Exploring the Impact of Reconstituted Skim Milk Pre-treatments on Partitioning of Caseins and Rennet Activity During Cheese-making

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ABSTRACT

Cheese-making offers the opportunity to exploit the unique flavour and health benefits of fermented milk proteins. The process involves several operations including milk pre-treatments, gelation/curdling and transforming curd into intended cheese. Curdling resulting from destabilisation of casein (CN) micelles is a critical step of the process with the key for many cheeses being the hydrolysis of κ-CN by rennet into para-κ-CN and caseino/glycomacropeptide.

A review of the literature shows that the extent/degree of κ-CN hydrolysis that is required to induce gelation of bovine milk during renneting under normal cheese-making conditions is <80%, despite partial estimation of total macropeptide release in most studies. Estimating the % fraction of hydrolysed κ-CN in the whole sample by mass balance (initial minus the residual amounts measured e.g., by Reverse-phase high performance liquid chromatography (RP-HPLC)) would be the most accurate approach. The accuracy of measuring the degree of κ-CN hydrolysis has implications on the precision of the data in relation to κ-CN's partitioning. Understanding and controlling the partitioning of κ- and other CNs (αs_1-, αs_2- and β- CNs), fat (in full–low fat cheeses) and rennet activity (RA) during cheese-making is essential for preserving the intrinsic cheese quality and improving curd structure, yield and whey quality. Partitioning is mainly influenced by milk pre-treatments and renneting conditions. Regression analysis of existing data on Cheddar cheese suggests that partitioning of CN and fat, cheese yield and whey composition could be improved by standardising milk CN and fat to both optimum levels and ratio. However, while studies on CN partitioning are many, information on partitioning of individual CNs is very limited. What drives partitioning of both CNs and RA is also not well understood, and the clotting

assay for quantifying RA partitioning is very subjective. Therefore, experimental studies evaluating the effect of some reconstituted skim milk pre-treatments (standardisation (8–26% total solids (TS), pre-salting (0–3 *M*, NaCl) and pre-acidification (pH 5–7)) were designed to address some of these gaps.

First, a small amplitude oscillatory method was developed to improve the milk clotting assay. This involves the creation of a calibration curve of objective rennet coagulation time (RCT) vs. the inverse of RA, requiring one spike level only to estimate the unknown residual RA in whey. Interestingly, the concentration of residual RA (International milk clotting units, IMCU/mL whey) appeared to be the same as the initial concentration added to milk. A common RP-HPLC method, modified for whey samples, confirmed the finding which became the basis for developing a general model to estimate RA partitioning. The modified RP-HPLC method was used in a second study examining the effect of reconstituted skim milk concentration under both natural and slight acidification conditions aiming to reveal the driving force. For all milk protein levels, findings were consistent with the first study despite linear positive relationships between protein or moisture and RA retentions, indicating that RA partitioning is not controlled by enzymes-CNs associations.

In the third and fourth studies, the impact of reconstituted skim milk concentration on partitioning of individual CNs and curd structure under natural and slight acidification conditions was examined. Partitioning was assessed by RP-HPLC and Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), whereas curd structure was tested by performing dynamic rheometry and light scattering measurements. Low levels of κ- and α_{s2}- CNs only were found in whey and were attributed to heat-induced complexation of these CNs and whey proteins as confirmed by SDS-PAGE. Their concentrations in whey increased

with increasing milk TS whereas partitioning into whey decreased. This could be explained partly by increased hydrophobic interactions and restricted rearrangements during curd aging as revealed in the fourth study.

In a fifth study, the impact of pre-acidification and pre-salting of reconstituted skim milk was also assessed by RP-HPLC and SDS-PAGE, and as per study three, small amounts of κ- & α_{s2}- CNs (<11%) were detected in rennet whey from unsalted or salted milk samples with ionic strength ≤ 0.9 *M*, NaCl. Greatest losses of all CNs occurred at 3 *M*, NaCl when pH was adjusted to 7 and 5.5, and for this type of milk, pH 6 was found to be optimal. Varying levels of para-κ-CN found in rennet whey from pre-salted milk demonstrate that losses of CNs into rennet whey were caused by an ionic strength- and pH- dependent dissociating effect of NaCl on CN micelles, inducing further κ-/α_{s2}-CNs-whey protein complexation, and inhibitory effect on para-CN aggregation.

In conclusion, current research advances the understanding the effect of pre-treatments of reconstituted skim milk in relation to partitioning of individual CNs and RA during cheese making. Findings could be particularly useful for cheese yield prediction modelling and quality control of both cheese and whey co-products. While CN-CN interactions appeared the driving force for RA partitioning, their role in relation to individual CNs partitioning was partially examined, thus, a full understanding remains to be established in future studies.

Declaration

"I, Joseph Flambeau Kayihura, declare that the PhD thesis entitled "Exploring the Impact of Milk Pre-treatments on Partitioning of Caseins and Rennet Activity During Cheese-making" is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

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Chapter No.	Publication Title	Publication Status • Published • Accepted for publication • In revised and resubmit stage • Under review • Manuscript ready for submission	Publication Details • Citation, if published • Title, Journal, Date of acceptance letter and Corresponding editor's email address • Title, Journal, Date of submission
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List of Abbreviations

LF: Lactoferrin

BSA: Bovine serum albumin

Ig: Immunoglobulin

ANS: 1-anilinonaphthalene-8-sulfonic acid

CCP: Colloidal Ca-phosphate nanoclusters

CFR: Casein-to-fat ratio

CMP: caseinomacropeptide

CN: Casein

F/SNF: fat/Solids-not-fat

GDL: Glucono-δ-lactone

GMP: Caseinoglycomacropeptide

HCF: High concentration factor

IMCU: International milk clotting units

LCF: Low-concentration-factor

LC-MS: Liquid chromatography-Mass spectrometry

MCC: Micellar casein concentrate

MF: Microfiltration

MFC: Microfiltration concentrate

MFR: Microfiltration retentate

MNFS: Moisture-in-non-fat substance

MPC: Milk protein concentrates

NF: Nanofiltration

NPN: Nonprotein Nitrogen

NR: non-reducing

PC: Phosphocasein

PDI: Polydispersity index

PFR: Protein-to-fat ratio

R: Reducing

RA: Rennet activity

RCT: Rennet coagulation time

RFI: Relative fluorescent intensity

RP-HPLC: Reverse-phase high performance liquid chromatography

S/M: Salt-in-moisture

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TCA: Trichloroacetic acid

TFA: Trifluoroacetic acid

TS: Total solids

UF: Ultrafiltration

UFC: Ultrafiltration concentrate

UHT: Ultra-high temperature

WPC: Whey protein concentrate

WPI: Whey protein isolate

α-LA: α-Lactalbumin

α_{s1}- CN: Alpha-s1-casein

α_{s2}- CN: Alpha-s2-casein

B-CN: Beta-casein

β-LG: Beta lactoglobulin

κ-CN: Kappa-casein

Chapter 1 – Introduction to the thesis

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1. Background

Dairy industries wish to maximise their profit as well as improve sustainability and diminish environmental impact by increasing the yield of cheese and process the whey stream into other healthy and valuable products. On the other hand, consumers demand high quality cheeses in terms of flavour, texture and the keeping qualities. It is also important for cheese to have consistent and specific characteristics. This requires controlling milk composition to optimum proportions (Chapman, 1981) since it varies naturally with animal genetics, seasons, age, lactation stage and feeding (Myburgh *et al*., 2012; Stocco *et al*., 2019; Lim *et al*., 2021; Hayes *et al*., 2023; Olaniyan *et al*., 2023). New dairy ingredients of varying composition and functionalities are also constantly emerging from new technologies, such as membrane filtration (Xia *et al*., 2020), which present more opportunities for cheese industries. Both natural and processing induced modifications to composition or structure of proteins alter the cheese making properties of milk (Wedholm *et al*., 2006; Fekadu *et al*. 2005; Ozturk *et al*., 2015; Chapman, 1981; Guinee, Pudja & Mulholland, 1994; Lipkowitz *et al*., 2018; Xia *et al*., 2020).

Variations in milk composition as well as different cheese making protocols affect not only cheese, but also the composition and functionality of whey products (Guinee, O'Kennedy & Kelly, 2006; Outinen *et al*., 2009; Nishanthi, Chandrapala & Vasiljevic, 2017). The most influential milk constituents are fat, CN and Ca, but fat has a passive role during

curd formation although the amount trapped into the curd contributes much on the yield and sensory properties of full fat cheese (Van Slyke & Publow, 1909). CN and Ca on the other hand, play an active role in all cheeses by forming the CN network; hence, their levels in milk are essential for controlling curd structure and the partitioning of constituents. Not only milk composition, but also cheese making conditions such as temperature, renneting extent and pH can modify curd structure (Lucey *et al*., 2000, Madadlou, Khosroshahi & Mousavi, 2005; Ong *et al*., 2011; Liu *et al*., 2014), and may also affect the partitioning of constituents.

A great deal of work has been performed on the gelation properties of various milk ingredients including the impact on recovery of major milk constituents (Guinee, Pudja & Mulholland, 1994; Guinee *et al*., 2007; Everard *et al*., 2011; Xia *et al*., 2020; Kalit *et al*., 2021), and changes in cheese quality (Guinee, Pudja & Mulholland, 1994; Hickey *et al*., 2018; Lipkowitz *et al*., 2018). In addition, typical retentions of total CN have been studied (Bynum & Olson, 1982; Ernstrom, 1980; Hsieh & Pan 2012; Hallén *et al.* 2010; Franceschi *et al.* 2020). However, the current literature is still lacking extensive studies on the partitioning of individual CNs in relation to changes in milk pre-treatments and renneting conditions. There is also limited understanding of why there are variations in distribution of constituents between the curd and whey matrices.

The second most important ingredient during the processing of many cheeses, after milk, is rennet. Like milk, the quality of renn*et al*so varies due to a number of factors such as origin, type of enzymes and their specificity. Traditionally, rennet was meant to be an extract from calf abomasum, but there are many milk clotting enzymes of microbial, plant and recombinant types which are commercially available nowadays (Jaros & Rohm, 2017). The primary enzyme in calf rennet is chymosin (rennin), an aspartyl protease with specific

cleavage at the Phe_{105}–Met_{106} bond of bovine κ-CN. The type and amount of rennet affect cheese structure (Madadlou, Khosroshahi & Mousavi, 2005; Soodam *et al*., 2015), and the amount of active rennet retained into the cheese is essential to enhance the cheese ripening process, which is responsible for development of cheese flavour. However, although some variations occur depending on type of rennet, milk composition and cheese making conditions (Bansal, Fox & McSweeney, 2007), a large portion is recovered in whey, yet undesirable in whey products (Singh & Creamer, 1990), probably because of its hydrolytic effect on serum albumin and immunoglobulin (Jost, Monti & Hidalgo, 1976). Thus, assessment of the RA partitioning between the cheese curd and whey is also necessary for quality control purposes. However, like milk constituents, the literature still lacks clear explanations in regards to what the driving forces for RA partitioning are. Also, the milk clotting assay for quantifying RA appears to be very subjective. This research explores how partitioning of CNs and RA between the curd and whey are affected by some common pre-treatments applied to cheese-milk. The current research presumes that individual CNs behave differently and changes in CN-CN interactions are responsible for controlling the partitioning and curd structure. The present research, however, does not investigate the relationship between individual CNs' behaviour and different specific forms of CN interactions.

2. Research Aims

The major aim was to examine how partitioning of individual CNs and RA during cheese-making vary with some cheese milk pre-treatments. Specifically, the aim was to: (i) develop an objective milk clotting assay for the determination of RA partitioning into rennet whey, (ii) assess the impact of reconstituted skim milk concentration in the protein range of 3 – 9.4% on the partitioning of RA between the curd and whey, (iii) examine the impact of

reconstituted skim milk total solids concentration in the range 8 – 25% on the percentage distribution of individual CNs between rennet curd and whey, and (iv) evaluate the impact of pre-salting (0 – 3 M, NaCl) and pre-acidification (pH 5 – 7) on the percentage distribution of individual CNs between rennet curd and whey.

3. Thesis structure

The current thesis consists of eight chapters: Chapter 1: an introduction providing a brief background and aims of the research. Chapter 2: a review of previous research assessing the accuracy of quantitative approaches for the degree of κ-CN hydrolysis during the primary enzymatic phase, and how different milk standardisation approaches influence the partitioning of CN and fat into Cheddar cheese. Chapter 3: original research which attempts to develop a new method of examining the recovery of active rennet into cheese whey by means of small amplitude oscillatory measurements. Chapter 4: original research analysing the impact of reconstituted skim milk concentration on partitioning of residual RA into rennet whey in an attempt to reveal the driving force. Chapter 5: original research examining how individual CNs partition during renneting of concentrated milk. Chapter 6: original research assessing the structural changes occurring during formation and aging of rennet curd from concentrated milk. Chapter 7: original research looking at how individual CNs partition under different pre-acidification and pre-salting levels. Chapter 8: the general conclusion and future recommendations.

References

Bansal, N., Fox, P. F., & McSweeney, P. L. H. (2007). Factors affecting the retention of rennet in cheese curd. *Journal of Agriculture and Food Chemistry, 55, 9219–9225.*

Bynum, D. G., & Olson, N. F. (1982). Influence of curd firmness at cutting on cheddar cheese yield and recovery of milk constituents. *Journal Dairy Science, 65, 2281–2290.*

Chapman, H.R. (1981). Standardisation of milk for cheesemaking at research level. *Journal of the Society of Dairy Technology, 34, 147 – 152.*

Ernstrom, C.A. (1980). A workable pricing system based on cheese yield. *Utah State University 4th Biennial Cheese Industry Conference, Logan, UT.*

Everard, C. D., O'Callaghan, D. J., Mateo, M. J., Castillo, M., Payne, F. A., & O'Donnell, C. P. (2011). Effects of milk composition, stir-out time, and pressing duration on curd moisture and yield. *Journal of Dairy Science, 94, 2673–2679.*

Fekadu, B., Soryal, K., Zeng, S., Van Hekken, D., Bah, B., & Villaquiran, M. (2005). Changes in goat milk composition during lactation and their effect on yield and quality of hard and semi-hard cheeses. *Small Ruminant Research, 59, 55–63.*

Franceschi, P., Malacarne, M., Formaggioni, P., Faccia, M., & Summer, A. (2020). Quantification of the effect of the cattle breed on milk cheese yield: comparison between Italian brown Swiss and Italian Friesian. *Animals, 10, 1331*.

Guinee, T. P., Mulholland, E. O., Kelly, J., & Callaghan, D. J. O. (2007). Effect of Protein-to-Fat Ratio of Milk on the Composition, Manufacturing Efficiency, and Yield of Cheddar Cheese. *Journal of Dairy Science, 90, 110–123.*

Guinee, T. P., O'Kennedy, B. T., & Kelly, P. M. (2006). Effect of Milk Protein Standardization Using Different Methods on the Composition and Yields of Cheddar Cheese. *Journal of Dairy Science, 89, 468–482.*

Guinee, T. P., Pudja, P. D., & Mulholland, E. O. (1994). Effect of milk protein standardization by ultrafiltration on the manufacture, composition and maturation of Cheddar cheese. *Journal of Dairy Research,* 61, 117-131.

Hallén, E., Lundén, A., Allmere, T., & Andrén, A. (2010). Casein retention in curd and loss of casein into whey at chymosin-induced coagulation of milk. *Journal of Dairy Research, 77, 71–76.*

Hayes, E., Wallace, D., O'Donnell, C., Greene, D., Hennessy, D., O'Shea, N., Tobin, J. T., & Fenelon, M. A. (2023). Trend analysis and prediction of seasonal changes in milk composition from a pasture-based dairy research herd. *Journal Dairy Science, 106, 2326–2337.*

Hickey, C. D., O'Sullivan, M. G., Davis, J., Scholz, D., Kilcawley, K. N., Wilkinson, M. G., & Sheehan, J. J. (2018). The effect of buttermilk or buttermilk powder addition on functionality, textural, sensory and volatile characteristics of Cheddar-style cheese. *Food Research International, 103, 468–477.*

Hsieh, J., & Pan, P. (2012). Proteomic profiling of the coagulation of milk proteins induced by chymosin. *Journal of Agricultural and Food Chemistry, 60, 2039−2045.*

Jaros, D., & Rohm, H. (2017). Rennets: Applied Aspects. In P. L. H., McSweeney, P. F., Fox, P. D., Cotter, & D. W. Everett (Eds.). *Cheese: Chemistry, Physics and Microbiology* (pp. 53). London: Elsevier.

Jost, R., Monti, J. C., & Hidalgo, J. (1976). Natural proteolysis in whey and susceptibility of whey proteins to acidic proteases of rennet. *Journal of Dairy Science, 59, 1568–1573.*

Kalit, S., Kalit, M. T., Špehar, I. D., Salajpal, K., Samaržija, D., Anušić, J., & Rako, A. (2021). The influence of milk standardization on chemical composition, fat and protein recovery, yield and sensory properties of Croatian PGI Lički Škripavac cheese. *Foods, 10, 690.*

Lim, D., Mayakrishnan, V., Ki, K., Kim, Y., & Kim, T. (2021). The effect of seasonal thermal stress on milk production and milk compositions of Korean Holstein and Jersey cows. *Animal Bioscience, 34, 567–574.*

Lipkowitz, J. B., Ross, C. F., Diako, C., & Smith D. M. (2018). Discriminating aging and protein-to-fat ratio in Cheddar cheese using sensory analysis and a potentiometric electronic tongue. *Journal of Dairy Science, 101, 1990–2004.*

Liu, X. T., Zhang, H., Wang, F., Luo, J., Guo, H. Y., & Ren, F. Z. (2014). Rheological and structural properties of differently acidified and renneted milk gels. *Journal of Dairy Science, 97, 3292–3299.*

Lucey, J. A., Tamehana, M., Singh, H., & Munro, P. A. (2000). Rheological properties of milk gels formed by a combination of rennet and glucono-δ-lactone. *Journal of Dairy Research, 67, 415–427.*

Madadlou, A., Khosroshahi, A., & Mousavi, M.E., 2005. Rheology, microstructure, and functionality of low-fat Iranian white cheese made with different concentrations of rennet. . *Journal of Dairy Science, 88, 3052–3062.*

Myburgh, J., Osthoff, G., Hugo, A., de Wit, M., Nel, K., & Fourie, D. (2012). Comparison of the milk composition of free-ranging indigenous African cattle breeds. *South African Journal of Animal Science, 42, 1–14.*

Nishanthi, M., Chandrapala J., & Vasiljevic, T. (2017). Properties of whey protein concentrate powders obtained by spray drying of sweet, salty and acid whey under varying storage conditions. *Journal of Food Engineering, 214, 137–146.*

Olaniyan, O. F., Kaya, I., & Secka, A. (2023). Assessment of composition and physical properties of the Gambian N'Dama cow milk. *Journal of Food Composition and Analysis, 115, 104961.*

Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. (2013). Microstructure and Composition of Full Fat Cheddar Cheese Made with Ultrafiltered Milk Retentate. *Foods, 2, 310-331.*

Outinen, M., Rantam¨aki, P. & Heino, A. (2009). Effect of milk pre-treatment on the whey composition and whey powder functionality. *Journal of Food Science, 00, 1–10.*

Ozturk, M., Govindasamy-Lucey, S., Jaeggi, J. J., Johnson, M. E., & Lucey, J. A. (2015). Low-sodium Cheddar cheese: Effect of fortification of cheese milk with ultrafiltration retentate and high-hydrostatic pressure treatment of cheese. *Journal of Dairy Science, 98, 6713–6726.*

Singh, H., & Creamer, L. K. (1990). A sensitive quantitative assay for milk coagulants in cheese and whey products. *Journal of Dairy Science, 73, 1158–1165.*

Soodam, K., Ong, L., Powell, I. B., Kentish, S. E., & Gras, S. L. (2015). Effect of rennet on the composition, proteolysis and microstructure of reduced-fat Cheddar cheese during ripening. *Dairy Science and Technology, 95, 665–686.*

Stocco, G., Summer, A., Malacarne, M., Cecchinato, A., & Bittante, G. (2019). Detailed macro- and micromineral profile of milk: Effects of herd productivity, parity, and stage of lactation of cows of 6 dairy and dual-purpose breeds. *Journal Dairy Science, 102, 9727–9739.*

Van Slyke, L. L., & Publow, C. A. (1909). The science and practice of cheese-making. *Orange Judd Company, New York.*

Wedholm, A., Larsen, L. B., Lindmark-Månsson, H., Karlsson, A. H., & Andrén, A. (2006). Effect of protein composition on the cheese-making properties of milk from individual dairy cows. *Journal of Dairy Science, 89, 3296–3305.*

Xia, X., Tobin, J. T., Sharma, P., Fenelon, M., McSweeney, P. L. H., & 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 110, 104796.*

Chapter 2 – Literature review

This chapter is constituted of two review articles: the first article (Chapter 2A) entitled "Extent of κ-casein hydrolysis during renneting of bovine milk: a critical assessment of the analytical and estimation approaches" has been published by Joseph F. Kayihura in a peer-reviewed journal: Food Science and Nutrition (2023). (https://doi.org/10.1002/fsn3.3868). The second article (Chapter 2B) entitled "Partitioning of casein and fat in Cheddar cheese manufacturing as affected by cheese milk standardisation: A review" has been published by Joseph F. Kayihura in a peer-reviewed journal: International Journal of Dairy Technology (2023). (https://doi.org/10.1111/1471-0307.13009).

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REVIEW

Extent of κ-casein hydrolysis during renneting of bovine milk: A critical assessment of the analytical and estimation approaches

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Abstract

Renneting is an enzymatic process that turns milk into curd which is then transformed into cheese. Rennet-induced coagulation of caseins (CNs) is the critical step during this process and the key is the primary hydrolysis of κ-CN's Phe_{105}-Met_{106} bond by chymosin. This article comprehensively reviews the existing data on the extent/degree of κ-CN hydrolysis during renneting of bovine milk and critically evaluates its determination methods. The data show that under normal cheese-making conditions, milk gelation occurs at a degree of κ-CN hydrolysis <80%, which varies due to several factors including analytical and estimation approaches. The common approach involves isolating the macropeptides released, by precipitating whey proteins and residual CN in 1%–12% trichloroacetic acid (TCA), then assuming that the maximum amount obtained is 100% κ-CN hydrolysis. The drawback is that the estimated degree of κ-CN hydrolysis may be higher than the actual value as TCA partially precipitates the macropeptide fractions. Moreover, macropeptide isolation seems unnecessary based on current advances in chromatographic and electrophoretic techniques. The present work proposes a simple mass balance-based approach that will provide accurate estimates in future studies. The accuracy of measuring the degree of κ-CN hydrolysis has implications on the precision of the data in relation to its partitioning (% distribution between the curd and whey) which is essential for improving whey quality.

KEYWORDS
bovine milk, caseinoglycomacropeptide, caseinomacropeptide, cheese, rennet, κ-CN hydrolysis

1 | INTRODUCTION

Rennet-induced coagulation of CNs (approximately 80% of bovine milk proteins) is the critical step during the processing of many cheese varieties. The key to achieving the coagulation of CNs is the primary proteolytic/hydrolytic action of rennet on κ-CN. CNs (41% α_{s1}, 8% α_{s2}, 37% β, and 14% κ) (Kayihura, 2023c) in milk occur primarily in the form of a stable colloidal dispersion owing to κ-CN's functional role as a (i) chain terminator during micellar assembly due to its lack of phosphoserine clusters that limit further networking and (ii) micellar stabilizer (Huppertz, 2013; Lucey, 2022). κ-CN located mainly on the surface as a hairy layer (or brush) provides micellar stability by steric and electrostatic repulsive interactions between its hydrophilic C-terminal moieties (Mackinlay & Wake, 1971; Vreeman et al., 1986). The layer was estimated to be 4.5–7 nm thick (Holt & Horne, 1996; van Hooydonk,

Hagedoorn, & Boerrigter, 1986) although 10–12 nm has also been reported (Dalgleish, 1998; Sandra et al., 2007).

The discovery of κ-CN as a stabilizer or protective colloid dates back to 1950s (Waugh & von Hippel, 1956) whereas the hairy layer model was proposed in 1996 but later supported by experiments in 1981 (Holt & Horne, 1996; Walstra et al., 1981). Thus, a suitable explanation for the rennet-induced destabilization of CN micelles resulting in milk-curd formation is that rennet enzymes cut off the stabilizing hairy layer allowing the hydrophobic and Ca-sensitive core components to aggregate (Horne & Lucey, 2017; Huppertz et al., 2018). Chymosin, the main enzyme in rennet, specifically hydrolyzes κ-CN's Phe_{105}-Met_{106} bond producing two peptides, namely, the hydrophobic N-terminal fraction (f1-105) known as para-κ-CN and the hydrophilic C-terminal fraction (f106-169) referred to as caseinomacropeptide (CMP) also called aglycosylated caseinomacropeptide (aCMP) if no carbohydrate, or glycomacropeptide (GMP) also called caseinoglycomacropeptide (cGMP) or glycosylated caseinomacropeptide (gCMP) if one or more carbohydrates are attached (Boutrou et al., 2008; Karimidastjerd & Gulsunoglu-Konuskan, 2023; Lucey, 2022; Sunds et al., 2019). The primary structures of κ-CN and both of its peptides have been established (Mercier et al., 1973) and proportions of glycosylated forms may vary depending on genetic factors (Bonfatti et al., 2014).

It is believed that there is a minimum degree of κ-CN hydrolysis necessary to induce milk gelation. Therefore, the primary enzymatic phase and subsequent aggregation of the para-CN micelles also referred to as the secondary phase are fundamental to cheese-making (Kalan & Woychik, 1965; Kelly et al., 2008; Lucey, 2022). However, quantitative aspects require careful assessment as there are marked variations in renneting conditions some of which are irrelevant to cheese-making, and most methods available lead to partial estimates. This review aims to comprehensively analyze the existing data on the primary phase of rennet action on κ-CN, underline the major factors, and provide a critical assessment of the analytical and estimation approaches. Specifically, the article attempts to answer the following questions: (a) what is the proportion (%) of the C-terminal macropeptide (CMP and GMP) fraction of κ-CN? (b) what is the distribution (%) of GMP? (c) what is the extent of hydrolysis (%) necessary to induce milk gelation under normal cheese-making conditions? (d) what is the most reliable method to determine the degree of κ-CN hydrolysis during renneting? The criteria for the articles selected (Tables 1 and 2) were as follows: (1) original articles, (2) sample type: bovine milk, (3) coagulant: calf rennet, chymosin, or recombinant chymosin, and (4) the coagulant strength (IMCU) indicated.

2 | PROPORTION OF TOTAL MACROPEPTIDE FRACTION OF BOVINE κ-CN

κ-CN in bovine milk is 12%–15% of total CN or 3–4 g/L of milk (Donnelly & Barry, 1983; Léonil & Mollé, 1991; Lucey, 2022; Nilsson et al., 2020; Phelan, 1981; Wake, 1959; Waugh & von Hippel, 1956) and the macropeptide in cheese whey is 1.2–1.68 g/L (Doultani et al., 2003; Manso & López-Fandiño, 2004). Therefore, if the actual cheese yield (Cheddar and Gouda, as examples) is 10%–11% of milk, the total macropeptide would be approximately 35.6%–37.8% of κ-CN. This is in line with 36.4%–37.9% based on the monomeric molecular weights of 6.8 KDa for CMP and ~8 KDa for GMP (Reddy & Kinsella, 1990; Shin & Jang, 2002; Swaisgood, 1975) and 19–22 KDa for nonglycosylated and glycosylated κ-CN (Huppertz, 2013; Swaisgood, 1975; Swaisgood et al., 1964), considering up to five trisaccharide units each contributing 0.657 KDa (Swaisgood, 1975). Both ranges are close to theoretical values of 4% of total CN or 1/3 of κ-CN equivalent to 33% (Chapman, 1981), 30% of total κ-CN nitrogen (Beeby & Nitschmann, 1963) as well as the 4%–5% of total CN nitrogen (Alais et al., 1953) equivalent to a maximum of 33.3% based on κ-CN content of 15%. This suggests that the degree of hydrolysis reported in terms of macropeptide (CMP and GMP) release (% of maximum) must demonstrate that the maximum was estimated based on initial κ-CN content and was approximately 36%–38% of total κ-CN. However, although values would vary depending on the genetic variant and distribution of GMP discussed below, this was not clearly elucidated in all relevant studies found in the literature.

3 | DISTRIBUTION OF GMP IN BOVINE κ-CN

According to literature (Caroli et al., 2009), bovine κ-CN appears classified into 14 genetic variants (A, A^I, B, B^2, C, D, E, F^1, F^2, G^1, G^2, H, I, and J) of which AA and BB are the most common, GG and HH are common while the rest are rather common or rare. Heterozygous variants such as AB, AE, and BE have also been identified (Hallén et al., 2010; Jensen et al., 2015). The AA variant is considered the parent protein with 169 amino acids whereas its caseinomacropeptide fraction consists of 64 amino acids (f106–169) and contains all the posttranslational phosphorylation and glycosylation. The phosphorylation and glycosylation levels vary from 0 to 3P and 0–6 residues, respectively (Huppertz, 2013; Sheng et al., 2022), whereas up to nine glycan residues were identified for BB variant (Vreeman et al., 1986). The five major glycoforms have been reported (Sunds et al., 2019) and all glycans are known to be attached to threonine residues (Huppertz, 2013). The distribution of GMP appears to be important because numerous studies have shown that the rate of GMP cleavage is slower in comparison with the CMP isoform, and this behavior was attributed to higher electronegativity of the former that retards the rennet access to the active site (Ferron-Baumy et al., 1992; Jensen et al., 2015; Shin & Jang, 2002; van Hooydonk et al., 1984; Wheelock & Knight, 1969). Although some studies agree on 40%–50% with BB variant exhibiting the highest levels (Bonfatti et al., 2014; Thomä et al., 2006), the degree of glycosylation found in literature is not consistent and ranges from <20% (Sheng et al., 2022) to >90% (Vasbinder et al., 2003). It varies with total κ-CN content and is dependent on animal genetics and isolation techniques; hence, the actual distribution may still be controversial.

TABLE 1 Degree of κ-CN hydrolysis (%) during renneting, stage or time of measurement, type of milk sample, amount of rennet (International milk clotting units, IMCU/mL of milk) as well as type and strength of rennet used.

Degree (%)	Stage and/or time (min)	Sample type	Rennet (IMCU/mL)	Rennet type and strength	References
60	Gelation	Nonfat dry milk, 12% TS	0.00768	>95% chymosin	He (1990)
~68	Gelation	Nonfat dry milk, 36% TS	0.00768		
<60	Gelation	Above samples with 0.01–0.80 M $CaCl_2$	0.00768		
<35	Gelation	Nonfat dry milk, 12% TS with 0.05 M $CaCl_2$	0.00768		
85	Gelation (4 h)	Fresh skim milk	0.0075	Rennet, 150 IMCU/mL	Vasbinder et al. (2003)
85	Gelation (4 h)	Heated skim milk (70°C/10 min)	0.0075		
67–95	After 4 h	Heated skim milk (90°C/10 min)	0.0075		
75	Gelation at pH 5.8	Reconstituted skim milk, 10% TS	0.09975	Chymostar, 570 IMCU/mL	Li and Dalgleish (2006)
55	Gelation at pH 5.4	Same sample above	0.01653		
100	After 1 h	Commercial pasteurized skim milk (3.4% protein)	0.18	Fermentation produced chymosin, 180 IMCU/mL	Bansal et al. (2007)
50	Coagulation (~15 min)	Skim milk with 2.75% CN, pH 5.8	0.01	Chy-Max Extra, strength not shown	Karlsson et al. (2007)
<20	Coagulation (27 min)	Ultra-filtered (UF) skim milk with 19.8% CN, pH 5.8	0.01		
60–67	After 7 h	Reconstituted skim milk (10%)	0.0036	Recombinant chymosin, 180 IMCU/mL	Renan et al. (2007)
50	After 7 h	Reconstituted skim milk (10%) and heated (90°C/10 min)	0.0036		
65–70	Flocculation	Skim milk	0.018–0.07	Chymostar, double strength	Sandra et al. (2007)
>90	Gelation (45–143 min)	Skim milk	0.018–0.07		
100	Formation of stiff gel	Raw milk	0.058	Chymogen, 290 IMCU/mL	Taylor and Woonton (2009)
32.7–85.4	Formation of stiff gel depending on heat (1–3 h)	Raw milk heated at: 65°C/15 s; 72°C/15 s; 90°C/15 s; 100°C/10 min	0.058		
11	Gelation, pH 5.4	Preheated (85°C/300 s) skim milk	0.000314	Chymostar, strength not shown	Cooper et al. (2010)
26	Gelation, pH 5.3	Preheated skim milk (85°C/20 s)	0.00126		
80, 95, 100	Flocculation, gelation (39.3 min), ~40 min	Fresh skim milk	0.018	Chymostar, single strength rennet	Titapiccolo, Corredig, and Alexander (2010)
80, 95, 100	Flocculation, gelation (37.5 min), final	Skim milk	0.018	Chymostar, single strength rennet	Titapiccolo, Alexander, and Corredig (2010)
75, 90, 100	Flocculation, gelation (25.6 min), final	Homogenized milk (34.5 MPa)	0.018		
78–82	Gelation (109 min)	Pasteurized skim milk (74°C/23 s) & 2, 3× UF concentrates	0.00296	Chy-max Ultra, 790 IMCU/mL	Salvatore et al. (2011)
90	Gelation (40–50 min)	Skim milk (2.9% protein) and 3, 5× UF concentrates	0.034	Chy-max Ultra, 790 IMCU/mL	Sandra et al. (2011)

(Continues)

TABLE 1 (Continued)

Degree (%)	Stage and/or time (min)	Sample type	Rennet (IMCU/mL)	Rennet type and strength	References
>90	Gelation point (30.3 min)	Fresh skim milk	0.018	Chymostar, single strength	Gaygadzhiev et al. (2012)
94–98	Gelation (53–54 min)	Skim milk	0.034	Chy-Max Ultra, 790 IMCU/mL	Sandra et al. (2012)
85–90	Gelation (32–39 min)	Skim milk with 1 mM $CaCl_2$	0.034		
100	~60 min	Skim milk without or with 1 mM $CaCl_2$	0.034		
80–85	40–45 min	Milk protein concentrate (MPC) in water, 6% protein	0.034	Chy-max Ultra, 790 IMCU/mL	Sandra and Corredig (2013)
40, 85	Gelation (10.2 min), >60 min	MPC in water, 6% protein, with 0.04% $CaCl_2$	0.034		
>85	Gelation (38.6 min)	MPC in skim milk, 6% protein	0.034		
85	Gelation (51.5 min)	MPC in water (6% protein), dialyzed against skim milk	0.034		
<70	Gelation (21 min)	Commercial pasteurized skim milk (3.2% protein), pH 6	0.034	Chy-max Ultra, 790 IMCU/mL	Eshpari et al. (2015)
85	After ~70 min	Commercial pasteurized skim milk (3.2% protein), pH 6	0.034		
>90	After 40 min	Individual Jersey and Holstein skim milk with different genetic variants of κ-CN	0.035	Chy-max Extra, 615 IMCU/mL	Jensen et al. (2015)
10, 20, 25	15 min	Commercial pasteurized skim milk, pH 6.62	0.01, 0.02, 0.03	Chy-Max Plus, 200 IMCU/mL	Sinaga et al. (2016)
50	30 min	Same sample above	0.03		
>70	After 60 min	Same sample above	0.03		
100	After 90 min	Same sample above	0.03		
60	Aggregation point	Same sample above	0.03		
85	Gelation (59–60 min)	Commercial skim milk (3.29% protein, pH 6.66) and 3, 5× UF concentrates	0.031	Chy-max Ultra, 790 IMCU/mL	Zhao and Corredig (2016)
85–90	Gelation (80–100 min)	Same samples above with 300 mM NaCl	0.031		
70	Gelation (>30 min)	CN solution (2.64%, pH 6.6–6.8) with whey protein ratio of 4:1	0.035	Chy-max Plus FPC, 200 IMCU/mL	Gamlath et al. (2018)
70	Gelation (>30 min)	Same sample above with whey protein ratio of 4:1	0.07		
50	Gelation (<14 min)	Same sample above with whey protein ratio of 0.03:1	0.035		
67	40 min	Fresh skim milk, pH 6.5	0.09 IMCU/mL	Chymax Plus, 200 IMCU/mL	Nilsson et al. (2020)
164	40 min	Fresh skim milk, pH 6.5	0.09 IMCU/mL		
52	40 min	Fresh skim milk, pH 6.5	0.09 IMCU/mL		

TABLE 2 Isolation and test methods applied to quantify the extent of κ-CN hydrolysis by rennet and the results presented as an indication of the calculation/estimation approach.

