquid samples*

The total solids, fat, ash and total protein contents as well as concentrations of non-casein nitrogen and non-protein nitrogen in the LHSMP, MCC, LB MCC and milk permeate powders were determined as described by Niro (1978), while those in cheesemilk and cheese whey were measured as described by Xia et al. (2020a).

5.3.7 *Rennet coagulation characterisation*

A 20 mL volume of cheesemilk was loaded to the conical concentric cylinder geometry attached to a rheometer (AR-G2 rheometer; TA Instruments, New Castle, DE, USA) 90 s after rennet addition; a time sweep was carried out and the test temperature was maintained at 32 °C. The values of rennet coagulation time (RCT), maximum gel firming rate (MGFR), A_{40}, tan δ_{40}, set to cut time (K_{35}) and K_{70} were determined and calculated from the storage modulus (G')-time (s) curve as described by Panthi et al. (2019).

5.3.8 *Cheese composition*

Total solids, fat, protein, salt, ash and total calcium contents as well as pH in cheese at day 14 of ripening were determined as per Xia et al. (2020a).

5.3.9 *Cheese yield*

Actual and composition adjusted cheese yields including actual cheese yield, moisture adjusted cheese yield, moisture adjusted cheese yield with fat and protein adjusted to reference levels as well as moisture adjusted cheese yield with fat and casein adjusted to reference levels were calculated as described by Guinee et al. (2006).

5.3.10 *Statistical analysis*

Quadruplicate trials were carried out for Emmental cheese manufacture within a one month period. The effect of β-casein removal and high heat treatment of MCC on the composition and rennet coagulability of cheesemilk as well as on the composition, pH and yield of the resultant cheeses were compared with least-squares difference (LSD) at 95 % significance level by one-way ANOVA using SPSS 24.0 (IBM Corp., 2016, Chicago, IL, USA).

5.4 Results and discussion

5.4.1 *Protein profile of membrane streams*

β-Casein is stabilised within casein micelles through hydrophobic interactions in addition to the role of colloidal calcium phosphate formed between calcium and phosphoserine clusters. Cooling of milk to ≤ 10 °C leads to dissociation of β-casein from casein micelles to the milk serum phase due to reduced hydrophobic interactions as well as a decrease in colloidal calcium caused by the cold temperature (Holland et al., 2011; Lucey and Horne, 2018). Microfiltration of cold milk at temperatures < 10 °C results in partition of some β-casein to the MF permeate and produces MCC of a lower β-casein content to that produced by warm MF, as found in this research (Table 5.2) and as reported by other researchers (Holland et al., 2011; O'Mahony et al., 2014; McCarthy et al., 2017; Schäfer et al., 2019).

Application of prolonged cold storage (1 °C × 24 h), microfiltration at 8.5 °C and diafiltration with chilled water (3 °C) in this study resulted in a removal of 4.25 % of β-casein from the feed milk, higher than that reported by Zulewska et al. (2018), who found ≤ 1 %. However, such levels were lower than those achieved by some other studies (i.e., 20%) (Holland et al., 2011; O'Mahony et al., 2014; Schäfer et al., 2019). Application of further

process steps which may enhance β-casein reduction include dilution of feed milk with chilled water, longer cold storage time before cold MF (maybe 40 – 48 h) as well as cold storage for 2 h after adding diafiltrant (Holland et al., 2011; O'Mahony et al., 2014; McCarthy et al., 2017; Schäfer et al., 2019).

A low level of β-casein removal (1.83 %) was also observed in MCC manufactured by warm MF where feed milk was cold stored for 48 hours at 4 °C prior heating to 47 °C and microfiltration. This suggests that a longer holding time at 47 °C than that used in this research (about 30 min) should be allowed to the heated feed milk to achieve full re-association of β-casein to casein micelles.

Warm MF retained less α-lactalbumin in the resultant MCC than cold MF (Table 5.2), contrary to that reported by McCarthy et al. (2017), where MCC produced from warm MF contained less β- lactoglobulin (β-LG) than that from cold MF. The difference between these two studies may be linked to the use of different membranes. Similarly, further research is required to identify how MF processing parameters affect the composition of whey proteins in MCC.

Table 5.2. Protein profile of pasteurised skim milk, micellar casein concentrate and β-casein reduced micellar casein concentrate[1]

Compositional parameters	Pasteurised skim milk	MCC[2]	LB MCC[2]
Casein (as a percentage of total casein, %)			
α_s+ κ-Casein	60.03	60.76	61.73
β-Casein	39.97	39.24	38.27
Whey protein (as a percentage of total whey protein, %)			
α-Lactalbumin	14.72	11.89	13.88
β-Lactoglobulin A	43.36	44.23	43.95
β-Lactoglobulin B	41.92	43.88	42.17

[1]Only one 'membrane filtration-spray drying' trial was conducted in this research, the above result was obtained from this trial.
[2]Abbreviation: micellar casein concentrate (MCC) and β-casein reduced micellar casein concentrate (LB MCC).

5.4.2 *Composition and pH of cheesemilks*

As cheesemilk standardisation was undertaken on a casein content basis, the total solids and protein contents in LH CM was higher than the other cheesemilks due to its significantly higher whey protein content (Table 5.3). Similar contents of casein, fat and ash as well as casein to fat ratio in all four cheesemilks were achieved. Cheesemilk formulated from HHT LB milk had a significantly lower native whey protein content compared to its pasteurised counterparts suggesting heat induced whey protein denaturation (Table 5.3). The pH of cheesemilk made from LHSMP was significantly lower than those made from MCC or LB MCC powder, possibly due to the higher pH in MCC (pH = 6.95) or LB MCC (pH = 6.95) than that in feed milk (pH = 6.62) as well as the high buffering capacity of the two MCCs.

Table 5.3. Composition and pH of cheesemilk prepared from low heat skim milk powder, micellar casein concentrate powder and β-casein depleted micellar casein concentrate powder[1]

Compositional parameters	LH CM[2]	MCC CM[2]	LB CM[2]	HHT LB CM[2]
Total solids (%, wt/wt)	12.35[a]	12.07[a]	12.01[a]	11.92[a]
Protein content (%, wt/wt)	3.53[a]	3.24[b]	3.08[b]	3.14[b]
Casein content (% , wt/wt)	2.76[a]	2.72[a]	2.59[a]	2.78[a]
Native whey protein content (% , wt/wt)	0.77[a]	0.52[b]	0.50[b]	0.36[c]
Fat content (% , wt/wt)	3.18[a]	3.19[a]	3.18[a]	3.17[a]
Ash content (% , wt/wt)	0.74[a]	0.70[a]	0.65[a]	0.68[a]
pH	6.61[b]	6.83[a]	6.83[a]	6.81[a]

[1]Results are means of quadruplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2]Cheesemilk were prepared from low heat skim milk powder (LH CM), micellar casein concentrate powder (MCC CM) or β-casein reduced micellar casein concentrate powder without (LB CM) or with high heat treatment (HHT LB CM).

5.4.3 *Calcium contents in cheesemilks*

Previous studies have reported contradictory information regarding rennet coagulability of LHSMP. Martin et al. (2008) suggested that due to the non-reversible association of soluble casein to casein micelles during powder manufacture, the rennet coagulability of LHSMP was impaired to such a degree that $CaCl_2$ addition is necessary to achieve rennet coagulation properties similar to those of raw milk. However, Lin et al. (2018) found that the dehydration procedure did not affect the rennet coagulability of rehydrated LHSMP. In preliminary research in the current study (data not shown), the set to cut time in cheesemilk prepared from LHSMP decreased from ~ 60 min to a more typical value (38 min) as reported by Xia et al. (2020a) after addition of 0.1 % (v/w) of 1 m mol kg^{-1} $CaCl_2$ prior to rennet addition. This suggests that

the powder manufacture process may have impaired the rennet coagulability of rehydrated LHSMP but this can be easily reversed by addition of $CaCl_2$.

The calcium to casein ratio in the MCC powder was 26.96 % (Table 5.4) lower than that in LHSMP, suggesting a significant amount of calcium removal during warm MF (47 °C) and diafiltration (DF) with RO water in line with the findings of Xia et al. (2020a). The calcium loss through cold MF (8.5 °C) along with DF with water was more pronounced than that in warm MF as demonstrated by a 32.33 % decrease in the calcium to casein ratio when comparing LB MCC powder to LHSMP. During cold storage (1 °C) of feed milk as well as cold MF and DF, an increased amount of colloidal calcium shifts to the milk serum phase as a result of temperature-induced changes in the calcium equilibrium (Gaucheron, 2005), leading to increased calcium removal from MF retentate compared to warm MF, as also reported by Holland et al. (2011) and McCarthy et al. (2017). Since calcium especially in its ionic form is crucial for rennet coagulation (Shalabi and Fox, 1982; Choi et al., 2007; Fox et al., 2017b), the ash contents in milk reconstituted from MCC or LB MCC powder were standardised to the same level in LHSMP milk by addition of milk permeate powder and $CaCl_2$ (Table 5.1, 5.3) to mitigate any gelation issues. As a result of ash standardisation, the total-, colloidal-, soluble- and ionic calcium contents as well as colloidal calcium to casein ratio in cheesemilk prepared from MCC or LB MCC powder were no less than those in the LH cheesemilk (Figure. 5.1). In preliminary experiments, and contrary to expectation, a gel was not formed without addition of calcium chloride to the MCC-, LB- and HHT LB cheesemilk before rennet addition (data not shown). An acceptable set to cut time was not achieved unless 0.6 % (v/w) of 1 m mol kg^{-1} $CaCl_2$ was added to these cheesemilks. After addition of calcium chloride, calcium in each form especially in the colloidal form (Fig 5.1b and f) increased significantly. Increased colloidal calcium levels can decrease the electrostatic repulsion between casein micelles, thus facilitating the aggregation of para-casein micelles (Choi et al., 2007). Paraskevi et al. (2021)

reported that skim milk standardized with permerate powder has reduced size, integrity and zeta potential of casein micelles compared to those standardised with liquid permeate. Decreased casein micelle integrity and zeta potential can impair the rennet coagulability of cheese milk (Lin et al. 2017), adding milk permeate powder instead of liquid UF permeate to cheesemilks might have caused the damaged rennet coagulation ability found in MCC, LB and HHT LB cheesemilks, further research is still required to confirm this assumption.

Table 5.4. Composition of LHSMP, MCC and LB MCC powders[1, 2]

Compositional parameters	LHSMP	MCC powder	LB MCC powder	Milk permeate powder
Total solids (%, wt/wt)	97.07	94.82	95.13	97.81
Ash (%, wt/ wt)	7.65	7.63	7.44	8.03
Total protein (%, wt/wt)	37.10	73.73	72.29	0
Casein content (%, wt/wt)	30.08	67.94	66.62	0
Casein number (%)	81.08	92.14	92.15	N/A
Total Ca (mmol kg^{-1})	304.20	501.81	455.92	230.43
Calcium/casein (mmol/g casein)	101.13	73.86	68.43	N/A

[1]Result from one membrane filtration-spray drying trial;
[2]Abbreviation: low heat skim milk powder (LHSMP), micellar casein concentrate (MCC) and β-casein reduced micellar casein concentrate (LB MCC).