Isolation method	Test method	Results presented/reported	References
8% TCA	Beckman high-performance analyzer, System 6300 & spectrophotometric	Moles of amino acids/mL and % hydrolysis at clotting, but calculations are not clearly shown	He (1990)
2% Acetic acid/ Na-acetate	RP-HPLC	Degree of hydrolysis (CMP, % of values obtained for fresh milk)	Vasbinder et al. (2003)
2, 8 & 12% TCA	RP-HPLC	CMP (% of values obtained for fresh milk)	
	RP-HPLC	Degree of hydrolysis (% of values obtained for fresh milk)	
8% TCA	RP-HPLC	GMP (% of maximum value for unheated milk)	Li and Dalgleish (2006)
8% TCA	RP-HPLC	Peak area vs. time (maximum peak considered 100%)	Bansal et al. (2007)
12% TCA	RP-HPLC	Storage modulus vs. hydrolysis of κ-CN (%), calculation not shown	Karlsson et al. (2007)
pH 4.6	RP-HPLC	CMP (% of values obtained using unheated milk for 24 h)	Renan et al. (2007)
8% TCA	RP-HPLC	CMP (% calculated using the fit of the first-order reaction)	Sandra et al. (2007)
Cation-exchange	RP-HPLC	GMP (% of GMP released from raw milk)	Taylor and Woonton (2009)
2% TCA	RP-HPLC	CMP (% of maximum peak area for unheated milk with 0.0351 IMCU/mL rennet)	Cooper et al. (2010)
2% TCA	RP-HPLC	CMP (% of the maximum assumed to be 100%)	Titapiccolo, Corredig, and Alexander (2010)
2% TCA	RP-HPLC	CMP (% of maximum area of control taken as 100%)	Titapiccolo, Alexander, and Corredig (2010)
2% TCA	RP-HPLC	CMP peak area (% of maximum peak area produced with 0.0710 IMCU/mL)	Salvatore et al. (2011)
2% TCA	RP-HPLC	CMP (% of maximum peak area considered 100%)	Sandra et al. (2011)
2% TCA	RP-HPLC	CMP (% relative to the maximum peak area for each sample)	Gaygadzhiev et al. (2012)
2% TCA	Referred to Chaplin and Green (1980)	CMP (% of maximum peak area for each sample considered 100%)	Sandra et al. (2012)
2% TCA	RP-HPLC	CMP (% of maximum peak area for each sample considered 100%)	Sandra and Corredig (2013)
2% PCA	RP-HPLC	CMP (% of maximum peak area for each sample considered 100%)	Eshpari et al. (2015)
1% TCA	LC–MS	Reported hydrolyzed κ-CN (% of intact κ-CN) but actual results presented are extracted ion chromatographic area vs. time	Jensen et al. (2015)
6% TCA	RP-HPLC	CMP (% of maximum peak area considered as 100%)	Sinaga et al. (2016)
2% TCA	RP-HPLC	CMP (% of maximum peak area considered as 100%)	Zhao and Corredig (2016)
4.1% TCA	RP-HPLC	CMP concentration (normalized to the maximum)	Gamlath et al. (2018)
	CE	ratios of para-κ-CN, CMP and intact κ-CN to initial κ-CN	Nilsson et al. (2020)

4 | DEGREE OF κ-CN HYDROLYSIS UNDER CONDITIONS RELEVANT TO CHEESE-MAKING

Cheese-making generally involves three major stages (Figure 1a): (1) milk pretreatments including standardization (e.g., casein-to-fat ratio), pasteurization (e.g., 72°C/15 s), cooling to renneting temperature (e.g., 30–32°C), addition of $CaCl_2$ and preacidification (e.g., to pH 6.5); (2) gelation or curd formation involving the primary enzymatic phase, secondary phase (aggregation of CNs in presence of Ca ions which neutralize the negatively charged residues and form crosslinks between para-casein micelles) and milk clotting; (3) post-gelation treatments that turn the curd into the final consumer product (cheese). As mentioned earlier, the enzymatic hydrolysis of κ-CN (Figure 1b) is the most critical step during cheese-making because it is the prerequisite for curd formation. Thus, fully understanding the

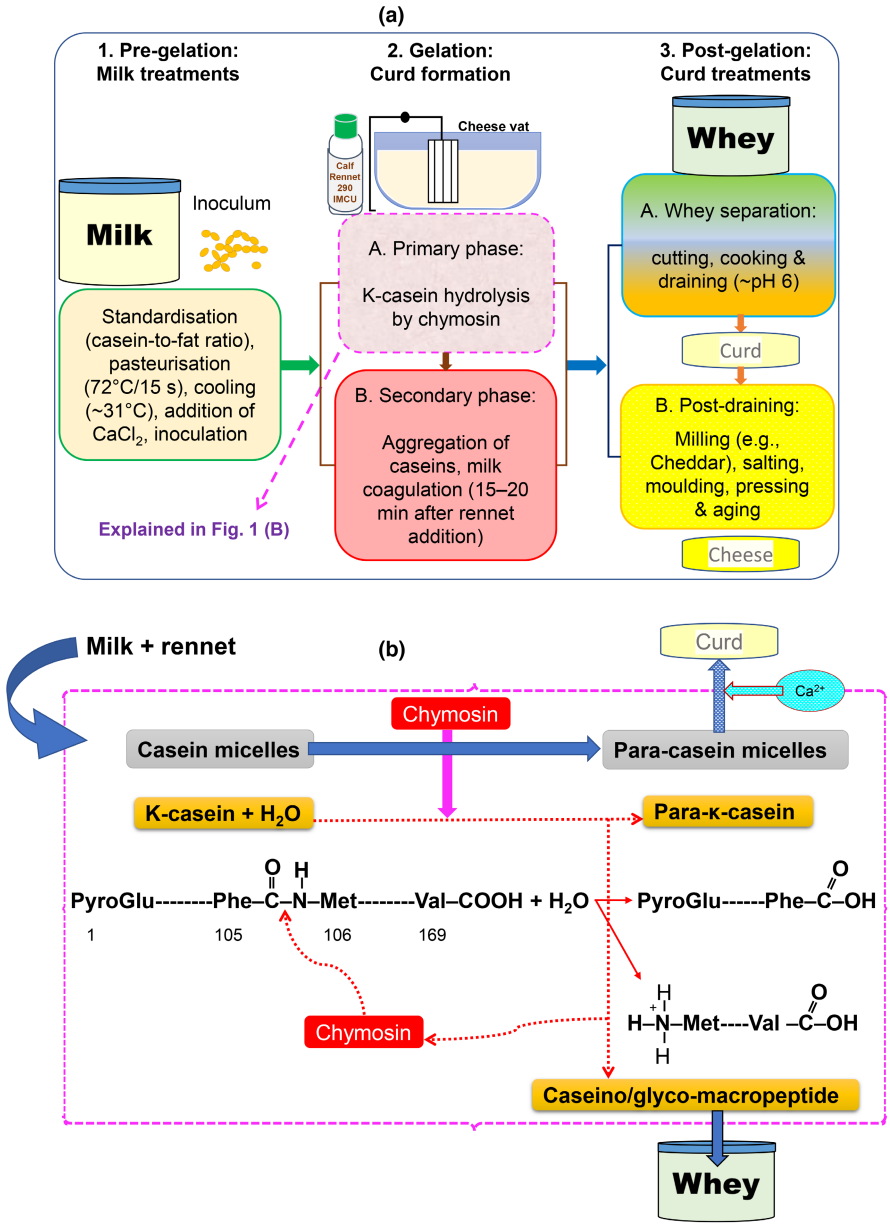

FIGURE 1 (a): Major stages of the general aged cheese-making process. (b): Primary enzymatic phase of κ-CN hydrolysis by chymosin at Phe105-Met106 bond which releases the caseinomacropeptide [soluble C-terminal fraction (f106–169)] into whey allowing para-κ-CN [hydrophobic fraction (f1–105)] together with other micellar components (collectively referred to as para-casein micelles) to aggregate. Further details of the reaction mechanism are available (Palmer et al., 2010; Yegin & Dekker, 2013.

extent and kinetics of rennet action on κ-CN is essential. Most results presented in the literature were obtained at gelation point which is one of the parameters commonly used to characterize the rennet coagulation behavior of milk (Lu et al., 2017; Lucey & Fox, 1992; Zhao & Corredig, 2016) as well as to test the rennet activity and its partitioning (Kayihura et al., 2022). Values at this phase are, therefore, very important as they represent the extent of hydrolysis necessary to induce CN aggregation. The values obtained at different times along the enzymatic phase can also reflect the conversion rate.

It is generally assumed that over 85% κ-CN hydrolysis is necessary to induce the aggregation phase (Dalgleish, 1979; Green et al., 1978) or nearly complete at clotting stage (McMahon et al., 1984; Wilson & Wheelock, 1972). However, as shown in Table 1 and other studies not considered in this review (Table S1), values vary widely due to several factors discussed below. Moreover, considering conditions relevant to cheese-making such as bovine whole or skim milk with at least 10% solids, pH 6–6.7, a normal clotting time of ~20 min after chymosin addition (Lu et al., 2017), or presence of 0.01%–0.02% $CaCl_2$, the experimental data found in the literature provide sufficient evidence to support a degree of κ-CN hydrolysis <80%. For example, a recent study on fresh milks at pH 6.5 indicates a degree of κ-CN hydrolysis of 52%–67% after 40 min (Nilsson et al., 2020). Similarly, ~80% para-κ-CN release found after 40 min indicates that gelation occurred at a much lower degree of κ-CN hydrolysis based on the clotting time (6–7 min) shown for unheated reconstituted skim milk (Anema et al., 2007). Eshpari et al. (2015) also found a degree <70% CMP release at gelation point of 21 min. Furthermore, although Lieske et al. (1996) reported 90% GMP release estimated at the time milk clotted, CMP release was 60%, indicating that the total macropeptide release was about 70% considering a GMP proportion of 38%–39% indicated by the same authors. According to He (1990) and van Hooydonk, Boerrigter, and Hagedoorn (1986), the degree of

κ-CN hydrolysis necessary for the onset of aggregation at a standard milk concentration (12% TS) is 60%, also in agreement with Sinaga et al. (2016) for a commercial pasteurized skim milk at pH 6.62.

He (1990) argued that higher degree of κ-CN hydrolysis quoted in many studies based on a kinetic study by Dalgleish (1979) is only applicable to highly diluted milk. Under very dilute conditions, He (1990) and others (Bringe & Kinsella, 1986a, 1986b; Dalgleish, 1979; Dalgleish et al., 1981; Pierre, 1983) agree on a minimum ~90% hydrolysis at clotting although at a normal renneting temperature of 30°C, Carlson et al. (1987b) also showed a critical conversion of 60% for reconstituted skim milk with 2% solids. In addition, another most cited study is that of Green et al. (1978) in which 86% hydrolysis at the start of rise in viscosity was reported based on an increase in Abs_{217}; however, the macropeptide isolated was GMP only. In general, the degree of κ-CN hydrolysis >80% at gelation point is most likely due to partial estimates of the total amount of the macropeptides released, conditions retarding the aggregation phase allowing longer reaction times, or maximum values considered 100% (Figure 2). For longer reaction times, 100% were reported in several studies (Table 1) but with no evidence that these are actually total values as they were not estimated based on initial κ-CN content in milk. This is very important because as discussed later, calculations based on an underestimated total value could certainly lead to higher estimates of the degree of κ-CN hydrolysis than actual values.

5 | FACTORS AFFECTING THE ESTIMATED DEGREE OF κ-CN HYDROLYSIS

5.1 | Milk, coagulant, and additive concentrations and pH

Enzyme-to-substrate ratio is a critical factor when studying the kinetics of κ-CN hydrolysis as the rate was found to increase proportionally with the amount of rennet (Bingham, 1975; Castle & Wheelock, 1972; Sandra et al., 2007; van Hooydonk et al., 1984) and decreases linearly with an increase in CN concentration (Le Feunteun et al., 2012). For a model solution of 1% κ-CN and rennet applied at a rate of 0.1 μg/mL, about 40% of intact κ-CN was found after 30 min of reaction whereas a considerable reduction was evident when rennet was increased to 1 μg/mL (Bingham, 1975). On the other hand, at a constant pH of 5.8 and rennet concentration of 0.01 IMCU/mL, a degree of κ-CN hydrolysis <20% was reported for skim milk concentrated 7× (19.8% CN content) by ultrafiltration (UF) (Karlsson et al., 2007). van Hooydonk et al. (1984) attributed the retarded enzymatic phase for UF-concentrated milk to low effective diffusion rate of the enzyme. In contrast, it was reported (Sandra et al., 2011; Zhao & Corredig, 2016) that skim milk concentration by UF up to 5× did not affect the enzymatic phase. Furthermore, using the same rennet concentration of 0.00768 IMCU/mL, He (1990) showed that the extent of hydrolysis at gelation point (68%) for reconstituted skim milk with 36% solids was slightly higher than that obtained with a 12% TS sample (60%). Similar results (60%–67%) were obtained with 10% reconstituted skim milk using 0.0036 IMCU/mL (Renan et al., 2007). Unlike disagreements between some studies regarding the degree of κ-CN hydrolysis at gelation point, the maximum is independent of both milk protein and coagulant concentrations (Castle & Wheelock, 1972; Garnot & Corre, 1980).

Increasing ionic strength of milk by the addition of $CaCl_2$ or NaCl may promote or retard both phases of renneting depending on the concentration (Famelart, 1994; Famelart et al., 1999; Klandar et al., 2007). Adding NaCl to cheese milk is most common in Domiati-style cheeses only, whereas for most other cheeses, $CaCl_2$ is often added to milk before renneting in order to increase the aggregation rate of para-CN micelles, reduce the gelation time, and improve curd firmness and yield (Sandra et al., 2012). The degree of κ-CN hydrolysis at gelation point decreases with increasing concentration of $CaCl_2$ to values ≤ 0.05 M. A reduction estimated at that point was about 3%–10% when 0.6–1.8 mM $CaCl_2$ was added to skim milk although the effect was not found when the pH was readjusted to 6.7 (He, 1990; Sandra et al., 2012; van Hooydonk, Hagedoorn, & Boerrigter, 1986). The authors believed that this was

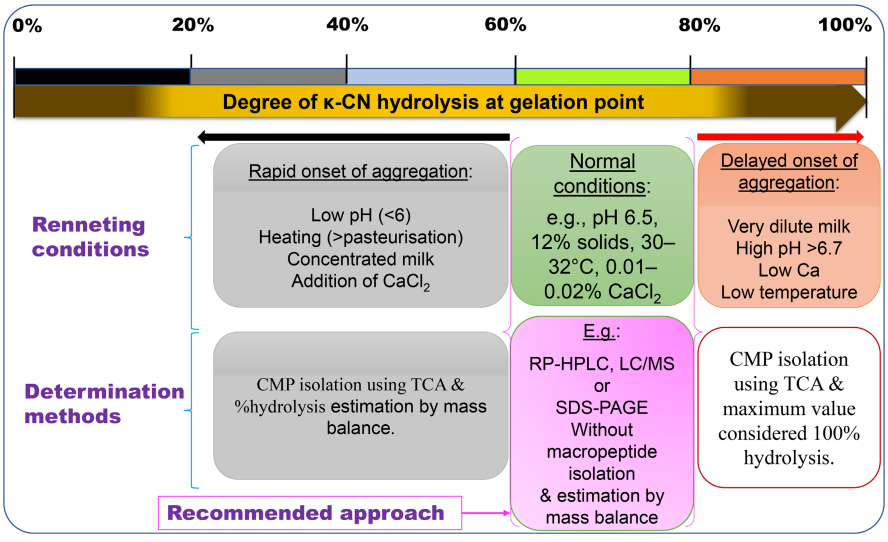

FIGURE 2 Perspectives on factors affecting the degree of κ-CN hydrolysis estimated at gelation point.

because of a rapid aggregation as gelation time decreased linearly with Ca concentration, and a reduction in pH is also clearly one of the reasons as suggested in other studies (He, 1990; van Hooydonk, Hagedoorn, & Boerrigter, 1986; Zhao & Corredig, 2016). With the addition of 10–120 mM $CaCl_2$, values could fall to 41%–56% for skim milk with 12%–36% solids (He, 1990), 45% for native phosphocaseinate (Famelart et al., 1999) or 40% for milk protein concentrate (Sandra & Corredig, 2013). In contrast, 0.12 M NaCl showed no effect (Famelart et al., 1999) whereas 0.3 M had a negative impact (Zhao & Corredig, 2016).

The rate of enzymatic hydrolysis appears to increase with both lowering gelation pH (in the range 6.2–6.7) and increasing gelation temperature (2–32°C) (Carlson et al., 1987a) and it was also suggested that slight acidification (to pH 6.3 and 6.5) makes the effect of concentration greater (He, 1990). In contrast, lowering pH in the range below 6 decreased the degree of the macropeptide release found at gelation point of skim milk to 75% (Li & Dalgleish, 2006) or 50% (Karlsson et al., 2007) at pH 5.8, 43% at pH 5.2 (Pierre, 1983), 55% at pH 5.4 (Li & Dalgleish, 2006) and pH 5.6 (van Hooydonk, Boerrigter, & Hagedoorn, 1986), or 11%–26% at pH 5.4–5.3 (Cooper et al., 2010). As indicated in Table 2, the differences in %CMP release shown by different authors at pH 5.4 and 5.8 could be due to different isolation techniques. It is noteworthy that gelation at low pH occurs at a relatively lower degree of hydrolysis because of a synergistic destabilizing action of the two coagulants (acid and rennet) on CN micelles.

5.2 | Preheating of milk

The impact of heating milk appears to have been studied extensively and was also reviewed (Britten & Giroux, 2022; Kethireddipalli & Hill, 2015) but the effects of renneting conditions and test methods were not comprehensively discussed. It is generally believed that pasteurization or preheating milk up to 90°C/30 min has little impact on the primary phase/κ-CN hydrolysis (Anema et al., 2007, 2011; Marshall, 1986; Vasbinder et al., 2003). However, Calvo (1995) reported that even 60°C/30 min in the presence of whey proteins inhibited CMP release from micellar CN, and up to 47% reduction was found when 80°C was applied whereas at 85°C/10 min, about 26% reduction was found (Reddy & Kinsella, 1990).

Similarly, 80°C/5 min considerably slowed down the rate of soluble nitrogen and GMP-carbohydrate groups released during renneting (Beeby & Nitschmann, 1963; Hindle & Wheelock, 1970). In addition, it has been shown that both preheating and preheating followed by homogenization of skim milk reduced the %CMP release by 10%–15% (Sandra & Dalgleish, 2007). A 5%–30% reduction in degree of hydrolysis after 4 h of renneting for milk pre-heated at 90°C/10 min was also reported depending on the isolation method (Vasbinder et al., 2003). The authors concluded that the reduction was very slight, $Ca_3(PO4)_2$ precipitation has no impact, and whey protein denaturation retards the kinetics of aggregation stage only, which contradicts Calvo (1995) as indicated above. Using half of the rennet concentration used by Vasbinder et al. (2003), Renan et al. (2007) found 10%–17% reduction after 7 h of renneting for reconstituted skim milk heated at 90°C/10 min. It is very clear that the rennet concentrations applied in these two studies were very low based on the normal 20-min clotting time mentioned earlier. When milk was heated to 100°C for 10 min, the amount of GMP released at the formation of a stiff gel was only 32.7% of the value obtained with unheated milk (Taylor & Woonton, 2009).

Contrary to Vasbinder et al. (2003), several other studies (Calvo, 1995; Lieske, 1997; Reddy & Kinsella, 1990; Wilson & Wheelock, 1972) had suggested that denatured β-LG and dissociated κ-CN complexation via hydrophobic interactions and covalent (disulfide) bonds (Anema, 2020; Reddy & Kinsella, 1990) and changes in Ca distribution were the reasons for inhibition of the primary phase due to partial inaccessibility of part of κ-CN to the enzyme. In addition, van Hooydonk et al. (1987) also concluded that denatured β-LG/κ-CN complexation has an adverse effect. Not only as a consequence of heat treatment but also native whey proteins have been confirmed to possess inhibitory properties on κ-CN hydrolysis (Gamlath et al., 2018). Similar to results shown by Renan et al. (2007), the reduction in the enzymatic rate or CMP release has been shown to be 18% for temperatures ≥90°C but up to 25%–45% have also been found for ultra-high-temperature (UHT)-treated milk (Ferron-Baumy et al., 1991; Leaver et al., 1995; van Hooydonk et al., 1987). A 25% reduction in CMP release was also found for high-heat reconstituted milk relative to that of raw milk (Lieske, 1997) and medium-heat reconstituted milk compared with low-heat milk (Klandar et al., 2007). For GMP, UHT caused a 40% reduction in the final release compared with raw milk whereas CMP was not impacted (Ferron-Baumy et al., 1992). For this reason, the authors concluded that complex formation with denatured β-LG involves only the glycosylated form of κ-CN. This study also confirms the 17%–18% reduction shown above based on the proportion of GMP indicated (42% of total macropeptide). According to the results reported by Lieske (1997), CMP was the most affected which contradicts Ferron-Baumy et al. (1992). Based on the isolation techniques, findings of Hindle and Wheelock (1970) also clearly indicate that GMP was less affected by milk sterilization. However, the final amount of total macropeptide and changes in the final amount of GMP-carbohydrate groups (except D-galactose, Gal) were lower, indicating that both isoforms decreased.

5.3 | Methods used to determine the degree of κ-CN hydrolysis

As shown in Table 2 and other studies not considered in this review (Table S1), measuring the degree of κ-CN hydrolysis can be achieved using different methods most of which are highly sensitive and provide accurate measurements, for example, reverse-phase high-performance liquid chromatography (RP-HPLC) which is the most common (Table 2) and liquid chromatography coupled with mass

spectrometry (LC–MS) (Jensen et al., 2015; Mollé & Léonil, 2005). Other analytical techniques also include cation-exchange chromatography (Léonil & Mollé, 1991); Kjeldahl (Klandar et al., 2007); sodium dodecyl sulfate/urea polyacrylamide gel electrophoresis (SDS/urea-PAGE) (Anema et al., 2007; Brinkhuis & Payens, 1985; Chen et al., 2021); and capillary electrophoresis (CE) (Leite Júnior et al., 2019; Nilsson et al., 2020). Nonetheless, the approaches or experimental designs (especially CMP and/or GMP isolation techniques) and calculations found in numerous studies show some drawbacks.

The most common isolation technique involves treatment of renneted samples with 2, 8, or 12% TCA. This precipitates whey proteins and residual CN and also stops the enzymatic reaction (Jensen et al., 2015; Klandar et al., 2007; Sandra et al., 2012). The reaction may also be stopped using pepstatin solution (Brinkhuis & Payens, 1985; Vasbinder et al., 2003). After stirring, the sample–TCA mixture may be incubated for ≥30 min (Klandar et al., 2007; Sandra et al., 2012; Vasbinder et al., 2003) or not incubated (Jensen et al., 2015), and then centrifuged and filtered (0.22 or 0.45 μm). The supernatants (supposedly containing all the macropeptides released from κ-CN) are collected and used for CMP and/or GMP analysis, for example, using RP-HPLC or LCMS. As indicated in Table 2, the maximum peak area (for each sample or for fresh milk) is often considered 100% macropeptide release. However, there are experimental evidence indicating that macropeptide isolation influences the amount of the macropeptide obtained and can result in ~30%–35% variation in the degree of hydrolysis estimated (Taylor & Woonton, 2009; van Hooydonk et al., 1987; Vasbinder et al., 2003). For example, although a TCA concentration of 8% was reported as the optimum (van Hooydonk & Olieman, 1982; van Hooydonk et al., 1987), Léonil and Mollé (1991) reported a macropeptide recovery of 30%–75% and van Hooydonk et al. (1984) found a decrease in the amount of CMP recovered of 61%, of that obtained using 2% TCA. Likewise, the results reported by Beeby and Nitschmann (1963), Mackinlay and Wake (1971), and Garnot and Corre (1980) also indicate that 12% TCA isolated only 25%–37.5% of total macropeptide obtained using 2% TCA or at pH 4.7.

It is believed that 6% TCA (+Na_2SO_4) and 12% TCA selectively isolate CMP and GMP, respectively, whereas 2% TCA isolates total macropeptide (Boutrou et al., 2008; Lieske, 1997; Lieske et al., 1996; Mackinlay & Wake, 1971; Pierre, 1983; Shin & Jang, 2002; Vasbinder et al., 2003). However, Vasbinder et al. (2003) showed that some CMP A were present in 12% TCA. In addition, the amount of carbohydrates in GMP (N-acetyl neuraminic acid (NeuAc), D-Gal and 2-acetamido-2-deoxy-D-galactos e) estimated using 12% TCA were lower than that found in 2% TCA (Hindle & Wheelock, 1970; Shin & Jang, 2002), an indication that GMP may also be underestimated when 12% TCA is used. Furthermore, although the initial rate of increase in 2% TCA-soluble NeuAc was slightly greater than that obtained by 10% TCA, the final amount was almost the same whereas differences in the amount of nitrogen released were higher (Wheelock & Knight, 1969). On the other hand, Vreeman et al. (1986) indicated that the optimum concentration of TCA varies depending on the κ-CN isoform where, for example, 3, 7, and 12% TCA were optimal for κ-CN BB-1P, κ-CN BB-1P,3NeuAc, and κ-CN BB-1,6NeuAc, respectively. However, studies using any amount of TCA are inaccurate according to Thomä et al. (2006), because even 1% TCA showed 10% CMP loss. Léonil and Mollé (1991) and Mollé and Léonil (2005) also agree with Thomä et al. (2006) that due to variations in sensitivities of different isoforms, TCA does not isolate total macropeptide.

All studies above clearly indicate that the isolation method is one of the major factors influencing the degree of κ-CN hydrolysis reported, but another issue is also how the results are calculated and presented. As shown in Table 2, most studies have presented the results as % of values obtained using unheated/fresh milk or the maximum peak areas for each sample assumed to be 100% (Bansal et al., 2007; Kethireddipalli et al., 2011; Nair & Corredig, 2015; Sandra & Dalgleish, 2007; Sandra et al., 2012; Sinaga et al., 2016; Taylor & Woonton, 2009; Titapiccolo, Alexander, & Corredig, 2010; Titapiccolo, Corredig, & Alexander, 2010). Nonetheless, CMP at a plateau or maximum peak area is not necessarily total macropeptide (100% hydrolysis), especially for reconstituted or heated milk in which complexes containing intact κ-CN (Kayihura, 2023a, 2023b) may inhibit complete hydrolysis. A good example to explain this is the results presented by Rocha et al. (2021) which indicate that CMP release began to plateau after 30 min but the degree of κ-CN hydrolysis estimated after 120 min was still <10% because calculations were based on initial total protein in samples. The problem, however, is that Rocha et al. (2021) used 10% TCA which does not recover total macropeptide as discussed above. Another example is a study on pepsin-induced hydrolysis using RP-HPLC in which the peak area of para-κ-CN produced after 8 h was considered 100% hydrolysis; however, chromatograms indicate the presence of intact κ-CN especially the B variant (Yang et al., 2022). It becomes clear, therefore, that the measurement procedures and calculation approaches need some improvements.

Since advanced analytical techniques such as SDS/urea-PAGE, CE, RP-HPLC, or LC–MS are capable of simultaneously separating individual CNs (including residual κ-CN), serum proteins and the peptides (para-κ-CN & CMP/GMP) in the renneted milk sample, macropeptide isolation seems unnecessary. This is because sample preparation for residual κ-CN or para-κ-CN analysis does not isolate those from other milk components. Instead, aliquots of a whole denaturing buffer-sample mixture are injected, for example, into HPLC or polyacrylamide gel (Anema et al., 2007; Nilsson et al., 2020; Thomä et al., 2006; Yang et al., 2022). Therefore, the degree of κ-CN hydrolysis can be estimated based on the amount of residual intact κ-CN or para-κ-CN instead of CMP/GMP as explained below. Moreover, one mole of κ-CN produces one mole of each of the hydrolysates (para-κ-CN and CMP or GMP) as shown in Figure 1b; thus, the best and provable approach to determine the degree of κ-CN hydrolysis is to do calculations by mass balance:

$$\kappa\text{-CN}_h = \kappa\text{-CN}_l - \kappa\text{-CN}_r \text{ Approach} - 1$$

and

$$\kappa CN_h = \sum_{i=1}^{3} P_i \text{Approach} - 2$$

where κCN_l is the initial κ-CN content (mg/g of milk), κCN_h is the hydrolyzed fraction of κ-CN (mg/g of milk), κCN_r is the residual intact κ-CN (mg/g of milk), and P_i is the total concentration (mg/g of milk) of all the peptides produced (para-κ-CN, CMP, and GMP).

Then, the degree of κ-CN hydrolysis is simply the % ratio of the hydrolyzed fraction of κ-CN to the initial amount in milk and can be calculated as:

$$100\left(\frac{\kappa CN_h}{\kappa CN_l}\right) = 100\left(1 - \frac{\kappa CN_r}{\kappa CN_l}\right)$$

Note that the whole renneted sample (without whey separation) should be used to analyze κCN_r. Also, the rennet action must be stopped at a specific stage/time when the degree of hydrolysis is to be determined. Approach-1 would give a more direct and the most accurate estimate since κCN_l and κCN_r can be determined using the same analytical method as mentioned above. The major drawback of approach-2 as mentioned earlier is that accurate determination of total CMP + GMP released is challenging. As stated above, para-κ-CN can also be determined by the same analytical method used for intact κ-CN (again in the whole renneted sample); therefore, another reliable alternative approach would be based on molar ratios, that is, κCN_h can be estimated by multiplying its molecular weight by moles of para-κ-CN since one mole of κ-CN produces one mole of para-κ-CN as mentioned above. The degree of κ-CN hydrolysis can also be expressed as a % ratio of the peak area of para-κ-CN at a specific time to the total peak area as shown by Yang et al. (2022, 2023), but complete hydrolysis must be achieved and verified by mass balance calculations, that is, $\kappa CN_l = \sum_{i=1}^{3} P_i + \kappa CN_r$.

To express the degree of κ-CN hydrolysis in terms of % macropeptide (CMP + GMP) release, development of an accurate approach for determination of total macropeptide (% of κ-CN) is required. For unheated milk, complete hydrolysis could be achieved by renneting part of the same milk (control) at low temperature (to inhibit micellar aggregation) until no intact κ-CN is remaining. This should also be verified by mass balance calculations.

6 | CONCLUSIONS AND FUTURE RECOMMENDATIONS

A full understanding of the extent and kinetics of rennet action on κ-CN is essential for proper control of the cheese-making process and determination of κ-CN's partitioning between cheese and whey. The literature revealed that the degree of κ-CN hydrolysis estimated at gelation point of bovine milk renneted under conditions relevant to cheese-making is <80% and varies depending on three major factors: compositional (e.g., enzyme-to-substrate ratio), pretreatments (e.g., changing ionic strength and preheating), and test and estimation approaches (e.g., TCA concentration and considering the maximum macropeptide released 100% κ-CN hydrolysis). The literature also shows that there appears to be little advancement in analytical and estimation approaches since 1950s; thus, the following are recommended for future studies: (1) using advanced analytical techniques (e.g., RP-HPLC or LC–MS) without macropeptide isolation (i.e., analyzing the residual intact κ-CN or para- κ-CN in the whole renneted milk instead of isolated CMP and/or GMP), (2) estimating the degree of κ-CN hydrolysis by performing mass balance calculations (based on initial κ-CN content in milk), (3) improving an approach to determine total macropeptide release especially in heated and reconstituted milks also remains to be established since there is no clear evidence that complete hydrolysis could be achieved due to κ–/αs_2-CNs-whey protein complexation, and (4) the best method (sample preparation, analytical technique, and procedure) and the actual extent of κ-CN hydrolysis (%) necessary to induce gelation in various milk systems or renneting conditions (e.g., for specific cheese varieties) will also need to be established.

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The author declares no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new datasets were created.

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REFERENCES

Alais, C., Mocquot, G., Nitschmann, H. S., & Zahler, P. (1953). Das Lab und seine Wirkung auf das Casein der Milch. VII. Über die Abspaltung von Nicht-Protein-Stickstoff (NPN) aus Casein durch Lab und ihre Beziehung zur Primärreaktion der Labgerinnung der Milch. *Helvetica Chimica Acta*, *36*, 1955–1968. https://doi.org/10.1002/hlca.19530360735

Anema, S. G. (2020). The whey proteins in milk: Thermal denaturation, physical interactions, and effects on the functional properties of milk. In M. Boland & H. Singh (Eds.), *Milk proteins, from expression to food* (3rd ed., pp. 325–384). Elsevier. https://doi.org/10.1016/B978-0-12-815251-5.00009-8

Anema, S. G., Lee, S. K., & Klostermeyer, H. (2007). Effect of pH at heat treatment on the hydrolysis of κ-casein and the gelation of skim milk by chymosin. *LWT-Food Science and Technology*, *40*, 99–106. https://doi.org/10.1016/j.lwt.2005.08.002

Anema, S. G., Lee, S. K., & Klostermeyer, H. (2011). Rennet-induced aggregation of heated pH-adjusted skim milk. *Journal of Agricultural*

and Food Chemistry, *59*, 8413–8422. https://doi.org/10.1021/jf201198s

Bansal, N., Fox, P. F., & MCSweeney, P. L. H. (2007). Aggregation of rennet-altered casein micelles at low temperatures. *Journal of Agricultural and Food Chemistry*, *2007*(55), 3120–3126. https://doi.org/10.1021/jf0631427

Beeby, R., & Nitschmann, H. (1963). The action of rennin on casein the disruption of the κ-casein complex. *Journal of Dairy Research*, *30*, 7–16. https://doi.org/10.1017/s0022029900011201

Bingham, E. W. (1975). Action of rennin on κ-casein. *Journal of Dairy Science*, *58*, 11–18.