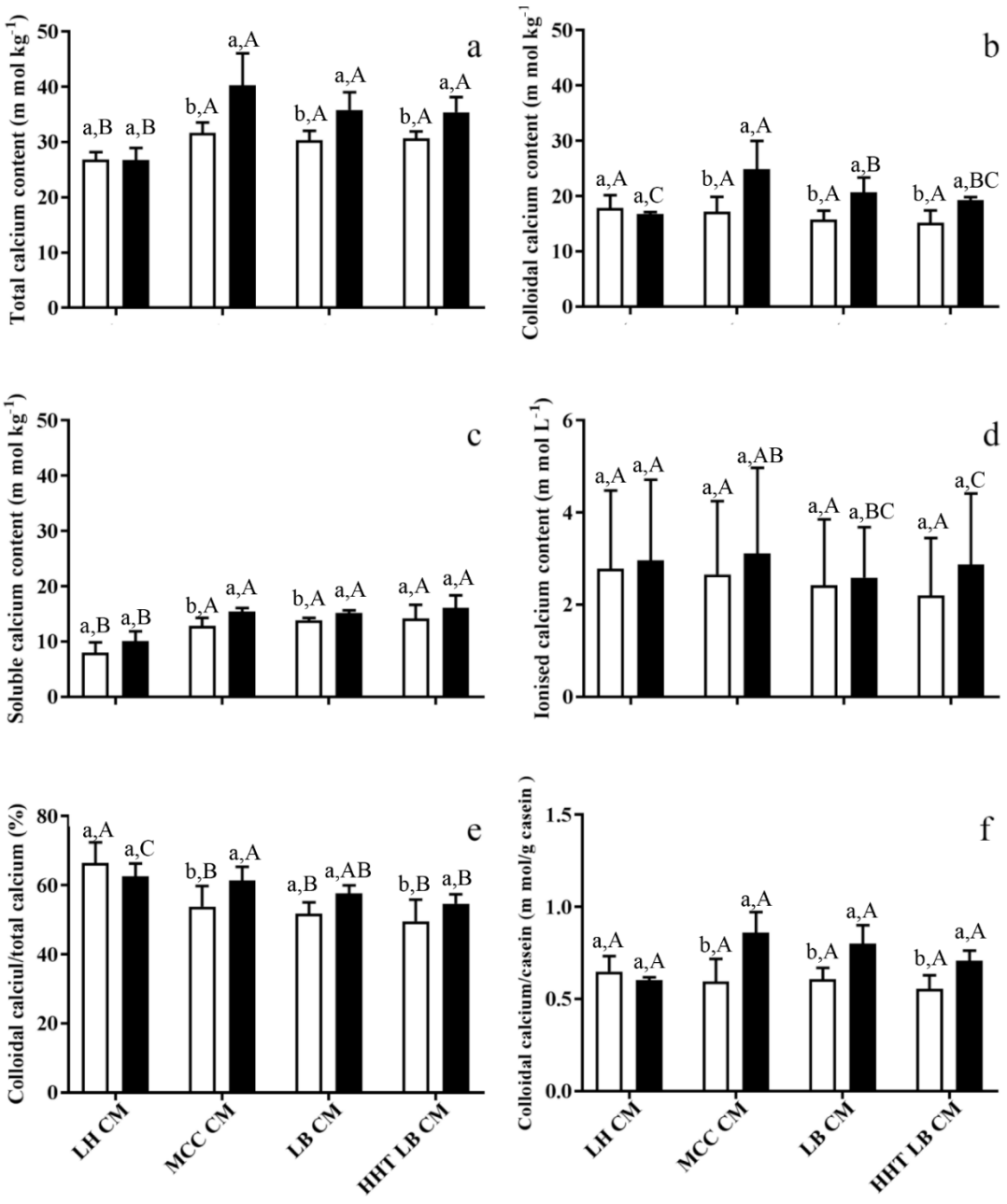

Figure 5.1. Total calcium (a), colloidal calcium (b), soluble calcium (c) and ionic calcium (d) contents as well as colloidal calcium as percentage of total calcium (e) and colloidal calcium per casein (f) in cheesemilk prepared from low heat skim milk powder (LH CM), micellar casein concentrate powder (MCC CM) or β-casein reduced micellar casein concentrate powder with different thermal history (LB CM or HHT LB CM) before (□) or after (■) $CaCl_2$ addition. ^{a,b}For each cheese type, values not sharing a common lowercase superscript means that significant difference exist between calcium contents before or after $CaCl_2$ addition ($P < 0.05$). ^{A,B}For calcium contents before or after $CaCl_2$ addition, values not sharing a common uppercase superscript means that significant difference exist between different cheese types ($P < 0.05$).

5.4.4. *Rennet coagulation property*

The rennet coagulation time (RCT), time required to reach the same gel firmness (K_{35} and K_{75}) in MCC- and LB cheesemilks were significantly shorter, and maximum gel firming rate (MGFR) and gel firmness at a given time (A_{40}) were significantly higher than those in LH cheesemilk (Table 5.5), suggesting a faster gel firming rate caused by the higher calcium contents in MCC- and LB cheesemilk as a result of $CaCl_2$ addition (Figure 5.1). Application of high heat treatment (HHT, 120 °C × 15 s) to LB milk significantly reduced the rennet coagulation properties of cheesemilk demonstrated by the significantly higher RCT, K_{35}, K_{75} values as well as the significantly lower MCFR and A_{40} levels in HHT LB cheesemilk compared to those in LB cheesemilk (Table 5.5). It is well known that heat-induced whey protein denaturation can impede the aggregation of para-casein by forming aggregates of β-LG-para-casein, β-LG-κ-casein and β-LG- β-LG (Kethireddipalli et al., 2010), even though the whey protein content in HHT LB cheesemilk was significantly lower than that in control milk, denatured whey protein may have partially impaired the rennet coagulation in HHT LB cheesemilk. However, Bulca and Kulozik (2004) suggested that casein dissociation and crosslinking might account for the significantly reduced gel formation observed in high heat treated (120 °C × 20 s) whey protein free cheesemilk compared to that in pasteurised milk. Futher research is required to determine the relative influence of denatured whey proteins compared to casein dissociation and crosslinking responsible for the reduced coagulability of the HHT LB milk (120 °C × 15 s). For cheesemilk of typical whey protein content, the A40 value of the rennet induced gel is around 5 Pa after subjecting the milk to 120 °C for 4 s (Waungana et al., 1996); extending the holding time to 15 s may have further lowered this value.

The much higher A_{40} value in HHT LB cheesemilk (24.92 Pa) obtained in this study again proved that the heat stability of cheesemilk can be improved by partial or complete removal of whey protein; milk of low whey protein content can be subjected to high heat treatment without losing rennet coagulability, in line with other studies (Schreiber, 2001; Bulca and Kulozik, 2004; Renhe and Corredig, 2018; Xia et al., 2020b). Spray drying can also significantly reduce the heat stability of reconstituted MCC (Beliciu et al., 2012), suggesting that milk prepared from fresh LB MCC would have better heat stability than that made from LB MCC powder, though more research is required to prove this assumption.

It is also expected that the syneresis properties of the resultant gels may also have been significantly reduced due to the significantly higher tan δ_{40} value in HHT LB cheesemilk compared to the other cheesemilks (Table 5.5) (Panthi et al., 2019). Denatured β-LG-*para*-casein aggregates can impede the fusion and re-arrangement of *para*-casein leading to a decrease in syneresis of the rennet induced gel (Guinee et al., 1995; Rynne et al., 2004).

Overall, the difference in β-casein content between MCC- and LB cheesemilk in the present study did not affect their gel forming properties and set to cut time (K_{35}) in agreement with Holland et al. (2011).

Table 5.5. Gel forming properties of cheesemilk prepared from low heat skim milk powder, micellar casein concentrate powder and β-casein depleted micellar casein concentrate powder [1]

Parameters	LH CM[2]	MCC CM[2]	LB CM[2]	HHT LB CM[2]
RCT (min)[3]	20.58^{b}	11.73^{c}	11.37^{c}	23.61^{a}
MCFR (Pa/ min)	2.84^{c}	13.85^{a}	9.91^{b}	2.60^{c}
A_{40} (Pa)[4]	40.33^{c}	164.53^{a}	125.27^{b}	24.92^{c}
$Tan\delta_{40}$[4]	0.26^{b}	0.27^{b}	0.28^{b}	0.32^{a}
K_{35} (min)[5]	38.23^{b}	16.40^{c}	17.95^{c}	43.21^{a}
K_{70} (min)[5]	54.52^{b}	19.46^{c}	22.66^{c}	64.47^{a}

[1]Results are means of quadruplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2] Cheesemilk were prepared from low heat skim milk powder (LH CM), micellar casein concentrate powder (MCC CM) or β-casein reduced micellar casein concentrate powder with different thermal history (LB CM or HHT LB CM).
[3]RCT: the time required for the G' to reach the value of 0.1 Pa after rennet addition.
[4]A_{40} and $tan\delta_{40}$: the storage modulus (G') and tan δ of gel 40 min after rennet addition.
[5]K_{35} or K_{70}: the time it take for the G' to reach the value of 35 or 70 Pa respectively after rennet addition.

5.5 Conclusions

LB MCC powder produced under cold MF retained less β-casein and colloidal calcium compared to the MCC powder manufactured by warm MF. Rennet coagulation time and gel firming rate were not influenced by the β-casein concentration in the cheesemilk, and similarly for the contents of protein, fat, moisture and salt as well as the yield of Emmental cheese. Even though the ash content in every cheesemilk was standardised to the same level, the concentration of colloidal calcium in LB MCC cheesemilk was lower than that in the MCC cheesemilk. As a result, the ash content and buffering capacity in LB MCC cheese were also lower, leading to the significantly lower pH than that in MCC cheese.

Denatured whey protein and associated aggregates associated with HHT (120 °C × 15 s) of the cheesemilk impaired rennet coagulation properties in HHT LB cheesemilk, however, an acceptable set to cut time (48 min) was still achieved, attributed to whey protein reduction before heat treatment. The levels of protein, moisture, ash and pH in HHT LB cheese was similar to those in LB cheese. However, lower contents of fat and FDM in cheese, and the significantly higher level of milk fat lost to cheese whey during manufacture of HHT LB cheese were attributed to increased curd breakage during stirring in the cheese vat. A higher portion of protein was recovered from cheesemilk to HHT LB cheese compared to LB cheese due to whey protein denaturation, and as a result, cheese yield was not influenced by HHT.

Further research is ongoing to evaluate how use of β-casein reduced milk and HHT (120 °C × 15 s) influences the proteolysis, texture and volatile compounds in matured Emmental cheese. Overall, application of cold MF before cheese manufacture can produce β-casein in addition to native whey protein without compromising rennet

coagulability of cheesemilk or the composition and yield of subsequent cheeses. Whey protein-reduced milk can be subjected to high heat treatment up to 120 °C × 15 s without losing rennet coagulability or impairing composition or yield in Emmental cheese. High heat treatment can give substrate milk elongated shelf life compared to pasteurisation, allowing extended preservation or long distance transportation of MCC reserved for cheesemaking.

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Chapter 6: Effect of β-casein reduction and high heat treatment of micellar casein concentrate on proteolysis, texture and the volatile profile of Emmental cheese during ripening

6.1 Abstract

Using microfiltration (MF), 53.60-56.38% of whey protein was removed from milk, generating micellar casein concentrate (MCC). MF at 8.5 °C reduced β-casein by 4.25 % producing β-casein-reduced (LB) MCC. Four fat-free milks were prepared from low heat skim milk powder (LH), MCC powder (MCC) and LB MCC powder (LB- or HHT LB) respectively, where LH-, MCC- and LB milks were pasteurised (72 °C × 15 s) and HHT LB milk was high heat treated (120 °C × 15 s). After standardization with pasteurized cream, Emmental cheeses were manufactured and ripened for 120 days. Plasmin activity (120 days) and flowability (70 and 120 days) in HHT LB cheeses were significantly reduced in comparison to LH-, MCC- and LB cheeses, and redness increased. Overall, β-casein reduction of cheesemilk did not affect the maturation indices of Emmental cheese; however, high heat treatment of whey protein-reduced milk impaired the functionality and appearance of the resultant cheeses.

6.2 Introduction

Microfiltration (MF) can selectively separate native whey protein from milk and produce micellar casein concentrate (MCC) as retentate and a virgin whey protein stream as permeate (Ardisson-Korat and Rizvi, 2004). The virgin whey protein is free from fat, chymosin, starter culture, cheese fines, cheese colorant and caseinomacropeptide, and has been called ‘ideal whey’ due to its superior technological and functional properties compared to cheese whey (Bacher and Kønigsfeldt, 2000). MF is usually carried out at 45 – 55 °C (warm MF) to achieve high permeate flux (Holland et al., 2011). However microfiltration of refrigerated milk at low temperature (4 – 15 °C, cold MF) is attracting more research interest, as it can minimise bacteria growth and heat damage to protein (Raghunath and Hibbard, 1997;

France et al., 2021). Since the association of β-casein to casein micelles is temperature dependent, holding milk at 1 – 4 °C for 12 – 48 hours can weaken the hydrophobic interaction between β-casein and casein micelles and liberate β-casein from the casein micelles to the milk serum phase (O'Mahony et al., 2014). As a result, in addition to native whey protein, cold MF can also remove a portion of β-casein from milk in the permeate (O'Mahony et al., 2014). β-Casein is (1) an effective stabilizer for emulsion and foam system (Li et al., 2016), (2) a precursor for bioactive compounds (Kamiński et al., 2007) and (3) an important component in infant milk formulae (Li et al., 2019), thus isolation of β-casein from milk is desired. As the by-product of both β-casein isolate and whey protein isolate, β-casein-reduced MCC increases the meltability of model Cheddar cheese made therefrom on heating (O'Mahony et al., 2014). However, the effect of β-casein-reduced MCC on the primary proteolysis, volatile profile and other maturation indexes of such cheeses have not been reported previously.

Plasmin mainly acts on β-casein and is the most important milk-borne proteinase for cheese ripening, especially so for cheese types made with high cooking temperature (≥ 50 °C) like Emmental (Ardö et al., 2017). After heat treatment, the free thiol- disulphide groups exposed on the surface of denatured β- lactoglobulin (β- LG) can form covalent bonds with the thiol-rich region on plasmin and plasminogen, and the activity of plasmin and plasminogen is reduced subsequently (Aaltonen and Ollikainen, 2011). Subjecting milk to heat treatment at 90 °C for 15 s largely decreased the plasmin activity in the resultant cheese (Benfeldt et al., 1997). However, thermal inactivation of plasmin is avoided by partly removing whey protein from milk using MF before heating (95 °C × 15 s) (Aaltonen and Ollikainen, 2011). In the current research, it was of interest to determine if the plasmin activity of whey protein-reduced

milk produced by MF remains unaffected after receiving heat treatment higher than 95 °C × 15 s, in this case 120 °C × 15 s.

In Chapter 5, it was found that partially removing β-casein from milk did not influence the rennet coagulability of cheesemilk as well as the composition and yield of Emmental cheeses made therefrom; while applying high heat treatment (120 °C × 15 s) to whey protein-reduced milk significantly impaired its rennet coagulation properties without affecting the composition (except fat content) and yield of cheese made therefrom. The objective of the current study is to determine the effect of β-casein reduction and high heat treatment of micellar casein concentrate on the proteolysis, texture and volatile profile of resultant Emmental cheese during ripening.

6.3 Materials and methods

6.3.1 *Preparation of Emmental cheese*

Mini (500 g) Emmental cheeses were manufactured from low heat skim milk powder (LHSMP), micellar casein concentrate powder (MCC powder) and β-casein reduced micellar casein concentrate powder (LB MCC powder) at pilot scale at Moorepark Technology Limited, Fermoy, Co. Cork, Ireland as described in Chapter 5.

Powder production: Pasteurised skim milk (1000 kg) was divided into three portions, where one portion (400 kg) of milk was evaporated and spray-dried, the powder generated therefrom was called LHSMP. The second portion (300 kg) was microfiltered at 47 °C, with 56.38 % of whey protein removed and the resultant whey protein reduced-milk was called MCC. The third portion (300 kg) was microfiltered at 8.5 °C depleting 53.60 % of whey protein and 4.25 % of β-casein from the milk to generate LB MCC. Both MCC and LB MCC were subsequently evaporated and spray-

dried producing MCC or LB MCC powder respectively. All powders were packed, sealed and stored at 4 °C after production.

Cheese manufacture: On day 1, four fat-free milks, i.e., LH-, MCC-, LB- and HHT LB milk, were recombined from LHSMP, MCC, LB MCC and milk permeate powders (Profile^{TM} P8 permeate powder W463-25kg, Kerry Group, Listowel, Ireland) as well as CaCl2 (calcium chloride anhydrous powder Reag. Ph Eur, Merck Chemicals Ltd, Nottingham, United Kingdom) and RO water. The contents of casein, lactose and ash in milks were standardised to 2.89 %, 5.07 % and 0.77 %, respectively. On day 2, the LH-, MCC- and LB MCC milks were pasteurised at 72 °C for 15 s and HHT LB milk was high heat treated at 120 °C for 15 s. On day 3, pasteurised cream was added to each milk facilitating standardisation of the cheesemilks (CMs) to a casein to fat ratio of 0.79:1. Emmental cheeses were then manufactured in 10 L cheese vats as described in Chapter 5. Subsequent to pressing and brining, the cheeses were vacuum packed and ripened at 8 °C from day 0 to day 14 (pre-ripening), at 22 °C from day 15 to 70 (warm room ripening) and at 4 °C from day 71 to 120 (cold room ripening).

6.3.2 *Texture, pH, colour and flowability*

Emmental cheeses were sampled at day 1, 14, 70 and 120 of ripening, where the texture profile (fracture stress, fracture strain and firmness, tested at day 1 and 14 of maturation), pH, colour (levels of L*, a* and b*) and flowability (tested at day 70 and 120 of maturation) of cheese samples were measured as described by Hou et al. (2012), Fenelon et al. (2000), Sheehan et al. (2009) and McCarthy et al. (2016), respectively.

6.3.3 *Plasmin activity*

The plasmin activity in LB MCC milks (unheated, pasteurised or high heat treated), cheesemilks and experimental cheeses at day 120 of maturation was monitored by the coumarin peptide method (Richardson and Pearce, 1981). Results were expressed as nmol AMC mL-1 min-1, equalling one unit of plasmin activity.

6.3.4 *Proteolysis*

Emmental cheeses were sampled at day 1, 14, 70 and 120 of ripening, and primary proteolysis levels were determined (1) by urea-polyacrylamide gel electrophoresis (PAGE) on a cheese weight basis as described by Lamichhane et al. (2019) and in Chapter 4; (2) by measuring levels of % pH 4.6 soluble nitrogen in a fixed weight of cheese (pH 4.6) as described by Fenelon et al. (2000) and (3) by analysing the peptide profile of the pH 4.6-soluble extracts using reverse-phase high-performance liquid chromatography (RP-HPLC) as outlined by Rohm et al. (1996), adapted for use with a shorter column.

The HPLC system used consisted of an Agilent 1200s with multi-wavelength detector and ChemStation software (Agilent Technologies Irl. Ltd., Little Island Cork, Ireland). Mobile phase A consisted of 0.1 % Trifluoroacetic acid (TFA) in MQ water and B consisted of 0.1 % TFA in acetonitrile. The stationary phase consisted of a 4.6 × 150 mm Zorbax 300-SB C8 column (Agilent Technologies). A gradient of between 0 - 95% B was generated over 47 min at flow rate of 0.75 mL/ min before returning to the starting conditions. Total run time was 59 min. The pH 4.6-soluble extracts were filtered through a 0.45 um cellulose acetate filter (Lab unlimited, Carl Stuart Group, Dublin 24, Ireland) prior to the injection of 5 µl of sample onto the HPLC system.

6.3.5 *Volatile compounds*

The volatile profiles of each cheeses were measured at day 120 of ripening by solid phase micro extraction (SPME) - gas chromatography mass spec (GCMS) as described by Lamichhane et al. (2018b). All tests were performed in triplicate.

6.3.6 *Statistical analysis*

Emmental cheeses were manufactured in quadruplicate pilot plant trials within 5 weeks. One-way ANOVA analysis was carried out to compare the effect of treatments on the plasmin activities in milks, cheesemilks and cheeses as well as on the volatile compounds in experimental cheeses, using IBM SPSS statistics 24.0 (IBM Corp., 2016, Chicago, IL, USA). The effect of treatments, ripening time and their interactions on the levels of pH, pH 4.6-SN, texture profile, flowability and colour in cheeses were determined by a split-plot design, split-plot design analysis was carried out by using the PROC MIXED procedure of SAS software version 9.4 (SAS Institute, 2012). The level of significance ($P < 0.05$) between means were determined by Tukey's test.

6.4 Results and discussion

6.4.1 *Composition of cheese*

The gross composition and pH of experimental cheeses at day 14 of maturation are reported in detail in Chapter 5 and in Supplementary Table 6.1. Among the four cheeses types, LH cheese had the highest contents of moisture and MNFS as well as the lowest contents of calcium and calcium per gram of protein, MCC cheese had the highest pH and HHT LB cheese the lowest FDM content. The level of protein, salt and S/M were similar between the experimental cheeses.

6.4.2 *pH*

Curd washing with water is often applied to decrease the residual lactose content in Emmental cheeses (Heino, 2008; Fröhlich-Wyder et al., 2017), and as a result of this and of metabolism of lactate, the pH of Emmental cheeses often increases from ~5.2 in day 1 to 5.6-5.7 by day 52-180 (Lawrence et al., 1987; Lopez et al., 2007; Lamichhane et al., 2018a). However, the pH of experimental Emmental cheeses in the current study decreased significantly from 5.30-5.40 on day1 to 5.10-5.25 on day 120 of ripening ($P < 0.0001$, Table 6.1, Figure 6.1). The lactose content of the four experimental milks was standardised to 5.07 %, which is slightly higher than the average lactose content in bovine milk (4.9 %) (Fox et al., 2015). The absence of curd washing to control final cheese pH in this research should have led to the presence of residual lactose in the experimental cheeses, resulting in a pH decrease in cheese over maturation due to bacterial fermentation of residual lactose (O'Sullivan et al., 2016).

The pH of cheese can influence cheese texture by affecting the level of casein hydration and the levels of primary proteolysis in cheese through modulating the activity of plasmin or residual rennet (Hickey et al., 2017; Li et al., 2020). Similarly, levels of primary proteolysis can also affect the intensity of flavour in cheese (McSweeney and Sousa, 2000) through the production of substrate for secondary proteolysis. By obtaining the desired pH value for a given cheese type, cheesemakers can produce cheeses with desired texture, taste and flavour characteristics. During the formulation of MCC-, LB- and HHT LB milks, milk permeate powder was added to fortify the lactose content in milk. By adding a lower quantity of milk permeate powder, the lactose content in these milks might be decreased from 5.07 % to a level where water addition can be eliminated to achieve a desired cheese pH (Heino, 2008).

Although the MCC cheese had a significantly higher pH at d 14 of ripening, when mean pH levels across ripening was considered, there was no significant effect of reduction of β-casein levels in the cheesemilk nor application of high heat treatment (120°C ×15s) to the milk on cheese pH during maturation, which may be due to their similar contents of protein, moisture and ash (Table S 6.1), thus leading to similar buffering capacities.

Table 6.1. Statistical significances (adjusted P-values) for mean changes of maturation indexes during ripening in Emmental cheeses under the effects of treatments, time as well as interactive effect of treatment and time[1]

Parameters	Treatment	Time	Interactive effect (treatment × time)
pH	NS	***	NS
% pH4.6SN/ 100g cheese	NS	***	NS
Firmness (N)	*	NS	*
Fracture stress (N)	NS	NS	NS
Fracture strain	NS	NS	NS
Flowability (%)	***	NS	NS
L* (whiteness)	NS	***	NS
a* (redness)	***	***	NS
b* (yellowness)	NS	***	NS

[1]Significant levels: NS, P > 0.05; *< 0.05; ** P < 0.01; *** P < 0.001.

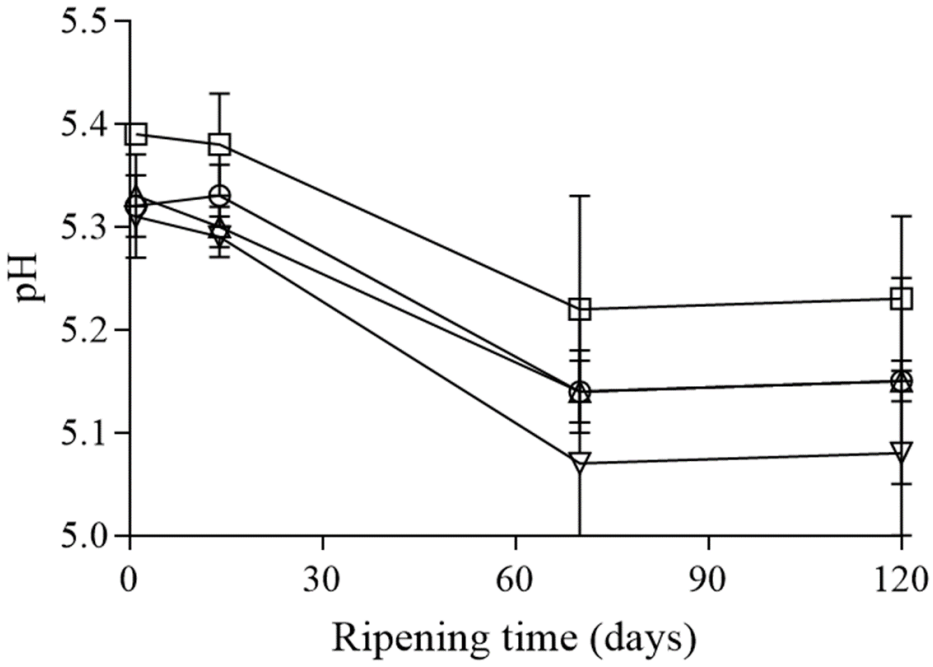

Figure 6.1. pH during ripening of Emmental cheeses manufactured from low heat skim milk powder (○), micellar casein concentrate powder (□) or β-casein reduced micellar casein concentrate powder which were pasteurised (Δ) or high heat treated (▽).

6.4.3 *Plasmin activity*

Keeping the holding time of heat treatment of LB MCC unchanged at 15 s, the plasmin activity in LB MCC decreased significantly on increasing the heat treatment from no heat treatment to pasteurisation (72°C) to HHT (120°C) (Figure 6.2A). Upon heat treatment, β-LG can be denatured and form disulphide bonds with both plasmin and plasminogen, thereby decreasing plasmin activity in heated milk, and increasing heating temperature and holding time can enhance this effect (Benfeldt et al., 1997). Since only 53.60% of whey proteins were removed from LB MCC by microfiltration, heat-induced denaturation of β-LG and binding with plasmin and plasminogen might explain the very low plasmin activity in HHT LB MCC. As a result, the cheese milk and Emmental cheese made from HHT LB MCC also had a much lower plasmin activity compared to those prepared from PS LB MCC (Figure 6.2B, C).