Bonfatti, V., Chiarot, G., & Carnier, P. (2014). Glycosylation of κ-casein: Genetic and nongenetic variation and effects on rennet coagulation properties of milk. *Journal of Dairy Science*, *97*, 1961–1969. https://doi.org/10.3168/jds.2013-7418

Boutrou, R., Jardin, J., Blais, A., Tomé, D., & Léonil, J. (2008). Glycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *Journal of Agricultural and Food Chemistry*, *56*, 8166–8173. https://doi.org/10.1021/jf801140d

Bringe, N. A., & Kinsella, J. E. (1986a). Use of platelet Aggregometer to monitor the chymosin-initiated coagulation of casein micelles. *Journal of Dairy Research*, *53*, 359–370. https://doi.org/10.1017/s0022029900024985

Bringe, N. A., & Kinsella, J. E. (1986b). Influence of calcium chloride on the chymosin-initiated coagulation of casein micelles. *Journal of Dairy Research*, *53*, 371–379. https://doi.org/10.1017/s0022029900024997

Brinkhuis, J., & Payens, T. A. (1985). The rennet-induced clotting of Para-κ-casein revisited: Inhibiton experiments with pepstatin a. *Biochimica et Biophysica Acta*, *832*, 331–336. https://doi.org/10.1016/0167-4838(85)90267-5

Britten, M., & Giroux, H. J. (2022). Rennet coagulation of heated milk: A review. *International Dairy Journal*, *124*, 105179. https://doi.org/10.1016/j.idairyj.2021.105179

Calvo, M. M. (1995). Heat-induced interactions between serum albumin, immunoglobulin, and κ-casein inhibit the primary phase of renneting. *Journal of Agricultural and Food Chemistry*, *43*, 2823–2827. https://doi.org/10.1021/jf00059a010

Carlson, A., Hill, C. G., & Olson, N. F. (1987a). Kinetics of milk coagulation: I. The kinetics of kappa casein hydrolysis in the presence of enzyme deactivation. *Biotechnology and Bioengineering*, *29*, 582–589. https://doi.org/10.1002/bit.260290507

Carlson, A., Hill, C. G., & Olson, N. F. (1987b). Kinetics of Milk coagulation: III. Mathematical modeling of the kinetics of curd formation following enzymatic hydrolysis of κ-casein-parameter estimation. *Biotechnology and Bioengineering*, *29*, 601–611. https://doi.org/10.1002/bit.260290509

Caroli, A. M., Chessa, S., & Erhardt, G. J. (2009). *Invited review*: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science*, *92*, 5335–5352. https://doi.org/10.3168/jds.2009-2461

Castle, A. V., & Wheelock, J. V. (1972). Effect of varying enzyme concentration on the action of rennin on whole milk. *Journal of Dairy Research*, *39*, 15–22. https://doi.org/10.1017/s0022029900013790

Chaplin, B., & Green, M. L. (1980). Determination of the proportion of κ-casein hydrolysed by rennet on coagulation of skim-milk. *Journal of Dairy Research*, *47*, 351–358. https://doi.org/10.1017/s0022029900021245

Chapman, H. R. (1981). Standardisation of milk and milk products. *Journal of the Society of Dairy Technology*, *34*, 147–152. https://doi.org/10.1111/j.1471-0307.1981.tb01514.x

Chen, C.-C., Chen, L.-Y., Li, W.-T., Chang, K.-L., Kuo, M.-I., Chen, C.-J., & Hsieh, J.-F. (2021). Influence of Chymosin on physicochemical and hydrolysis characteristics of casein micelles and individual caseins. *Nanomaterials*, *11*, 2594. https://doi.org/10.3390/nano11102594

Cooper, C., Corredig, M., & Alexander, M. (2010). Investigation of the colloidal interactions at play in combined acidification and rennet of different heat-treated milks. *Journal of Agricultural and Food Chemistry*, *58*, 4915–4922. https://doi.org/10.1021/jf100372b

Dalgleish, D. G. (1979). Proteolysis and aggregation of casein micelles treated with immobilized or soluble chymosin. *Journal of Dairy Research*, *46*, 653–661. https://doi.org/10.1017/s0022029900020719

Dalgleish, D. G. (1998). Casein micelles as colloids: Surface structures and stabilities. *Journal of Dairy Science*, *81*, 3013–3018. https://doi.org/10.3168/jds.s0022-0302(98)75865-5

Dalgleish, D. G., Brinkhuis, J., & Payens, T. A. J. (1981). The coagulation of differently sized casein micelles by rennet. *European Journal of Biochemistry*, *119*, 257–261. https://doi.org/10.1111/j.1432-1033.1981.tb05602.x

Donnelly, W. J., & Barry, J. G. (1983). Casein compositional studies. III. Changes in Irish milk for manufacturing and role of milk proteinase. *Journal of Dairy Research*, *50*, 433–441. https://doi.org/10.1017/s0022029900032660

Doultani, S., Turhan, K. N., & Etzel, M. R. (2003). Whey protein isolate and glycomacropeptide recovery from whey using ion exchange chromatography. *Journal of Food Science*, *68*, 1389–1395. https://doi.org/10.1111/j.1365-2621.2003.tb09655.x

Eshpari, H., Jimenez-Flores, R., Tong, P. S., & Corredig, M. (2015). Partial calcium depletion during membrane filtration affects gelation of reconstituted milk protein concentrates. *Journal of Dairy Science*, *98*, 8454–8463. https://doi.org/10.3168/jds.2015-9856

Famelart, M. H. (1994). Rennet coagulation of milk in the presence of sucrose. *Journal of Dairy Research*, *61*, 473–483. https://doi.org/10.1017/s0022029900028405

Famelart, M. H., Le Graet, Y., & Raulot, K. (1999). Casein micelle dispersions into water, NaCl and $CaCl_2$: Physicochemical characteristics of micelles and rennet coagulation. *International Dairy Journal*, *9*, 293–297. https://doi.org/10.1016/s0958-6946(99)00077-1

Ferron-Baumy, C., Maubois, J. L., Garric, G., & Quiblier, J. P. (1991). Coagulation présure du lait et des retentats d'ultrafiltration. Effets de divers traitements thermiques. *Le Lait*, *71*, 423–434. https://doi.org/10.1051/lait:1991432

Ferron-Baumy, C., Mollé, D., Garric, G., & Maubois, J.-L. (1992). Characterization of caseinomacropeptides released from renneted raw and UHT treated milks. *Le Lait*, *72*, 165–173. https://doi.org/10.1051/lait:1992211

Gamlath, C. J., Leong, T. S. H., Ashokkumar, M., & Martin, G. J. O. (2018). The inhibitory roles of native whey protein on the rennet gelation of bovine milk. *Food Chemistry*, *244*, 36–43. https://doi.org/10.1016/j.foodchem.2017.10.029

Garnot, P., & Corre, C. (1980). Influence of milk protein concentration on the gelling activity of chymosin and bovine pepsin. *Journal of Dairy Research*, *47*, 103–111. https://doi.org/10.1017/s0022029900020938

Gaygadzhiev, Z., Massel, V., Alexander, M., & Corredig, M. (2012). Addition of sodium caseinate to skim milk inhibits rennet-induced aggregation of casein micelles. *Food Hydrocolloids*, *26*, 405–411. https://doi.org/10.1016/j.foodhyd.2011.02.015

Green, M. L., Hobbs, D. G., Morant, S. V., & Hill, V. A. (1978). Intermicellar relationships in rennet-treated separated milk. II. Process of gel assembly. *Journal of Dairy Research*, *45*, 413–422. https://doi.org/10.1017/s0022029900016630

Hallén, E., Lundén, A., Allmere, T., & Andrén, A. (2010). Casein retention in curd and loss of casein into whey at chymosin-induced coagulation of milk. *Journal of Dairy Research*, *77*, 71–76. https://doi.org/10.1017/s0022029909990434

He, F. (1990). *Substrate concentration, calcium concentration and κ-CN hydrolysis in milk coagulation*. [Ph.D. thesis]. (pp. Xii–144). Utah State University. https://doi.org/10.26076/A2E3-B88C

Hindle, E. J., & Wheelock, J. V. (1970). The primary phase of rennin action in heat-sterilized milk. *Journal of Dairy Research*, *37*, 389–396. https://doi.org/10.1017/s0022029900013418

Holt, C., & Horne, D. S. (1996). The hairy casein micelle: Evolution of the concept and its implications for dairy technology. *Netherlands Milk & Dairy Journal*, *50*, 85–111.

Horne, D. S., & Lucey, J. A. (2017). Rennet-induced coagulation of milk. In *Cheese: Chemistry, physics and microbiology, Vol. 1. General aspects* (4th ed., pp. 115–143). Elsevier. https://doi.org/10.1016/B978-0-12-417012-4.00005-3

Huppertz, T. (2013). Chemistry of the caseins. In *Advanced dairy chemistry* (4th ed., pp. 135–160). Springer. https://doi.org/10.1007/978-1-4614-4714-6_4

Huppertz, T., Fox, P., & Kelly, A. L. (2018). The caseins: Structure, stability, and functionality. In *Proteins in food processing* (2nd ed., pp. 49–92). Elsevier. https://doi.org/10.1016/B978-0-08-100722-8.00004-8

Jensen, H. B., Pedersen, K. S., Johansen, L. B., Poulsen, N. A., Bakman, M., Chatterton, D. E. W., & Larsen, L. B. (2015). Genetic variation and posttranslational modification of bovine κ-casein: Effects on caseino-macropeptide release during renneting. *Journal of Dairy Science*, *98*, 747–758. https://doi.org/10.3168/jds.2014-8678

Kalan, E. B., & Woychik, J. H. (1965). Action of rennin on κ-casein, the amino acid compositions of the Para-κ-casein, and glycomacropeptide fractions. *Journal of Dairy Science*, *48*, 1423–1428. https://doi.org/10.3168/jds.s0022-0302(65)88492-2

Karimidastjerd, A., & Gulsunoglu-Konuskan, Z. (2023). Biological, functional and nutritional properties of caseinomacropeptide from sweet whey. *Critical Reviews in Food Science and Nutrition*, *63*, 4261–4273. https://doi.org/10.1080/10408398.2021.2000360

Karlsson, A. O., Ipsen, R., & Ardö, Y. (2007). Rheological properties and microstructure during rennet induced coagulation of UF concentrated skim milk. *International Dairy Journal*, *17*, 674–682. https://doi.org/10.1016/j.idairyj.2006.08.002

Kayihura, J. F. (2023a). Reconstituted skim milk concentration affects partitioning of some individual caseins between rennet curd and whey. *International Journal of Dairy Technology*, *76*, 852–860. https://doi.org/10.1111/1471-0307.13003

Kayihura, J. F. (2023b). Partitioning of individual caseins between rennet curd and whey: Impact of pre-acidification and pre-salting of reconstituted skim milk. *Food and Humanity*, *1*, 905–911. https://doi.org/10.1016/j.foohum.2023.08.014

Kayihura, J. F. (2023c). Partitioning of casein and fat in Cheddar cheese manufacturing as affected by cheese milk standardisation: A review. *International Journal of Dairy Technology*. Advance online publication. https://doi.org/10.1111/1471-0307.13009

Kayihura, J. F., Huppertz, T., & Vasiljevic, T. (2022). Application of small amplitude oscillatory rheology measurements for estimating residual rennet activity in rennet whey. *International Dairy Journal*, *134*, 105451. https://doi.org/10.1016/j.idairyj.2022.105451

Kelly, A. L., Huppertz, T., & Sheehan, J. J. (2008). Pre-treatment of cheese milk: Principles and developments. *Dairy Science and Technology*, *88*, 549–572. https://doi.org/10.1051/dst:2008017

Kethireddipalli, P., & Hill, A. R. (2015). Rennet coagulation and cheesemaking properties of thermally processed milk: Overview and recent developments. *Journal of Agriculture and Food Chemistry*, *63*, 9389–9403. https://doi.org/10.1021/jf504167v

Kethireddipalli, P., Hill, A. R., & Dalgleish, D. G. (2011). Interaction between casein micelles and whey protein/κ-casein complexes during renneting of heat-treated reconstituted skim milk powder and casein micelle/serum mixtures. *Journal of Agriculture and Food Chemistry*, *59*, 1442–1448. https://doi.org/10.1021/jf103943e

Klandar, A. H., Lagaude, A., & Chevalier-Lucia, D. (2007). Assessment of the rennet coagulation of skim milk: A comparison of methods. *International Dairy Journal*, *17*, 1151–1160. https://doi.org/10.1016/j.idairyj.2007.03.005

Le Feunteun, S., Ouethrani, M., & Mariette, F. (2012). The rennet coagulation mechanisms of a concentrated casein suspension as observed by PFG-NMR diffusion measurements. *Food Hydrocolloids*, *27*, 456–463. https://doi.org/10.1016/j.foodhyd.2011.09.008

Leaver, J., Law, A. J. R., Horne, D. S., & Banks, J. M. (1995). Influence of heating regime and pH on the primary phase of renneting of whole milk. *International Dairy Journal*, *5*, 129–140. https://doi.org/10.1016/0958-6946(95)92206-j

Leite Júnior, B. R. d. C., Tribst, A. A. L., Ribeiro, L. R., & Cristianini, M. (2019). High pressure processing impacts on the hydrolytic profile of milk coagulants. *Food Bioscience*, *31*, 100449. https://doi.org/10.1016/j.fbio.2019.100449

Léonil, J., & Mollé, D. (1991). A method for determination of macropeptide by cation exchange fast protein liquid chromatography and its use for following the action of chymosin in milk. *Journal of Dairy Research*, *58*, 321–328. https://doi.org/10.1017/s0022029900029897

Li, J., & Dalgleish, D. G. (2006). Controlled proteolysis and the properties of milk gels. *Journal of Agricultural and Food Chemistry*, *54*, 4687–4695. https://doi.org/10.1021/jf060284s

Lieske, B. (1997). Influence of preliminary treatments on structural properties of casein micelles affecting the rennetability. *Le Lait*, *77*, 201–209. https://doi.org/10.1051/lait:1997114

Lieske, B., Faber, W., & Konrad, G. (1996). A new spectrophotometric approach for following the release of caseinomacropeptide during renneting of milk. *Milchwissenschaft*, *51*, 548–551.

Lu, Y., McMahon, D. J., & Vollmer, A. H. (2017). Investigating rennet coagulation properties of recombined highly concentrated micellar casein concentrate and cream for use in cheese making. *Journal of Dairy Science*, *100*, 892–900. https://doi.org/10.3168/jds.2016-11648

Lucey, J. A. (2022). Cheese: Rennet coagulation of milk. In P. L. H. McSweeney & J. P. McNamara (Eds.), *Encyclopedia of dairy sciences* (Vol. *3*, 3rd ed., pp. 309–315). Elsevier. https://doi.org/10.1016/B978-0-12-818766-1.00209-9

Lucey, J. A., & Fox, P. F. (1992). Rennet coagulation properties of late-lactation milk: Effect of pH adjustment, addition of $CaCl_2$, variation in rennet level and blending with mid-lactation milk. *Irish Journal of Agricultural and Food Research*, *31*, 173–184. https://www.jstor.org/stable/25562189

Mackinlay, A. G., & Wake, R. G. (1971). κ-Casein and its attack by rennin (chymosin). In H. A. McKenzie (Ed.), *Milk Proteins* (Vol. *2*, 4th ed., pp. 175–215). Elsevier. https://doi.org/10.1016/B978-0-12-485202-0.50010-5

Manso, M. A., & López-Fandiño, R. (2004). κ-Casein macropeptides from cheese whey: Physicochemical, biological, nutritional, and technological features for possible uses. *Food Reviews International*, *20*, 329–355. https://doi.org/10.1081/fri-200033456

Marshall, R. J. (1986). Increasing cheese yields by high heat treatment of milk. *Journal of Dairy Research*, *53*, 313–322. https://doi.org/10.1017/s0022029900024912

McMahon, D. J., Brown, R. J., & Ernstrom, C. A. (1984). Enzymic coagulation of milk casein micelles. *Journal of Dairy Science*, *67*, 745–748. https://doi.org/10.3168/jds.s0022-0302(84)81364-8

Mercier, J. C., Brignon, G., & Ribadeau-Dumas, B. (1973). Structure primaire de la caséine κB bovine séquence complète. *European Journal of Biochemistry*, *35*, 222–235. https://doi.org/10.1111/j.1432-1033.1973.tb02829.x

Mollé, D., & Léonil, J. (2005). Quantitative determination of bovine κ-casein macropeptide in dairy products by liquid chromatography/electrospray coupled to mass spectrometry (LC-ESI/MS) and liquid chromatography/electrospray coupled to tandem mass spectrometry (LC-ESI/MS/MS). *International Dairy Journal*, *15*, 419–428. https://doi.org/10.1016/j.idairyj.2004.08.013

Nair, P. K., & Corredig, M. (2015). Rennet-induced gelation of concentrated milk in the presence of sodium caseinate: Differences

between milk concentration using ultrafiltration and osmotic stressing. *Journal of Dairy Science*, *98*, 27–36. https://doi.org/10.3168/jds.2014-8356

Nilsson, K., Abdelghani, A., Burleigh, S., Johansen, L. B., Lindmark-Månsson, H., Paulsson, M., & Glantz, M. (2020). An investigation of the enzymatic cleavage of κ-casein in non-coagulating milk. *International Dairy Journal*, *109*, 104754. https://doi.org/10.1016/j.idairyj.2020.104754

Palmer, D. S., Christensen, A. U., Sørensen, J., Celik, L., Qvist, K. B., & Schiøtt, B. (2010). Bovine chymosin: A computational study of recognition and binding of bovine κ-casein. *Biochemistry*, *49*, 2563–2573. https://doi.org/10.1021/bi902193u

Phelan, J. A. (1981). Standardisation of milk for cheesemaking at factory level. *Journal of the Society of Dairy Technology*, *34*, 152–156. https://doi.org/10.1111/j.1471-0307.1981.tb01515.x

Pierre, A. (1983). Influence of a change in micellar charge on the proportion of caseinomacropeptide released by rennet on coagulation of milk. *Le Lait*, *63*, 217–229. https://doi.org/10.1051/lait:1983627-62815

Reddy, I. M., & Kinsella, J. E. (1990). Interaction of β-lactoglobulin with κ-casein in micelles as assessed by chymosin hydrolysis. Effects of added reagents. *Journal of Agriculture and Food Chemistry*, *38*, 366–372. https://doi.org/10.1021/jf00092a005

Renan, M., Guyomarc'h, F., Chatriot, M., Gamerre, V., & Famelart, M. H. (2007). Limited enzymatic treatment of skim milk using chymosin affects the micelle/serum distribution of the heat-induced whey protein/κ-casein aggregates. *Journal of Agriculture and Food Chemistry*, *55*, 6736–6745. https://doi.org/10.1021/jf0705771

Rocha, G. F., Cotabarren, J., Obregón, W. D., Fernández, G., Rosso, A. M., & Parisi, M. G. (2021). Milk-clotting and hydrolytic activities of an aspartic protease from *Salpichroa origanifolia* fruits on individual caseins. *International Journal of Biological Macromolecules*, *192*, 931–938. https://doi.org/10.1016/j.ijbiomac.2021.10.004

Salvatore, E., Pirisi, A., & Corredig, M. (2011). Gelation properties of casein micelles during combined renneting and bacterial fermentation: Effect of concentration by ultrafiltration. *International Dairy Journal*, *21*, 848–856. https://doi.org/10.1016/j.idairyj.2011.05.006

Sandra, S., Alexander, M., & Dalgleish, D. G. (2007). The rennet coagulation mechanism of skim milk as observed by transmission diffusing wave spectroscopy. *Journal of Colloid and Interface Science*, *308*, 364–373. https://doi.org/10.1016/j.jcis.2007.01.021

Sandra, S., Cooper, C., Alexander, M., & Corredig, M. (2011). Coagulation properties of ultrafiltered milk retentates measured using rheology and diffusing wave spectroscopy. *Food Research International*, *44*, 951–956. https://doi.org/10.1016/j.foodres.2011.02.018

Sandra, S., & Corredig, M. (2013). Rennet induced gelation of reconstituted milk protein concentrates: The role of calcium and soluble proteins during reconstitution. *International Dairy Journal*, *29*, 68–74. https://doi.org/10.1016/j.idairyj.2012.10.011

Sandra, S., & Dalgleish, D. G. (2007). The effect of ultra-high-pressure homogenization (UHPH) on rennet coagulation properties of unheated and heated fresh skimmed milk. *International Dairy Journal*, *17*, 1043–1052. https://doi.org/10.1016/j.idairyj.2007.01.005

Sandra, S., Ho, M., Alexander, M., & Corredig, M. (2012). Effect of soluble calcium on the renneting properties of casein micelles as measured by rheology and diffusing wave spectroscopy. *Journal of Dairy Science*, *95*, 75–82. https://doi.org/10.3168/jds.2011-4713

Sheng, B., Thesbjerg, M. N., Glantz, M., Paulsson, M., Nielsen, S. D., Poulsen, N. A., & Larsen, L. B. (2022). Phosphorylation and glycosylation isoforms of bovine κ-casein variant E in homozygous Swedish red cow milk detected by liquid chromatography-electrospray ionization mass spectrometry. *Journal of Dairy Science*, *105*, 1959–1965. https://doi.org/10.3168/jds.2021-21172

Shin, S. C., & Jang, H. D. (2002). Effect of trichloroacetic acid on the solubility of caseinomacropeptide. *Preventive Nutrition and Food Science*, *7*, 43–47. https://doi.org/10.3746/jfn.2002.7.1.043

Sinaga, H., Bansal, N., & Bhandari, B. (2016). Partial renneting of pasteurised bovine milk: Casein micelle size, heat and storage stability. *Food Research International*, *84*, 52–60. https://doi.org/10.1016/j.foodres.2016.02.021

Sunds, A. V., Poulsen, N. A., & Larsen, L. B. (2019). Short communication: Application of proteomics for characterization of caseinomacropeptide isoforms before and after desialidation. *Journal of Dairy Science*, *102*, 8696–8703. https://doi.org/10.3168/jds.2019-16617

Swaisgood, H. (1975). Primary sequence of kappa-casein. *Journal of Dairy Science*, *58*, 583–592. https://doi.org/10.3168/jds.s0022-0302(75)84614-5

Swaisgood, H. E., Brunner, J. R., & Lillevik, H. A. (1964). Physical parameters of κ-casein from cow's milk. *Biochemistry*, *3*, 1616–1623. https://doi.org/10.1021/bi00899a003

Taylor, C. M., & Woonton, B. W. (2009). Quantity and carbohydrate content of glycomacropeptide fractions isolated from raw and heat-treated milk. *International Dairy Journal*, *19*, 709–714. https://doi.org/10.1016/j.idairyj.2009.06.010

Thomä, C., Krause, I., & Kulozik, U. (2006). Precipitation behaviour of caseinomacropeptides and their simultaneous determination with whey proteins by RP-HPLC. *International Dairy Journal*, *16*, 285–293. https://doi.org/10.1016/j.idairyj.2005.05.003

Titapiccolo, G. I., Alexander, M., & Corredig, M. (2010). Rennet-induced aggregation of homogenized milk: Impact of the presence of fat globules on the structure of casein gels. *Dairy Science and Technology*, *90*, 623–639. https://doi.org/10.1051/dst/2010023

Titapiccolo, G. I., Corredig, M., & Alexander, M. (2010). Modification to the renneting functionality of casein micelles caused by non-ionic surfactants. *Journal of Dairy Science*, *93*, 506–514. https://doi.org/10.3168/jds.2009-2629

van Hooydonk, A. C. M., Boerrigter, I. J., & Hagedoorn, H. G. (1986). pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 2. Effect of pH on renneting of milk. *Netherland Milk Dairy Journal*, *40*, 297–313.

van Hooydonk, A. C. M., de Koster, P. G., & Boerrigter, I. J. (1987). The renneting properties of heated milk. *Netherland Milk Dairy Journal*, *41*, 3–18.

van Hooydonk, A. C. M., Hagedoorn, H. G., & Boerrigter, I. J. (1986). The effect of various cations on the renneting of milk. *Netherland Milk Dairy Journal*, *40*, 369–390.

van Hooydonk, A. C. M., & Olieman, C. (1982). A rapid and sensitive high-performance liquid chromatography method of following the action of chymosin in milk. *Netherland Milk Dairy Journal*, *36*, 153–158.

van Hooydonk, A. C. M., Olieman, C., & Hagedoorn, H. G. (1984). Kinetics of the chymosin-catalysed proteolysis of κ-casein in milk. *Netherland Milk Dairy Journal*, *38*, 207–222.

Vasbinder, A. J., Rollema, H. S., & de Kruif, C. G. (2003). Impaired renneting ability of heated milk; study of enzymatic hydrolysis and gelation kinetics. *Journal of Dairy Science*, *86*, 1548–1555. https://doi.org/10.3168/jds.s0022-0302(03)73740-0

Vreeman, H. J., Visser, S., Slangen, C. J., & Van Riel, J. A. M. (1986). Characterization of bovine κ-casein fractions and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography. *Biochemical Journal*, *240*, 87–97. https://doi.org/10.1042/bj2400087

Wake, R. G. (1959). Studies of casein. V. The action of rennin on casein. *Australian Journal of Biological Sciences*, *12*, 479–489. https://doi.org/10.1071/bi9590479

Walstra, P., Bloomfield, V. A., Wei, G. J., & Jenness, R. (1981). Effect of chymosin action on the hydrodynamic diameter of casein micelles. *Biochimica et Biophysica Acta*, *669*, 258–259. https://doi.org/10.1016/0005-2795(81)90249-x

Waugh, D. F., & von Hippel, P. H. (1956). κ-Casein and the stabilization of casein micelles. *Journal of the American Chemical Society*, *78*, 4576–4582. https://doi.org/10.1021/ja01599a017

Wheelock, J. V., & Knight, D. J. (1969). The action of rennet on whole milk. *Journal of Dairy Research*, *36*, 183–190. https://doi.org/10.1017/s0022029900012681

Wilson, G. A., & Wheelock, J. V. (1972). Factors affecting the action of rennin in heated milk. *Journal of Dairy Research*, *39*, 413–419. https://doi.org/10.1017/s0022029900014254

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2022). Kinetics of pepsin-induced hydrolysis and the coagulation of milk proteins. *Journal of Dairy Science*, *105*, 990–1003. https://doi.org/10.3168/jds.2021-21177

Yang, M., Ye, A., Yang, Z., Everett, D. W., Gilbert, E. P., & Singh, H. (2023). Pepsin-induced coagulation of casein micelles: Effect of whey proteins and heat treatment. *Food Chemistry*, *402*, 134214. https://doi.org/10.1016/j.foodchem.2022.134214

Yegin, S., & Dekker, P. (2013). Progress in the field of aspartic proteinases in cheese manufacturing: Structures, functions, catalytic mechanism, inhibition and engineering. *Dairy Science and Technology*, *93*, 565–594. https://doi.org/10.1007/s13594-013-0137-2

Zhao, Z., & Corredig, M. (2016). Influence of sodium chloride on the colloidal and rennet coagulation properties of concentrated casein micelle suspensions. *Journal of Dairy Science*, *99*, 6036–6045. https://doi.org/10.3168/jds.2015-10622

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Chapter 3 – Application of small amplitude oscillatory measurement for estimating the remaining rennet activity in cheese-whey

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This chapter has been published as an original research paper entitled "Application of Small Amplitude Oscillatory Measurement for Estimating the Remaining Rennet Activity in Cheese-Whey" by Joseph F. Kayihura, Thom Huppertz and Todor Vasiljevic in a peer-reviewed journal: International Dairy Journal, 134 (2022) 105451. (https://doi.org/10.1016/j.idairyj.2022.105451). The findings obtained using the newly developed assay were confirmed by comparison with a RP-HPLC method developed by Hurley et al. ((1999) https://doi.org/10.1016/S0958-6946(99)00118-1). The procedure of the RP-HPLC was also modified for its application to determine the residual rennet activity levels in whey as recommended by the authors, and was applied in the next study (Chapter 4).

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Application of small amplitude oscillatory rheology measurements for estimating residual rennet activity in rennet whey

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ABSTRACT

The relationship between (very low) rennet activity (RA) levels and rennet coagulation time (RCT) is non-linear, and its determination is time consuming, with accuracy depending on an objective RCT. Here, RCT was determined by small amplitude oscillatory rheology and the Carlson, Hill and Olson's spiking technique was modified to estimate the unknown residual RA directly from a linear calibration curve of RCT against 1/RA created using the serum phase of the reconstituted skim milk (RSM) utilised for preparation of the rennet whey. The estimated residual RA mL^{-1} of the rennet whey could be obtained by spiking the rennet whey with a known RA derived from the linear range of the calibration curve. This observation was confirmed by a commonly used reverse-phase high-performance liquid chromatography (RP-HPLC) method. Using this information, a simple general model that estimates RA partitioning based on total volume of whey separated from the curd could be proposed.

1. Introduction

Rennet is a general term referring to a mixture of proteases used for cheese production by converting milk from a stable colloidal dispersion into a gel. Rennet strength varies due to a number of factors such as origin, type of enzymes and their specificity. Rennet was traditionally extracted from calf abomasum, but these days, a vast number of other rennet types are also available, i.e., microbial, plant and recombinant milk clotting enzymes (Jaros & Rohm, 2017). The primary enzyme in calf rennet is chymosin, an aspartyl protease with greater milk clotting than general proteolytic activity owing to its specificity. Chymosin specifically cleaves the Phe_{105}—Met_{106} bond of bovine κ-casein and releases the 64-amino acid C-terminal peptide Met_{106}—Val_{169}, known as casein-omacropeptide (CMP). CMP-depleted para-casein micelles become unstable and aggregate to form a milk coagulum (Lefebvre-cases et al., 1998).

Rennet partitioning between the curd and whey during cheesemaking is affected by several factors, including type of rennet and composition, milk pH, casein micelle size, ionic strength and cooking temperature (Bansal, Fox, & McSweeney, 2007). A large proportion of rennet (typically ≥85%) is recovered in whey and has to be inactivated for being undesirable in whey products (Fox, 1989; Singh & Creamer, 1990; Sousa, Ardo, & McSweeney, 2001). Active rennet retained in cheese contributes to texture and flavour changes during cheese ripening. For example; cheese softening results from the action of rennet on the Phe_{23}—Phe_{24} bond of α_{S1}-casein, releasing α_{S1}-CN (f1—23) and α_{S1}-I-CN (Hynes, Aparo, & Candioti, 2004). Rennet partitioning tests are, therefore, necessary for quality control of both cheese and whey products.

A number of methods have been described for determining rennet activity in cheese or whey, including a casein-agar technique (Holmes, Duersch, & Ernstrom, 1978), the accuracy of which appears to be moderate and time consuming, immunochemical tests (Boudjellab, Rolet-Repecaud, & Collin, 1994), which are expensive and some may include inactive enzymes in the estimated activity, and a RP-HPLC method using a synthetic heptapeptide (Pro—Thr—Glu—Phe— [NO_2—Phe] —Arg—Leu) as a substrate (Hurley, O'Driscoll, Kelly, & McSweeney, 1999).

Quantitative determination of active rennet relied formerly on changes in rennet clotting time (RCT) with enzyme concentration (Berridge, 1952), but the lengthy time for very low activity levels and the subjectivity of determining RCT visually were two major challenges, requiring improvements. The former was addressed by Carlson, Hill, and Olson (1985), who developed an assay that was

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later optimised by Singh and Creamer (1990) for its application to whey protein concentrates. This method is based on a linear relationship between pre-incubation times with an unknown low activity and the clotting times after a known activity is added. Basically, the test sample containing unknown rennet activity (RA) is pre-incubated with skim milk for a known time initially, followed by several additions of a known amount of the enzyme of established activity, which would clot the milk quickly. This decrease in clotting time is proportional to RA in the original test sample and can be inferred from a slope of the decline in clotting time.

Small amplitude oscillatory rheology is a technique used nowadays to objectively study rennet-induced coagulation of milk (Curcio et al., 2001; Douillard, 1973; Karlsson, Ipsen, & Ardö, 2007; Lu, McMahon, & Vollmer, 2017; Mishra, Govindasamy-Lucey, & Lucey, 2005; Nicolau, Buffa, O'Callaghan, Guamis, & Castillo, 2015; O'Callaghan & Guinee, 1996; Panthi et al., 2019; Sandra, Cooper, Alexander, & Corredig, 2011); however, its application in determining residual rennet activity and rennet partitioning has not been tested. Thus, the current research aimed to apply this technique in assessing the rennet activity remaining in rennet whey with modification to the method developed by Carlson et al. (1985) by determining an unknown RA directly from a linear calibration curve of RCT against the inverse of RA. In addition, this could make this approach more feasible considering determination time (Carlson et al., 1985) and omission of extraction buffers (Singh & Creamer, 1990).

2. Materials and methods

2.1. Rennet whey preparation

The experimental design is summarised in Fig. 1. Two commercially available rennet types including fermentation produced chymosin (FPC; 200 international milk-clotting units (IMCU) mL^{-1}, Chymax Plus, Melbourne Food Ingredient Depot, Melbourne, Australia) and calf rennet (CR; 290 IMCU mL^{-1}, Cheeselinks, Lara, Australia) were used in this study. Reconstituted skim milk (RSM) was prepared by dispersing low-heat skim milk powder (Warrnambool Cheese and Butter - Saputo, Warrnambool, Victoria, Australia) in 8 mM $CaCl_2$ solution at a level of 110 g L^{-1} and was used to prepare the rennet whey. Rennet whey was prepared by adding 0.25 g glucono-δ-lactone (GDL) to 300 mL of RSM at 31 °C, to achieve pH at whey drainage of ~6.15, followed by immediate addition of freshly diluted rennet solutions (0.010 IMCU mL^{-1} RSM). The milk gels were cut 30 min after rennet addition, followed by cooking at 38 °C for 30 min, cooling at room temperature and whey drainage through a double layer cheese cloth for 30 min. Drain whey was collected directly into measuring cylinders. The curd was consequently centrifuged (Avanti J-26XP Centrifuge, Beckman Instruments Australia Pty. Ltd, Gladesville, Australia) at 5000 × *g* and collected (pressed) whey was mixed with the drain whey to obtain the total volume of the rennet whey. The rennet whey obtained was then ultra-centrifuged for 1 h at 100,000 × *g* at 20 °C using a Beckman Ultra L-70 type centrifuge (Beckman Coulter Inc., Brea, CA, USA) with a 70.1 TI Rotor, the centrifuged whey was kept refrigerated until determination of residual RA.

2.2. Determination of residual rennet activity in the rennet whey and rennet partitioning

2.2.1. Creation of calibration curves

Calibration curves were created by performing clotting tests on standard RSM [0.12 g skim milk powder mL^{-1} 0.1 M $CaCl_2$ solution (Berridge, 1945)] using rennet standards prepared in ultra-centrifugal control whey (milk serum). Ultra-centrifugal control whey was prepared from the corresponding RSM used for rennet whey preparation and was treated in the same way as the one used for preparation of rennet whey, excluding the renneting step only (i.e., addition of GDL, 30 min incubation at 31 °C, heating to 38 °C for 30 min and cooling to room temperature for 30 min). Treated RSM was also ultra-centrifuged at 100,000 × *g* for 1 h and 20 °C, and the serum was kept refrigerated upon collection.

Clotting tests were performed by adding exactly 2 mL of the standard rennet preparations (0.04–0.59 and 0.04–0.34 IMCU mL^{-1} milk serum for FPC and CR, respectively) to 19 mL of pre-warmed standard RSM at 30 °C in the rheometer cup, and performing dynamic oscillatory tests at 30 °C, at constant frequency of 1 Hz and constant strain of 0.5% (Sinaga, Bansal, & Bhandari, 2017) using a cup and bob geometry of a MCR 301CS/CR rheometer (Anton Paar, GmbH, Ostfildern, Germany). Upon rennet addition and lowering of the rheometer probe, which took approximately 90 s, the test started by applying a shear of 500 s^{-1} for 15 s, and subsequently, changes in rheological firmness (storage modulus, G′) with time were recorded until a G′ value ≥ 1 Pa was reached (Guinee, Pudja, & Mulholland, 1994).

Several calibration curves were created by plotting the renneting time (during small amplitude measurements) for every G′ value along the enzymatic phase (from 0.1 to 1.1 Pa) against the inverse of RA of the standard preparations. Finally, the calibration curve at G′ = 1 Pa (Fig. 2) was selected and used to estimate the residual RA in rennet whey and rennet partitioning presented in the current study although all calibration curves including those that did not satisfy the criteria resulted in similar results (Table 1). This selection was based on relevance to a specific point at which an important physical change occurs, i.e., the transition from the enzymatic to aggregation phase also termed as the RCT or clotting time which is frequently defined as the first time point at which G′ ≥ 1 Pa (Lu et al., 2017; Mishra et al., 2005; Nicolau et al., 2015).

2.2.2. Determination of residual RA in whey

Different known RA of each rennet (0.07–0.28 IMCU mL^{-1} of ultra-centrifugal control whey for FPC and 0.12–0.24 IMCU mL^{-1} of ultra-centrifugal control whey for CR) based on the linear range of the calibration curves were prepared. Several samples of the rennet whey containing an unknown residual RA were then spiked with these known RA levels. Two millilitres of such rennet whey samples were then used to clot the standard RSM and corresponding RCTs were obtained using the rheometer as described above. The total RA in the rennet whey samples was then estimated from the calibration curves and the unknown residual RA in the rennet whey was calculated as a difference between the estimated values for each spiking level [i.e., (total RA in the rennet whey) − (control RA in the ultra-centrifugal control whey)]. The RA partitioning, i.e., proportion of RA remaining in the whey and retained in the curd as percentages of the total RA added to the RSM was then calculated using equations (1) and (2) below:

$$\text{RA in the rennet whey } (RA_w, \%) = \left(\frac{RA_m \times V_w}{RA_T}\right) \times 100 \quad (1)$$

and the RA retained in the curd was given by:

$$\text{RA retained in the curd } (\%) = 100 - RA_w \quad (2)$$

where RA_w is % RA estimated in whey, RA_m is RA added per 1 mL of RSM, V_w presents total volume of rennet whey in mL (drained + pressed whey), RA_T is total RA added to RSM and V_m presents total volume of RSM in mL.