No significant difference in plasmin activity was observed between MCC- and LB CM and their cheeses as expected (Figure 6.2B). Whey proteins especially β-LG can inhibit the activity of plasmin and plasminogen activator in milk and, as both plasmin and plasminogen are associated with casein micelles (France et al., 2021), they can be retained in whey protein-reduced MCC after microfiltration, resulting in MCC with a higher plasmin activity than its original feed milk, as shown by Aaltonen and Ollikainen (2011). Thus, it was not surprising to observe a significantly lower plasmin activity in LH CM compared to those in MCC- and LB CM (Figure 6.2B), with similar results also reported by Li et al. (2020). However this difference was not carried forward to their cheeses, contrary to the findings of Li et al. (2020). Further research is required to determine the reason for this.

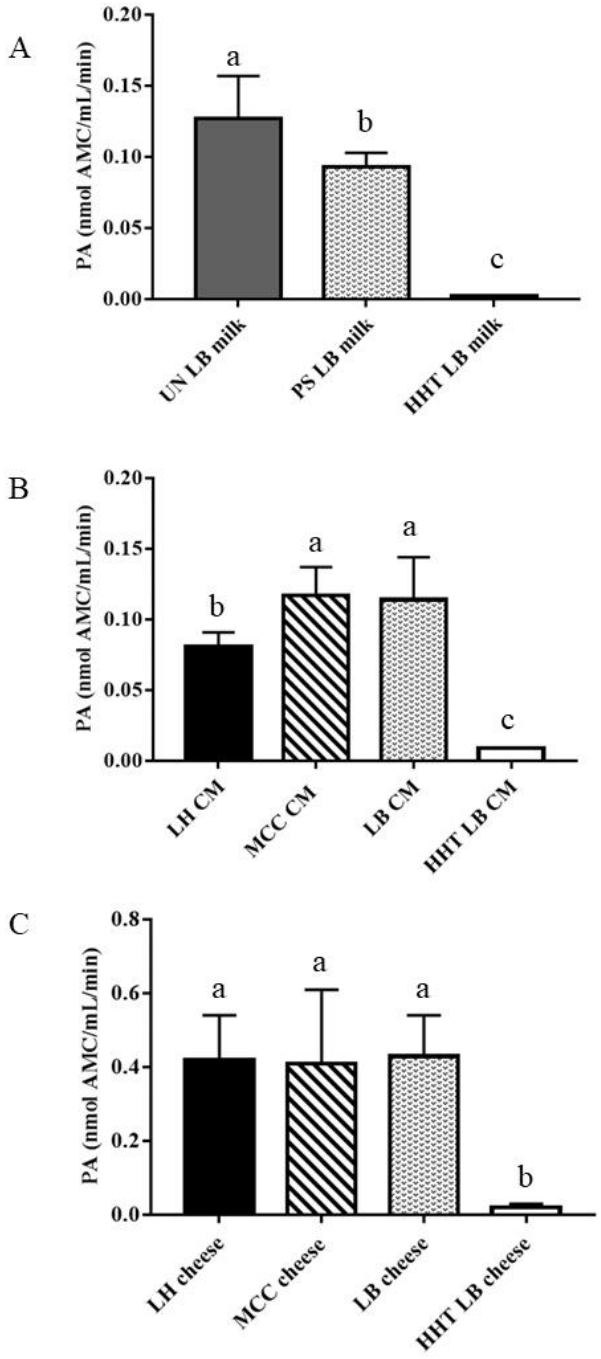

Figure 6.2. Plasmin activity in micellar casein concentrates (A), whey protein reduced cheese milks (B) and Emmental cheeses made there from at day 120 of ripening (C). Results are means of quadruplicate trials, values within a figure not sharing the same superscript differ significantly ($P < 0.05$). Abbreviations: plasmin activity (PA); milk prepared from β-casein reduced micellar casein concentrate were not heated (UN LB milk), pasteurised (PS LB milk) or high heat treated (HHT LB milk); cheese milks were prepared from low heat skim milk powder (LH CM), micellar casein concentrate powder (MCC CM) or β-casein reduced micellar casein concentrate powder which were pasteurised (LB CM) or high heat treated (HHT LB CM), the Emmental cheeses made therefrom were called LH-, MCC-, LB- ot HHT LB cheese respectively.

6.4.4 *Urea-PAGE*

The patterns of proteolysis of α_{s1}- and β-caseins in Emmental cheeses were evaluated using urea-polyacrylamide gel electrophoresis, where cheese samples were of similar protein levels and were loaded on weight basis (Figure 3). For each treatment, significant hydrolysis of α_{s1}- and β-caseins was observed during warm room ripening, with little further breakdown during cold room ripening. The high temperature (22°C) of the warm ripening can accelerate the hydrolysis of both α_{s1}- and β-caseins, as reported by Sheehan et al. (2008) and Lamichhane et al. (2019).

From day 14 of maturation onwards, the proteolysis of α_{s1}- and β-caseins in MCC-, LB- and HHT LB cheeses were much more extensive than those in the LH cheese. Plasmin has been widely considered as the main proteinase that hydrolyses β-casein during the course of cheese maturation (Ardö et al., 2017). The level of intact β-casein in LH cheese was much higher than those in cheeses MCC- and LB, suggesting a lower plasmin activity in LH cheese as a result of lower plasmin activity in its cheese milk. A much higher level of β-casein breakdown was expected in the MCC- and LB cheeses than that in the HHT LB cheese due to their much higher plasmin activities (Figure 2C); however, similar levels of proteolysis of β-casein in these three cheeses suggest that an analytical approaches more sensitive than urea-PAGE may be required to discriminate the effect of treatments on cheese proteolysis. It is suggested that LCMS analysis should be applied to determine the peptides produced and the potential origin of those peptides which would also provide suggestions as to the potential enzymes producing such peptides.

Hydrolysis of α_{s1}-casein was also observed in four experimental cheeses. Both Cryphonectria parasitica proteinase (also called Endothia parasitica proteinase, used

as coagulant in this research) (Gagnaire et al., 2001) and cathepsin D (Hays et al., 2001) can degrade α_{s1}-casein during cheese ripening. Even though it is reported that the activity of *Cryphonectria parasitica* proteinase in the resulting cheese can be totally inactivated after being cooked at 50 -55 °C for 30 - 60 min (Garnot and Mollé, 1987; Winwood, 1989). In this research, the curds were cooked to 50 °C and were drained immediately after reaching this temperature, and the size of the derived Emmental cheeses were 0.5 kg per cheese, much smaller than those of Emmental produced industrially (75 and 120 kg) (Fröhlich-Wyder et al., 2017). As a result, the temperature of experimental cheese might have decreased from 50 °C to lower temperatures shortly after cooking, and for this reason, the activity of *Cryphonectria parasitica* proteinase in experimental cheeses might only be partly inactivated.

As a milk borne proteinase, cathepsin D can partly survive pasteurisation of cheesemilk (72 °C × 15 s, 8 % survival) and cooking in cheesemaking (55 °C × 30 min, 45 % survival) (Hays et al., 2001), it was possible that cathepsin D has also played an important role in the hydrolysis of α_{s1}-casein in experimental cheeses during maturation. The levels of αs1-casein hydrolysis in MCC-, LB- and HHT LB cheeses were much higher than that in LH cheese. However, more experiments are required to (1) elucidate which enzyme(s) is responsible for the hydrolysis of α_{s1}-casein in this research and to (2) explain why LH cheese had higher level of intact α_{s1}-casein.

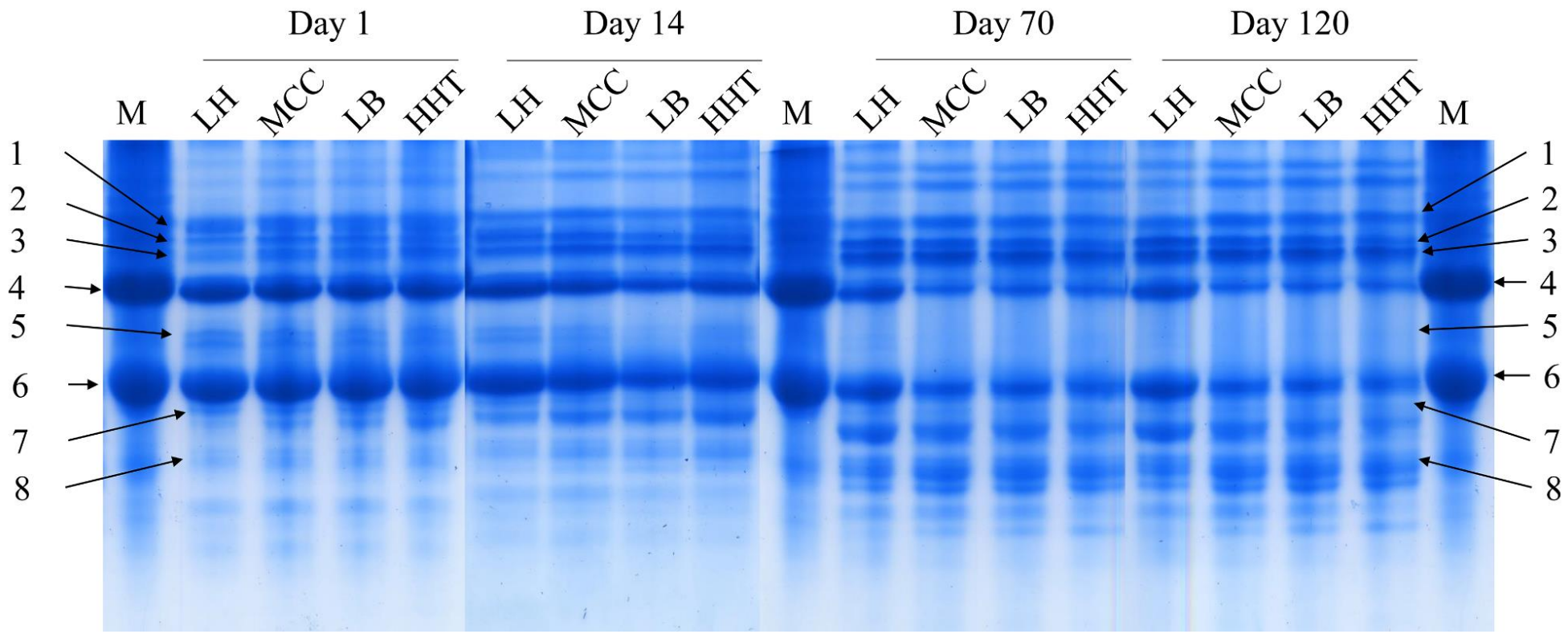

Figure 3. Urea-polyacrylamide gel electrophoretograms of Emmental cheeses at day 1, 14, 70 and 120 of ripening. Abbreviation: M, Marker (sodium caseinate); Emmental cheeses manufactured from low heat skim milk powder (LH), micellar casein concentrate powder (MCC) or β-casein reduced micellar casein concentrate powder which were pasteurised (LB) or high heat treated (HHT). Number: 1, β-casein (f106-209); 2, β-casein (f29-209); 3, β-casein (f108-209); 4, β-casein; 5, β-casein (f1-192); 6, α_{s1}-casein; 7, α_{s1}-casein (f102-199); 8, α_{s1}-casein (f24-199).

6.4.5 *pH4.6SN/100 g cheese*

The level of primary proteolysis in cheese was expressed as a percentage of pH4.6 soluble nitrogen (SN) per 100 g of cheese, where a higher content of pH4.6SN per 100g cheese indicates a higher level of primary proteolysis in experimental cheese (McCarthy et al., 2017). The levels of pH4.6-SN per 100 g cheese in all experimental cheeses increased significantly during the hot room period (day 14 to day 70, 22°C, $P < 0.0001$), in line with the previous reports for Swiss-type (Sheehan et al., 2008, O'Sullivan et al., 2016) and Maasdam cheeses (Lamichhane et al., 2018a). However the change was not significant from day 1 to day 14 (8°C, $P > 0.05$) or from day 70 to day 120 (4°C, $P > 0.05$) of maturation (Table 6.1, Figure 6.4), due to the lower ripening temperature and shorter ripening duration.

Variations in the contents of whey protein, β-casein and in the thermal history of the cheese milk did not affect the levels of pH4.6SN per 100 g cheese ($P > 0.05$), suggesting that these treatments did not affect levels of primary proteolysis.