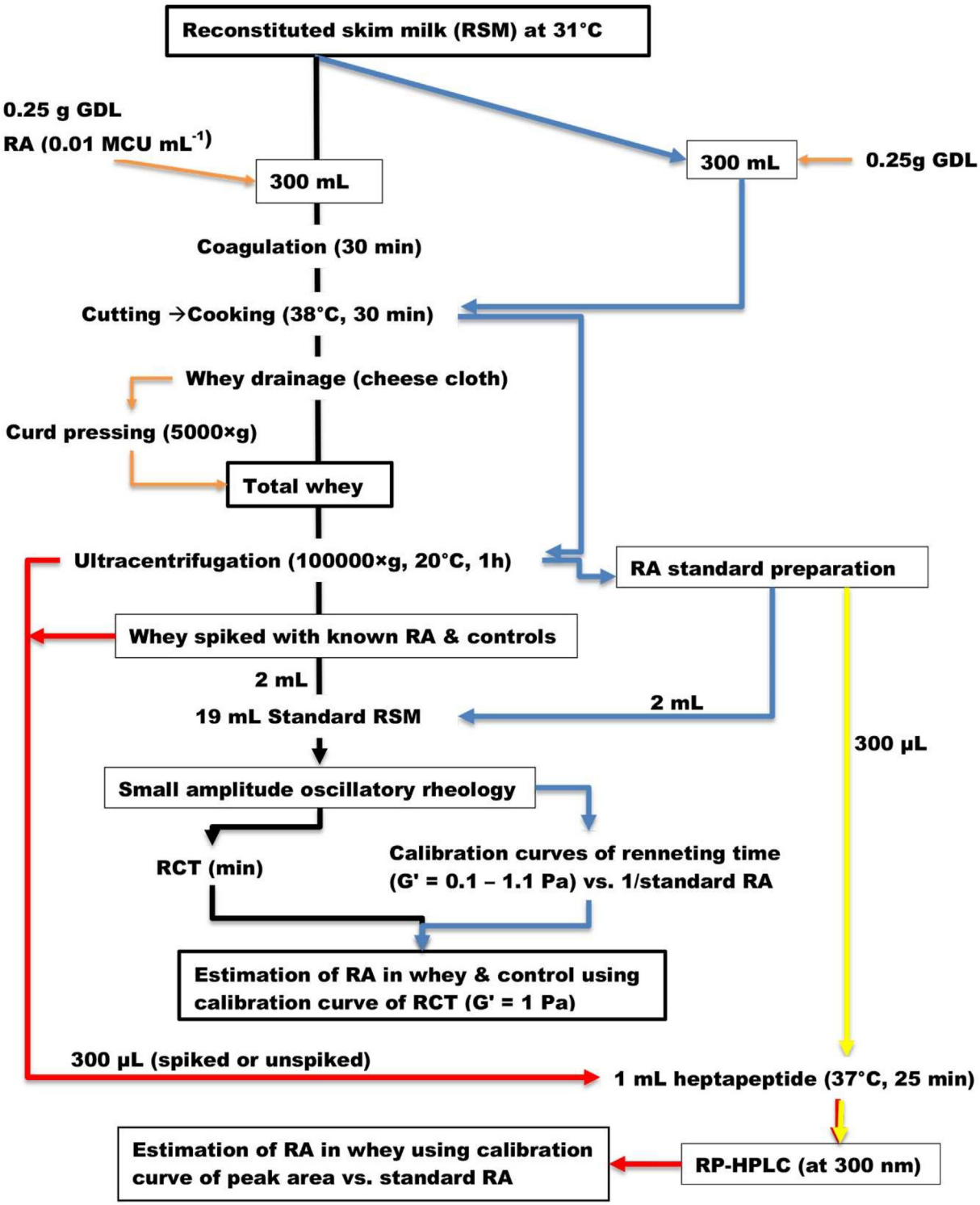

Fig. 1. Summary of the experimental design.

2.3. Comparison with the RP-HPLC method

The results obtained by small amplitude oscillatory measurements were compared to a RP-HPLC method developed by Hurley et al. (1999) with some modifications. A synthetic heptapetide (Pro–Thr–Glu–Phe– [NO_2–Phe] –Arg–Leu) was purchased from Mimotopes (Mulgrave, Victoria, Australia) and used as a substrate. The substrate was prepared by dissolving 10 mg of the heptapetide in 10 mL of Milli-Q water and made to 100 mL with sodium formate buffer (100 mM, pH 3.2).

For establishing the RA using the RP-HPLC, stock solutions of rennet standards were prepared by adding 2.8 IMCU for FPC or 4.7 IMCU for CR to 4.96 mL of ultra-centrifugal control whey. From these, standard solutions of 0.002–0.022 IMCU mL^{-1} or 0.004–0.038 IMCU mL^{-1} of ultra-centrifugal control whey were respectively prepared for establishing calibration curves. Several

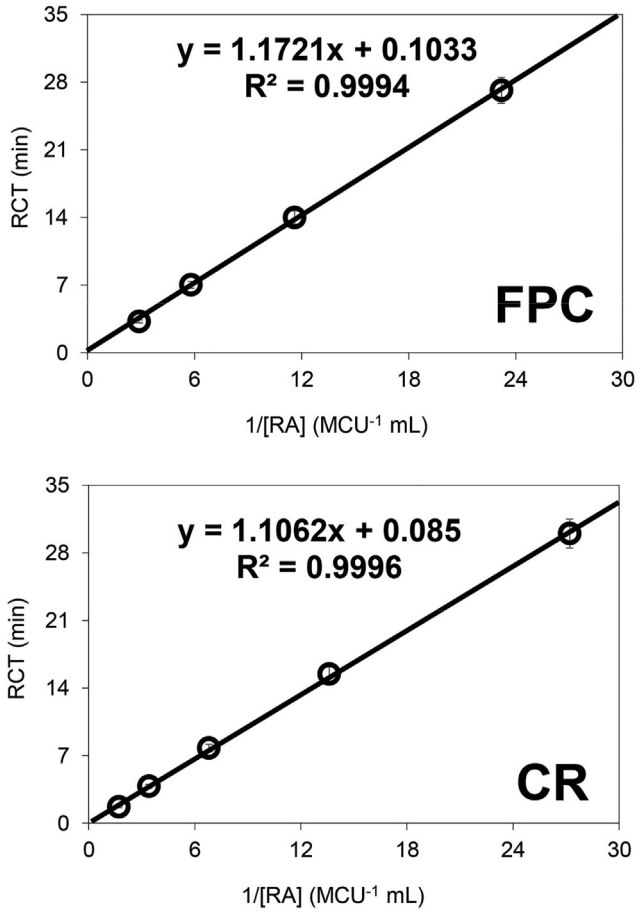

Fig. 2. Calibration curves of rennet coagulation time (RCT at G′ = 1 Pa) against the inverse of rennet activity (RA) obtained by addition of known RA of FPC or CR preparation to the serum phase of the RSM obtained by ultracentrifugation.

whey samples were prepared by either spiking the rennet whey with an unknown residual RA or without it. The rennet whey samples were spiked in triplicates with two levels of RA (i.e., 0.007 and 0.014 IMCU for FPC or 0.012 and 0.024 IMCU for CR).

For each standard and test sample, 1 mL of the substrate solution was pipetted into a 5 mL micro tube. The substrate, standards and test samples were placed in a water bath at 37 °C and incubated for 10 min. After the incubation period, 300 μL of standard or test sample solution were added to 1 mL of the substrate solution and the timer was immediately started. The mixture was vortexed for 7 s and incubated at 37 °C in the water bath for 25 min. Then, 26 μL pepstatin (Sigma–Aldrich Pty. Ltd, Macquarie Park, Australia) solution (0.68 mg mL^{-1} ethanol) were added to stop the reaction. The mixture was again vortexed for 7 s and filtered through a 0.45 μm membrane filter prior to direct injection into an RP-HPLC system (LC-2030C, Prominence-I, Shimadzu Corporation, Kyoto, Japan) and analysed using a C_{18} column (Jupiter® 5 μm, 300 Å, 250 × 10 mm, Phenomenex, Macclesfield, UK) with 0.1% trifluoroacetic acid (TFA, Sigma–Aldrich) in Milli-Q water and 0.1% TFA in 90% acetonitrile (Sigma–Aldrich) as mobile phase A and B, respectively. The elution gradient was started with 10% B for 1 min, increased linearly to 66.6% B over 15 min and back to 10% B that was maintained for 14 min, oven temperature was set at 25 °C and the flow rate was 0.7 mL min^{-1}. The parent peptide and its hydrolysate were detected at 300 nm using a UV-detector (Shimadzu). A calibration curve of peak area of the hydrolysate against RA was drawn (Fig. 3) and used to estimate the RA in cheese-whey which for spiked samples was calculated as described above, i.e., total RA − control (spike level).

2.4. Statistical analysis

The whole approach was at least replicated with subsampling. The individual observations were analysed using a general linear model (GLM) with rennet source and spiking levels as the main factors by SAS software (v. 9.1). The level of significance was set at $p \leq 0.05$. To determine whether there were significant differences (95% confidence interval) in the RA mL^{-1} of rennet whey and RA partitioning between the curd and whey estimated using different spike levels, a pairwise comparison of duplicate measurements was performed in MS Excel 2016 by performing an unpaired, two tailed t-test assuming unequal variance.

3. Results

3.1. Residual rennet activity in rennet whey determined by small amplitude oscillatory rheology

Creation of several calibration curves for every G′ value along the enzymatic phase (from 0.1 to 1.1 Pa) was performed to determine how the essential calibration parameters, i.e., slope, Y-intercept and R^2 change as a function of G′ in order to select the most reliable calibration curve for the estimation of the RA in the rennet whey. The selection criteria included a positive slope (indicating the linearity defined by Foltman, 1959), high R^2 value and renneting time ≥ 0 at the highest RA value (i.e., a positive Y-intercept). A number of calibration curves satisfied all the criteria as shown in Table 1.

The estimated concentration of residual RA mL^{-1} of rennet whey using all calibration curves for every G′ value (0.1–1.1 Pa) along the enzymatic phase was found to be the same (Table 2), indicating that determination of RA partitioning using small amplitude oscillatory measurement is feasible as shown in Table 3. An important part of the approach was establishment of a

Table 1
Changes in major calibration parameters (slope, y-intercept and R^2) as a function of G′ from the calibration curves of renneting time at every G′ value from 0.1 to 1.1 Pa versus the inverse of rennet activity (RA) of the rennet standard preparations.[a]

Rennet type	Calibration parameter	G′ (Pa)										
		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1
FPC	Slope	1.0916	1.1036	1.1133	1.1242	1.1333	1.1415	1.1487	1.1567	1.1649	1.1734	1.1799
	Intercept	−0.2479	−0.1795	−0.1341	−0.1054	−0.0730	−0.0408	−0.0092	0.0218	0.0532	0.0757	0.1070
	R^2	0.9995	0.9994	0.9994	0.9994	0.9994	0.9994	0.9994	0.9994	0.9994	0.9994	0.9994
CR	Slope	1.0031	1.0239	1.04	1.051	1.0629	1.073	1.0816	1.0896	1.098	1.1062	1.1128
	Intercept	−0.1503	−0.1213	−0.1017	−0.0707	−0.0489	−0.0283	0.0013	0.0317	0.0606	0.085	0.1247
	R^2	0.9997	0.9996	0.9996	0.9996	0.9996	0.9996	0.9996	0.9996	0.9995	0.9996	0.9995

[a] FPC, fermentation produced chymosin; CR, calf rennet.

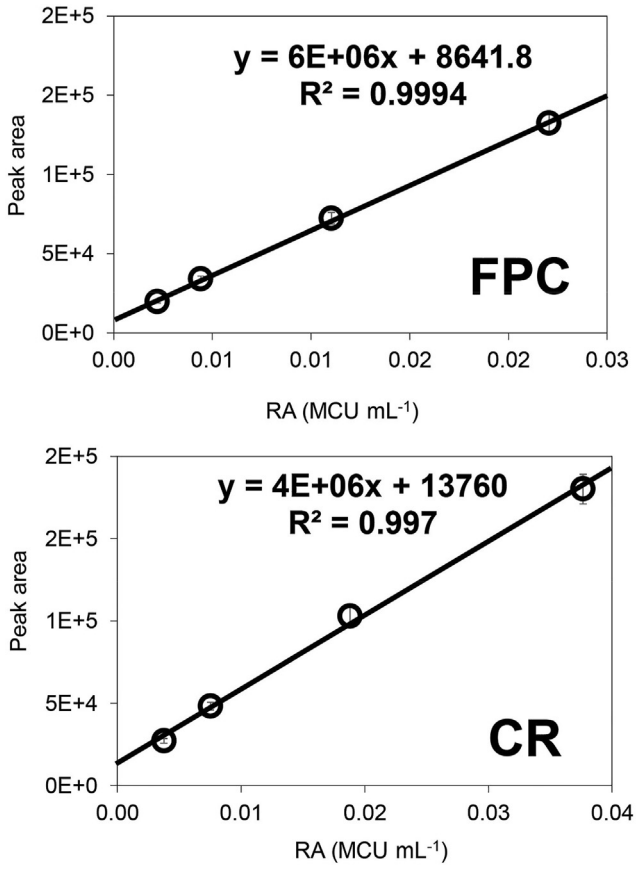

Fig. 3. Calibration curves of peak area of the heptapeptide hydrolysate against the rennet activity (RA) of FPC or CR preparation obtained by a standard RP-HPLC method.

Table 2
Estimated residual rennet activity (IMCU $mL^{-1}$of rennet whey) using calibration curves for every G′ value (0.1–1.1 Pa) along the enzymatic phase and every spiking level.[a]

G′ (Pa)	Spiking level (IMCU mL^{-1} of ultra-centrifuge control serum)							
	Fermentation produced chymosin			Calf rennet				
	0.28	0.14	0.007	0.24	0.21	0.18	0.15	0.12
0.1	0.0099	0.0096	0.0094	0.0099	0.0103	0.0092	0.0095	0.0096
0.2	0.0104	0.0102	0.0103	0.0095	0.0100	0.0099	0.0095	0.0099
0.3	0.0098	0.0095	0.0093	0.0091	0.0102	0.0099	0.0093	0.0102
0.4	0.0100	0.0097	0.0094	0.0106	0.0098	0.0094	0.0096	0.0102
0.5	0.0102	0.0103	0.0099	0.0089	0.0101	0.0098	0.0101	0.0103
0.6	0.0103	0.0104	0.0101	0.0090	0.0101	0.0102	0.0090	0.0099
0.7	0.0100	0.0112	0.0103	0.0096	0.0100	0.0114	0.0094	0.0109
0.8	0.0101	0.0106	0.0099	0.0096	0.0103	0.0109	0.0098	0.0099
0.9	0.0103	0.0105	0.0094	0.0091	0.0098	0.0104	0.0098	0.0104
1	0.0100	0.0098	0.0099	0.0085	0.0098	0.0100	0.0097	0.0097
1.1	0.0105	0.0102	0.0089	0.0087	0.0101	0.0105	0.0102	0.0101
SEM	0.0011			0.0008				

[a] SEM, standard error of the mean; all means were similar at 95% confidence ($p > 0.05$).

calibration curve (Fig. 1) within a linear region of enzymatic activity. For this reason, spiking samples with known activities within this range resulted in the same value of the residual RA of approximately 0.01 IMCU mL^{-1} shown in Table 2. This infers that once a calibration curve is created, a sample containing unknown residual RA can be spiked with a single level of known activity to establish this unknown residual level. Furthermore, a calculated RSD of 0.00% is likely an indication that the residual RA (quantity

Table 3
Rennet activity (RA) partitioned between the rennet whey and curd using a small amplitude oscillatory measurement and applying the model depicted by equations (1) and (2).[a]

Rennet type	Spiking level	RA recovered
FPC	0.28	86.7 ± 3.8
	0.14	
	0.07	
CR	0.24	87.2 ± 3.5
	0.21	
	0.18	
	0.15	
	0.12	

[a] FPC, fermentation produced chymosin; CR, calf rennet. Spiking level is the rennet activity added to whey (IMCU mL^{-1}); RA recovered is estimated RA recovered in whey (% of total). All values are means of at least duplicated tests; the results are presented as means ± standard deviation.

retained in the curd) depends on the degree of syneresis and pressing. This relationship can be described by a simple model depicted by equations (1) and (2), which estimates RA partitioning based on the total volume of the whey separated from the curd. This model showed that 86.7% and 87.2% of total RA added to milk for FPC and CR, respectively, remained in the whey.

Our observations appear in agreement with Bansal et al. (2007) and De Roos (1999), who stated that approximately 5–10% is usually retained in the cheese curd. On the other hand, Holmes et al. (1978) reported 72% residual RA in whey from freshly coagulated milk. In addition, 13.3% and 12.8% of total RA retained into the curd correspond to 19 and 18.9 IMCU kg^{-1} of the curd which is also in strong agreement with previous findings of 11.1–20.1 residual RA units kg^{-1} of commercial Cheddar cheeses (Hurley et al., 1999; Singh & Creamer, 1990).

3.2. Comparison with RP-HPLC results

As shown in Table 4, two approaches (with and without spiking) were used to verify our findings. For both approaches the RP-HPLC results confirmed that in terms of unknown residual RA recovered in the rennet whey, the values were practically the same to those determined by small amplitude oscillatory measurement (Table 3). The two methods differ in terms of sensitivity as it is clear that unlike the small amplitude oscillatory measurement approach where very low spike levels would result in a lengthy testing time, RP-HPLC method does not necessarily require the rennet whey to be spiked. Even without spiking, changes in the peak area of the heptapetide hydrolysate could still be detected and accurately used to predict the unknown RA. Therefore, obtaining the same results despite differences in protocols followed confirms the reliability of the small amplitude oscillatory measurement approach. In addition to confirming the feasibility of the small amplitude oscillatory measurement approach, this also confirmed that the model depicted by equations (1) and (2) could be effectively used to estimate RA partitioning. The maximum RSD was found to be 5.5% for spiked and 5.9% for non-spiked FPC (n = 3) samples whereas the maximum RSD for CR (n = 3) was 2.4%.

4. Discussion

The residual unknown RA in the rennet whey was measured using a small amplitude oscillatory measurement in an attempt to further improve the method of Carlson et al. (1985). The improvement includes the application of an objective and reliable RCT determination technique, i.e., the small amplitude oscillatory measurement, the creation of a calibration curve of RCT as a

Table 4
Estimated residual rennet activity (RA) in the rennet whey using a standard RP-HPLC method.[a]

Rennet type	Spiking level	Residual RA in whey (IMCU mL^{-1})		RSD (%, n = 3)
		Total	Unknown	
FPC	0.014	0.0243[a]	0.0105[a]	1.7
	0.007	0.0187[b]	0.0118[a]	5.5
	none	0.0109[c]	0.0109[a]	5.9
SEM		0.0004		
CR	0.024	0.0345[a]	0.0109[a]	1.4
	0.012	0.0223[b]	0.0105[a]	1.6
	none	0.0116[c]	0.0116[a]	2.4
SEM		0.0003		

[a] Standard RP-HPLC method as in Hurley et al. (1999). Spiking level is the rennet activity added to whey (IMCU mL^{-1}): FPC, fermentation produced chymosin; CR, calf rennet. All values are means of at least triplicate tests; different superscript letters indicate significant difference among the means for individual rennet type (p <0.05): SEM, standard error of the mean.

function of inverse of known RA in a serum phase of the same RSM used for preparation of rennet whey and thereby needing one spike level only for direct estimate of very low unknown RA.

Compared with the reference method (Carlson et al., 1985) and the standard RP-HPLC (Hurley et al., 1999) approach, the current technique may be more feasible on sites with an available rheometer and that lack analytical tools such as HPLC. Another advantage of the current approach is that there are several alternatives by which the creation of calibration curves and the subsequent estimates of the unknown residual RA could be done. One of these is the fact that the same results were obtained using renneting times at different G′ values along the enzymatic phase (Table 2). In addition, since RCT is defined either as the time at which G′ $\geq$ 1 Pa (Lu et al., 2017; Mishra et al., 2005; Nicolau et al., 2015) or G′ = loss modulus (G″) (Lu et al., 2017), the creation of calibration curves and the subsequent estimates of residual RA could be also possibly achieved based on tan $\delta = 1$ as a rheometer provides both G′ and G″ values. The calibration curve was also obtained in a linear region resulting in a high R^2, which allowed estimate of an unknown residual RA in the rennet whey using a single spiking level.

This is not the first time such a linearity has been observed although Singh and Creamer (1990) noted this for a relationship between clotting time (t_c) and incubation time (t_i) used for estimation of residual RA in the equation outlined by Carlson et al. (1985). Clotting activity of a rennet preparation, like curd firming rate, is not a property of the enzyme alone, but is also governed by other factors (Lomholt & Qvist, 1999) including milk protein content, pH, temperature, salts and presence various inhibitors in milk (Bansal, Fox, & McSweeney, 2010a,b; Panthi et al., 2019). However, it is known that the level of rennet activity effects both the rate of enzymatic reaction and the curd firming rate (Lomholt & Qvist, 1997; Soodam et al., 2015). Furthermore, a correlation between the cutting time and rheological properties was observed previously (López, Lomholt, & Qvist, 1998). Therefore, even though what is measured in both approaches (i.e., using G′ values along the enzymatic phase as presented in the current study or the former incubation time-clotting time relationship approach (Carlson et al., 1985; Singh & Creamer, 1990) is not purely enzymatic, they both correlate well with changes in RA and appeared to be effective in estimating the unknown RA.

As outlined above, properties of rennet preparations including the source and specific activity appear to be important factors affecting the clotting time. However, for determination of residual RA in cheese whey, other factors may likely play a role as it appears that the determination needs to be performed under well-defined conditions, i.e., the calibration curve would need to be created from the serum phase of the milk used in the cheesemaking. When water was used, lower RA standards produced higher RCT than expected (according to the relationship: enzyme concentration $\times$ (RCT − intercept) = constant; Foltman, 1959) and appeared to affect the linearity of the calibration curve, indicating that higher standards and spike levels would be required. Compared with what was observed using water, when the serum phase of the RSM, used for obtaining the rennet whey, was used for calibration, a much more linear relationship (higher R^2) between RCT and 1/RA was obtained irrespective of the enzyme origin.

Another important observation is that a chosen spiking level should be within a linear range. This has been confirmed using one of the common methods for determination of residual RA (Table 4). The noted recovery of unknown RA mL^{-1} in the rennet whey (Tables 2 and 4) appears highly likely since chymosin is soluble in the serum phase and as a catalyst does not form any strong and stable bond with the casein micelles. Instead, temporary, weak and short-range interactions take place, which facilitate the hydrolysis of the scissile peptide bond by a water molecule activated by an aspartyl residue in the enzyme cleft (Palmer et al., 2010; Yegin & Dekker, 2013). An intermediate complex formed during simultaneous transfer of protons between the dyad, water molecule and the peptide bond breaks down rapidly by an acid-base reaction leaving the enzyme free for further catalysis (Chitpinityol & Crabbe, 1998; Yegin & Dekker, 2013). In addition, based on its specificity and limited access to other sites of caseins due to limited diffusion and conformation of caseins once the curd is formed, chymosin may be considered free at the end of gelation since almost none of its binding site on κ-casein remains available. Therefore, the enzyme can easily be washed away during whey drainage and pressing. Thus, in terms of concentration, the activity of rennet per mL of the medium (the whey) into which the catalytic reaction takes place remains unchanged.

Our calculations of RA partitioning appear in agreement with previous studies, such as that of Bansal et al. (2007) who reported that the retention of chymosin in the cheese curd was independent of both casein content and average casein micelle size, showing that the enzyme only facilitates the hydrolysis of κ-casein while remaining physically as part of the serum phase. Bansal et al. (2007) also suggested that the effect of ionic strength may be important as chymosin retention increased with an increase in ionic strength from 0.2 to 0.8 M NaCl, which they believed was to be due to an increase in binding of chymosin to the casein micelles as a result of decreased surface potential. In addition, chymosin binding to para-κ-casein was also reported to occur when the enzyme was immobilised onto soya-oil emulsion droplets stabilised with κ-casein, but not with other caseins (De Roos, 1999). The association was believed to be via electrostatic interactions as it was found to be enhanced by low pH, temperature and ionic strength. Similarly, Reyes (1971) argued that rennin binds to cheese curd at low milk coagulation pH based on a reduced distribution in cheese-whey and its release from the curd during extraction.

Awad (2007), on the other hand, claimed that NaCl has no influence on the aggregation of hydrolysed casein micelles, instead, only that the enzymatic phase is affected where the RCT is reduced up to 3 mM NaCl addition while higher concentrations produce an inhibitory effect. Therefore, the effect of ionic strength on rennet partitioning could be related to its influence on water retention rather than on chymosin binding claimed by Bansal et al. (2007). Furthermore, no association with adsorbed caseins other than κ-casein was found, and in the same study, in solutions of κ-casein or mixtures of caseins in imidazole buffer the enzyme was found to dissociate after a certain time which was attributed to its proteolytic activity as well as to its competition with other caseins for association with para-κ-casein (De Roos, 1999). This is in agreement

with the current study, implying that the enzyme-κ-casein complex formed under cheesemaking conditions is temporary, which implies that the proteolytic activity of the enzyme on other caseins would continue during cheese ageing and storage contributing to textural and flavour changes (Fox, 1989; Hynes, Aparo, & Candioti, 2004; Visser & de Groot-Mostert, 1977). Furthermore, as lower RA was detected in whey at pH 5.2–5.9 (Holmes et al., 1978; Wang, 1969) while whey separation increases as pH decreases up to 5 (Liu et al., 2014; Patel, Lund, & Olson, 1971), contrasting findings obtained in the current study at a final pH of ~5.4 could be attributed to differences in the sensitivity of the methods under various conditions. The results of the current research suggest that the retention of chymosin into cheese curd may rather be promoted by conditions that control whey removal unless the enzyme is denatured. This information may have important technological implications with regard to attempts to increase chymosin retention to accelerate cheese ripening while preserving the intrinsic cheese quality.

The retention of the two rennets at a similar proportion in the current study was not surprising since the enzymatic properties of calf chymosin which makes up >90% in calf rennet (Kumar, Grover, Sharma, & Batish, 2010) and recombinant chymosin appear indistinguishable (Meisel & Frister, 1988). Higher RA retention into the curd than whey was reported at low renneting pH of 5.2 (Holmes et al., 1978; Reyes, 1971) as well as pH 5.9 and 5.7 (Wang, 1969) while the residual RA detected into Cheddar cheese for a typical renneting pH of 6.6 ranged from 6 to 18.4% (Holmes et al., 1978; Wang, 1969) which is also in agreement with the present study. The retention was, however, greater than 2–3% retention for microbial rennets whereas porcine pepsin was found to be extensively denatured during cheesemaking (Holmes et al., 1978). Pepsin or microbial rennets do not favour low pH in cheese (Creamer, Lawrence, & Gilles, 1985) which is in contrast to chymosin as aforementioned. Greater general proteolytic power of microbial as well as plant rennets leads to production of bitter peptides in bovine milk cheeses. Therefore, low retention rates would be advantageous as it may help to control excessive bitterness. At the same pH some plant rennets and recombinant chymosin showed similar clotting activities and, unlike bovine milk, some were confirmed as suitable substitutes for cheese production from goat milk (Esteves, Lucey, Wang, & Pires, 2003; García et al., 2012).

5. Conclusion

In this research, we investigated an application of small amplitude oscillatory measurement for estimating residual RA in the rennet whey produced using either a calf rennet or fermentation produced chymosin. The findings suggest that under the conditions indicated including creation of a calibration curve in the serum phase of corresponding cheese milk, the approach could be reliably used to estimate the unknown RA. The partitioning of RA between the rennet whey and the curd appears to be dependent on the quantity of whey removed, provided enzymes are not denatured by the cheese making conditions. The advantage of this approach lies in the fact that the Berridge method commonly used for determination of residual RA in an industrial setting could be objectified by replacing visual observation with determination of RCT using the spiking technique and a small amplitude oscillatory rheology. The approach tested, however, covers only one set of variables (source of rennet) and would require confirmation by applying various industry relevant conditions (for example pH, milk composition, degree of pressing or syneresis) to arrive to establishment of an objective, simple and reliable technique for determination of RA partitioning using rheology.

Declaration of competing interest

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

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References

Awad, S. (2007). Effect of sodium chloride and pH on the rennet coagulation and gel firmness. *LWT - Food Science and Technology, 40*, 220–224.
Bansal, N., Fox, P. F., & McSweeney, P. L. H. (2007). Factors affecting the retention of rennet in cheese curd. *Journal of Agricultural and Food Chemistry, 55*, 9219–9225.
Bansal, N., Fox, P. F., & McSweeney, P. L. H. (2010a). Inhibition of rennet activity in cheese using equine blood serum. *Dairy Science & Technology, 90*, 673–685.
Bansal, N., Fox, P. F., & McSweeney, P. L. H. (2010b). Inhibition of rennets by blood serum. *International Journal of Dairy Technology, 63*, 381–386.
Berridge, N. J. (1945). The purification and crystallization of rennin. *Biochemical Journal, 39*, 179–186.
Berridge, N. J. (1952). Some observations on the determination of the activity of rennet. *Analyst, 77*, 57–62.
Boudjellab, N., Rolet-Repecaud, O., & Collin, J. (1994). Detection of residual chymosin in cheese by an enzyme-linked immunosorbent assay. *Journal of Dairy Research, 61*, 101–109.
Carlson, A., Hill, C. G. J. R., & Olson, N. F. (1985). Improved assay procedure for determination of milk-clotting enzymes. *Journal of Dairy Science, 68*, 290–299.
Chitpinityol, S., & Crabbe, M. J. C. (1998). Chymosin and aspartic proteinases. *Food Chemistry, 61*, 395–418.
Creamer, L. K., Lawrence, R. C., & Gilles, J. (1985). Effect of acidification of cheese milk on the resultant Cheddar cheese. *New Zealand Journal of Dairy Science & Technology, 20*, 185–203.
Curcio, S., Gabriele, D., Giordano, V., Galabrò, V., Cindio, B., & Iorio, G. (2001). A rheological approach to the study of concentrated milk clotting. *Rheologica Acta, 40*, 154–161.
De Roos, A. L. (1999). *The adsorption of chymosin and lysozyme onto emulsion droplets and their association with casein* (PhD thesis). Wageningen, The Netherlands: Wageningen Agricultural University.
Douillard, R. (1973). Rheological analysis of curd formation. *Journal of Texture Studies, 4*, 158–165.
Esteves, C. L. C., Lucey, J. A., Wang, T., & Pires, E. M. V. (2003). Effect of pH on the gelation properties of skim milk gels made from plant coagulants and chymosin. *Journal of Dairy Science, 86*, 2558–2567.
Foltman, B. (1959). On the enzymatic and coagulation stages of the renneting process. In *15th International Dairy Congress, London, 2* pp. 655–661).
Fox, P. F. (1989). Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science, 72*, 1379–1400.
García, V., Rovira, S., Teruel, R., Boutoial, K., Rodríguez, J., Roa, I., et al. (2012). Effect of vegetable coagulant, microbial coagulant and calf rennet on physicochemical, proteolysis, sensory and texture profiles of fresh goats cheese. *Dairy Science & Technology, 92*, 691–707.
Guinee, T. P., Pudja, P. D., & Mulholland, E. O. (1994). Effect of milk protein standardization by ultrafiltration on the manufacture, composition and maturation of Cheddar cheese. *Journal of Dairy Research, 61*, 117–131.
Holmes, D. G., Duersch, J. W., & Ernstrom, C. A. (1978). Distribution of milk clotting enzymes between curd and whey and their survival during Cheddar cheese making. *Journal of Dairy Science, 60*, 862–869.
Hurley, M. J., O'Driscoll, B. M., Kelly, A. L., & McSweeney, P. L. H. (1999). Novel assay for the determination of residual coagulant activity in cheese. *International Dairy Journal, 9*, 553–558.
Hynes, E. R., Aparo, L., & Candioti, M. C. (2004). Influence of residual milk-clotting enzyme on α_{S1}-casein hydrolysis during ripening of Reggianito Argentino cheese. *Journal of Dairy Science, 87*, 565–573.
Jaros, D., & Rohm, H. (2017). Rennets: Applied aspects. In P. L. H., P. F. McSweeney, P. D. Fox, Cotter, & D. W. Everett (Eds.), *Cheese: Chemistry, physics and microbiology* (p. 53). London, UK: Elsevier.
Karlsson, A. O., Ipsen, R., & Ardö, Y. (2007). Rheological properties and microstructure during rennet induced coagulation of UF concentrated skim milk. *International Dairy Journal, 17*, 674–682.
Kumar, A., Grover, S., Sharma, J., & Batish, V. K. (2010). Chymosin and other milk coagulants: Sources and biotechnological interventions. *Critical Reviews in Biotechnology, 30*, 243–258.
Lefebvre-cases, E., Gastaldi, E., Vidal, V., Marchesseau, S., Lagaude, A., Cuq, J.-L., et al. (1998). Identification of interactions among casein gels using dissociating chemical agents. *Journal of Dairy Science, 81*, 932–938.

Liu, X. T., Zhang, H., Wang, F., Luo, J., Guo, H. Y., & Ren, F. Z. (2014). Rheological and structural properties of differently acidified and renneted milk gels. *Journal of Dairy Science, 97*, 3292–3299.
Lomholt, S. B., & Qvist, K. B. (1997). Relationship between rheological properties and degree of κ-casein proteolysis during renneting of milk. *Journal of Dairy Research, 64*, 541–549.
Lomholt, S. B., & Qvist, K. B. (1999). Gel firming rate of rennet curd as a function of rennet concentration. *International Dairy Journal, 9*, 417–418.
López, M. B., Lomholt, S. B., & Qvist, K. B. (1998). Rheological properties and cutting time of rennet gels. Effect of pH and enzyme concentration. *International Dairy Journal, 8*, 289–293.
Lu, Y., McMahon, D. J., & Vollmer, A. H. (2017). Investigating rennet coagulation properties of recombined highly concentrated micellar casein concentrate and cream for use in cheese making. *Journal of Dairy Science, 100*, 892–900.
Meisel, H., & Frister, H. (1988). Chemical characterization of a casein-ophosphopeptide isolated from in vivo digests of a casein diet. *Biological Chemistry Hoppe-Seyler, 369*, 1275–1279.
Mishra, R., Govindasamy-Lucey, S., & Lucey, J. A. (2005). Rheological properties of rennet-induced gels during the coagulation and cutting process: Impact of processing conditions. *Journal of Texture Studies, 36*, 190–212.
Nicolau, N., Buffa, M., O'Callaghan, D. J., Guamis, B., & Castillo, M. (2015). Estimation of clotting and cutting times in sheep cheese manufacture using NIR light backscatter. *Journal of Dairy Science and Technology, 95*, 495–507.
O'Callaghan, D. J., & Guinee, T. P. (1996). Comparison of mathematical models applied to the rennet coagulation of skim milks. *Journal of Texture Studies, 26*, 607–633.
Palmer, D. S., Christensen, A. U., Sørensen, J., Celik, L., Qvist, K. B., & Schiøtt, B. (2010). Bovine chymosin: A computational study of recognition and binding of bovine κ-casein. *Biochemistry, 49*, 2563–2573.
Panthi, R. R., Kelly, A. L., McMahon, D. J., Dai, X., Vollmer, A. H., & Sheehan, J. J. (2019). Response surface methodology modeling of protein concentration coagulum cut size, and set temperature on curd moisture loss kinetics during curd stirring. *Journal of Dairy Science, 102*, 4989–5004.
Patel, M. C., Lund, D. B., & Olson, N. F. (1971). Factors affecting syneresis of renneted milk gels. *Journal of Dairy Science, 55*, 913–918.
Reyes, J. (1971). *A procedure for measuring residual rennin activity in whey and curd from freshly coagulated milk* (MSc thesis). Logan, Utah, USA: Utah State University.
Sandra, S., Cooper, C., Alexander, M., & Corredig, M. (2011). Coagulation properties of ultrafiltered milk retentates measured using rheology and diffusing wave spectroscopy. *Food Research International, 44*, 951–956.
Sinaga, H., Bansal, N., & Bhandari, B. (2017). Effects of milk pH alteration on casein micelle size and gelation properties of milk. *International Journal of Food Properties, 20*, 179–197.
Singh, H., & Creamer, L. K. (1990). A sensitive quantitative assay for milk coagulants in cheese and whey products. *Journal of Dairy Science, 73*, 1158–1165.
Sousa, M. J., Ardo, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal, 11*, 327–345.
Visser, F. M. W., & de Groot-Mostert, A. E. A. (1977). Contribution of enzymes from rennet starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 4. Protein breakdown: A gel electrophoretical study. *Netherlands Milk and Dairy Journal, 31*, 247–264.
Wang, J. T. (1969). *Survival and distribution of rennin during Cheddar cheese manufacture* (MSc Thesis). Logan, Utah, USA: Utah State University.
Yegin, S., & Dekker, P. (2013). Progress in the field of aspartic proteinases in cheese manufacturing: Structures, functions, catalytic mechanism, inhibition and engineering. *Dairy Science & Technology, 93*, 565–594.

Chapter 4– Impact of reconstituted skim milk concentration on partitioning of rennet activity between rennet curd and whey

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This chapter has been submitted as an original research manuscript entitled "Impact of reconstituted skim milk concentration on partitioning of rennet activity between rennet curd and whey" by Joseph F. Kayihura for consideration in a peer-reviewed journal and it is currently in the revised form. As described in Chapter 3 (Kayihura et al. 2022; 10.1016/j.idairyj.2022.105451), the modified procedure of the RP-HPLC method originally developed by Hurley et al. ((1999) https://doi.org/10.1016/S0958-6946(99)00118-1) was applied in this study to assess the relationship between the reconstituted skim milk protein content and concentration of the residual rennet activity in rennet whey (International Milk Clotting Units, IMCU/mL) in order to reveal whether or not partitioning is driven by enzymes-caseins associations. This was tested as a hypothesis based on the findings of the previous study (Chapter 3) where the residual rennet activity (IMCU/mL of whey) was equivalent to the initial concentration of rennet activity (IMCU/mL) added to a milk sample with ~3.5% protein.

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Joseph Flambeau Kayihura	100%	Conceptualisation, design, experimentation, writing, submission, revision & editing	[redacted]	01/10/2023

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Impact of reconstituted skim milk concentration on partitioning of rennet activity between rennet curd and whey

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ABSTRACT

The amount of rennet used for milk coagulation during cheesemaking is partitioned between the curd and whey. Active rennet retained into the curd contributes towards flavour and texture development in aged cheeses. The aim of the present study was to evaluate the impact of increasing the reconstituted skim milk concentration (3–9.4% protein) particularly on the concentration of residual rennet activity (RA) lost in whey and the %RA partitioned/retained into the curds produced by renting only and renneting under slow acidification achieved using glucono-δ-lactone (GDL). The RA partitioned into curds increased with skim milk concentration from 9.2 to 31.4% under renneting without acidification or 7.2 to 20.2% under renneting combined with slow acidification and appeared to be proportional to moisture and protein retentions. Contrary, the concentration of residual RA in whey (international milk clotting units, IMCU/mL) was not affected and was the same as the initial IMCU/mL of milk. The implication is that partitioning of RA between rennet curd and whey is not driven by the enzymes–caseins association. Instead, the strength of casein-casein interactions could be the driving force, although this remains to be evaluated. This information may be useful for studies dealing with improving the cheese ripening process.

Key words: Calf rennet; Milk concentration; Rennet activity partitioning; Rennet curd; Rennet whey.

1. Introduction

Gelation of milk is an essential step during the manufacture of most cheeses. This process requires the application of one or a combination of coagulants (mainly clotting enzymes and acids) to destabilise caseins which naturally occur in milk as stable colloidal dispersions. Calf rennet is the oldest form of milk clotting enzymes used for cheesemaking (McSweeney, 2022). Depending on the age and feeding, commercial rennets may contain 50–95% chymosin which is known to hydrolyse a specific bond on κ-casein resulting into aggregation of casein micelles and milk clotting (Broome & Limsowtin, 1998; Kumar, Grover, Sharma & Batish, 2010; Jacob, Jaros & Rohm, 2011; McSweeney, 2022). Thus, the strength or clotting activity of calf rennet depends on chymosin concentration and purity (Fish, 1957; López *et al*.,1997), however, since it also contains pepsin whose proteolytic and clotting activities vary compared to chymosin, RA would preferably be quantified in terms of individual activities (% or mg/L) (Andrén, 2011). RA is quantitatively assessed by milk clotting assays i.e., based on a linear inverse relationship between rennet concentration and the time it takes to clot milk under defined conditions (Berridge, 1952; Carlson, Hill & Olson, 1985). Former conditions include a standard Berridge substrate of 12% reconstituted skim milk in 0.02 N $CaCl_2$ solution and a renneting temperature of 30°C (Berridge, 1945). The newly suggested international standard conditions for total RA assessment are 0.05% (v/w) $CaCl_2$, pH 6.5, 32°C and reference rennet powders with 1000 IMCU/g (McSweeney, 2022).

Like milk constituents, the RA used for milk coagulation during cheesemaking is partitioned between the curd and whey, and if not denatured by the cheesemaking conditions, the fraction retained remains active and its proteolytic action especially on α_s-casein contributes towards flavour and texture development in aged cheeses (Hynes, Aparo & Candioti, 2004). The common method used for determination of the RA partitioning (retained into cheese or lost into

whey) is a RP-HPLC assay involving the use of a synthetic heptapeptide (Hurley, O'Driscoll, Kelly & McSweeney, 1999). Theoretically, 5–7% of the total RA would be retained in cheese curd (Dunnewind, de Roos & Geurts, 1996; Broome & Limsowtin, 1998) but empirical values vary widely depending on the cheese variety, rennet type and milk concentration. For example, 6% retention into pressed Cheddar cheese (Holmes, Duersch & Ernstrom, 1978), 55% retention into Camembert cheese (Garnot, Molle & Piot, 1987) and 100% retention into Feta cheese from ultrafiltered milk (Bansal, Fox & McSweeney, 2009) were reported. In our previous study (Kayihura, Huppertz & Vasiljevic, 2022), about 87% of the total RA added to milk was found in a mixture of fresh drain & pressed (mimicked by centrifugation) rennet whey while in terms of concentration the RA in whey (IMCU/mL of whey) was the same as initially added to milk (IMCU/mL of milk). The aim of the present study was to test whether the same could be found when milk concentration changes with an attempt to reveal what drives the RA partitioning during milk renneting under two conditions i.e., rennet only or rennet combined with slow acidification.

2. Materials and methods

2.1.Preparation of reconstituted skim milk samples

The experimental design of this study is shown in Fig. 1. Reconstituted skim milk samples with 3, 4.5, 6.1, 7.7 or 9.4% protein content were prepared from low heat skim milk powder (Warrnambool Cheese and Butter - Saputo, Warrnambool, Victoria, Australia). Pre-determined amounts of the skim milk powder were slowly added into 1 L of Milli-Q water in a beaker placed on a magnetic stirrer. After 2 hrs of continuous stirring at room temperature, the reconstituted skim milk samples were refrigerated overnight to complete the hydration of proteins.

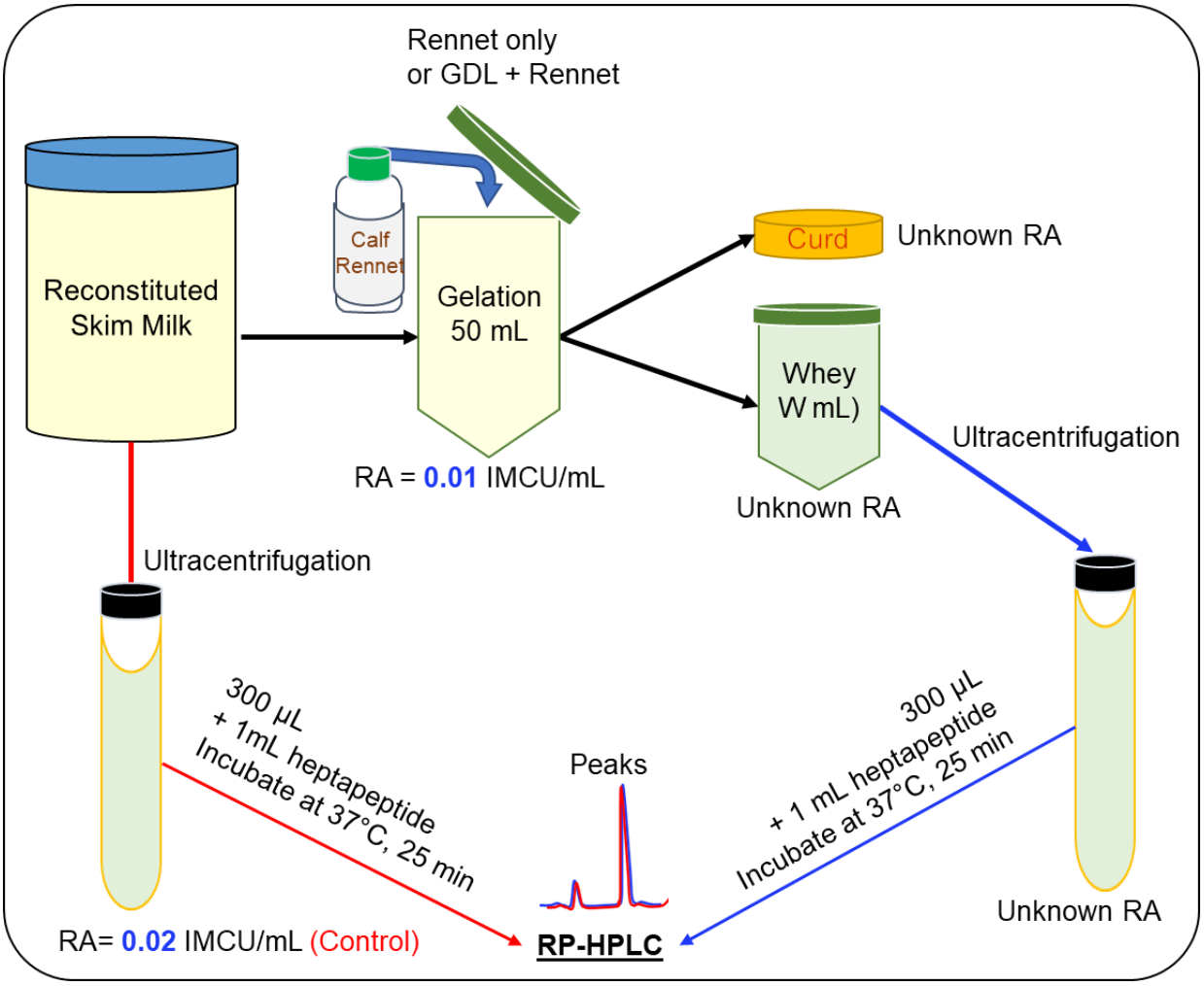


Fig. 1. Experimental design.

2.2.Rennet coagulation of reconstituted skim milk samples

Reconstituted skim milk curds and whey samples were prepared in duplicates (two independent replicates) as described previously (Kayihura, 2023). Each reconstituted skim milk sample was divided into two sets of 50 mL each. The first set was coagulated by renneting under slow acidification using GDL which was adjusted so that the drain whey pH was 6.0 and the second set was coagulated by renneting only. All samples were pre-warmed to a renneting temperature of 31°C in a water bath and coagulation was induced by addition of 0.05 IMCU of freshly diluted (10×) solutions of calf rennet (290 IMCU/mL, Cheeselinks, Lara, Victoria, Australia). Samples with GDL set faster and were coagulated for 30 min whereas samples without GDL set slowly and were coagulated for 50 min. Then, all the coagula were cut vertically (3 × 3) using a thin knife (Kayihura, 2023), cooked at 38°C for 30 min and whey separated at 3200×g using a 5810R Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany).

2.3.Protein and moisture content determination

The total protein (T-Protein) content of each sample was determined in triplicate by using an ultrasonic milk analyser with an accuracy of ± 0.15 (Milkotronic Ltd, Nova Zagora, 8900 Bulgaria) as described previously (Daniloski *et al*., 2022) and the moisture content was determined by overnight drying of 10 g of each milk or whey and 1.0 g of each curd samples at 105°C according to Forsbäck *et al*. (2011). T-protein partitioning into curds was calculated as ((grams of T-protein in milk – grams of T-protein lost in whey)/grams of T-protein in milk)×100, and moisture partitioning into curds was calculated as (grams of moisture in curds/grams of moisture in milk)×100.

2.4.Determination of residual RA in rennet whey and its partitioning into curds

The concentration of residual RA in rennet whey samples was determined by a RP-HPLC method developed by Hurley, O'Driscoll, Kelly & McSweeney (1999) following a modified procedure described by Kayihura, Huppertz & Vasiljevic (2022). Part of each milk used for rennet coagulation and each corresponding whey sample were ultra-centrifuged in duplicate at 100,000×g and 20°C for 1 h using a Beckman Ultra L-70 type centrifuge (Beckman Coulter Inc., Brea, California, USA) as described in other studies (Bansal, Fox & McSweeney, 2007; Bogahawaththa, Chandrapala & Vasiljevic, 2019). A known RA was added to each of the supernatants obtained from skim milk samples (0.02 IMCU/mL of supernatant) and these were used as controls. It is worth noting that milk fractions used for controls were prepared in the same way as renneted samples excluding the renneting step as described previously (Kayihura, Huppertz & Vasiljevic, 2022). Aliquots (300 µL) of each of the controls and their corresponding whey (test samples) were separately added to a 1 mL solution of a synthetic heptapeptide (Pro–Thr–Glu–Phe–[NO_2–Phe]–Arg–Leu) from Mimotopes (Mulgrave, Victoria, Australia), all pre-incubated at 37°C. The mixtures were vortexed and incubated at 37°C for 25 min after which the reaction was stopped by addition of 26 µL of pepstatin solution (SigmaeAldrich Pty. Ltd, Macquarie Park, Australia). The reaction time was determined in preliminary tests to ensure that the heptapeptide is not completely hydrolysed as this would result into the same peak area of the hydrolysate. It is noteworthy that timing (pre-incubation, reaction time, pepstatin addition & all vortexing steps) is very important for this experiment as delaying for few seconds can significantly influence the results. After pepstatin addition, samples were immediately vortexed, filtered (0.45 µm) and the filtrates analysed using RP-HPLC (LC-2030C, Prominence-I, Shimadzu Corporation, Kyoto, Japan) under the following conditions: C_{18} column (Jupiter® 5 mm, 300 Å, 250 × 10 mm, Phenomenex,

Macclesfield, UK), oven temperature: 25°C, UV-detector (Shimadzu): 300 nm, mobile phase A: 0.1% trifluoroacetic acid (TFA, SigmaeAldrich) in Milli-Q water and B: 0.1% TFA in 90% acetonitrile (SigmaeAldrich), flow rate: 0.7 mL/min, elution gradient: 1 min at 10% B, increase to 66.6% B at a constant rate over 15 min and finally 14 min at 10% B. Duplicate RA measurements for each coagulation experiment were performed and the unknown residual RA in whey was estimated based on peak ratios using the equation [1] below:

$$(PA_c/PA_w) = (0.02/RA_w) \qquad [1]$$

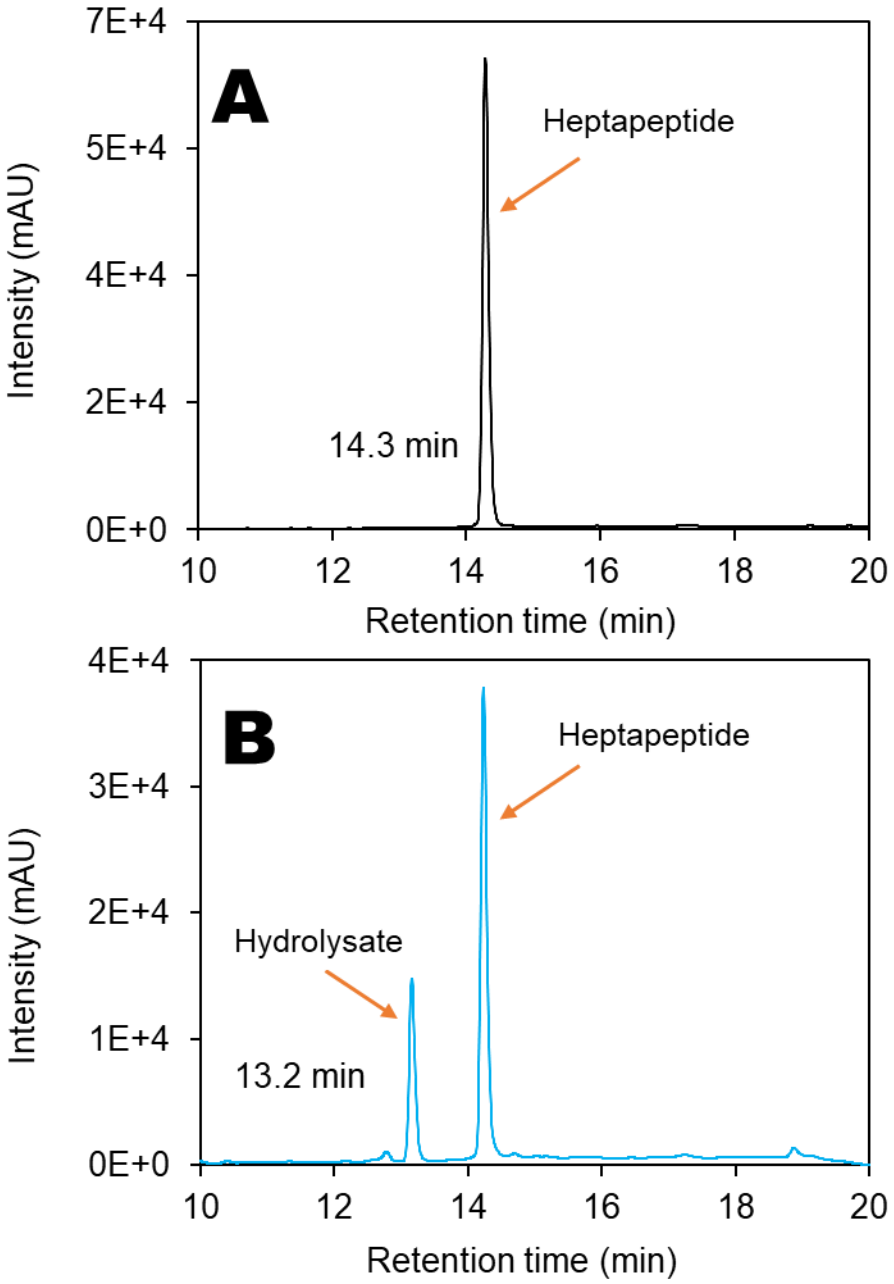
Where PA_c is the peak area of the hydrolysate ([NO_2–Phe]–Arg–Leu) produced by the control (known RA added to milk supernatant), PA_w is the peak area of the hydrolysate produced by unknown RA in rennet whey, 0.02 is the know concentration (IMCU/mL) of RA in the control sample and RA_w is the unknown concentration (IMCU/mL) of RA in whey.

The % RA partitioned into curds was then calculated as the difference between the initial total RA added to milk and total RA found in whey divided by the initial total RA added to milk and multiplied the value obtained by 100. Total RA in milk or whey were obtained by multiplying their respective RA concentrations (IMCU/mL) and their respective total volumes (mL).

3. Results and discussion

3.1.Hydrolysis of heptapeptide by calf rennet under various conditions

Understanding how RA is partitioned during cheesemaking is important in order to control the quality of both aged cheeses and whey-derived products because proteolysis is desirable in the former but not in the latter. Calf RA partitioning into freshly produced curds from reconstituted skim milks of varying concentrations was studied by comparing the hydrolytic action of unknown



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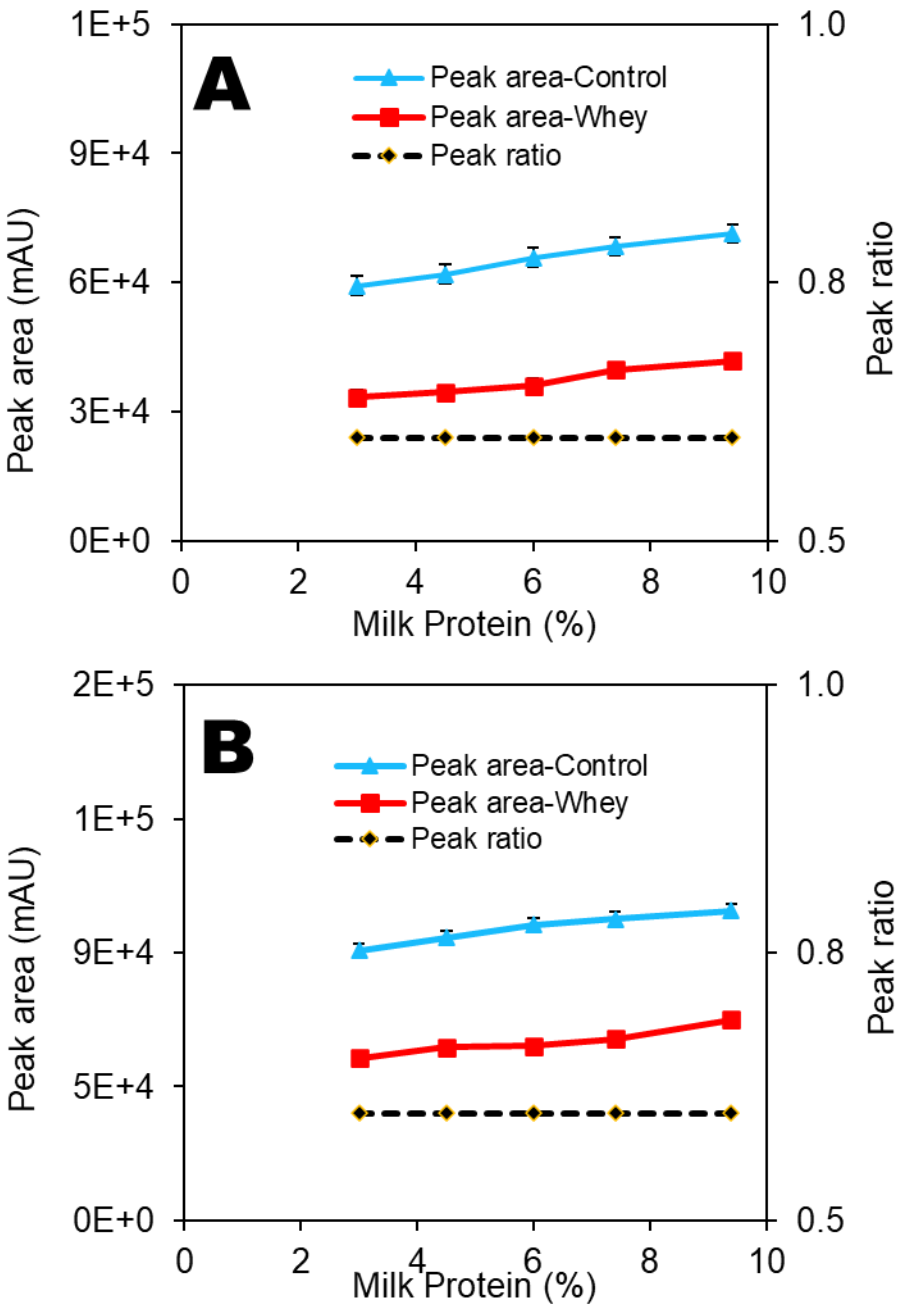
144 **Fig. 2.** Typical chromatograms: (A) initial heptapeptide (Pro–Thr–Glu–Phe–[NO_2–Phe]–Arg–Leu)
145 and (B) heptapeptide remaining & the hydrolysate produced ([NO_2–Phe]–Arg–Leu) after
146 incubation at 37°C for 25 min with ultracentrifugal supernatant of reconstituted skim milk
147 containing 0.02 International Milk Clotting Units (IMCU) of calf rennet. GDL: glucono-δ-lactone;
148 RA: rennet activity.

RA recovered in whey against that of a known control RA on a synthetic heptapeptide. Fig. 2 indicates a clear single peak of the initial heptapeptide (Fig. 2A) used as well as its reduction and the hydrolysate produced from it (Fig. 2B) after 25 min of rennet action at the Phe_4– [NO_2 – Phe_5] bond. The elution profile of the heptapeptide and its hydrolytic product is the same as shown in the original method (Hurley, O'Driscoll, Kelly & McSweeney, 1999) except that the retention times were shorter due to changes made in the procedure such as column type and size, solvent concentration and elution gradient. Fig. 3 indicates changes in peak areas of the hydrolysate produced by the control and unknown residual RA in whey as indicative of the hydrolytic activity of calf rennet under various milk concentrations and two pH levels i.e., pH 6 for acidified samples using GDL (Fig. 3A) and natural pH (Fig. 3B). It is clear that the peak areas increase as the concentration of milk used to prepare the control and rennet whey increased, but values obtained under acidification (3A) are not the same as obtained without acidification (3B). A decrease in peak areas of the hydrolysate due to acidification was also found by Børsting, Qvist & Ardö (2014). This implies that the hydrolytic activity of calf rennet on the heptapeptide is influenced by sample concentration and pH and differences explain why standards (control) should be prepared under conditions similar to those of the test samples (whey). This is also the case when applying the milk clotting assays (Kayihura, Huppertz & Todor, 2022), because concentration and renneting conditions such as pH and temperature modulate the aggregation rate of caseins which influences the milk clotting time. Moreover, types and concentration of enzymes and additives such as $CaCl_2$ or NaCl in addition to milk concentration and renneting conditions have been found to impact the rate of rennet action on κ-casein (Carlson, Hill & Olson, 1987; He, 1990; Karlsson, Ipsen & Ardö, 2007; Sandra, Alexander & Dalgleish, 2007; Cooper, Corredig & Alexander, 2010; Sandra & Corredig, 2013; Zhao & Corredig, 2016). Therefore, when the RA is determined either by milk



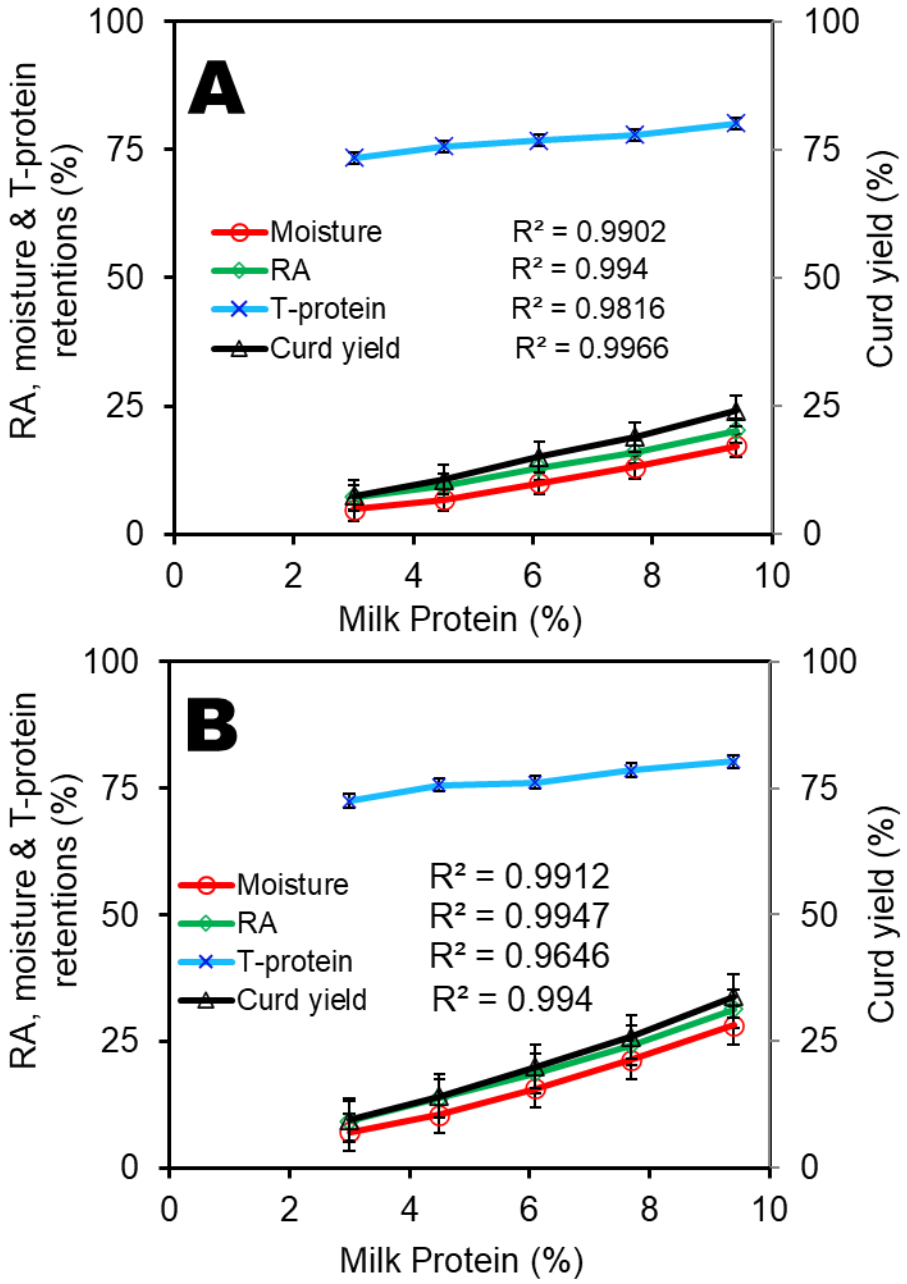
172

173 **Fig. 3.** Changes in peak areas of the synthetic heptapeptide hydrolysate due to the action of calf
174 rennet added to ultracentrifugal supernatants (peak area-control) or recovered in whey (peak area-
175 whey) and residual rennet activity concentration [RA, International milk clotting units (IMCU)]
176 estimated in whey produced from reconstituted skim milk of varying protein concentrations. (A):
177 samples with glucono-δ-lactone (GDL) and (B): samples without GDL.

clotting or RP-HPLC assays and estimations are performed either using calibration curves or using peak ratios, it would be better to prepare the standards or controls using serums of the same milk from which the rennet whey was prepared.

3.2.RA, moisture & T- protein partitioning into rennet curd & curd yield

Changes in RA, moisture and T-protein partitioning into rennet curds and fresh curd yields (w/w) are presented in Fig. 4A for samples renneted under acidification and Fig. 4B for samples renneted without acidification. For all samples, it is clear that RA, moisture and T-protein retentions increased linearly with an increase in milk protein concentration, and larger coefficients ($R^2 > 96$ for T-protein and > 99 for RA and moisture) indicate higher predictive ability of the milk protein content. Furthermore, a positive linear increase in curd yield, as expected, could be directly related to both moisture and protein retentions. It is also revealed that as reconstituted skim milk concentration increases, RA retention into curds increase proportionally with moisture and T-protein retentions. The RA partitioned into curds under renneting combined with slow acidification increased from 7.2% of total RA added to milk with 3% protein to 20.2% of total RA added to milk with 9.4% protein. The RA partitioning at 3% milk protein is in strong agreement with the theoretical value of 6–7% of the final RA retention in cheese curd (Broome & Limsowtin, 1998) and close to 6% reported for pressed Cheddar cheese (Holmes, Duersch & Ernstrom, 1978). Without acidification, a similar pattern was also found but values were higher (9.2–31.4%) as less moisture was expelled. The RA retention values found in this study for curds from milk samples with 3–7.7% protein by renneting and acidification are within the range of 6–18.4% reported for typical Cheddar cheese (Holmes, Duersch & Ernstrom, 1978; Wang, 1969) which is also close to the range of 10–18% reported for miniature Cheddar cheese produced from whole milk with 3.35% protein (Bansal, Fox & McSweeney, 2009) as well as 13.99–18.26 RA units/kg of Cheddar cheese



201

202 **Fig. 4.** Changes in fresh curd yields calculated as (curd weight/milk weight)×100 and retentions
203 of total protein (T-Protein), moisture and rennet activity (RA) into rennet curds produced from
204 reconstituted skim milk of varying protein concentrations. (A): samples with glucono-δ-lactone
205 (GDL) and (B): samples without GDL. Values are means of two different gelation experiments
206 and error bars represent standard error of the mean.

(Fenelon & Guinee, 2000). In addition, findings are also in agreement with Bansal, Fox & McSweeney (2009) who also found higher residual RA in high moisture cheeses and an increase due to milk concentration was attributed to limited whey drainage. The relationship with moisture is also in line with results found for Cheddar cheeses of varying Ca, P & lactose (Upreti, Metzger & Hayes, 2006). However, Upreti, Metzger & Hayes (2006) reported 26% RA retention into Cheddar cheese from whole milk which is above the values found in the present study for samples produced under acidification as well as both ranges found in other studies as indicated above. Bansal, Fox & McSweeney (2009) attributed such variations occurring within the same variety to slight differences in manufacturing protocols applied.

While the current study is in line with Holmes, Duersch & Ernstrom (1978) regarding a decrease in curd yields due to acidification, lower RA retentions at low pH contradict their findings as well as those of Børsting, Qvist & Ardö (2014). It was generally believed that retention of RA due to binding of rennet to caseins occurs especially at low pH or high ionic strength (Reyes, 1971; Holmes, Duersch & Ernstrom, 1978; Dunnewind, de Roos & Geurts, 1996; Broome & Limsowtin, 1998; Bansal, Fox & McSweeney, 2007). However, the results shown herein suggest a different view. The concentration of the hydrolysate produced from the synthetic heptapeptide by calf rennet was previously found to be directly related to RA (Hurley, O'Driscoll, Kelly & McSweeney, 1999), thus, the peak ratios were considered in the present study as equivalent to RA rations as described above. The peak ratios of the unknown to the control RA were the same regardless of skim milk concentration and acidification (Fig. 3). Since controls contained the same RA, a constant peak ratio indicates that the concentration in test samples was also the same. Indeed, the results as shown in Table 1 revealed that the concentration of residual RA in rennet whey (IMCU/mL) neither changed with the reconstituted skim milk concentration nor with the acidification. These findings

Table 1

Concentration of residual rennet activity [RA, Intenrational Milk Clotting Units (IMCU)] estimated in rennet whey produced from reconstituted skim milk samples of varying protein concentrations under combined renneting and slow acidification achieved using glucono-δ-lactone (GDL) or by renneting only (without GDL).

Milk protein (%)	RA (IMCU/mL of whey) [a)]	
	With GDL	Without GDL
3	0.011 ± 0.001	0.012 ± 0.000
4.5	0.011 ± 0.000	0.011 ± 0.001
6.1	0.012 ± 0.001	0.011 ± 0.000
7.7	0.012 ± 0.001	0.011 ± 0.001
9.4	0.012 ± 0.001	0.011 ± 0.001

[a)] Values are means of duplicate measurements for two different experiments ± the standard deviations.

238 are the same as found in our previous study and are in accordance with Fenelon & Guinee (2000)
239 who found no change in residual RA/kg of protein as well as Bansal, Fox & McSweeney (2007)
240 who also found no change in the quantity of coagulant bound/g of casein. Moreover, unlike the
241 concentration of residual RA in rennet whey, both total casein and T-protein content in rennet
242 whey are directly related to reconstituted skim milk concentration (Kayihura, 2023). This
243 information in addition to the linear relationship between changes in RA retention with moisture
244 retention (Fig. 4) as well as a direct relationship between total RA and moisture content in cheese
245 reported in previous studies (Holmes, Duersch & Ernstrom, 1978; Garnot, Molle & Piot, 1987;
246 Bansal, Fox & McSweeney, 2009) provides sufficient evidence that the retention of RA into rennet
247 curds is not driven by the enzyme-casein associations. If this was the case, higher RA retention
248 would have been obtained for curds produced with acidification as suggested by Holmes, Duersch
249 & Ernstrom (1978). The results obtained in the present study, therefore, elucidate that the driving
250 force could rather be the same as the one controlling the retention of moisture and other milk
251 constituents and curd yield i.e., the strength of casein-casein interactions. However, the role of
252 specific casein-casein interactions remains to be unravelled in future studies.

253 **4. Conclusions and future directions**

254 The present study evaluated the effect of increasing the reconstituted skim milk
255 concentration up to 9.4% T-protein on the partitioning of RA during renneting only or renneting
256 combined with slow acidification achieved using GDL. It is revealed that although RA retention
257 into curds increases proportionally with moisture and T-protein retentions as protein concentration
258 in milk increased, the concentration of residual RA in whey (IMCU/mL of whey) remains
259 unchanged and is the same as the initial concentration (IMCU/mL of milk). This confirms that the
260 retention of RA into rennet curds is not driven by the enzyme-casein associations. The driving

force could rather be the strength of casein-casein interactions although the role of specific interactions remains unknown and requires further investigation in future research. It should be further emphasised that the standards and test samples must be prepared under similar conditions as RA could be affected by the chemical solutions used. This may provide important information for studies on acceleration of the ripening process of aged hard cheeses from concentrated milk systems. Studies on RA retention into rennet curds produced from other types of modified milk systems using various renneting conditions would also be interesting.