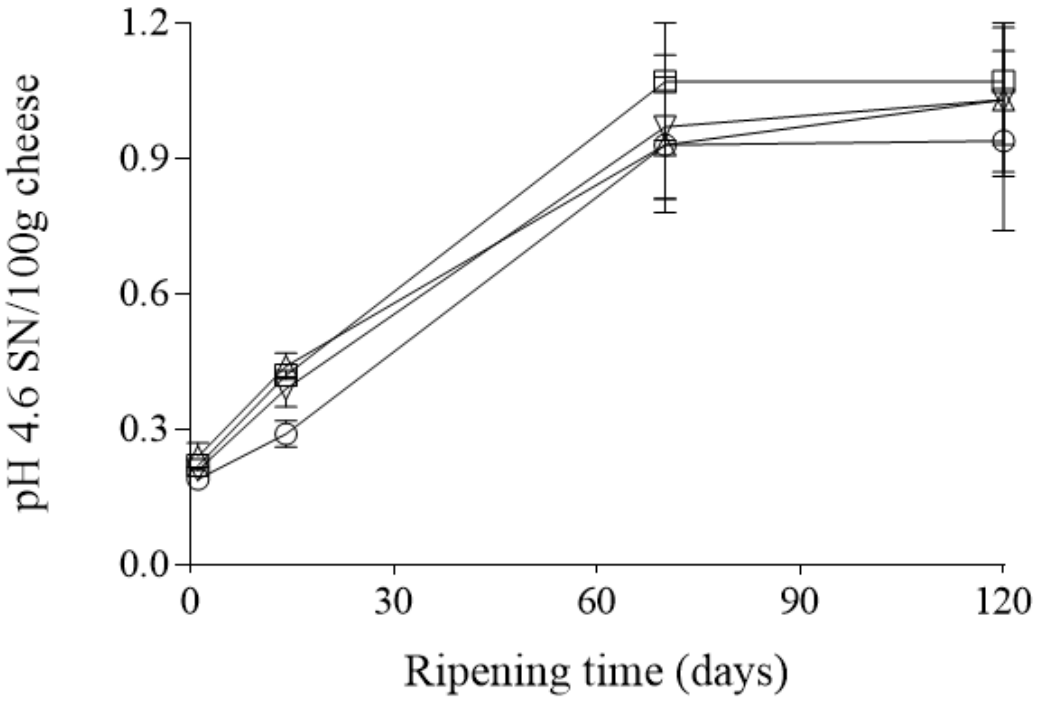

Figure 6.4. pH4.6SN levels expressed per 100g of Emmental cheeses manufactured from low heat skim milk powder (○), micellar casein concentrate powder (□) or β-casein reduced micellar casein concentrate powder which were pasteurised (Δ) or high heat treated (▽).

6.4.6 *HPLC*

The peptide profiles in pH4.6-soluble extracts was monitored by RP- HPLC during ripening (Figure 6.5). Since the degradation products of β-casein generated by plasmin are highly hydrophobic, these peptides typically elute after α-lactalbumin and β-LG (Upadhyay et al., 2004) and appeared between 30 to 32.5 min on the chromatograms (Figure 6.5). The peak area of β-casein hydrolysates as well as all the peptides present in pH4.6-soluble extracts from the experimental cheeses at different ripening time were calculated and compared (Figure 6.6). Even though the plasmin activity in LH cheese was much higher than that in HHT LB cheese, and similar to those in cheeses MCC and LB, the proteolysis products of β-casein from LH cheese were much lower than those from the other cheeses from day 1 to day 120 of ripening

(Figure 6.6a), agreeing with urea-PAGE results. Cheeses manufactured from MCC powder or LB MCC powder might be more accessible to plasmin in comparison to those made from LHSMP, though further research is required to clarify this suggestion. HHT LB cheeses also had lower levels of β-casein hydrolysis compared to MCC and LB cheeses as a result of lower plasmin activity (Figure 6.2C), this difference was not obvious from urea-PAGE, suggesting that urea-PAGE works better as a qualitative method rather than quantitative one.

For each experimental cheeses, the total peak area for all peptides in the pH4.6 soluble extracts increased as ripening progressed (Figure 6.6b), in line with the levels of pH4.6-SN per 100 g cheese. However, unlike pH4.6-SN expressed per 100 g cheese, the total peptides present in the pH4.6 soluble extracts for LH cheese was much lower than those for the other cheeses, which might be related to its lower hydrolysis of α_{s1}- and β-caseins as shown by urea-PAGE results. The levels of pH4.6-SN soluble in 100 g cheese measured the total nitrogen content in pH4.6 soluble extracts, while HPLC monitored the amount of peptides in this extract, HPLC provided a more reliable way to quantify and qualitatively examine proteolysis in cheese.

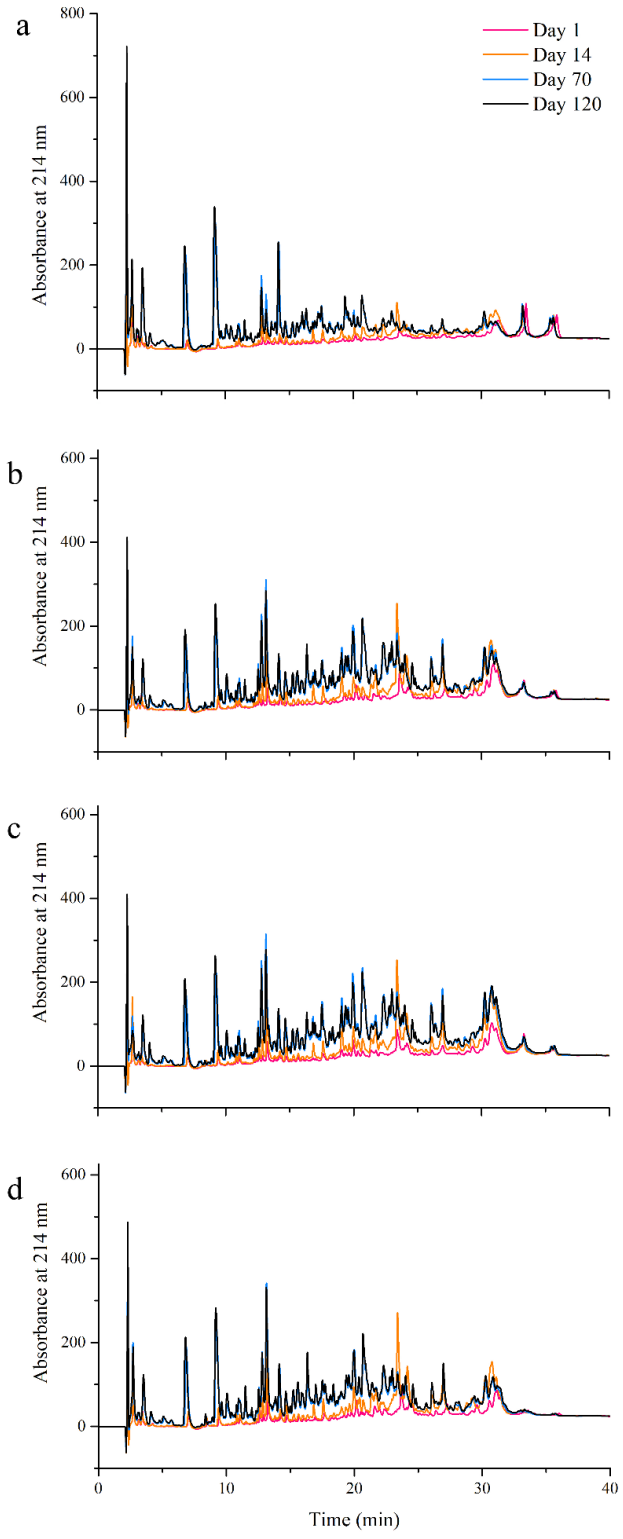

Figure 6.5. RP-HPLC chromatogram peptide profiles of pH4.6 soluble extracts in Emmental cheeses manufactured from low heat skim milk powder (a), micellar casein concentrate powder (b) or β-casein reduced micellar casein concentrate powder which were pasteurised (c) or high heat treated (d) at different ripening stage at 214 nm.

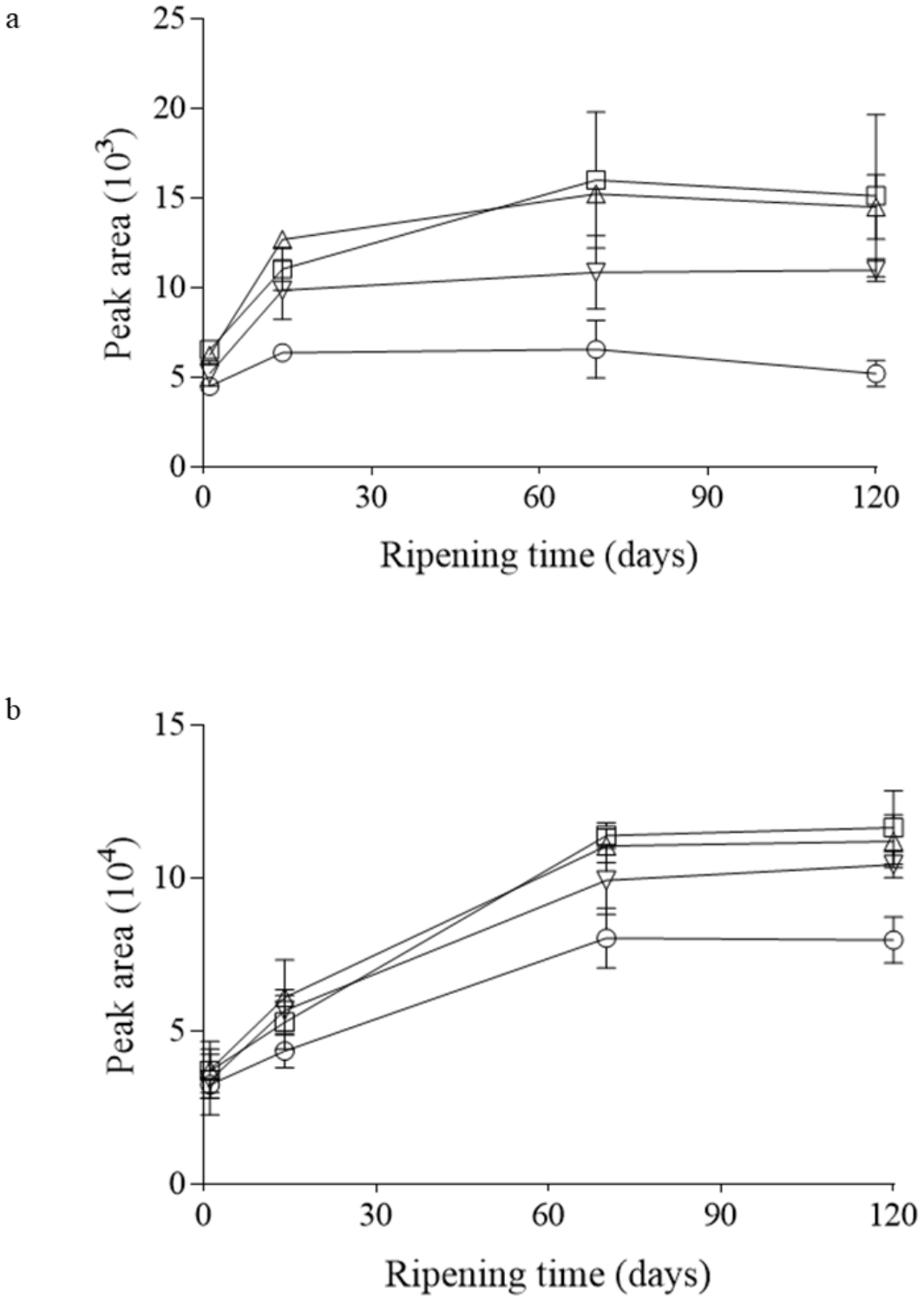

Figure 6.6. Peak area of β-casein hydrolysate (a) or all peptides (b) present in pH4.6-soluble extracts of Emmental cheeses manufactured from low heat skim milk powder (○), micellar casein concentrate powder (□) or β-casein reduced micellar casein concentrate powder which were pasteurised (Δ) or high heat treated (▽) at day 1 and day 14 of ripening.

6.4.7 *Texture*

The hardness of experimental cheeses sampled at day 1 of ripening were not significantly different from those sample at day 14 (Table 6.1), which was not surprising, since a pronounced decrease of hardness in brine-salted semi-hard cheeses is usually observed during warm room ripening (Lamichhane et al., 2019). Due to eye formation after 14 days of maturation, the texture of Emmental cheeses was not measured at later time points.

On both day 1 and day 14 of ripening, the firmness and fracture stress in MCC cheese were higher than the other cheeses (Figure 6.7), which might be related to the higher calcium content (Lawrence et al., 1984) and significantly higher pH level in this cheese (Table S 6.1). A higher cheese pH level can increase the amount of calcium bound to para-casein, resulting in greater levels of para-casein aggregation and a firmer cheese curd structure, and thus a greater cheese hardness (Hou et al., 2014). O'Mahony et al. (2014) reported that removing β-casein by 9 – 10% from milk significantly reduced the hardness of a model Cheddar cheese. However, and possibly due to the much lower level of β-casein reduction in our research (4.25%), no significant difference s in levels of fracture stress, fracture strain and hardness between LH cheese and cheeses LB- and HHT LB was observed ($P > 0.05$).