Declarations of interest: none

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5. References

Andrén, A. (2011). Rennets and Coagulants. In: McSweeney, P. L. H., & McNamara, J. P. (Eds.). *Encyclopedia of Dairy Sciences, 2nd Ed. Elsevier. Pp.573–578.*

Bansal, N., Fox, P. F., & McSweeney, P. L. H. (2007). Factors affecting the retention of rennet in cheese curd. *Journal of Agricultural and Food Chemistry, 55, 9219–9225.*

Bansal, N., Fox, P. F., & McSweeney, P. L. H. (2009). Comparison of the level of residual coagulant activity in different cheese varieties. *Journal of Dairy Research, 76, 290–293.*

Berridge, N. J. (1945). The purification and crystallization of rennin. *Biochemical Journal, 39, 179–186.*

Berridge, N. J. (1952). Some observations on the determination of the activity of rennet. *Analyst, 77, 57–62.*

Bogahawaththa, D., Chandrapala, J., & Vasiljevic, T. (2019). Thermal denaturation of bovine β-lactoglobulin in different protein mixtures in relation to antigenicity. *International Dairy Journal, 91, 89–97.*

Børsting, M. W., Qvist, K. B., & Ardö, Y. (2014). Effect of pH on retention of camel chymosin in curd. International Dairy *Journal, 38, 133–135.*

Broome, M. C., & Limsowtin, G. K. Y. (1998). Milk coagulants. *Australian Journal of Dairy Technology, 53, 188.*

Carlson, A., Hill, C. G. J. R., & Olson, N. F. (1985). Improved assay procedure for determination of milk-clotting enzymes. *Journal of Dairy Science, 68, 290–299.*

Carlson, A., Hill, C. G., & Olson, N. F. (1987). Kinetics of milk coagulation: I. The kinetics of kappa casein hydrolysis in the presence of enzyme deactivation. *Biotechnology and Bioengineering, 29, 582–589.*

Cooper, C., Corredig, M., & Alexander, M. (2010). Investigation of the colloidal interactions at play in combined acidification and rennet of different heat-treated milks. *Journal of Agricultural and Food Chemistry, 58, 4915–4922.*

Daniloski, D., McCarthy, N. A., Markoska, T., Auldist, M. J., & Vasiljevic, T. (2022). Conformational and physicochemical characteristics of bovine skim milk obtained from cows with different genetic variants of β-casein. *Food Hydrocolloids, 124, 107186.*

Dunnewind, B., de Roos, A. L., & Geurts, T. J. (1996). Association of chymosin with caseins in solution. *Netherlands Milk and Dairy Journal, 50, 121–133.*

Fenelon, M. A., & Guinee, T. P. (2000). Primary proteolysis and textural changes during ripening in Cheddar cheeses manufactured to different fat contents. *International Dairy Journal 10, 151–158.*

Fish, J. C. (1957). Activity and Specificity of Rennin. *Nature, 180, 345.*

Forsbäck, L., Lindmark-Månsson, H., Svennersten-Sjaunja, K., Bach Larsen, L., & Andrén, A. (2011). Effect of storage and separation of milk at udder quarter level on milk composition,

proteolysis, and coagulation properties in relation to somatic cell count. *Journal of Dairy Science, 94, 5341–5349.*

Garnot, P., Molle, D., & Piot, M. (1987). Influence of pH, type of enzyme and ultrafiltration on the retention of milk clotting enzymes in Camembert cheese. *Journal of Dairy Research, 54, 315–320.*

He, F. (1990). Substrate concentration, calcium concentration and κ-CN hydrolysis in milk coagulation. *Ph.D. thesis, Utah State University, Pp Xii–144.*

Holmes, D. G., Duersch, J. W., & Ernstrom, C. A. (1978). Distribution of milk clotting enzymes between curd and whey and their survival during Cheddar cheese making. *Journal of Dairy Science, 60, 862–869.*

Hurley, M. J., O'Driscoll, B. M., Kelly, A. L., & McSweeney, P. L. H. (1999). Novel assay for the determination of residual coagulant activity in cheese. *International Dairy Journal, 9, 553–558.*

Hynes, E. R., Aparo, L., & Candioti, M. C. (2004). Influence of residual milk-clotting enzyme on αs1 casein hydrolysis during ripening of Reggianito Argentino cheese. *Journal of Dairy Science, 87, 565–573.*

Jacob, M., Jaros, D., & Rohm H. (2011). Recent advances in milk clotting enzymes. *International Journal of Dairy Technology, 64, 14–33.*

Karlsson, A. O., Ipsen, R., & Ardö, Y. (2007). Rheological properties and microstructure during rennet induced coagulation of UF concentrated skim milk. *International Dairy Journal, 17, 674–682.*

Kayihura, J. F. (2023). Reconstituted skim milk concentration affects partitioning of some individual caseins between rennet curd and whey. *International Journal of Dairy Technology, 76, 852-860.*

Kayihura, J. F., Huppertz, T., & Vasiljevic T. (2022). Application of small amplitude oscillatory rheology measurements for estimating residual rennet activity in rennet whey. *International Dairy Journal, 134, 105451.*

Kumar, A., Grover, S., Sharma, J., & Batish, V. K. (2010). Chymosin and other milk coagulants: sources and biotechnological interventions. *Critical Reviews in Biotechnology, 30, 243–258.*

López, M. B., M.J. Jordan, Hellin, P., Castillo, M., & Laencina, J. (1997). Kinetics of κ-casein hydrolysis by different rennet and coagulant enzymes in murciano-granadlna goat milk. *Milchwissenschaft, 52, 370–373.* McSweeney, P. L. H. (2022). Rennets and Coagulants. In: McSweeney, P. L. H., & McNamara, J. P. (Eds.). *Encyclopedia of Dairy Sciences, 3rd Ed. Elsevier. Pp.316–320.*

Reyes, J. (1971). A procedure for measuring residual rennin activity in whey and curd from freshly coagulated milk (MSc thesis). *Logan, Utah, USA: Utah State University.*

Sandra, S., & Corredig, M. (2013). Rennet induced gelation of reconstituted milk protein concentrates: the role of calcium and soluble proteins during reconstitution. *International Dairy Journal, 29, 68–74.*

Sandra, S., Alexander, M., & Dalgleish, D. G. (2007). The rennet coagulation mechanism of skim milk as observed by transmission diffusing wave spectroscopy. *Journal of Colloid and Interface Science, 308, 364–373.*

Upreti, P., Metzger, L. E., & Hayes, K. D. (2006). Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on cheddar cheese quality: proteolysis during ripening. *Journal of Dairy Science, 89, 444–453.*

Wang, J. T. (1969). Survival and distribution of rennin during Cheddar cheese manufacture. *M.S. Thesis. Utah State University, Logan.*

Zhao, Z., & Corredig, M. (2016). Influence of sodium chloride on the colloidal and rennet coagulation properties of concentrated casein micelle suspensions. *Journal of Dairy Science, 99, 6036–6045.*

Chapter 5 - Reconstituted skim milk concentration affects partitioning of some individual caseins between rennet curd and whey

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This chapter has been published as an original research paper entitled “Reconstituted skim milk concentration affects partitioning of some individual caseins between rennet curd and whey” by Joseph F. Kayihura in a peer-reviewed journal: International Journal of Dairy Technology, 76 (2023) 852-860. (https://doi.org/10.1111/1471-0307.13003).

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Surname: Kayihura First name: Joseph Flambeau

Institute: Institute for Sustainable Industries and Liveab Candidate's Contribution (%): 100%

Status:

Accepted and in press: ☐ Date:

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Joseph Flambeau Kayihura	100%	Conceptualisation, study design, experimentation, writing, submission & revision	[redacted]	01/09/2023

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Chapter 6 - Structural dependence of concentrated skim milk curd on micellar restructuring

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This chapter has been published as an original research paper entitled “Structural dependence of concentrated skim milk curd on micellar restructuring” by Joseph F. Kayihura in a peer-reviewed journal: Heliyon, 10, (2024) e24046. (https://doi.org/10.1016/j.heliyon.2024.e24046). The chapter explores how reconstituted skim milk concentration affects changes occurring within the rennet curd matrix, which could partly help to explain some changes in partitioning of milk constituents observed in chapter 5.

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Joseph Flambeau Kayihura	100%	Conceptualisation, study design, experimentation, writing, submission & revision	[redacted]	09/01/2024

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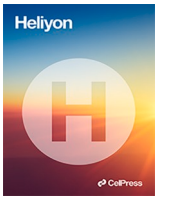

Research article

Structural dependence of concentrated skim milk curd on micellar restructuring

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ABSTRACT

This study was conducted to establish an understanding of how milk concentration modulates the rennet curd structure. Rennet-induced gelation and renneting under slow acidification achieved using glucono-δ-lactone (GDL) and structural properties of reconstituted skim milk gels at two concentration levels (9 and 25 % total solids) were studied by measuring variations in (a) viscoelastic behaviour, (b) micellar size, charge density, diffusivity, and (c) hydrophobicity using dynamic rheometry, dynamic light scattering and fluorimetry, respectively. Concentrated milk showed a greater estimated hydrodynamic radius of casein micelles, lower zeta (ζ)-potential, ratio of serum to total Calcium (Ca) and charge density and increased surface hydrophobicity, all supporting the view that micellar restructuring particularly sub-particle transfer takes place and contributes to rapid gelation. Moreover, hydrophobic interactions occurred very quickly (within 5 min in combined gels, 10 min for renneting only), demonstrating their pivotal role during the flocculation stage. All gels exhibited a solid viscoelastic character as the elastic modulus (G') was greater than loss modulus (G'') while both G' and tan δ (G''/G') were frequency-dependent. Frequency sweeps classified the concentrated gels into three stiffness categories caused by the level of rennet or GDL as rigid, hard and soft, whereas an increased flow-like behaviour (high tan δ), restricted diffusion and excessive water retention revealed limited structural rearrangements (contraction & macrosyneresis) during curd ageing. Acidification increased the diffusion rate in control curd, thus, enhanced contractive rearrangements, macrosyneresis and curd strength. Findings suggest that micellar restructuring induced by milk concentration is the principal modulator of the curd structure.

1. Introduction

In order to improve cheese quality or overcome inconsistencies due to natural variations in milk composition, intentional modifications are made [1]. The type of milk system selected as the starting material and the concentration level have different effects on curd structure, and consequently determine the qualities of both cheese and whey products. Guinee, Pudja & Mulholland [2] and Ong et al. [3] found excessive losses of fat and protein into whey when milk was concentrated to protein content over 5 %, and this was attributed to a porous gel structure. In addition to milk composition, cheese making conditions such as temperature, renneting extent and pH can also modify the curd structure [4–8].

Several studies have attempted to explain why milk composition and processing conditions affect gel structure. These include, for

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example; the dependence of curd strength on native casein micelle size [9], demineralisation of Ca and a lower net charge reported as the reason for a reduced density of the protein network during renneting of milk with decreasing pH [10] and a higher gelation temperature reported to be responsible for an unstructured and coarse network of larger casein aggregates [11]. Rearrangements in rennet gels from pre-heated milk were found to be limited due to crosslinking by denatured whey proteins [6], while using a fractal scaling model, Mellema [12] reported that the structure of skim milk curd at various pH and temperatures is related to rearrangements thought to be caused by particle fusion.

Furthermore, the cheesemaking properties of milk systems concentrated by various methods have been extensively researched and structural or compositional differences reported [13–21]. However, studies on structural changes occurring within the gel as a function of milk concentration during combined renneting and acidification, so far, seem to be limited. The first report appeared a decade ago and was on quark-type cheese [20]. Similar studies on phosphocaseinate and acidified milk are also available [22–24] but there appears to be none on conditions applied for the manufacturing of hard cheeses. Therefore, the underlying reason why milk concentration modulates curd formation and structural properties appears to have not been clearly elucidated. Based on some evidence already available in literature that micellar restructuring (changes involving particle transfer, secondary structure, aggregate formation & size increment) occurs during milk concentration [18,25–30], the present research attempted to establish its relationship to structural properties of renneted skim milk gel with varying concentration levels of total solids (9 and 25 %), examined by dynamic rheometry and dynamic light scattering measurements. Gelation was achieved by renneting or renneting combined with slow acidification (using GDL). In addition, changes in hydrophobic interactions and retention of different Ca fractions, ash, total solids and moisture into the curds were examined to further explain some structural rearrangements related to phase separation or syneresis [31].

2. Materials and methods

2.1. Preparation of reconstituted skim milk samples

Reconstituted skim milk samples were prepared from low heat skim milk powder (Warrnambool Cheese and Butter - Saputo, Warrnambool, Victoria, Australia) dispersed in Milli-Q water, stirred at room temperature (~20 °C) for at least 2 h and kept refrigerated overnight to fully hydrate the proteins. The dispersions were stirred and standardised to two concentration levels i.e., a concentrated skim milk of 25 % total solids (9.4 % protein) and a control of 9 % total solids (3.2 % protein). The concentrations were chosen as part of an undergoing project that covers the range of protein levels studied previously for the manufacture of Cheddar cheese [2]. Composition of skim milk during standardisation was tested using an ultrasonic milk analyser (Milkotronic Ltd, Nova Zagora, 8900 Bulgaria).

2.2. Preparation of rennet whey samples

Skim milk dispersions were coagulated in 50 mL tubes either by renneting only using a commercial calf rennet (290 International Milk Clotting Units (IMCU) mL^{-1}, Cheeselinks, Lara, Victoria, Australia) or renneting combined with slow acidification achieved by addition of glucono-δ-lactone (GDL). Milk samples were placed in a water bath and warmed to a coagulation temperature of 31 °C [32]. For combined renneting and acidification, GDL was added based on protein content (0.2 g/g) to mimic the slow acidification process of a starter culture, targeting a drainage pH of 6.0 ± 0.1, achieved after 1 h. Concentrated milk was divided into two sub-samples and rennet added to the first one based on milk volume i.e., the same as added to control (0.02 IMCU/mL) and to the second one based on protein content (0.47 IMCU/g). Samples were left undisturbed to coagulate for 30 min followed by cutting and then cooking at 38 °C for 30 min. Curd pressing was simulated by centrifugation for 1 h at 20 °C and 1700×*g* according to Shakeel-Ur-Rehman, McSweeney & Fox [33]. Another set of the same skim milk samples was also coagulated under the same conditions without GDL.

2.3. Total solids, ash and Ca fractions in milk and whey

Total solids, ash and three Ca fractions (total, serum and ionic Ca) were assessed on both milk and the resultant whey samples. Ten grams of each skim milk or whey were ultra-centrifuged for 1 h at 25 °C and 100000×*g* to obtain the serum fraction which was separately dried with another 10 g of the original milk or whey and ~2 g of pressed curd samples. Drying to a constant weight was performed in a hot airdrying oven at 105 °C to determine the total solids, and ash content determined by mineralising dry samples in a muffle furnace at 550 °C for at least 18h. The whole ash obtained was dissolved into 100 mL of 5 % nitric acid, filtered through 0.45 μm membrane filters and directly analysed using an inductively coupled plasma atomic emission spectrometer (Multitype, Shimadzu Corporation, Kyoto, Japan). Ionic Ca of skim milk and whey samples was measured using a Ca ion selective electrode of the laboratory research grade benchtop pH/mV/ISE meter – HI5222 series (Hanna Instruments Inc. Woonsocket, RI, USA). After addition of 500 μL ionic strength adjuster solution (HI 4004-00, Hanna Instruments Inc. Woonsocket, RI, USA) to 25 mL of each sample, records were taken with continuous stirring once stable values were displayed.

2.4. Dynamic rheometry measurements

The time course of gel formation was assessed by dynamic rheometry with a bob (25 mm diameter) and cup (27.11 mm diameter) geometry using a Physica MCR 301 rheometer (Anton Paar GmbH, Graz, Austria) as described previously [34] with some modifications. Each sample (19 mL) was loaded at 31 °C into a rheometer cup immediately after predetermined amounts of the coagulants

(rennet only or GDL + rennet) were added. Variations in the elastic (storage) modulus (G′) and the ratio of viscous (loss) to elastic moduli (G''/G′, also known as loss tangent or tan δ) with gelation time were monitored at constant temperature, frequency and strain of 31 °C, 1 Hz and 0.5 %, respectively. Rennet coagulation time (RCT) defined as the time at which G' ≥ 1 Pa [35], time to reach the cutting window defined according to Panthi et al. [19] as the time at which G' ≥ 35 Pa (k_{35}) and G′ after 30 min (G'_{30}, Pa) were all determined. After 1 h of gelation, curd properties were tested by frequency sweep (0.1–100 Hz) at a constant strain of 0.5 % which was within the linear viscoelastic region. Changes in G′, tan δ, complex viscosity (η*, Pa.s) and shear stress (τ, Pa) as a function of the oscillatory frequency were evaluated.

2.5. Dynamic light scattering and ζ-potential measurements

Dynamic light scattering measurements were performed on reconstituted skim milk samples prior to and during gelation. Both diffusion coefficients and ζ-potential were measured using a Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK) as described previously [27] except that samples were not diluted. Previously, zeta potential was linearly correlated to the particle size [36] thus a ratio of effective zeta potential to estimated average hydrodynamic radius of skim milk particles is nominally termed charge density. Charge density in milk samples was calculated after estimating the average hydrodynamic size of micelles using Stokes-Einstein equation [37–39] shown below:

$$r_h = \frac{k_b T}{6\pi\eta \mathrm{D}} \quad \text{or} \quad d_h = \frac{k_b T}{3\pi\eta \mathrm{D}}$$

where r_h is the hydrodynamic radius and d_h is diameter, k_b is the Boltzmann constant, T is the temperature (K), η is the viscosity of the solvent and D is the measured diffusion coefficient.

This calculation was based on previous reports that dynamic light scattering instruments in backscattering mode at 173° or 180° can measure particle size in concentrated suspensions [40] up to 40 % [41]. In addition, de Kruif [38] and de Kruif & Zhulina [42] applied the same technique and presented changes in micellar size during renneting. Further, it has been noted that casein micelles in concentrated milk systems behave like colloidal hard spheres up to >45 % [43], with constant dynamic mobility up to 4 × concentration [44] and free diffusing Brownian motion up to a volume fraction of 0.3 [45]. The viscosity (at 10/s) of the serum phase (prepared by ultracentrifugation at 100000×*g* at 25 °C for 1 h) was used as its components (water, whey proteins and salts) are considered a continuous medium in which casein micelles viewed as hard spherical particles are dispersed [38] and since milk (≤30 % total solids) or whey protein solutions (≤10 %) exhibit Newtonian behaviour [46–48] a shear rate of 10/s would have no impact on viscosity. Liu et al. [49] used the viscosity of milk serum prepared at 55000×*g*, 25 °C for 90 min. Changes in diffusion coefficients during curd formation were also tested for 1 h and the time to reach a polydispersity index of 1 (PDI_1) was also recorded. PDI is an important physical property used to describe micellar size distribution and an increase in both indicates the occurrence of aggregation [50,51]. Samples were prepared as described above for rheological measurement and gelation temperature was also set at 31 °C.

2.6. Surface hydrophobicity measurement

Surface hydrophobicity of skim milk samples was determined fluorometrically (Shimadzu RF-5301PC spectrofluorometer (Shimadzu Corp., Kyoto, Japan)) as described previously [52] using an 8 mM solution of 1-anilinonaphthalene-8-sulfonic acid (ANS) in 0.1 M phosphate buffer (pH 7) as the fluorescent probe at 5 nm for both emission and excitation slit widths and wavelengths of 390 and 470 nm, respectively. Skim milk samples were first diluted with Milli-Q water to stock solutions of 0.01 % protein content and further diluted to 0.0004–0.002 % in 0.1 M phosphate buffer. Measurements were taken 20 min after incubating 4 mL of each dilution with 20 μL of ANS solution in darkness and the intensity of ANS binding to hydrophobic sites was determined based on the slope of the relative fluorescent intensity (RFI) plotted against the protein content. A blank (4 mL buffer + 20 μL ANS) was measured and its RFI taken as the baseline before each set of control (no ANS) or test samples.

Changes in hydrophobic interactions during coagulation were also assessed following a method of Peri et al. [53] with some modifications. Briefly, three portions (20 mL) of each milk sample containing an initial concentration of 0.2 mM ANS were coagulated as described above and the rennet activity in the 1st, 2nd and 3rd portions was stopped after 5, 10 and 20 min, respectively, using 24.4 μL of pepstatin solution (0.99 mM in ethanol). The three renneted portions and 20 mL of the original skim milk (containing 0.2 mM ANS) were centrifuged for 30 min at 10000 rpm and 25 °C and supernatants collected were diluted 40 × with 1 % triton-x100 solution. A calibration curve of RFI (au.) vs. ANS concentration (mM) in 1 % triton-x100 solution was also generated ($y = 162992x + 3.6771$, $R^2 = 0.9998$) and used to estimate the concentration of ANS recovered in each supernatant. Then, the concentration of ANS retained into the curds after 5, 10 and 20 min as a percentage of initially added to milk was calculated and considered a quantitative index of hydrophobic interactions that occurred within each sample after different time intervals.

2.7. Statistics

Results of replicated measurements for each milk or whey sample were submitted to one-way analysis of variance (ANOVA) and comparisons were made by performing the Turkey test using a General Linear Model Procedure of the statistical analysis software (SAS Instrument, 1996). The level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Total solids, ash and Ca content in milk and whey and their retention into curds

As shown in Table 1, the concentration of concentrated skim milk samples was >2 times that of the control. Ca content of the control is in line with previously reported [54]. However, serum to total Ca ratio and all Ca fractions' ratios to total protein content appeared to be higher in the control than the concentrated milk. This observation is also consistent with previous studies such as Ferrer, Hill & Corredig [55]. As expected, whey from concentrated skim milk contained higher levels of solids and Ca compared to the control especially during combined renneting and acidification where GDL caused a significantly greater amount of Ca release. This appears in agreement with the previous report by Salvatore, Pirisi & Corredig [20]. As indicated in Table 2, the retention of total Ca and moisture into combined rennet curds (with GDL) were less than in those produced without GDL. During renneting only (without GDL), all constituents tested were apparently retained at a lower rate in control compared to concentrated milk curds.

3.2. Diffusion coefficients, particle size and charge density of milk samples

Diffusion coefficients were 0.9 μ^2/s for concentrated skim milk and 2.1 μ^2/s for control milk samples (Table 3). As diffusion coefficients depend on both viscosity and particle size [56] lower values in concentrated samples may reflect greater viscosity and presence of larger micelles. Indeed, the estimated hydrodynamic diameter (d_h) from these values indicates that concentrated skim milk samples contain larger micelles than the controls. In contrast, it was found that the control milk had greater (>3 ×) absolute value of negative charge density than concentrated skim milk samples.

3.3. Curd formation and structural properties

As expected, concentrated milk in which rennet was added based on protein content had shorter RCT and k_{35} and greater G'_{30} for both sets (with & without GDL) than those of the control (Table 4). Without GDL, the G'_{30} of both control and concentrated milk curds with the same amount of rennet were <1 Pa and both the RCT and k_{35} of the control were >60 min. During combined renneting and acidification (with GDL), however, the RCT of the control was shorter than that of concentrated milk with the same amount of rennet as the control, whereas the difference between k_{35} was not significant ($p > 0.05$). The same trend in coagulation times was also observed from dynamic light scattering measurements based on PDI_1.

Unlike the initial stages of curd formation, the final curd firmness of concentrated milk with the same amount of rennet as the control was higher than that of the control due to a greater curd firming rate as reflected by a steeper increase in G' (Fig. 1A). Tan δ remained stable throughout the primary phase (not shown as it makes the important part (Fig. 1B) unclear) until milk coagulation began where values declined continuously to < 1. Tan δ of the control (with GDL) reached a minimum and rose at a faster rate compared to concentrated milk curds (with GDL) which showed a plateauing behaviour above the control (Fig. 1B). However, tan δ of the control (without GDL) did not reach a minimum value as a thick gel had not formed yet while those of concentrated milk curds (without GDL) were lower than those observed in presence of GDL.

All curds exhibited a solid-like viscoelastic character as G′ was greater than G″ while both G′ and tan δ were frequency-dependent [57] with a pseudo plastic shear thinning behaviour at low frequency regimes. However, as shown in Fig. 2A–D, the frequency-dependency of the viscoelastic properties was remarkably able to categorise the curds into distinct groups which could be described mechanically as rigid (concentrated milk with rennet added based on protein), hard (concentrated milk with GDL & the same

Table 1
Total solids, ash and Ca fractions in control and concentrated skim milk samples and their respective wheys produced by combined renneting and slow acidification (using GDL) or renneting only.

Sample type	Coagulant	Total solids (mg/g)	Ash (mg/g)	Total Ca (mg/g)	Serum Ca (mg/g)	Ionic Ca (mg/g)
Control milk	NA	90.5 ± 1.5^{E}	7.1 ± 0.0^{E}	1.16 ± 0.01^{D}	0.39 ± 0.01^{E}	0.08 ± 0.00^{E}
Control whey	rennet only	64.9 ± 0.4^{G}	4.9 ± 0.2^{G}	0.35 ± 0.01^{G}	0.35 ± 0.00^{F}	0.06 ± 0.00^{F}
	rennet & GDL	71.8 ± 1.1^{F}	5.7 ± 0.1^{F}	0.64 ± 0.01^{F}	0.63 ± 0.01^{D}	0.25 ± 0.01^{C}
Concentrated milk	NA	246.8 ± 10.3^{A}	18.5 ± 0.1^{A}	2.81 ± 0.04^{A}	0.69 ± 0.01^{C}	0.13 ± 0.00^{D}
Concentrated milk Whey	same rennet as control & GDL	204.7 ± 8.0^{B}	15.2 ± 0.0^{B}	1.65 ± 0.02^{C}	1.62 ± 0.02^{B}	0.34 ± 0.00^{B}
	rennet & GDL based on protein content	204.3 ± 24.2^{B}	14.6 ± 1.3^{C}	1.71 ± 0.01^{B}	1.69 ± 0.02^{A}	0.36 ± 0.00^{A}
	same rennet as control	197.3 ± 11.6^{C}	12.5 ± 0.0^{D}	0.72 ± 0.00^{E}	0.71 ± 0.01^{C}	0.06 ± 0.00^{F}
	rennet based on protein content	190.6 ± 7.7^{D}	12.5 ± 0.4^{D}	0.70 ± 0.01^{E}	0.70 ± 0.01^{C}	0.06 ± 0.00^{F}

Results are presented as means of duplicate (total solids & ash) and triplicate (Ca fractions) measurements ($n \geq 2$) ± standard deviation.
Means in the same column with different superscripts are significantly different ($p < 0.05$).
NA: Not applicable.

Table 2
Retention (% of the original amount in milk) of total solids, moisture, ash and different Ca fractions into curds produced from control and concentrated skim milk samples by combined renneting and slow acidification (using GDL) or renneting only.

Milk type	Coagulant	Total solids (%)	Moisture (%)	Ash (%)	Total Ca (%)
Control	rennet & GDL	33.4 ± 0.5^{B}	6.1 ± 1.5^{C}	28.3 ± 1.6^{E}	50.3 ± 0.1^{F}
	rennet only	36.0 ± 1.7^{B}	8.4 ± 0.2^{C}	38.3 ± 0.3^{C}	75.3 ± 0.2^{C}
Concentrated	same rennet as control & GDL	36.1 ± 1.5^{B}	20.4 ± 2.4^{B}	33.8 ± 1.1^{D}	54.7 ± 0.2^{D}
	rennet & GDL based on protein content	40.4 ± 5.8^{B}	22.4 ± 4.9^{B}	37.9 ± 4.3^{C}	53.3 ± 0.3^{E}
	same rennet as control	54.3 ± 2.4^{A}	43.3 ± 0.8^{A}	54.9 ± 8.4^{AB}	86.0 ± 0.1^{B}
	rennet based on protein content	58.8 ± 4.1^{A}	48.1 ± 1.3^{A}	66.6 ± 5.0^{A}	86.7 ± 0.0^{A}

Results are presented as means of duplicate (total solids, moisture & ash) and triplicate (Ca) measurements ($n \geq 2$) ± standard deviation.
Means in the same column with different superscripts are significantly different ($p < 0.05$).

Table 3
Diffusion coefficients (D), estimated hydrodynamic diameter (d_h) and charge density of reconstituted skim milk samples.

Milk type	D (μ^2/s)	d_h (μm)	Charge density (mV/μm)
Control	2.1 ± 0.02^{A}	0.23 ± 0.00^{B}	-57.2 ± 1.4^{B}
Concentrated	0.9 ± 0.02^{B}	0.34 ± 0.01^{A}	-18.3 ± 0.6^{A}

Results are presented as means of at least duplicate measurements ($n \geq 2$) ± standard deviation.
Means in the same column with different superscripts are significantly different ($p < 0.05$).

Table 4
Gelation properties of control and concentrated skim milk samples under combined renneting and slow acidification (using GDL) or renneting only.

Milk type	Coagulant	RCT (min)	k_{35} (min)	G'_{30} (Pa)	PDI_1 (min)
Control	rennet & GDL	9.0 ± 0.5^{E}	18.8 ± 2.1^{C}	81.0 ± 15.1^{C}	9.4 ± 0.0^{E}
	rennet only	$>60^{A}$	$>60^{A}$	$<1^{D}$	45.9 ± 4.5^{A}
Concentrated	same rennet as control & GDL	14.4 ± 0.6^{C}	17.6 ± 0.6^{C}	333.5 ± 47.4^{B}	14.7 ± 0.4^{C}
	rennet & GDL based on protein content	5.9 ± 0.7^{F}	8.1 ± 1.3^{D}	583.5 ± 68.1^{A}	6.7 ± 0.4^{F}
	same rennet as control	35.4 ± 2.2^{B}	44.5 ± 2.4^{B}	$<1^{D}$	32.7 ± 2.5^{B}
	rennet based on protein content	11.2 ± 0.3^{D}	15.3 ± 0.5^{C}	226.5 ± 27.6^{B}	10.5 ± 0.4^{DE}

Results are presented as means of duplicate measurements ($n = 2$) ± standard deviation.
Means in the same column with different superscripts are significantly different ($p < 0.05$).

rennet as control), soft (control with GDL & concentrated milk with the same rennet as control without GDL) and very soft (control without GDL). The latter was completely deformed and returned to liquid milk-like state at frequencies ≥6.8 Hz. An increase in G', η^* and τ at higher frequencies indicates a network recovery probably due to limited time for bonds to relax [58] and new cluster formation by hydrodynamic interactions. The concentrated milk curd with GDL & the same rennet as control was not categorised as rigid because at >68 Hz tan δ increased to ~1/3 of the original value (i.e., $G'' > G'$). Soft curds were also significantly affected as shown by the rise in tan δ and η^*, but were not deformed completely until the frequency reached 100 Hz. The τ values of all samples increased with frequency, showing that more stress was required to maintain a constant strain.

Measurement of changes in diffusion coefficients during curd formation complemented rheological measurements. A synergistic action of rennet and GDL on promoting a rapid coagulation in combined gels was evident (Fig. 3A) compared with slow coagulation by renneting only (Fig. 3B). The three phases of curd formation could also be clearly shown: (1) slight increase in diffusion due to simultaneous enzymatic hydrolysis of κ-casein and dissolution of micellar $Ca_3(PO_4)_2$ caused by acidification, the opposite trend with the same interpretation was also seen previously for changes in particle size [9,38,42,51], (2) sharp drop due to aggregation of para-casein up to a minimum at PDI_1 (flocculation) indicating a condensed phase or sol-gel transition state and (3) slight rise which was much more apparent in combined control curd, an indication of extensive restructuring, and whose rate decreased as the curd strengthens and ages. An almost linear increase in diffusion coefficients ($R^2 = 0.74$) in the third phase of the control curd during combined renneting and acidification was observed. A similar change both with time and decreasing pH was also observed during acidification without rennet (Fig. 4A_1 & B_1). On the other hand, concentrated milk exhibited a slight change despite similar degree of acidification to that of control (Fig. 4A_2 & B_2).

3.4. Changes in hydrophobic interactions

A linear plot of RFI vs. protein content (figure not shown) indicates that the number of hydrophobic sites increased parallel to the milk concentration. The percentage of initial concentration of the fluorescent probe (ANS) retained into concentrated milk curds during coagulation was also found to be greater than observed in control curds (Fig. 5). The ANS retention appeared at ≥70 % for concentrated milk and 40 % for control with GDL, whereas control without GDL showed only 8 % retention of the initial ANS 20 min after addition of coagulants. It is also clear that the curves of ANS retention rose sharply immediately after adding coagulants, reaching

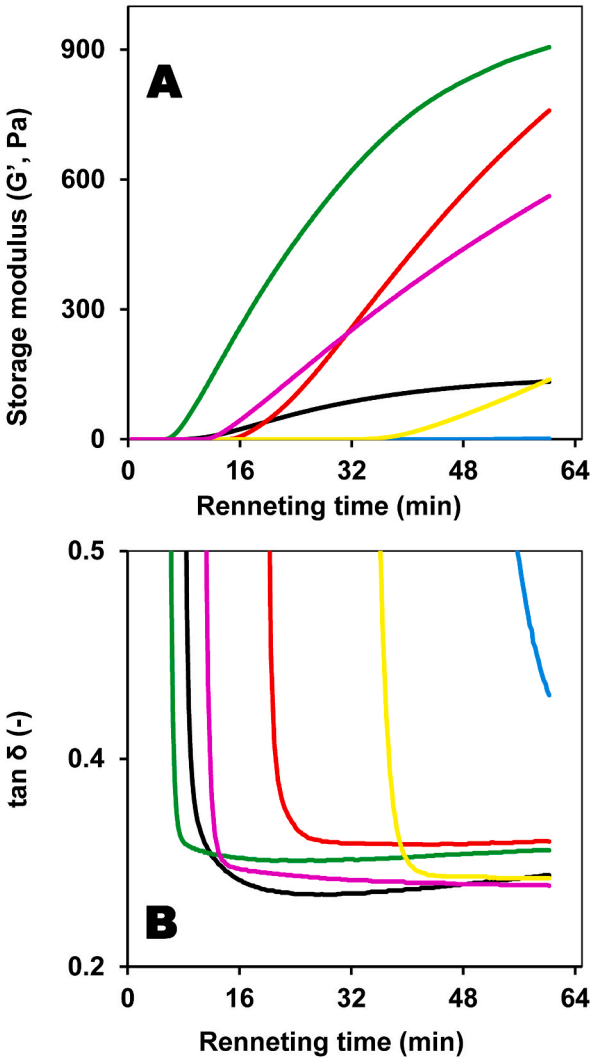

Fig. 1. Changes in G' (A) and tan δ (B) with time during rennet coagulation of skim milk samples (black line: control with GDL; blue line: control without GDL; green line: concentrated milk with GDL & rennet added based on protein content; purple line: concentrated milk without GDL & rennet added based on protein content; red line: concentrated milk with GDL & same rennet as control; yellow line: concentrated milk without GDL & same rennet as control).

a plateau within 5 min for samples containing GDL, and 10 min for concentrated milk without GDL while that of control without GDL showed a small increase only for a 20 min sub-sample. Similar results were reported [53]. This clearly explains that a slower curd firming rate observed from rheological and light scattering measurements was partly due to limited hydrophobic interactions and demonstrates their key role during early stages of curd formation. Moreover, the influence of milk concentration was much higher compared to that of pH.

4. Discussion

Concentrated milk systems are widely applied in cheese industries for cheese milk standardisation which helps to achieve consistent cheese composition and texture, and have the potential to improve cheese yield. Here, a series of changes occurring within the gel matrix under two rennet and acidification levels were assessed. Concentrated skim milk renneted based on protein content clotted faster due to an increased micellar size indicated by higher d_h (Table 3) usually as a consequence of greater sticking probability [59], and sufficient rennet/casein ratio. Although a certain proportion of κ-casein needs to be hydrolysed before clotting begins [60, 61], this stage appeared to have no significant effect on RCT compared to that of acidification. However, G'_{30} of concentrated milk (Table 4) indicates the dependence of flocculation time and curd firming rate on the renneting extent and milk concentration, respectively, which is in strong agreement with Karlsson, Ipsen & Ardö [62].