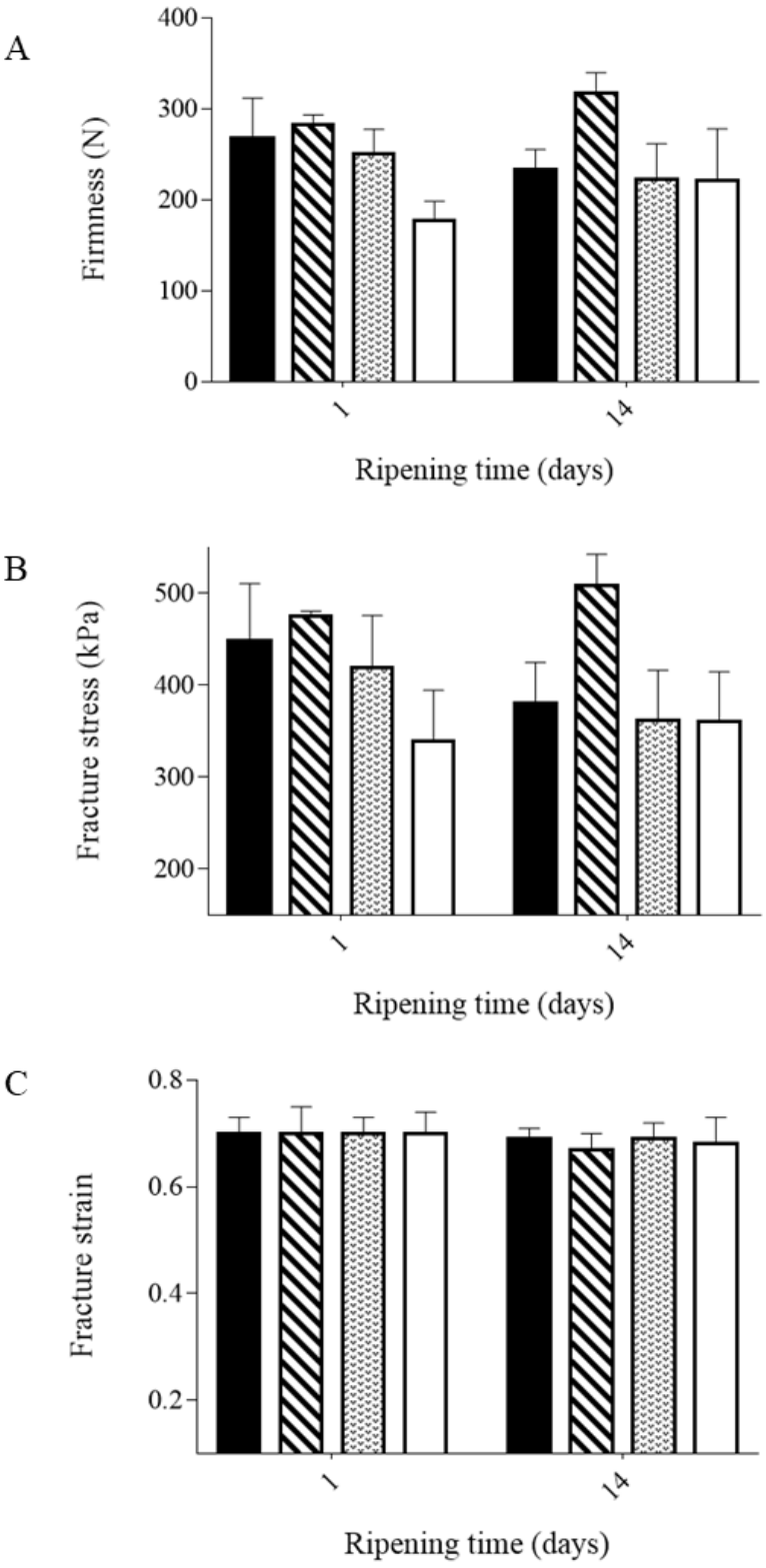

Figure 6.7. Fracture stress (A), fracture strain (B) and firmness (C)of Emmental cheeses manufactured from low heat skim milk powder (○), micellar casein concentrate powder (□) or β-casein reduced micellar casein concentrate powder which were pasteurised (Δ) or high heat treated (▽) at day 1 and day 14 of ripening.

6.4.8 *Flowability*

The flowability of the cheeses was measured at day 70 and day 120 of ripening, with no significant change observed in all treatments during this time (Table 6.1, Figure 6.8), possibly due to the absence of significant changes in levels of primary proteolysis as demonstrated by levels of pH4.6-SN per 100 g cheese. Increased levels of casein breakdown are conducive to increased levels of casein hydration, encouraging the heat-induced detachment of adjacent layers of the para-casein network upon heating (Sheehan and Guinee, 2004).

On increasing the heat treatment temperature of the LB milk from 72 to 120°C, the flowability of cheese decreased significantly at both day 70 and day 120 of ripening in comparison to the other cheeses, possibly due to the higher levels of denatured β-LG recovered in HHT LB cheese (Chapter 6.5). Rynne et al. (2004) also reported a negative impact of increasing heat temperature, i.e., from 72 to 87°C, of cheese milk on the flowability of half-fat Cheddar cheese. It was suggested that the continuity of the protein network in cheese made from HHT milk was enhanced, due to the inclusion of denatured β-LG in the cheese matrix through disulphide bonds formed between β-LG and para-casein, as well as increased hydrophobic interactions between hydrophobic sites on denatured β-LG in heated cheese (Rynne et al., 2004). Reduction in the level of (intact) β-casein in the cheese through partly depleting β-casein from the cheesemilk (by 9 – 10 %) can enhance its meltability upon heating (O'Mahony et al., 2014), however the lower amount of β-casein reduction in LB MCC (by 4.25 %) achieved in our study did not result in cheeses with a significantly higher flowablity than the LH- and MCC ($P > 0.05$, Figure 6.8) cheeses.

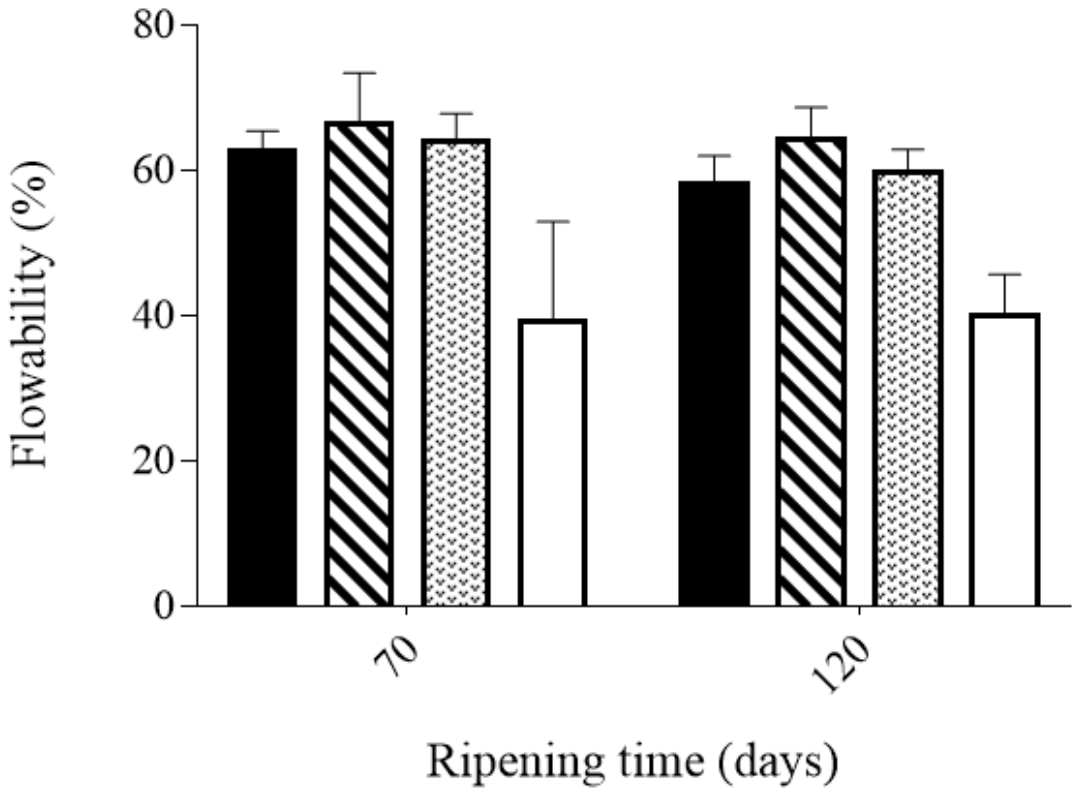

Figure 6.8. Flowability of Emmental cheeses manufactured from low heat skim milk powder (black), micellar casein concentrate powder (stripe) or β-casein reduced micellar casein concentrate powder which were pasteurised (dot) or high heat treated (white) at day 70 and 120 of maturation.

6.4.9 *Colour*

The colour of Emmental cheeses were monitored over 120 days of maturation, and was expressed as L* (lightness, 0-100, from black to white), a*(-100 to 100, from green to red) and b* values (-100 to 100, from blue to yellow) (Table 1, Figure 9). For cheeses subjected to different treatments, the levels of L*, a* and b* were significantly affected by ripening time ($P < 0.0001$) (Table 6.1). The whiteness (L*) ($P < 0.0001$) and redness (a*) ($P < 0.0001$) of experimental cheeses increased significantly during warm room period, however the yellowness (b*) decreased significantly during both warm room ($P < 0.05$) and the subsequent cold room ripening period ($P < 0.01$).

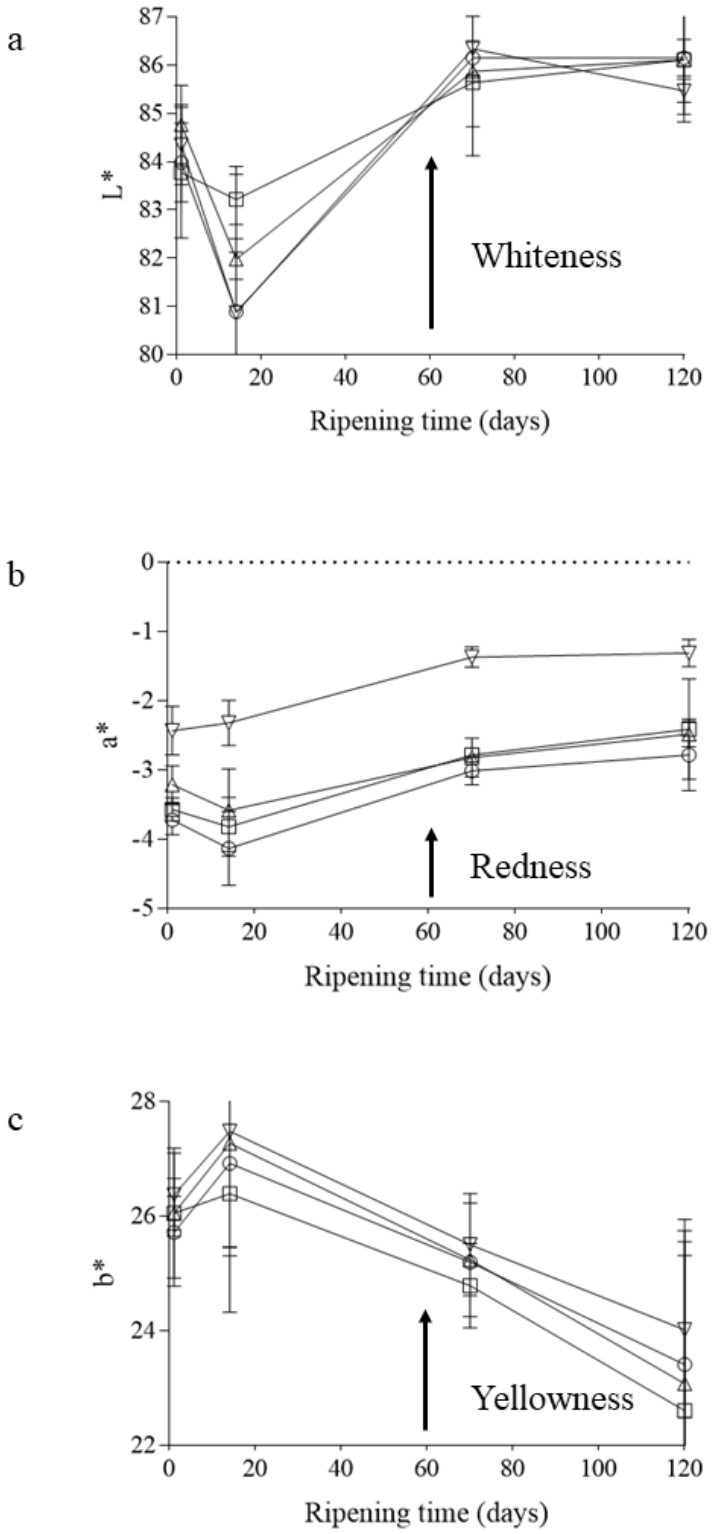

Figure 6.9. Colour expressed as L* (a), a* (b) and b* (c) values of Emmental cheeses manufactured from low heat skim milk powder (○), micellar casein concentrate powder (□) or β-casein reduced micellar casein concentrate powder which were pasteurised (Δ) or high heat treated (▽)at different ripening stage.

The hue for each cheeses, on visual observation, were yellow at day 14 and turned more red at day 70 of ripening, contrary to Rohm and Jaros (1996), who found that the yellowness (b*) of Emmental cheese increased after 10 weeks of maturation giving the cheese a slightly orange appearance. Sulejmani and Hayaloglu (2016) suggested that the concentration of β-carotene in cheese during storage caused by moisture loss might explain the increased yellowness over cheese maturation. Dairy products, including heat treated milk, powder and cheese, darken upon intensive heat treatment (temperature and holding time) and storage due to Maillard Reaction, where lactose and lysine rich protein react and generate protein bound-brown compounds (Morales and Van Boekel, 1998; Al-Saadi et al., 2013). Due to the absence of water addition in our trials, the residual lactose content in the resultant cheeses might be much higher compared to that in typical Emmental cheese as was also postulated previously. Reducing sugars, like lactose, in cheese can make major contribution to cheese discolouration during storage through Maillard Reaction (Igoshi et al., 2017), while high storage temperature can also enhance Maillard Reaction and the formation of a brown pigment (Rufián-Henares et al., 2006).

From day 1 to day 120 of ripening, the redness (a*) of LH-, MCC- and LB cheeses was not significantly different from each other, however they were significantly lower than that in HHT LB cheese (Table 6.1, Figure 6.9b). Similar results were also reported by Aydemir and Dervisoglu (2010), who reported that Kulek cheese made from heat-treated milk (75 °C × 5 min) is redder than that made from raw milk. The increase of a* value indicated the formation of brown pigment in HHT LB cheese (Le et al., 2011), which may be correlated with the visually increased brownness observed in HHT LB cheese in comparison to those in the other cheeses. No significant difference was observed in colour between unheated, pasteurised and

high heat treated LB milk (data not shown), however HHT LB cheese was redder than the other cheeses from day 1 of ripening (Figure 6.9b). This suggests that low levels of MR might have occurred in HHT LB milk, but the level of browning reaction was so low that the measured milk colour was not changed after being subjected to 120°C for 15 s. Since the brown compounds produced from MR are bound to casein (Morales and Van Boekel, 1998), the colour formed in heat treated milk might be retained and concentrated in the resultant cheese, giving the fresh HHT LB cheese a brown look.

The Maillard reaction is undesirable for cheese makers due to discolouration and impaired nutritional value (measured by lysine loss) in cheese (Rufián-Henares et al., 2006; Aydemir and Dervisoglu, 2010; Patel et al., 2013). Studies show that, processed cheese made from natural cheese with darker appearance tends to show higher levels of discolouration (Arai et al., 2020). Since lactose is a limiting factor in MR, by normalizing the lactose content in cheese milk by adding less milk permeate powder (which provided lactose to the cheese milk in this study) or by applying HHT to milk before adding milk permeate, undesired Maillard reaction might be avoided, though future research is required to evaluate this assumption.

Regardless of treatment applied the whiteness and yellowness of experimental cheeses were not significantly affected (Table 6.1).

6.4.10 *Volatile compounds*

Thirty four volatile compounds were detected in experimental cheeses with HS-SPME GC-MS, which included 9 acids, 5 alcohols, 2 aldehydes, 6 esters, 8 ketones, 1 lactone, 2 sulphur compounds and 1 terpene (Table 6.2). LH cheese had higher levels of aldehydes (2 out of 2), ketones (8 out of 8) and sulphur (1 out of 2)

compounds than MCC-, LB- and HHT LB cheeses (Table 6.2), indicating higher levels of amino acid metabolism, lipid oxidation and lactose metabolism in LH cheese.

Principal component analysis was carried out to evaluate the effect of treatments on volatile profile in Emmental cheeses, the total variance was 83.3 % with PC-1 axis accounting for 50.6 % of difference and PC-2 axis 32.7 % difference (Figure 6.10). Cheeses made from β-casein reduced MCC, i.e., LB- and HHT LB cheeses, were located on the positive side of PC-2, and the other two cheeses on the negative side (Figure 6.10). LB- and HHT LB cheeses were characterised with higher levels of esters (5 out of 6) and ethanol as well as lower levels of isopropyl alcohol and propionic acid compared to LH- and MCC cheeses (Table 6.2). The high level of ethanol in cheeses LB- and HHT LB, which suggest high level of lactose degradation, should explain the high level of ethyl ester in these cheeses, since ethanol is often considered as the rate limiting step for ethyl ester formation (Thierry et al., 2006).

It was proposed earlier that a certain level of Maillard reaction might have taken place in the HHT LB cheese, however, MR related volatile compounds, such as furfural and furfuryl alcohol (Newton et al., 2012), were not detected in this cheese.

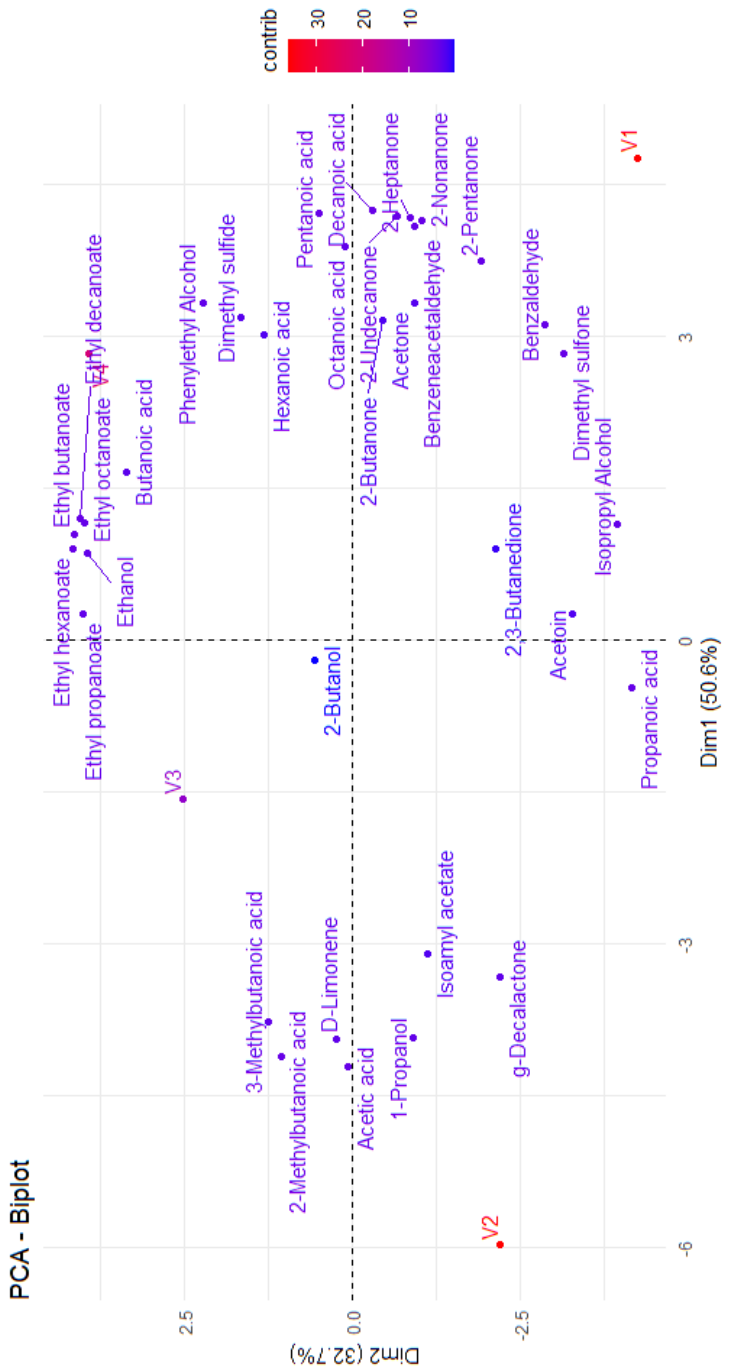

Figure 6.10. Principle component analysis Bi-plot of separation based on volatile profile of Emmental cheese at day 120. Results are means of triplicate trials. Emmental cheeses manufactured from low heat skim milk powder (LH cheese, V1), micellar casein concentrate powder (MCC cheese, V2) or β-casein reduced micellar casein concentrate powder which were pasteurised (LB cheese, V3) or high heat treated (HHT LB cheese, V4).

6.5 Conclusion

Reduction of β-casein from MCC by 4.25 % did not influence the pH, plasmin activity, primary proteolysis, texture profile, flowability and colour in Emmental cheeses made therefrom. However by partly removing whey proteins, inhibitors of plasmin and plasminogen activators, from pasteurised skim milk using microfiltration, the plasmin activity in LH cheesemilk was significantly lower than those in MCC- and LB cheesemilks, which eventually led to lower β-casein hydrolysis levels in that cheese (as indicated in results from urea-PAGE and HPLC analysis).

Though 53.60 % of whey protein was removed from feed milk prior to high heat treatment, the plasmin activity in HHT LB milk and its cheese was largely inhibited probably due to the formation of disulphide bonds between denatured β-LG and plasmin. As a result, the level of β-casein degradation in HHT LB cheese was lower compared to MCC- and LB cheeses (HPLC). HHT LB cheese also had significantly lower level of flowability as well as a higher level of redness than other treatments, possibly due to the enhanced hydrophobic interaction between denatured whey protein or early stage Maillard reaction, respectively.

Both β-casein and native whey protein are valuable dairy ingredients; based on our results, these two components can be partly removed from milk before cheesemaking without changing the cheese quality during ripening. Due to its impaired flowability on heating and colour, the Emmental cheese made from high heat treated whey protein reduced milk may not be appealing to consumers. By increasing the casein content on a total protein basis as well as decreasing the lactose content in whey protein-reduced-milk before high heat treatment, cheese with improved levels of plasmin activity, primary proteolysis and flowability together with less red colour

may be possible to manufacture. However, further research is suggested to validate this.

6.5 References

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Supplemental Table 6.1. Composition and pH of Emmental cheeses manufactured from whey protein reduced cheese milk of different thermal histories and β-casein contents at 14 days of ripening[1, 2]

Compositional parameters	LH cheese[2]	MCC cheese[2]	LB cheese[2]	HHT LB cheese[2]
Protein content (%)	23.53[a]	23.33[a]	22.78[a]	23.48[a]
Fat content (%)	26.49[b]	27.47[a,b]	28.07[a]	26.71[b]
Pro: fat ratio	0.89[a]	0.85[a,b]	0.81[b]	0.90[a]
Moisture content (%)	41.85[a]	39.80[b]	40.15[b]	40.81[a,b]
FDM (%)[3]	45.55[a,b]	45.73[a,b]	46.89[a]	44.94[b]
MNFS (%)[4]	56.92[a]	55.05[a]	55.81[a]	55.67[a]
Salt content (%)	1.33[a]	1.26[a]	1.23[a]	1.32[a]
S/M (%)[5]	3.17[a]	3.15[a]	3.06[a]	3.15[a]
Ash content (%)	3.61[c]	3.97[a]	3.72[b,c]	3.82[a,b]
Ca (mg/100g)	677.67[b]	876.07[a]	782.64[a]	783.75[a]
Calcium/protein (mg/g of protein)	28.78[c]	37.60[a]	34.34[a,b]	33.42[b]
pH	5.33[b]	5.38[a]	5.33[b]	5.31[b]

[1]Results are means of triplicate trials, values within a row not sharing the same superscript differ significantly ($P < 0.05$).
[2] Emmental cheeses were manufactured from low heat skim milk powder (LH cheese), micellar casein concentrate powder (MCC cheese) or β-casein reduced micellar casein concentrate powder which were pasteurised (LB cheese) or high heat treated (HHT LB cheese).
[3] FDM= fat in dry matter.
[4] MNFS=moisture in non-fat substance.
[5] S/M=salt in moisture.

Chapter 7: General conclusion

7.1 Introduction

During the microfiltration (MF) of skim milk, the serum phase of milk which contains small molecules like whey protein, lactose, soluble salts and soluble caseins are removed to permeate, and the casein micelles are retained and concentrated in retentate, called micellar casein concentrate (MCC) (Neocleous et al., 2002b). By increasing partition of colloidal calcium phosphate or β-casein from casein micelles to the serum phase through manipulation of the pH and temperature of skim milk before MF, MCC of decreased calcium or β-casein levels can be produced; these MCC streams can be reconstituted and used as cheesemilk (Brandsma and Rizvi, 1999). Since the whey protein produced by MF has superior technical and functional properties than that produced from cheese whey (Bacher and Kønigsfeldt, 2000), maximizing the removal of whey protein from milk to permeate before cheesemaking is desired. To achieve this, diafiltration (DF) with liquid media is often used, and increasing the number of DF steps can increase whey protein removal (Hausmann et al., 2013). The number of DF steps and the composition of DF media can also affect the composition of MCC and of the resultant cheesemilk (Nelson and Barbano, 2005; Amelia and Barbano, 2013).