In absence of an acidifying agent, low charge density, greater amount of soluble Ca fractions (Table 1) and increased hydrophobic

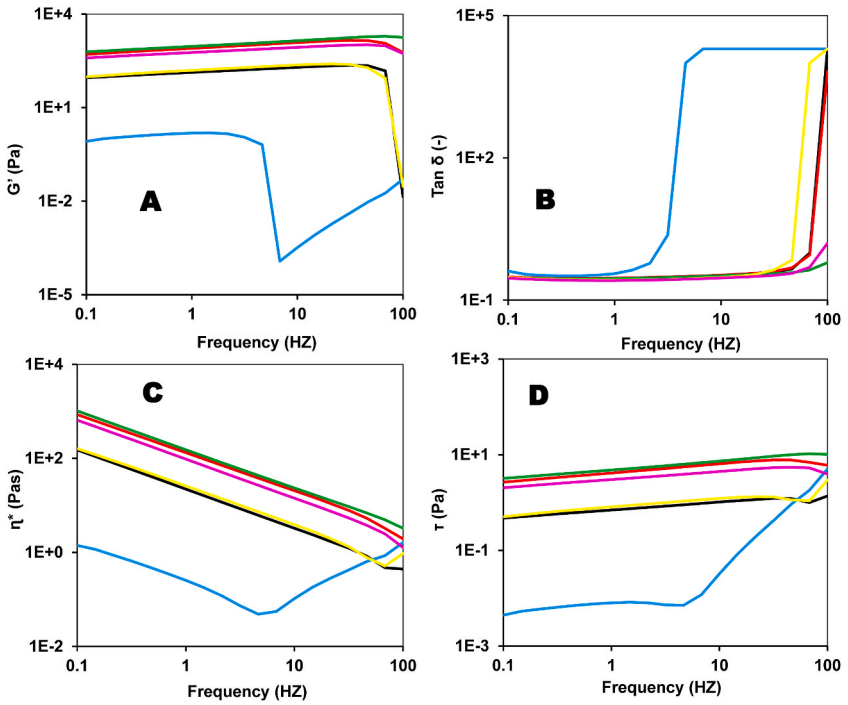

Fig. 2. (A) Storage modulus (G'), (B) tan δ, (C) complex viscosity (η*) and (D) shear stress (τ) as a function of oscillatory frequency for rennet coagulated skim milk curds (black line: control with GDL; blue line: control without GDL; green line: concentrated milk with GDL & rennet added based on protein content; purple line: concentrated milk without GDL & rennet added based on protein content; red line: concentrated milk with GDL & same rennet as control; yellow line: concentrated milk without GDL & same rennet as control).

interactions (Fig. 5) appear to have played a significant role in addition to a greater micellar size aforementioned. The rate of change in hydrophobic interactions observed in combined gels (within 5 min) demonstrates the role of pH reduction (from 6.8 for control or 6.4 for concentrated samples to 6.0 ± 0.1) in minimising the clotting time and increasing the curd firming rate and curd strength (Fig. 1A). This probably relies on a strong synergistic action of both coagulants towards reducing the charge and chain densities [42] which effectively drops the net repulsive forces or energy barrier between micelles, while stronger gels (higher G′) could be attributed to a highly branched and interconnected network [63].

According to Mellema [12], higher G′ values suggest a great number and strength of junctions between particles which increases the local compactness and size of the compact building blocks. It was also found that tan δ, spontaneous syneresis and pore sizes increase with decreasing pH [5]. However, despite higher G′ in combined gels from concentrated milk (Fig. 2A), higher tan δ (Fig. 2B), which accounts for conditions that promote structural changes, shows that concentrated milk curds have relatively weakly organised structures. Lucey et al. [6] suggested that high or low tan δ values are indicators of extensive or fewer large scale structural rearrangements, respectively. It had also been proposed [59] that a high tan δ value is an indication of shorter bonds relaxation time due to flow-like rearrangements. This would imply that concentrated milk curds underwent greater rearrangements than the control. In contrast, Karlsson et al. [62] reported a lower extent of rearrangements in highly concentrated UF skim milk curd, and this could be related to the concentration method. In the present study, an apparently continuous rise in tan δ in the later phase of the control compared to a plateauing behaviour shown by concentrated milk curds, is also a likely indication of more bond relaxation and a tendency for syneresis as suggested by van Vliet et al. [64], i.e., progressive rise in rearrangements and microphase separation due to changes in type and strength of bonds being formed during curd ageing.

Progressively higher structural rearrangements during curd ageing in combined control were confirmed by a continuous rise in diffusion coefficients (Fig. 3A). In acidified gels, demineralisation of colloidal $Ca_3(PO_4)_2$ [65] reduces the resistance to deformation due to lower bond energy and promotes restructuring and microsyneresis. This reflects an increased number of deformable bonds as particles have greater mobility. An important structural rearrangement taking place during curd ageing is particle fusion [12]. This leads to a progressive coarsening of gel structure and pore size increments that promote phase separation. Unlike controls, however, highly concentrated curds exhibit limited macroscopic contraction [66], thus, limited cluster fusion and macrosyneresis. The overall curd is stiff due to a higher solid to moisture ratio [67], but the rate of whey separation during curd ageing is likely greater in control. Therefore, as changes in diffusion coefficients in concentrated milk curds were similar regardless of pH (Fig. 3), they likely rather undergo slow microsyneresis and long-term rearrangements at bonding sites (primary particle level) due to an increasing number of unbonded particles, and perhaps responsible for more flow-like behaviour shown in Fig. 1B.

It is thought that the gel strength is strongly reduced by increasing the size of the primary aggregates [68], besides, milk from cows known to naturally have small casein micelles produce firmer gels [9,69]. According to Mezzenga & Fischer [70], the size of interacting

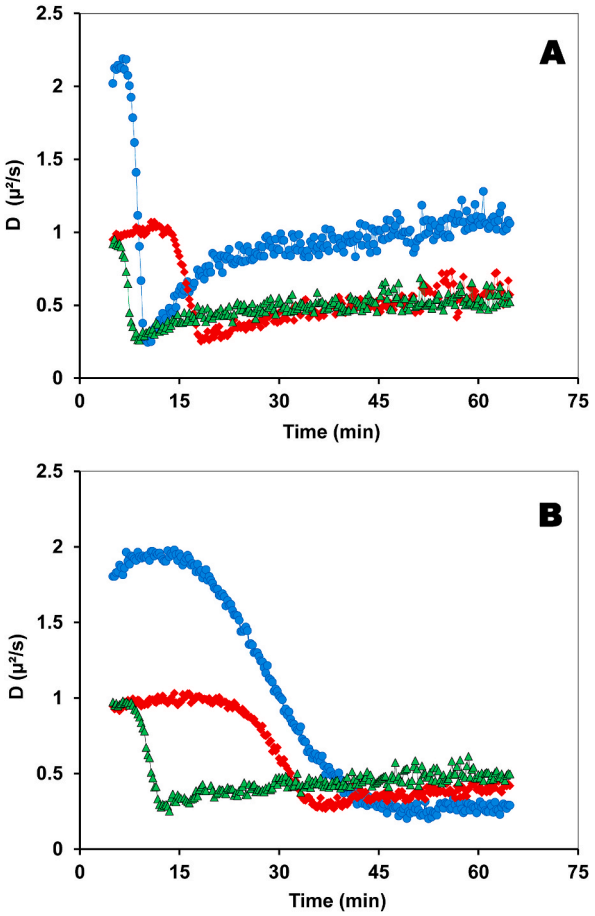

Fig. 3. Changes in diffusion coefficients (D) as a function of gelation time during renneting combined with acidification using GDL (A) or renneting only (B) of control (blue line) and concentrated skim milk samples with the same rennet as control (red line) or with rennet added based on protein content (green line).

colloids causes a large competition between electrostatic repulsive and hydrophobic attraction forces. However, small native micelles are usually associated with higher levels of κ-casein [71]. Moreover, it is clearly shown herein that the gel strength increased with milk concentration despite greater hydrodynamic radius of casein micelles in milk. Therefore, an estimated increase in hydrodynamic radius due to milk concentration appears one of the essential changes resulting from micellar restructuring. Micellar restructuring arises from shorter inter-particle distance and altered ionic equilibrium creating an imbalance in net repulsive forces causing some of the serum Ca and caseins to shift into the colloidal phase and some of micelles to partially adjoin [26,30] as repulsive forces are overcome by attraction. This is supported by low diffusion coefficients obtained (Table 3) which are not only indicative of greater hydrodynamic radius but also increased attractive protein-protein interactions [72]. Eventually the charge density also decreases (Table 3), contributing to the rise in surface hydrophobicity which plays a key role. Therefore, it may be this micellar restructuring-related changes that determine the type and strength of interactions formed during gelation and rearrangements, which according to Dickinson [73] the gel structure and pore size depend on. He argued that the pore size is particularly determined by the degree of attractive interconnections and phase separation.

In the current research, the rate of attractive rearrangements (specifically, floc contraction) and macroscopic phase separation during curd ageing in concentrated milk gels appears to be limited. This explains a higher moisture retention (Table 2) as an indication of impaired macrosyneresis. Higher retention of Ca and other solids may also be related to more serum solids associated with water retention rather than to greater Ca binding. In contrast, a significant decrease in curd moisture on increasing milk concentration up to 4 % [74], 4.6 % [16] or 6 % protein [19] was reported, showing that curd structure improves under optimum limits of milk concentration and explained by microstructural changes such as pore size [75]. However, fractures were observed in process cheese from concentrated milk up to 6 % protein which resulted in the failure to entrap fat [76], an indication of a less-cohesive matrix [11].

5. Conclusion

This study examined how skim milk total solids concentration modulates the rennet curd structure using dynamic rheometry,

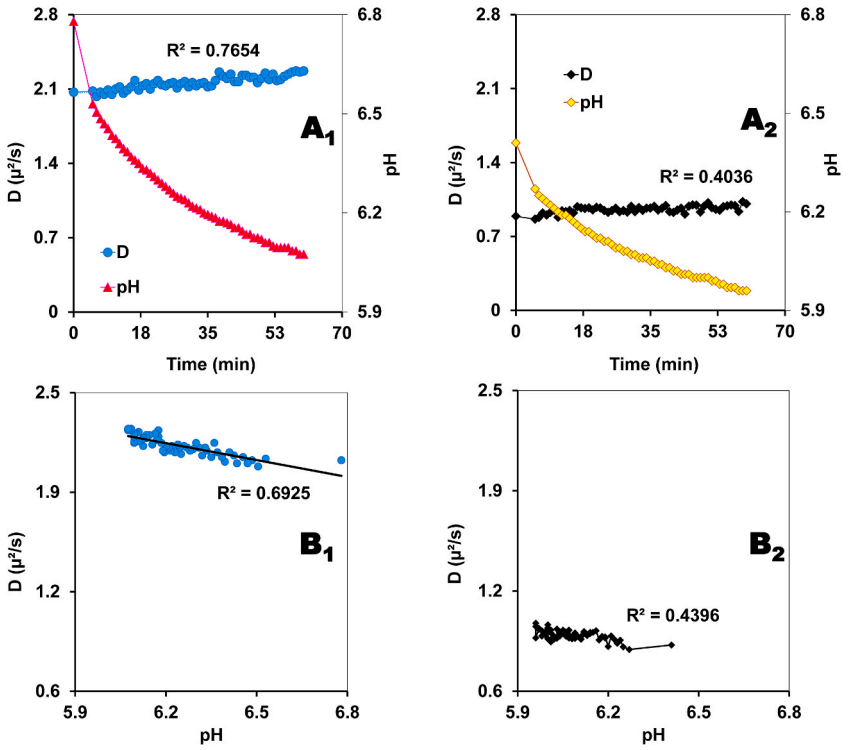

Fig. 4. Changes in diffusion coefficients (D) and pH as a function of time during 1 h of acidification using GDL for control (A_1) and concentrated (A_2) skim milk samples as well as changes in D with pH during 1 h of acidification using GDL for control (B_1) and concentrated (B_2) skim milk samples.

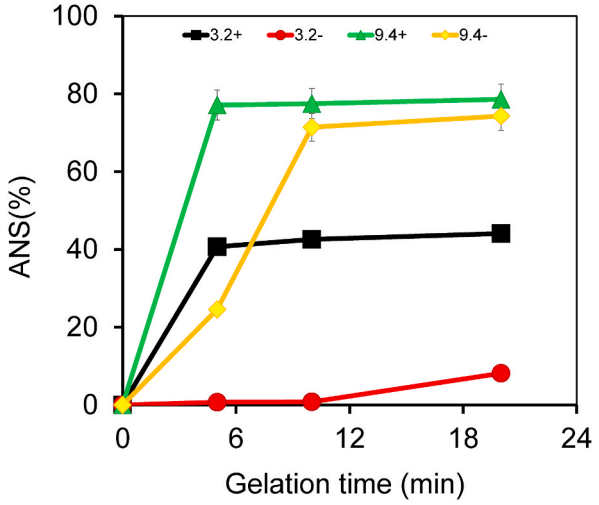

Fig. 5. ANS retained into the curds (% of initial concentration added to milk) at different time intervals during rennet coagulation of the control skim milk with GDL (black line with squares) or without GDL (red line with circles) and concentrated skim milk samples with GDL (green line with triangles) or without GDL (yellow line with rotated squares).

dynamic light scattering and fluorimetry. It is concluded that micellar restructuring induced by the change of net repulsion – as evidenced by increased micellar size, lower serum/total Ca ratio, charge density and diffusivity, and greater hydrophobicity – is the underlying reason for variations in rennet curd formation and ageing properties due to milk concentration. This paves the way for future research into the drivers of curd formation and matrix integrity, enzyme kinetics, and caseins, fat and rennet activity partitioning, which may help to better control yield, texture and maturation rate of cheeses made from milks of different concentrations or protein genetic variants. The key driver could probably be the change in the predominant type and/or strength of major casein

interactions which can be established by performing specific interactions/bond blocking or dissociation tests.

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Data availability statement

The data used are contained in tables and figures within the article.

CRediT authorship contribution statement

Joseph F. Kayihura: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The author declares no competing interests.

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References

[1] H.R. Chapman, Standardisation of milk for cheesemaking at research level, J. Soc. Dairy Technol. 34 (1981) 147–152, https://doi.org/10.1111/j.1471-0307.1981.tb01514.x.
[2] T.P. Guinee, P.D. Pudja, E.O. Mulholland, Effect of milk protein standardization by ultrafiltration on the manufacture, composition and maturation of Cheddar cheese, J. Dairy Res. 61 (1994) 117–131, https://doi.org/10.1017/S0022029900028119.
[3] L. Ong, R.R. Dagastine, S.E. Kentish, S.L. Gras, Microstructure and composition of full fat Cheddar cheese made with ultrafiltered milk retentate, Foods 2 (2013) 310–331, https://doi.org/10.3390/foods2030310.
[4] E. Lefebvre-cases, E. Gastaldi, V. Vidal, S. Marchesseau, A. Lagaude, J.-L. Cuq, B.T. De la Fuente, Identification of interactions among casein gels using dissociating chemical agents, J. Dairy Sci. 81 (1998) 932–938, https://doi.org/10.3168/jds.S0022-0302(98)75653-X.
[5] X.T. Liu, H. Zhang, F. Wang, J. Luo, H.Y. Guo, F.Z. Ren, Rheological and structural properties of differently acidified and renneted milk gels, J. Dairy Sci. 97 (2014) 3292–3299, https://doi.org/10.3168/jds.2013-7568.
[6] J.A. Lucey, M. Tamehana, H. Singh, P.A. Munro, Rheological properties of milk gels formed by a combination of rennet and glucono-δ-lactone, J. Dairy Res. 67 (2000) 415–427, https://doi.org/10.1017/S0022029900004246.
[7] A. Madadlou, A. Khosroshahi, M.E. Mousavi, Rheology, microstructure, and functionality of low-fat Iranian white cheese made with different concentrations of rennet, J. Dairy Sci. 88 (2005) 3052–3062, https://doi.org/10.3168/jds.S0022-0302(05)72986-6.
[8] K. Soodam, L. Ong, I.B. Powell, S.E. Kentish, S.L. Gras, Effect of rennet on the composition, proteolysis and microstructure of reduced-fat Cheddar cheese during ripening, Dairy Sci. Technol. 95 (2015) 665–686, https://doi.org/10.1007/s13594-015-0250-5.
[9] M. Glantz, T.G. Devold, G.E. Vegarud, H. Lindmark Månsson, H. Stålhammar, M. Paulsson, Importance of casein micelle size and milk composition for milk gelation, J. Dairy Sci. 93 (2010) 1444–1451, https://doi.org/10.3168/jds.2009-2856.
[10] J.C. Amaro-Hernández, G.I. Olivas, C.H. Acosta-Muñiz, N. Gutiérrez-Méndez, D.R. Sepulveda, Structure rearrangement during rennet coagulation of milk modifies curd density, J. Dairy Sci. 103 (2020) 3088–3094, https://doi.org/10.3168/jds.2019-16998.
[11] L. Ong, R.R. Dagastine, M.A.E. Auty, S.E. Kentish, S.L. Gras, Coagulation temperature affects the microstructure and composition of full fat Cheddar cheese, J. Dairy Sci. Technol. 91 (2011) 739–758, https://doi.org/10.1007/s13594-011-0033-6.
[12] M. Mellema, Scaling Relations between Structure and Rheology of Ageing Casein Particle Gels, PhD. Thesis, Wageningen University, Netherlands, 2000, https://edepot.wur.nl/192892.
[13] M.R. Acharya, V.V. Mistry, Comparison of effect of vacuum-condensed and ultrafiltered milk on Cheddar cheese, J. Dairy Sci. 87 (2004) 4004–4012, https://doi.org/10.3168/jds.S0022-0302(04)73541-9.
[14] D.L. Anderson, V.V. Mistry, R.L. Brandsma, K.A. Baldwin, Reduced fat Cheddar cheese from condensed milk. Manufacture, composition, and ripening, J. Dairy Sci. 76 (1993) 2832–2844, https://doi.org/10.3168/jds.S0022-0302(93)77622-5.
[15] M.M. Calvo, N.A. Espinoza, Syneresis rate of cow's, Ewe's, and goat's curd. Effect of thermal treatment and ultrafiltration, J. Agric. Food Chem. 47 (1999) 883–886, https://doi.org/10.1021/jf980783w.
[16] T.P. Guinee, D.J. O'callaghan, E.O. Mulholland, D. Harrington, Milk protein standardization by ultrafiltration for Cheddar cheese manufacture, J. Dairy Res. 63 (1996) 281–293, https://doi.org/10.1017/S0022029900031770.
[17] K.S. Kealey, F.V. Kosikowski, Cheddar cheese from ultrafiltered whole milk retentates in industrial cheese making, J. Dairy Sci. 68 (1985) 3148–3154, https://doi.org/10.3168/jds.S0022-0302(85)81220-0.
[18] P.K. Nair, M. Corredig, Rennet-induced gelation of concentrated milk in the presence of sodium caseinate: differences between milk concentration using ultrafiltration and osmotic stressing, J. Dairy Sci. 98 (2015) 27–36, https://doi.org/10.3168/jds.2014-8356.
[19] R.R. Panthi, A.L. Kelly, D.J. McMahon, X. Dai, A.H. Vollmer, J.J. Sheehan, Response surface methodology modeling of protein concentration coagulum cut size, and set temperature on curd moisture loss kinetics during curd stirring, J. Dairy Sci. 102 (2019) 4989–5004, https://doi.org/10.3168/jds.2018-15051.
[20] E. Salvatore, A. Pirisi, M. Corredig, Gelation properties of casein micelles during combined renneting and bacterial fermentation: effect of concentration by ultrafiltration, Int. Dairy J. 21 (2011) 848–856, https://doi.org/10.1016/j.idairyj.2011.05.006.
[21] P. Upreti, V.V. Mistry, M.R. Acharya, Characterization of rennet coagulation of milk concentrated by vacuum condensing and ultrafiltration, Dairy Sci. Technol. 91 (2011) 383–395, https://doi.org/10.1007/s13594-011-0004-y.
[22] S. Le Feunteun, F. Mariette, Effects of acidification with and without rennet on a concentrated casein system: a kinetic NMR probe diffusion study, Macromolecules 41 (2008) 2079–2086, https://doi.org/10.1021/ma702248z.

[23] S. Le Feunteun, M. Ouethrani, F. Mariette, The rennet coagulation mechanisms of a concentrated casein suspension as observed by PFG-NMR diffusion measurements, Food Hydrocoll 27 (2012) 456–463, https://doi.org/10.1016/j.foodhyd.2011.09.008.
[24] I. Sone, M. Hosoi, L.C. Geonzon, H. Jung, F.B. Descallar, H. Bingjie, S. Matsukawa, Gelation and network structure of acidified milk gel investigated at different length scales with and without addition of iota-carrageenan, Food Hydrocoll 123 (2022) 107170, https://doi.org/10.1016/j.foodhyd.2021.107170.
[25] S.G. Anema, Effect of milk concentration on heat-induced, pH-dependent dissociation of casein from micelles in reconstituted skim milk at temperatures between 20 and 120°C, J. Agric. Food Chem. 46 (1998) 2299–2305, https://doi.org/10.1021/jf970909+.
[26] D.Z. Liu, D.E. Dunstan, G.J.O. Martin, Evaporative concentration of skimmed milk: effect on casein micelle hydration, composition, and size, Food Chem. 134 (2012) 1446–1452, https://doi.org/10.1016/j.foodchem.2012.03.053.
[27] T. Markoska, T. Huppertz, M.K. Grewal, T. Vasiljevic, FTIR analysis of physiochemical changes in raw skim milk upon concentration, LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 102 (2019) 64–70, https://doi.org/10.1016/j.lwt.2018.12.011.
[28] G.J.O. Martin, R.P.W. Williams, D.E. Dunstan, Comparison of casein micelles in raw and reconstituted skim milk, J. Dairy Sci. 90 (2007) 4543–4551, https://doi.org/10.3168/jds.2007-0166.
[29] A.B. McKenna, Effect of Processing and Storage on Reconstitution Properties of Whole Milk and Ultrafiltered Skim Milk Powders, PhD. Thesis, Massy University, New Zealand, 2000.
[30] H. Singh, Interactions of milk proteins during the manufacture of milk powders, Lait 87 (2007) 413–423, https://doi.org/10.1051/lait:2007014.
[31] M. Mellema, P. Walstraa, J.H.J. van Opheusden, T. van Vliet, Effects of structural rearrangements on the rheology of rennet-induced casein particle gels, Adv. Colloid Interface Sci. 98 (2002) 25–50, https://doi.org/10.1016/S0001-8686(01)00089-6.
[32] M.A. Fenelon, T.P. Guinee, The effect of milk fat on Cheddar cheese yield and its prediction, using modifications of the Van Slyke cheese yield formula, J. Dairy Sci. 82 (1999) 2287–2299, https://doi.org/10.3168/jds.S0022-0302(99)75477-9.
[33] Shakeel-Ur-Rehman, P.L.H. McSweeney, P.F. Fox, Protocol for the manufacture of miniature cheeses, Lait 78 (1998) 607–620, https://doi.org/10.1051/lait:1998654.
[34] H. Li, C. Yang, C. Chen, F. Ren, Y. Li, Z. Mu, P. Wang, The use of tri-sodium citrate to improve the textural properties of acid-induced, transglutaminase-treated micellar casein gels, Molecules 23 (2018) 1632, https://doi.org/10.3390/molecules23071632.
[35] R. Mishra, S. Govindasamy-Lucey, J.A. Lucey, Rheological properties of rennet-induced gels during the coagulation and cutting process: impact of processing conditions, J. Texture Stud. 36 (2005) 190–212, https://doi.org/10.1111/j.1745-4603.2005.00011.x.
[36] Y. Nakatuka, H. Yoshida, K. Fukui, M. Matuzawa, The effect of particle size distribution on effective zeta-potential by use of the sedimentation method, Adv. Powder Technol. 26 (2015) 650–656, https://doi.org/10.1016/j.apt.2015.01.017.
[37] C.M. Beliciu, C.I. Moraru, Effect of solvent and temperature on the size distribution of casein micelles measured by dynamic light scattering, J. Dairy Sci. 92 (2009) 1829–1839, https://doi.org/10.3168/jds.2008-1467.
[38] C.G. De Kruif, Casein Micelles: diffusivity as a function of renneting time, Langmuir 8 (1992) 2932–2937, https://doi.org/10.1021/la00048a014.
[39] A.O. Karlsson, R. Ipsen, K. Schrader, Y. Ardö, Relationship between physical properties of casein micelles and rheology of skim milk concentrate, J. Dairy Sci. 88 (2005) 3784–3797, https://doi.org/10.3168/jds.S0022-0302(05)73064-2.
[40] M. De Kanter, J. Meyer-Kirschner, J. Viell, A. Mitsos, M. Kather, A. Pich, C. Janzen, Enabling the measurement of particle sizes in stirred colloidal suspensions by embedding dynamic light scattering into an automated probe head, Measurement 80 (2016) 92–98, https://doi.org/10.1016/j.measurement.2015.11.024.
[41] J. Stetefeld, S.A. McKenna, T.R. Patel, Dynamic light scattering: a practical guide and applications in biomedical sciences, Biophys. Rev. 8 (2016) 409–427, https://doi.org/10.1007/s12551-016-0218-6.
[42] C.G. De Kruif, E.B. Zhulina, κ-casein as a polyelectrolyte brush on the surface of casein micelles, Colloids Surf. A: Physicochem. Eng. Asp. 117 (1996) 151–159, https://doi.org/10.1016/0927-7757(96)03696-5.
[43] M. Alexander, L.F. Rojas-Ochoa, M. Leser, P. Schurtenberger, Structure, dynamics, and optical properties of concentrated milk suspensions: an analogy to hard-sphere liquids, J. Colloid Interface Sci. 253 (2002) 35–46, https://doi.org/10.1006/jcis.2002.8452.
[44] I. Gülseren, M. Alexander, M. Corredig, Probing the colloidal properties of skim milk using acoustic and electroacoustic spectroscopy. Effect of concentration, heating and acidification, J. Colloid Interface Sci. 351 (2010) 493–500, https://doi.org/10.1016/j.jcis.2010.07.078.
[45] P.K. Nair, M. Alexander, D. Dalgleish, M. Corredig, Physico-chemical properties of casein micelles in unheated skim milk concentrated by osmotic stressing: interactions and changes in the composition of the serum phase, Food Hydrocoll 34 (2014) 46–53, https://doi.org/10.1016/j.foodhyd.2013.01.001.
[46] S. Homer, L. Lundin, D.E. Dunstan, Heat-induced whey protein microparticulation under continuous shear in acidic conditions, Food Hydrocoll 121 (2021) 107044, https://doi.org/10.1016/j.foodhyd.2021.107044.
[47] P. González-tello, F. Camacho, E.M. Guadix, G. Luzón, A. González, Density, viscosity and surface tension of whey protein concentrate solutions, J. Food Process. Eng. 32 (2009) 235–247, https://doi.org/10.1111/j.1745-4530.2007.00213.x.
[48] B. Trinh, K.T. Trinh, D. Haisman, Effect of total solids content and temperature on the rheological behaviour of reconstituted whole milk concentrates, J. Dairy Res. 74 (2007) 116–123, https://doi.org/10.1017/S0022029906002287.
[49] Z. Liu, Y. Hemar, S. Tan, P. Sanguansri, J. Niere, R. Buckow, M.A. Augustin, Pulsed electric field treatment of reconstituted skim milks at alkaline pH or with added EDTA, J. Food Eng. 144 (2015) 112–118, https://doi.org/10.1016/j.jfoodeng.2014.06.033.
[50] S.G. Anema, S.K. Lee, H. Klostermeyer, Reconstituted skim milk: effect of re-dilution after heating concentrated milk at different pH and temperatures, Int. Dairy J. 131 (2022) 105368, https://doi.org/10.1016/j.idairyj.2022.105368.
[51] H. Sinaga, N. Bansal, B. Bhandari, Effects of milk pH alteration on casein micelle size and gelation properties of milk, Int. J. Food Prop. 20 (2017) 179–197, https://doi.org/10.1080/10942912.2016.1152480.
[52] N. Alizadeh-Pasdar, E.C. Li-Chan, Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes, J. Agric. Food Chem. 48 (2000) 328–334, https://doi.org/10.1021/jf990393p.
[53] C. Peri, E. Pagliarini, S. Iametti, F. Bonomi, A study of surface hydrophobicity of milk proteins during enzymic coagulation and curd hardening, J. Dairy Res. 57 (1990) 101–110, https://doi.org/10.1017/S0022029900026650.
[54] T. Huppertz, T.T. Lambers, Influence of micellar calcium phosphate on in vitro gastric coagulation and digestion of milk proteins in infant formula model systems, Int. Dairy J. 107 (2020) 104717, https://doi.org/10.1016/j.idairyj.2020.104717.
[55] M.A. Ferrer, A.R. Hill, M. Corredig, Rheological properties of rennet gels containing milk protein concentrates, J. Dairy Sci. 91 (2008) 959–969, https://doi.org/10.3168/jds.2007-0525.
[56] F. He, G.W. Becker, J.R. Litowski, L.O. Narhi, D.N. Brems, V.I. Razinkov, High-throughput dynamic light scattering method for measuring viscosity of concentrated protein solutions, Anal. Biochem. 399 (2010) 141–143, https://doi.org/10.1016/j.ab.2009.12.003.
[57] S.M. Loveday, L.K. Creamer, H. Singh, M.A. Rao, Phase and rheological behavior of high-concentration colloidal hard-sphere and protein dispersions, J. Food Sci. 72 (2007) R101–R107, https://doi.org/10.1111/j.1750-3841.2007.00452.x.
[58] J.A. Lucey, Formation and physical properties of milk protein gels, J. Dairy Sci. 85 (2002) 281–294, https://doi.org/10.3168/jds.S0022-0302(02)74078-2.
[59] L.G.B. Bremer, Fractal Aggregation in Relation to Formation and Properties of Particle Gels, PhD Thesis, Landbouwuniversiteit Wageningen, Netherlands, 1992, p. 189, https://edepot.wur.nl/201301.
[60] D.G. Dalgleish, Proteolysis and aggregation of casein micelles treated with immobilized or soluble chymosin, J. Dairy Res. 46 (1979) 653–661, https://doi.org/10.1017/S0022029900020719.
[61] S.B. Lomholt, K.B. Qvist, Relationship between rheological properties and degree of κ-casein proteolysis during renneting of milk, J. Dairy Res. 64 (1997) 541–549, https://doi.org/10.1017/S002202999700246X.
[62] A.O. Karlsson, R. Ipsen, Y. Ardö, Rheological properties and microstructure during rennet induced coagulation of UF concentrated skim milk, Int. Dairy J. 17 (2007) 674–682, https://doi.org/10.1016/j.idairyj.2006.08.002.
[63] J. Choi, D.S. Horne, J.A. Lucey, Effect of insoluble calcium concentration on rennet coagulation properties of milk, J. Dairy Sci. 90 (2007) 2612–2623, https://doi.org/10.3168/jds.2006-814.

[64] T. Van Vliet, H.J.M. van Dijk, P. Zoon, P. Walstra, Relation between syneresis and rheological properties of particle gels, Colloid Polym. Sci. 269 (1991) 620–627, https://doi.org/10.1007/BF00659917.
[65] N.N. Silva, M. Piot, A.F. de Carvalho, F. Violleau, A.L. Fameau, F. Gaucheron, PH–induced demineralization of casein micelles modifies their physico-chemical and foaming properties, Food Hydrocoll 32 (2013) 322–330, https://doi.org/10.1016/j.foodhyd.2013.01.004.
[66] E. Gastaldi, A. Lagaude, S. Marchesseau, B.T. de la Fuente, Acid milk gel formation as affected by total solids content, J. Food Sci. 62 (1997) 671–687, https://doi.org/10.1111/j.1365-2621.1997.tb15432.x.
[67] N. Zhou, S.J. Mulvaney, The effect of milk fat, the ratio of casein to water, and temperature on the viscoelastic properties of rennet casein gels, J. Dairy Sci. 81 (1998) 2561–2571, https://doi.org/10.3168/jds.S0022-0302(98)75813-8.
[68] D.S. Horne, Aspects of scaling behaviour in the kinetics of particle gel formation, J. Chim. Phys. 93 (1996) 977–986, https://doi.org/10.1051/jcp/1996930977.
[69] A. Logan, A. Leis, L. Day, S.K. Øiseth, A. Puvanenthiran, M.A. Augustin, Rennet gelation properties of milk: influence of natural variation in milk fat globule size and casein micelle size, Int. Dairy J. 46 (2015) 71–77, https://doi.org/10.1016/j.idairyj.2014.08.005.
[70] R. Mezzenga, P. Fischer, The self-assembly, aggregation and phase transitions off food protein systems in one, two and three dimensions, Rep. Prog. Phys. 76 (2013) 046601, https://doi.org/10.1088/0034-4885/76/4/046601.
[71] D.T. Davies, A.J.R. Law, Variation in the protein composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature, J. Dairy Res. 50 (1983) 67–75, https://doi.org/10.1017/S0022029900032532.
[72] A. Saluja, A.V. Badkar, D.L. Zeng, S. Nema, D.S. Kalonia, Ultrasonic storage modulus as a novel parameter for analyzing protein-protein interactions in high protein concentration solutions: correlation with static and dynamic light scattering measurements, Biophys. J. 92 (2007) 234–244, https://doi.org/10.1529/biophysj.106.095174.
[73] E. Dickinson, Computer simulation of particle gel formation, J. Chem. Soc., Faraday trans. 90 (1994) 173–180, https://doi.org/10.1039/FT9949000173.
[74] T.P. Guinee, B.T. O'Kennedy, P.M. Kelly, Effect of milk protein standardization using different methods on the composition and yields of Cheddar cheese, J. Dairy Sci. 89 (2006) 468–482, https://doi.org/10.3168/jds.S0022-0302(06)72110-5.
[75] G.H. Meletharayil, H.A. Patel, T. Huppertz, Rheological properties and microstructure of high protein acid gels prepared from reconstituted milk protein concentrate powders of different protein contents, Int. Dairy J. 47 (2015) 64–71, https://doi.org/10.1016/j.idairyj.2015.02.005.
[76] V.V. Mistry, A.N. Hassan, M.R. Acharya, Microstructure of pasteurized process cheese manufactured from vacuum condensed and ultrafiltered milk, Lait 86 (2006) 453–459, https://doi.org/10.1051/lait:2006018.

Chapter 7– Partitioning of individual caseins between rennet curd and whey: impact of pre-acidification and pre-salting of reconstituted skim milk

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This chapter has been published as an original research paper entitled “Partitioning of individual caseins between rennet curd and whey: impact of pre-acidification and pre-salting of reconstituted skim milk” by Joseph F. Kayihura in a peer-reviewed journal: Food and Humanity, 1 (2023) 905-911. (https://doi.org/10.1016/j.foohum.2023.08.014). In addition to modifying cheese-milk composition (standardisation), acidification and salting are other common pre-treatments applied to cheese-milk. Therefore, like chapter 5, this chapter also further the knowledge of variations in partitioning of caseins due to cheese-milk pre-treatments.

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1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book:	Partitioning of individual caseins between rennet curd and whey: impact of pre-acidification and pre-salting of reconstituted skim milk
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In the case of the above publication, the following authors contributed to the work as follows:

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Joseph Flambeau Kayihura	100%	Conceptualisation, study design, experimentation, writing, submission & revision	[redacted]	01/09/2023

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Partitioning of individual caseins between rennet curd and whey: Impact of pre-acidification and pre-salting of reconstituted skim milk

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Rennet whey

ABSTRACT

Acidification is one of the essential pre-treatments applied to milk for the manufacture of many cheese varieties while pre-salting is most common in Domiati-style cheeses. Both treatments play several safety and qualitative roles. This research examined the impact of pre-acidification (pH 5–7), pre-salting (0–3 *M,* NaCl) and combined pre-acidification and pre-salting (pH 5–7 at 3 *M*, NaCl) of reconstituted skim milk on partitioning of individual caseins between rennet curd and whey. No α_{s1}- and β-caseins were detected in rennet whey from unsalted or pre-salted milk with ionic strength $\leq$ 0.9 *M*, NaCl whereas κ- and α_{s2}- caseins were found at all pH and NaCl levels. The greatest loss of caseins (72.9 % of total-casein (T-casein)) into whey occurred when milk with 3 *M*, NaCl was adjusted to pH 7 whereas pH 6 (10 % T-casein loss) appeared optimal for this milk. Findings for unsalted samples are attributed to soluble κ-/α_{s2}-caseins-whey protein complexes as evidenced by gel electrophoresis. Notably, varying levels of para-κ-casein lost in rennet whey from pre-salted milk reveal inhibition of aggregation phase of renneting which was dependent on ionic strength and pH. Partial inhibition of the enzymatic phase was also evident in highly pre-salted samples (3 *M*, NaCl) at pH values above or below 6.