The objective of the research undertaken in this thesis were to (1) explore the application of MF to skim milk to facilitate the formulation of cheesemilk of target composition; (2) study the effect of cheesemilk composition (casein, whey protein and β-casein) on its rennet coagulability and the quality of resultant semi-hard cheeses (Cheddar or Emmental); (3) evaluate the effect of heat treatment (90 °C × 15 s or 120 °C × 15 s) of MCC on the rennet coagulation properties of resultant cheesemilk as well as on the quality of semi-hard cheeses made therefrom.

7.2 Overall results and discussion

7.2.1 *Factors influencing the composition of MCC*

In Chapter 2, MF of skim milk was carried out using a ceramic membrane of pore size 0.14 µm, in which two steps of DF with reverse osmosis (RO) water was applied to maximize the removal of whey protein from milk. MF without a DF step depleted the largest amount of small molecules (whey protein, lactose and minerals) from the serum phase of the feed milk, while one DF step removed a lesser amount and application of a second DF step remove the lowest amount of solutes from the milk serum phase. After two steps of DF with water, 60.81 % of whey protein in addition to nearly all lactose (94.14 %) and soluble calcium were removed from the skim milk to permeate.

The type of membrane used in MF can influence the ability to separate whey protein. By keeping other processing parameters during MF and DF constant, (i.e. temperature (47- 50 °C), volume concentration factor (VCF, 3), dilution factor (2), weight of feed milk (300- 350 kg), diafiltrant (water), number of DF steps (2) and membrane plant (GEA Model F filtration unit)), MF at ceramic membranes of pore size 0.10 µm (50 °C) and polyvinyldiene fluoride (PVDF) membranes at molecular weight cut-off of 800 kDa (47 °C) isolated 70.29 % or 56.38 % of whey protein from the feed milk to permeate, respectively (Chapter 3 & 5). Similarly, the temperature applied during MF processing can also affect fractionation of whey protein. For example, MF with an 800 kDa PVDF membrane removed 56.38 % of whey protein from milk when operated at 47 °C (warm MF); however this value dropped to 53.60 % when the processing temperature was held at 8.5 °C (cold MF, Chapter 5).

After storage of milk at refrigeration temperatures (1- 4 °C) for an extended period of time (12- 48 hours), both β-casein and colloidal calcium phosphate (CCP) can be partially liberated from casein micelles to the serum phase, due to reduced hydrophobic interactions and disturbance of the calcium balance respectively (Gaucheron, 2005; O'Mahony et al., 2014). As a result, cold MF reduced more β-casein (4.25 % vs 1.83 %) and calcium (68.43 vs 73.86 m mol/ g casein in the final MCC powders) from feed milk compared to warm MF (Chapter 5).

Regardless of the membrane type and the temperature at which MF is applied, after two steps of DF with water, the pH of the final retentate (MCC) was much higher than that in the feed milk (6.90- 6.96 vs 6.61– 6.76) (Chapter 2, 3 and Chapter 5). Since the pH of diafiltration water used in these studies was 7.00, the reduction of buffering salts from the feed milk as well as possible dissociation of colloidal calcium phosphate might explain the increased pH in MCC when water was used as diafiltrant (Chapter 2). Similar trends were also reported in other studies (Hurt et al., 2010; McCarthy et al., 2017; Boiani et al., 2018).

7.2.2 *Application of MF in the formulation of cheesemilk of target composition*

After MF, the MF permeate was further fractionated into ultrafiltration (UF) retentate (native whey protein), reverse osmosis (RO) retentate (lactose and minerals) and RO permeate (water) (Chapter 2). Nearly all the lactose and minerals originally present in MF permeate were separated and concentrated in RO retentate, and the RO permeate was pure water free from lactose and minerals. Whey protein reduced cheesemilks with the same or 1.5 times the casein content as the feed milk and with similar lactose and ash contents to the in feed milk were successfully formulated from MCC (MF retentate subjected to evaporation), RO retentate, RO permeate and

pasteurized cream (Chapter 2). Due to the high casein content of the MCC (13.14 %), cheesemilk (of lactose content = 4.32 %) of casein content up to 10.16 % can be prepared from membrane streams. The highest casein content that can be achieved in the recombined cheesemilk is determined by the casein content of the MCC and the lactose content in the RO retentate. The casein content in MCC can be increased by evaporation (Chapter 2), spray drying (Chapter 5) or by increasing the VCF during MF (Neocleous et al., 2002a).It is suggested that formulating cheesemilk of reduced lactose content (3.2- 3.9 %) for use in Emmental cheese manufacture, can be applied to avoid water addition or curd washing as measures to control lactose content while still achieving the desired pH in the final cheese (Heino, 2008). By adding less RO retentate, cheesemilk of lower level of lactose can also be formulated (Chapter 2). β-casein reduced cheesemilk can also be reconstituted from β-casein reduced MCC powder (Chapter 5).

7.2.3 *Effect of cheesemilk composition on cheese quality*

In this research thesis, cheesemilks with various levels of casein (Chapter 2, 3 & 4), whey protein (Chapter 2, 5 & 6) and β-casein (Chapter 5 & 6) were formulated through MF. Cheddar or Emmental type of cheeses were then manufactured and cheese ripening and quality indices were characterized.

By increasing the casein content in cheesemilk from 2.87 % to 4.32 % (Chapter 2) or from 3.09 % to 4.31 % (Chapters 3 & 4), the gel firming rate of renneted gels increased significantly. The total cheesemaking time of Cheddar cheese in concentrated cheesemilk was also significantly elongated due to the higher buffering capacity (Chapter 3), in agreement with the data of St-Gelais et al. (1998). Cheddar cheese made from concentrated cheesemilk had lower levels of moisture and MNFS

and higher contents of ash and protein (Chapter 2 & 3), which might in turn gave the cheese a lower level of lactose and lactate (Rynne et al., 2007), a higher buffering capacity (Lucey and Fox, 1993) and higher pH over 180 days of maturation (Chapter 4). In the present study (Chapter 2 & 3), chymosin was added on a milk weight basis rather than casein content basis, as a result, the hydrolysis of α_{s1}-casein (urea-polyacrylamide gel electrophoresis (PAGE)) decreased on increasing casein content in cheesemilk. The reduced levels of primary proteolysis (urea-PAGE and % pH4.6-SN/Total cheese) in cheese made from cheesemilk of higher casein content also explained the increased levels of hardness as well as decreased levels of flowability and volatile compounds over maturation (Chapter 4).

Removal of 53.60 – 60.81 % whey protein or 1.83 – 4.25 % β-casein from milk did not affect the rennet coagulation properties of the cheesemilk, or the composition, pH, texture profile, yield, flowability upon heating and color in the Cheddar- or Emmental- type cheeses made therefrom (Chapter 2, 5 & 6). However, since both plasmin and plasminogen activator are associated with casein micelles, and β-lactoglobulin (β-LG) is an inhibitor of both enzymes (France et al., 2021), whey protein reduced-cheesemilk had a significantly lower plasmin activity than control cheesemilks (Chapter 6). Due to the lower plasmin activity of the whey protein-reduced cheesemilk, the extent of β-casein (urea-PAGE and HPLC) in this cheese were significantly reduced (Chapter 6).

7.2.4 *Heat stability of MCC and its effect on cheesemilk derived therefrom*

Subjecting milk to heat treatments higher than 70 °C denature whey protein including β-LG (Bulca and Kulozik, 2004). The free thiol group exposed on the surface of denatured β-LG can form disulphide bonds with either casein micelles,

soluble κ-casein and other denatured β-LG (Kethireddipalli et al., 2010) or plasmin and plasminogen (Aaltonen and Ollikainen, 2011). As a result, the rennet coagulability and plasmin activity in milk without whey protein reduction are largely reduced after receiving heat treatment of 88 °C × 15 s (Guinee et al., 1997) or 90 °C × 15 s (Benfeldt et al., 1997) respectively, the syneresis levels of such cheese curds are also impaired, leading to increased levels of moisture, pH and proteolysis in the Cheddar cheese made therefrom (Guinee et al., 1997). Due to inclusion of denatured whey protein in the final Cheddar cheese, cheese made from milk heated to 87 °C × 26 s has lower flowability upon heating in comparison to cheese made from pasteurized milk (Rynne et al., 2004). However, after removing 70.29 % of whey protein from the feed milk, the resultant MCC was heated to 90 °C for 15 s without significantly decreasing the rennet coagulation properties and the plasmin activity in the cheesemilk formulated from the MCC. Similarly, the syneresis properties of the cheese curd, the composition, pH, yield, primary proteolysis, texture profile, flowability on heating and volatile compounds in the resultant Cheddar cheeses were also unaffected (Chapters 3 & 4).

However, for the recombined milk formulated from whey protein-reduced MCC powder (53.60 %), heat treatment at 120 °C for 15 s significantly inhibited its rennet coagulation properties and almost eliminated its plasmin activity in cheesemilk (Chapter 5). The contents of fat and FDM, β-casein hydrolysis and flowability in the resultant Emmental cheeses were decreased, and the redness in cheese was increased (Chapter 5 & 6). Since spray drying can impair the heat stability of MCC solution (Beliciu et al., 2012), increasing the whey protein depletion as well as eliminating spray drying should be able to improve the rennet coagulation ability and cheese quality in heat treated whey protein reduced milk.

7.3 Overall conclusions

- The composition of MCC was influenced by the type of membrane, temperature, diafiltrant and number of DF steps during MF. By manipulating these factors, MCC with desired contents of whey protein, β-casein, lactose and minerals can be produced.
- Cheesemilk with target levels of casein, whey protein, lactose and β-casein can be readily formulated from MCC and other membrane streams.
- Increasing the casein content in cheesemilk increased its gel firming rate and total cheesemaking time and also increased the levels of ash, protein and hardness in the resultant cheese. Conversely, the levels of moisture, pH, hydrolysis of α_{s1}-casein, flowability and volatile compounds in the resultant Cheddar cheese were reduced. The reduction of whey protein or β-casein from cheesemilk did not influence its rennet coagulability or indices of cheese quality, except that the whey protein-reduced cheesemilk had lower plasmin activity and its cheese had reduced level of primary proteolysis.
- Heat treatment up to 90 °C × 15 s can be safely applied to whey protein-reduced milk (70.29 %) without negatively affecting its rennet coagulation as well as the quality of the resulting Cheddar cheese;
- The rennet coagulation properties in addition to Emmental cheese qualities were significantly impaired after subjecting the whey protein reduced milk (53.60 %) reconstituted from spray dried-powder to a heat treatment of 120 °C for 15 s.

7.4 Future Research

To expand our knowledge on the (1) effect of MF and DF on the composition of MCC and (2) how to better utilize MF in cheesemaking, future research as described below is recommended:

- When water was used as a diafiltrant, and added to the MF retentate before DF, due to the loss of soluble calcium from MF retentate during the last MF or DF step, the addition of water could induce the solubilisation of CCP from casein micelles to the serum phase of MF retentate (Fox et al., 2015b). Since DF was initiated immediately after water addition, and the dissociation of CCP takes time, even though several researchers suggested that a certain amount of CCP might be removed from the feed milk after DF with water, research is still required to prove this assumption.
- Selectively separating and concentrating casein micelles as MCC for use in cheesemaking, can not only standardize and formulate cheesemilk to a desired casein content, but also mitigate any shortage of milk supply caused by seasonal or regional conditions. Spray drying, freezing and high heat treatment are all good methods to preserve MCC. However, to find out the most suitable method, the following factors need to be evaluated: (1) the shelf life and reconstitutability of MCC stabilized by the various methods; (2) rennet coagulability and plasmin activity of the formulated cheesemilk; (3) the yield, primary proteolysis and other characteristics of the resultant cheeses as well as (4) the comparative energy consumption and cost generated by pre-treatment (spray drying, freezing or high heat treatment), storage, distribution and reconstitution.

- MCC with a β-casein reduction greater than 4.25 % should be prepared, to see if a lower β-casein content in cheesemilk can change the profile of flowability, proteolysis, color and volatile compounds in resultant semi-hard cheeses.
- An up-to-date research based on the work of Papadatos et al. (2003) is required to evaluate if it is profitable, or how profitable it is, to apply MF along with other membrane filtration (UF and RO) approaches as described in this thesis to manufacture cheese and high value dairy ingredients (native whey protein and β-casein) at the same time. The capital costs of the necessary plant and facilities, energy consumption, and retention of technical and trained staff to operate and maintain the membrane equipment used should also be considered.

Overall, as dairy processes evolve, there is a need to understand the effects of such processes on milk and the dairy products manufactured therefrom. This is of particular importance given the complex nature of the relationship between cheese production and maintaining the economic value of whey streams, particularly for use in nutritional applications. Advances in membrane and microfiltration technologies are an important component of this. It is envisaged that the research described in this thesis will help to better inform the research community and dairy processors alike.

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Appendix: Reprints of published articles