1. Introduction

Milk gel formation due to destabilisation of casein micelles achieved by enzymatic (e.g., rennet) and/or acidic reactions on κ-casein is fundamental to cheesemaking (Kelly et al., 2008). κ-casein appears mostly on the surface of the micelles as a hairy layer and provides stability owing to its negatively charged and hydrophilic C-terminal peptide that extends into the serum phase (De Kruif, 1999; Horne & Lucey, 2017; Huppertz, 2013). Rennet gelation is initiated by enzymatic cleavage and the release of that peptide (Huppertz & de Kruif, 2007) while acidification neutralises the negative charges and dissolves the colloidal calcium (Ca) phosphate (CCP) (de Kruif, 1999; Le Graët & Gaucheron, 1999). This causes the collapse of the protective hairy layer and subsequent aggregation of caseins (de Kruif, 1999).

Rennet-induced and acid-induced gels are used mainly in production of hard ripened and fresh soft cheeses, respectively (Lucey, 2017), though there are fresh acid cheeses produced with minor enzymatic hydrolysis or heating (Farkye, 2017). Lowering pH increases the gelation rate, suppresses pathogenic microorganisms and enhances syneresis, cheese composition and flavour (McSweeney, 2007). $CaCl_2$ is also often added which further improves the aggregation rate and curd properties while salting (addition of NaCl) in most cheese varieties is normally performed after curd formation either directly to milled curd, during or after moulding (dry salting) or by immersion of pressed curds in 18–27 % NaCl solution (brine salting) (de Kruif, 1999; Guinee & Fox, 2017; McSweeney, 2007; Sandra et al., 2012). Cheese salting plays several similar roles to those of acidification stated above (Guinee & Fox, 2017; McSweeney, 2007).

Unlike most cheeses as mentioned above, an appreciable amount of NaCl (6–15 %, w/w) is added directly to cheese milk for Domiati and similar traditional cheeses in eastern countries (Awad, 2007; Ramet et al., 1983). Like $CaCl_2$, addition of NaCl modifies the ionic strength and pH (Huppertz & Fox, 2006; Zhao & Corredig, 2016). Both pH and ionic strength affect the proportion of solubilised proteins and Ca and it was reported that an elevated amount of soluble proteins slow the gelation while soluble Ca produce the opposite effect (Gaygadzhiev et al., 2012; Sandra & Corredig, 2013). Furthermore, some previous studies have also indicated that the pH at renneting and ionic strength impact the retention of some milk constituents, curd microstructure and yield (Choi et al., 2015; Daviau et al., 2000; Lauzin et al., 2020; Ong et al., 2012). However, as far as is known at present, there is little information regarding how individual caseins' partitioning are affected. Therefore,

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the present study was designed to assess how pre-acidification (pH 5–7), pre-salting (0–3 *M* or 0–15 %, NaCl, w/w) and combined pre-acidification and pre-salting (pH 5–7 at 3 *M*, NaCl) of reconstituted skim milk affect the partitioning of individual caseins between rennet curd and whey. Findings of this study could be particularly important in cheese yield prediction modelling and quality control of both cheese and whey co-products.

2. Materials and methods

2.1. Preparation of skim milk samples

The skim milk used was prepared by reconstituting low heat skim milk powder (Warrnambool Cheese and Butter - Saputo, Warrnambool, Victoria, Australia) in Milli-Q water. After mixing using a magnetic stirrer for 2 h at room temperature, reconstituted skim milk was refrigerated overnight. The reconstituted milk had a total solids (TS) content of 9.2 % and was used as a control. From this, three other sets of test samples were prepared: (1) pH-adjusted samples (pH 5, 5.5, 6, 6.5 & 7) using 6 *M*, HCl or 1 *M*, NaOH (for pH 7), (2) pre-salted samples (0.1, 0.3, 0.9, 2.7 & 3 *M*, NaCl) and (3) samples of 3 *M*, NaCl adjusted to different pH levels (pH 5, 5.5, 6, 6.5 & 7). Part of each test sample was used for preparation of rennet curds and the other part ultra-centrifuged according to Bogahawaththa et al. (2019) i.e., for 1 h at 100,000g, using a Beckman Ultra L-70 type centrifuge (Beckman Coulter Inc., Brea, California, USA). The supernatants obtained were used to assess the changes in serum fractions of caseins and Ca.

2.2. Rennet gelation

The same volume (50 mL) of milk samples was placed in centrifuge tubes, weighed and heated to 50 °C in a water bath, then, cooled to gelation temperature (31 °C). Ten microliters of a 10 × diluted calf rennet (Cheeselinks, Lara, Victoria, Australia) per 1 mL of milk were used to coagulate the milk samples (Awad, 2007). Fifty minutes after rennet addition samples were heated for 30 min at 38 °C and whey separation was achieved by centrifugation for 1 h at 3200g and 20 °C using a 5810R Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany). Part of whole whey collected was also ultra-centrifuged as mentioned above.

2.3. Determination of total and soluble fractions of proteins and Ca

Total protein content of all samples was determined by the Kjeldahl method (Daniloski et al., 2022; Dissanayake & Vasiljevic, 2009). Following an overnight drying of 10 g of milk or whey samples and their supernatants (from 10 g each) at 105 °C (Forsbäck et al., 2011), ashing was performed at 550 °C for a minimum of 18 h. The ash was cooled to room temperature and transferred with several washings using 5 % nitric acid solution into 100 mL volumetric flasks, filled to volume and the mixture swirled. Aliquots were filtered (0.45 μm) and Ca content analysed using an inductively coupled plasma atomic emission spectrometer (Multitype, Shimadzu Corporation, Kyoto, Japan).

Total and serum fractions of individual caseins in skim milk and whey samples were measured following a reverse-phase liquid chromatography (RP-HPLC) method described previously (Aprianita et al., 2014; Daniloski et al., 2022) with few modifications. Samples were diluted five times in an 8 M urea solution containing 165 mM of Tris, 44 mM of sodium citrate and 0·3 % (v/v) β-Mercaptoethanol. Twenty microliters of filtered (0·45 μm) samples were directly analysed by the RP-HPLC system. Separation of individual proteins was achieved using a C_4 column (Jupiter® 5 μm, 300 Å, 250×4.6 mm, Macclesfield, UK) at a detection wavelength of 220 nm. Elution was carried out using two mobile phases: (A) 0·1 % trifluoroacetic acid (TFA) in water and (B) 0·1 % TFA in acetonitrile which increased linearly from 30 % to 50 %, 50–100 %, kept constant at 100 %, decreased back to 30 % and held at this rate for 40, 2, 1, 3 and 5 min, respectively. The flow rate was kept constant at 0·8 mL/min throughout the entire run time. Identification of individual caseins and whey proteins was achieved by running the standards (Sigma-Aldrich, St. Louis, MO) through the instrument under the same conditions and also based on literature (Daniloski et al., 2022).

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Changes in caseins were also qualitatively assessed by both reducing (using β-mercaptoethanol) and non-reducing SDS-PAGE using polyacrylamide gels (30 % acrylamide and 10 % SDS) as described elsewhere (Bogahawaththa et al., 2017; Dissanayake & Vasiljevic, 2009). All samples were diluted in SDS buffer to 1 mg of total protein/mL for milk samples or 3 mg of total protein/mL for rennet whey and both milk and whey supernatants. Staining was achieved using Coomassie Brilliant Blue. Following a de-staining procedure, gels were cleaned with Milli-Q water and then, scanned using a ChemiDoc Imaging System (Bio-Rad Laboratories, Gladesville, NSW, Australia). Identification of individual caseins and para-κ-casein was based on literature (Paludetti et al., 2020).

3. Results

3.1. Composition of milk samples

On average, the total solids (TS), T-protein, total casein (T-casein) and total Ca (T-Ca) content in the control reconstituted skim milk (pH 6.7) were 91.9, 32, 28.6 and 1.04 mg/g of sample, respectively. T-casein was 84.8 % of the T-protein and consisted of 39.9 % α_{s1}-casein, 8.3 % α_{s2}-casein, 38.4 % β-casein and 13.4 % κ-casein. These proportions of individual caseins are close to theoretical values of 4:1:3.5:1.5, respectively (Fox, 2003; Huppertz, 2013; Huppertz et al., 2018). As the HCl used for pH adjustments had a high concentration, few drops were enough to reach the target pH, and thus, the composition of milk was not affected. On the other hand, the concentration of TS and ash increased while other constituents decreased with increasing amount of NaCl added, but the ratios of T-casein to T-protein or individual caseins to T-casein were not changed.

3.2. Changes in soluble fractions with pH and pre-salting levels

Changes in soluble fractions of major constituents in treated and untreated skim milk samples are shown in Fig. 1 and Fig. 2. The control and pre-salted samples showed higher levels of soluble fractions (% of individual total amount) of κ- and α_{s2}- caseins than α_{s1}- and β-caseins

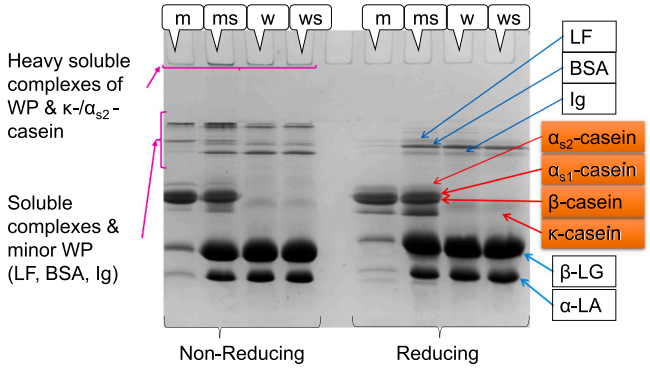

Fig. 1. SDS-PAGE image of control reconstituted skim milk (m), control milk supernatant (ms), control rennet whey (w) and control rennet whey supernatant (ws) under non-reducing and reducing conditions. LF: lactoferrin, BSA: Bovine serum albumin, Ig: immunoglobulin, α_{s1}, α_{s2}-, β- or κ-casein: Individual caseins, β-LG: β-Lactoglobulin, α-LA: α-Lactalbumin, WP: whey proteins.

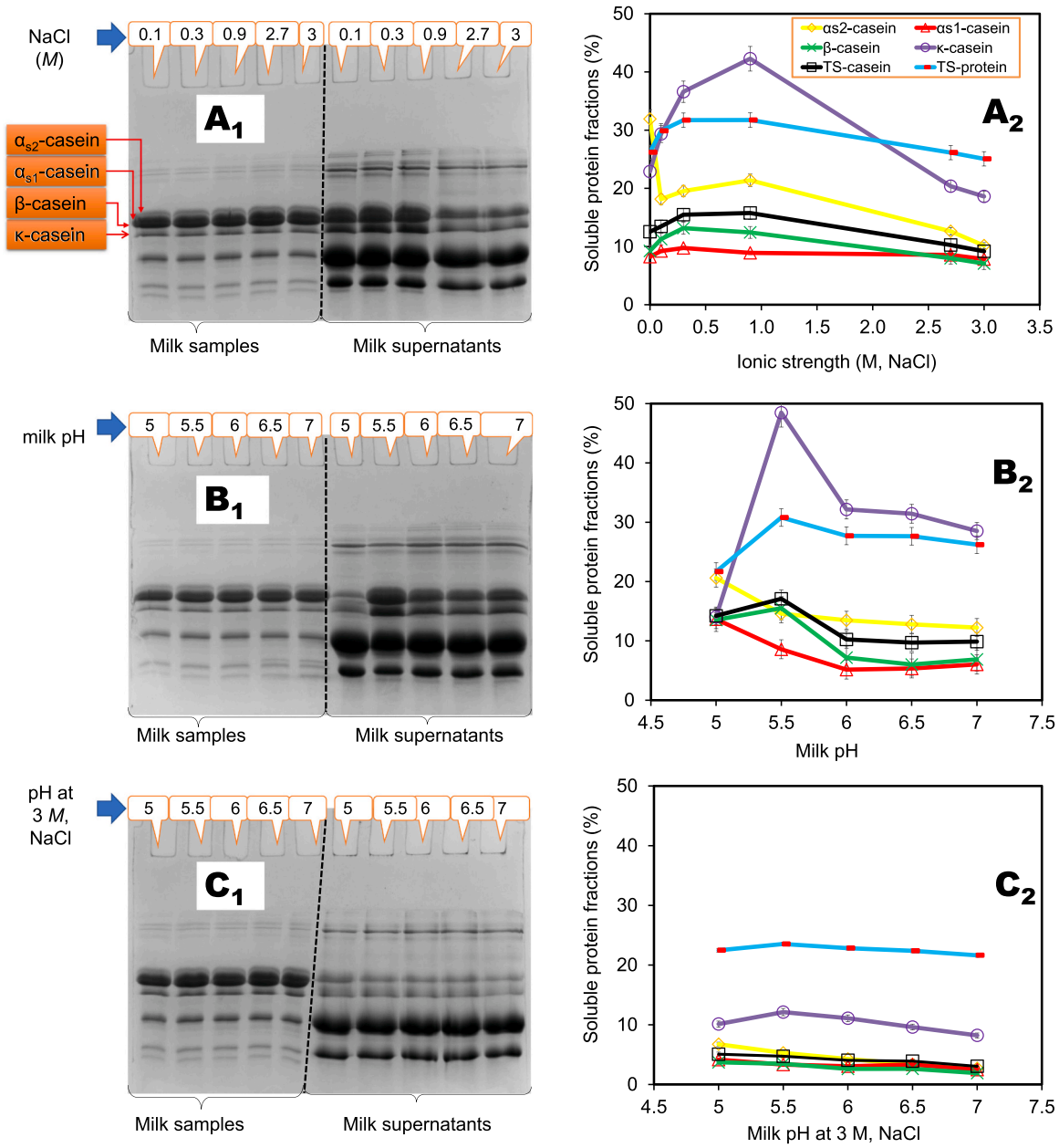

Fig. 2. Reducing SDS-PAGE images (A_1, B_1, C_1) and RP-HPLC results (A_2, B_2, C_2) showing changes in soluble fractions of individual caseins (α_{s1}, α_{s2}-, β- & κ-caseins), total casein (T-casein) & total protein (TS-protein) in reconstituted skim milk samples of varying ionic strength (A_1, A_2), pH (B_1, B_2) & pH at an ionic strength of 3 *M*, NaCl (C_1, C_2).

(Fig. 1 & Fig. 2A). Fig. 1 (reducing SDS-PAGE) indicates the presence of soluble aggregates which are likely heat-induced complexes with denatured whey proteins (Kethireddipalli et al., 2011). Fig. 2A_1 & C_1 indicated that pre-salting increased soluble aggregates including heavy ones (stuck into the wells), revealing the dissociating/solubilising effect of NaCl on casein micelles and further complexation of dissociated caseins with denatured whey proteins. It is also clear that the bands (including β-LG & α-LA) diminished at higher NaCl levels (>0.9 *M*) probably due to formation of even heavier insoluble aggregates. Solubilisation of κ-casein increased with ionic strength reaching a maximum at 0.9 *M*, NaCl, then decreased on further increasing the ionic strength (Fig. 2A_2). Changes in total soluble proteins (TS-protein) and total soluble casein (TS-casein) were similar to those of individual caseins with a plateau between 0.3 and 0.9 *M*.

The maximum TS-protein and TS-casein for pH-adjusted samples were found at pH 5.5 (Fig. 2B) which is the same as reported by Gülseren et al. (2010) and very close to pH 5.6 reported by Van Hooydonk et al. (1986a). α_s-caseins (α_{s1}-casein & α_{s2}-casein) showed higher dissociation at pH 5 whereas κ- and β- caseins exhibited maximum dissociation at pH 5.5 and decreased at pH 5 indicating that partial aggregation involving these caseins occurred at pH 5. The same observation was made for TS-casein and TS-protein. The trend observed is in agreement with previous studies (Anema, 1998; Anema & Klostermeyer, 1997; Anema & Li, 2015; Dalgleish & Law, 1988; Schiffer et al., 2021). Adjusting pH for highly pre-salted milk caused similar changes to those of unsalted milk although values were comparatively lower, but as aforementioned, heavy complexes were observed (Fig. 2C_1). As shown in Fig. 3 (A_1, B_1, C_1), total soluble Ca (TS-Ca, % of T-Ca) increased with ionic strength and reached a maximum at 2.7 *M*. Similarly, TS-Ca increased linearly with decreasing pH for both unsalted and highly pre-salted samples. This was expected because of CCP dissociation which is in line with other studies (Law & Leaver, 1998; Schiffer et al., 2021). Similar changes were also

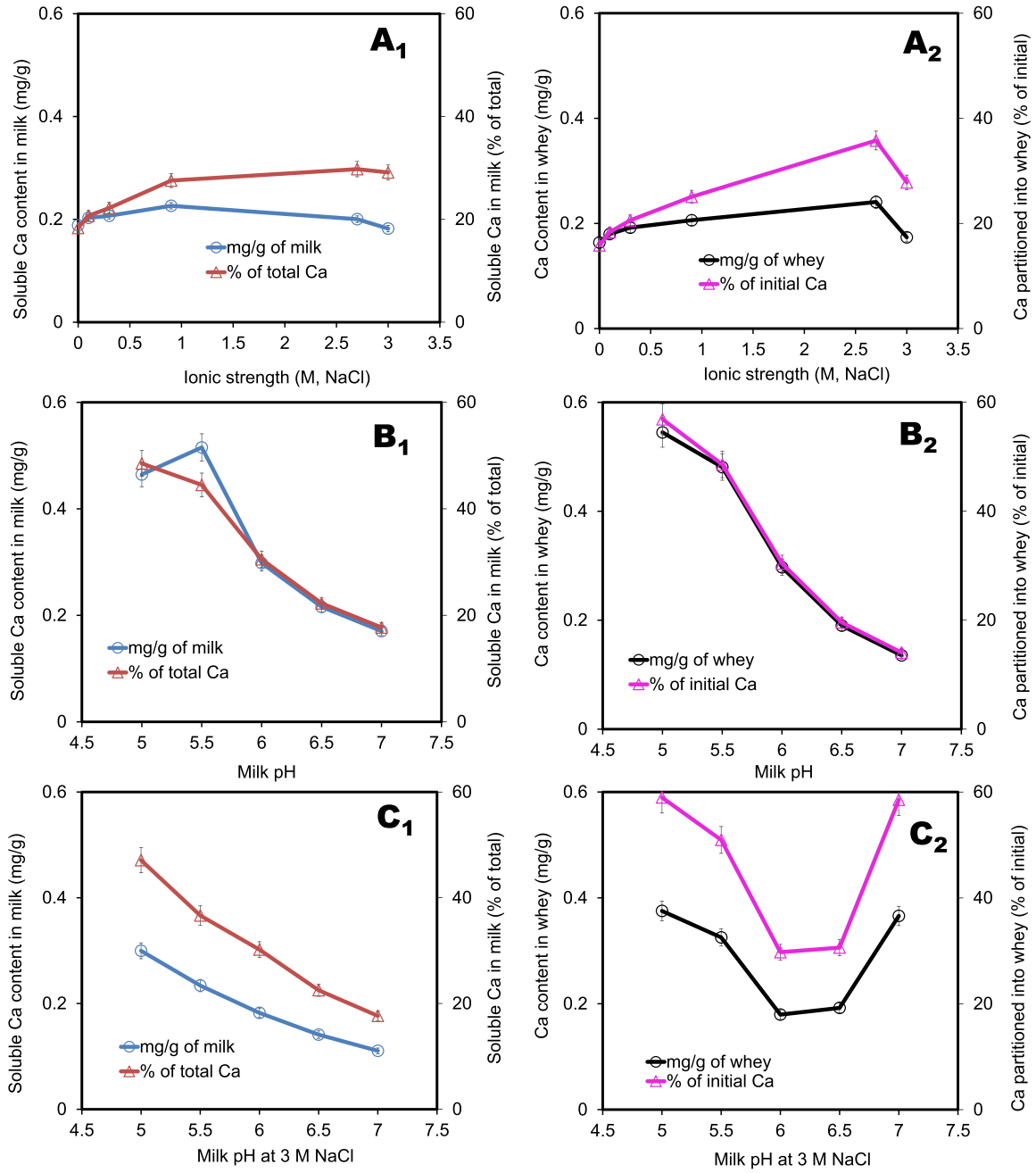

Fig. 3. Changes in soluble calcium (Ca) content in reconstituted skim milk samples [expressed as mg/g of milk & % of total Ca (A_1, B_1, C_1)] and changes in Ca content as well as Ca partitioning (A_2, B_2, C_2) into rennet whey produced from reconstituted skim milk samples of varying ionic strength (A_1, A_2), pH (B_1, B_2) & pH at an ionic strength of 3 *M*, NaCl (C_1, C_2).

observed in terms of TS-Ca concentration (mg/g of milk).

3.3. Partitioning of individual & total caseins, total protein and Ca

As indicated in Fig. 4A & B, α_{s1}- and β-caseins were 100 % retained into rennet curds from all unsalted milk samples (pH 5 – 7) and pre-salted milk up to an ionic strength of 0.9 *M*, NaCl. Based on both SDS-PAGE (Fig. 4A_1) and RP-HPLC (Fig. 4A_2) results, it is also shown that this level of pre-salting had no clear effect on partitioning of κ- and α_{s2}-caseins or T-casein into rennet whey. For κ-casein, no change was observed up to 2.7 *M*, NaCl due to rennet action, whereas significant amounts of other caseins (26.4 % of α_{s1}-casein, 21.5 % α_{s2}-casein, 26.2 β-casein & 30.1 % T-casein) were lost at 2.7 *M*, NaCl. Loss of all caseins decreased when ionic strength was further increased to 3 *M*. The same decrease in whey protein bands (both minor and major) and presence of soluble and insoluble aggregates as observed in milk samples pre-salted to ionic strength > 0.9 *M* is also clear in whey samples (Fig. 4A_1). Another important observation is that higher levels of para-κ-casein were detected in rennet whey especially at 2.7 *M*, NaCl. Changes in total protein partitioning followed the same trend as T-casein. Fig. 4B shows that pre-acidification of reconstituted skim milk below pH 6.5 increased the loss of κ- and α_{s2}- caseins as well as T-casein and total protein into rennet whey with a maximum at pH 5.5. These results are similar to previous report by Liu et al. (2014) who also found more casein losses at pH 5.2, 5.5 & 6.2 compared to pH 4.8. The amounts of T-protein retained are consistent with previously reported in Cheddar cheese (Ong et al., 2012).

Different pH levels of a highly pre-salted (3 *M*, NaCl) milk sample

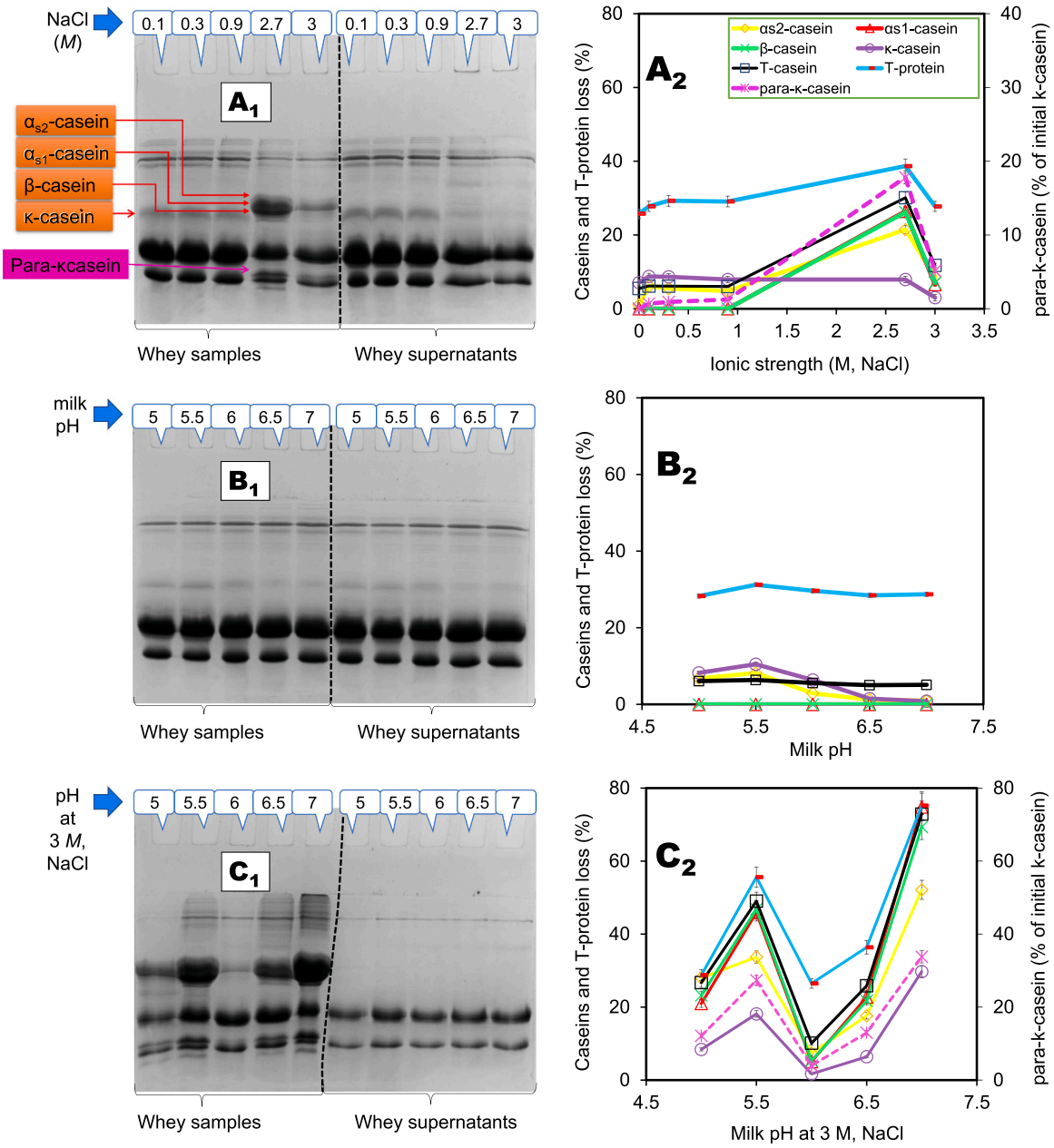

Fig. 4. Reducing SDS-PAGE images (A_1, B_1, C_1) and RP-HPLC results (A_2, B_2, C_2) showing changes in individual caseins (α_{s1}, α_{s2}-, β- & κ-caseins), total casein (T-casein) & total protein (T-protein) partitioned as well as para-κ-casein lost into rennet whey produced from reconstituted skim milk samples of varying ionic strength (A_1, A_2), pH (B_1, B_2) & pH at an ionic strength of 3 *M*, NaCl (C_1, C_2). T-casein includes also both para-κ-casein & the caseino-macropeptide (CMP).

showed tremendous changes in partitioning of individual caseins (Fig. 4C_1 & C_2). All caseins and para-κ-casein showed a similar trend with a minimum loss (10.1 % of T-casein) observed at pH 6 implying a maximum retention into rennet curds. The greatest loss of all caseins (72.9 % of T-casein) into rennet whey occurred when pH was adjusted to 7 followed by pH 5.5 (49 % of T-casein). SDS-PAGE (Fig. 4C_1) revealed diminished bands for all whey proteins especially lactoferrin (LF) and immunoglobulin (Ig). All caseins and lactoferrin together with the aggregates observed in whole whey samples disappeared completely from the supernatant. This clearly tells as mentioned earlier that higher NaCl levels and especially when HCl or NaOH were added may have induced other complex reactions between dissociated caseins and denature whey proteins which formed visible clots that were insoluble in SDS-sample buffer (Supplementary material, Fig. S1). These did not even stick into the wells on top of the stacking gel and were washed away. It is not clear whether para-κ-casein was also involved but the extent to which major whey protein bands diminished and some minor proteins disappeared (Fig. 4A_1 & C_1) compared to milk supernatants (Fig. 2A_1 & C_1) indicate the likelihood.

As indicated in Fig. 3 (A_2, B_2, C_2) the greatest loss of T-Ca occurred at pH 5 regardless of whether samples are salted or not. The highest T-Ca losses were 35.8 % of initial amount in milk at 2.7 *M*, NaCl without pH adjustment, 56.9 % at pH 5 for unsalted samples and 59 % at pH 5 of the highly pre-salted sample. The latter showed maximum Ca retention into rennet curds at pH values 6 (29.7 %) and 6.5 (30.6 %) which correlates with retention of caseins (Fig. 4C).

4. Discussion

To ensure safety and product consistency, cheese milk may undergo a series of pre-treatments prior to coagulation with rennet. These may include thermisation (e.g., 63 °C for 15 s), chilling (4–7 °C),

standardisation (e.g., defined casein to fat ratio), heating (e.g., full pasteurisation at 72 °C for 15 s), addition of $CaCl_2$ and pre-acidification (Kelly et al., 2008; McSweeney, 2007). In Domiati-style cheeses, NaCl is also added directly to cheese milk before renneting. The impact of pH and the level of NaCl added to reconstituted skim milk before renneting on distribution of individual caseins between rennet curd and whey is established in the current research.

Solubilisation of some caseins at low pH occurs as a result of CCP dissociation from micelles (Griffin et al., 1988; Sinaga et al., 2017). Dissociation of CCP is understood as the loss of micellar crosslinks and the rise in electrostatic repulsion between exposed phosphoserine moieties (Choi et al., 2007; Lucey, 2002). Thus, an observed increase in the soluble caseins with acidification up to pH 5.5 (Fig. 2B) was caused by dissociation of some individual caseins as a result of loosened internal micellar structure (Van Hooydonk et al., 1986b). On the contrary, reduced levels of κ- and β- caseins in the serum phase at pH 5 mark the beginning of acid-induced aggregation when pH falls below 5.5. Partial re-association of soluble caseins and denatured whey proteins with casein micelles and rapid aggregation were reported to take place around pH 5.4 and pH < 5.4, respectively (Anema & Li, 2015). Renneting at pH values around this range accelerate the aggregation of caseins, and indeed van Hooydonk et al. (1986b) noticed the beginning of aggregation at < 30 % κ-casein hydrolysis unlike 60 – 70 % at pH 6.7. However, curds renneted at low pH were found to be more porous and syneresis increased with the degree of acidification in the pH range 6.2 – 5 (Liu et al., 2014) which explains higher losses of intact κ-casein and α_{s2}-casein at pH values < 6.5 (Fig. 4B_2). As renneting pH approaches the isoelectric point, the curd behaves more like an acid-induced gel with reduced syneresis probably due to higher hydrogen bonds (Lefebvre--cases et al., 1998). Thence, the present study also found more soluble κ- and α_{s2}- caseins retained at pH 5 compared to pH 5.5 and 6 as also shown by Liu et al. (2014) for pH 4.8. These findings could also be related to curd strength since rennet gels made at pH ≤ 5.8 are generally weaker than those made at pH > 6 (Awad, 2007; Choi et al., 2007; Liu et al., 2014) which is due to limited Ca crosslinking as shown by low Ca retention (Fig. 3B_2). Indeed, the minimum gel strength was reported to be at ~pH 5–5.3 (Awad, 2007; van Hooydonk et al., 1986b) while a maximum was found at pH values 6.2–6.4 (Awad, 2007; Choi et al., 2007). For this reason, in addition to the impact on rennet activity, cheese milk is mildly pre-acidified unless it is intended to make fresh soft cheeses. Therefore, selection of renneting pH would depend on the specific desired outcome which in the present study was the maximum retention of T-casein into the curd matrix and appeared at pH 6.5.

Pre-salting of reconstituted skim milk induced an impact on micellar stability similar to that of acidification, and it was suggested to be mainly via alterations in micellar surface charge (Huppertz & Fox, 2006). Some of the caseins solubilised (Fig. 2A) were as a response to slight reduction in pH and others were due to replacement of divalent micellar Ca ions by monovalent Na ions which was said to weaken the internal micellar structure (Awad, 2007; Zhao & Corredig, 2015). Similar to what happened in unsalted milk adjusted to pH 5 (Fig. 2B), partial re-association might have occurred in samples with NaCl levels higher than 0.9 *M* (Fig. 2A) where pH fell to 6.3 for 2.7 *M* or 6.09 for 3 *M* as opposed to resolubilisation of caseins found at higher acidification levels i.e., pH < 4 (Strange et al., 1994). Whereas complete Ca dissociation due to acidification was reported to take place around the isoelectric point (Le Graët & Gaucheron, 1999), acid-induced aggregation occurs at this point because interactions other than Ca bonding are involved (Lefebvre-cases et al., 1998; Liu et al., 2014). In contrast, maximum dissociation (Fig. 3A_1) and lowest retention (Fig. 3A_2) of Ca found in the current research at 2.7 *M*, NaCl, demonstrate that poor coagulation and more importantly, higher losses of all caseins and higher levels of para-κ-casein lost in rennet whey from this sample (Fig. 4A) are due to inhibited para-casein aggregation. This might have been via limited Ca binding and hydrophobic interactions as these were found to be related (Grewal et al., 2021). This appears also in agreement with Dalgleish (1983). In addition, higher concentration of monovalent cations delays the secondary (aggregation) phase (Okigbo et al., 1985; Ramet et al., 1983) probably via electrosteric stabilisation (Dalgleish, 1983; Payens & Both, 1980; Zhao & Corredig, 2015). As mentioned above, electrostatic repulsion between exposed phosphoserine residues due to dissociation of CCP is expected (Choi et al., 2007). Therefore, electrostatic interactions may also have a potential role. On the other hand, diminished bands (Fig. 2A_1 & Fig. 4A_1) which likely indicate the formation of heavy insoluble complexes (Supplementary material, Fig. S1) might be an explanation for improved retention of caseins into rennet curds from milk samples with 3 *M* compared to 2.7 *M*, NaCl.

When pre-salting and pre-acidification were applied together on a concentrated casein solution, the impact of NaCl on CCP solubilisation was masked by that of pH (Le Graët & Gaucheron, 1999). This agrees with the present study as lowering the pH of milk at 3 *M*, NaCl caused similar trends in dissociation of Ca and caseins to those of unsalted samples (Fig. 2B_2 & C_2 and Fig. 3B_2 & C_2). However, the presence of various levels of all types of caseins and para-κ-casein in rennet whey from salted milk with 3 *M*, NaCl at all pH levels in contrast to their unsalted counterparts indicates a pH-dependent inhibitory effect of NaCl on the secondary phase of renneting i.e., para-casein aggregation. Retarded hydrolysis of κ-casein by chymosin due to increased NaCl levels was also reported previously (Famelart et al., 1999). This is evidenced in the current study by diminished and/or disappeared bands (Fig. 2A_1 & Fig. 4C_1) as indicators of further κ-/α_{s2}-caseins-whey protein complexation which are known to interfere with the rennet action on entrapped κ-casein (Calvo et al., 1995; Kannan & Jenness, 1961; Leaver et al., 1995). Based on levels of κ-casein loss shown in Fig. 4A_1 compared to those in Fig. 4C_1, it appears that the inhibitory effect of NaCl on the primary enzymatic phase of renneting (κ-casein hydrolysis) is also pH-dependent, and pH 6 is the optimum for the highly pre-salted milk.

5. Conclusions

The impact of pre-acidification and pre-salting of reconstituted skim milk on partitioning of individual caseins between rennet curd and whey is established. This study demonstrates that the proportion of individual caseins lost into rennet whey is apparently increased by increasing ionic strength above 0.9 *M*, NaCl as well as lowering the renneting pH below 6.5. For highly pre-salted milk (3 *M or* 15 % NaCl), pH 6 appears to be the optimum. Levels of κ-casein and α_{s2}-casein found in rennet whey from unsalted milk are attributed to their heat-induced complexes with denatured whey proteins whereas pre-salting results in partial inhibition of both enzymatic and aggregation phases of renneting and both effects are pH-dependent. The inhibitory effect on aggregation phase also depends on ionic strength probably via electrosteric stabilisation as well as limited Ca crosslinking and hydrophobic interactions. Future studies using whole milk to further understand the impact on fat partitioning and the mechanisms involved would also be interesting.

Declaration of Competing Interest

The author declares no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.foohum.2023.08.014.

References

Anema, S. G. (1998). Effect of milk concentration on heat-induced, pH-dependent dissociation of casein from micelles in reconstituted skim milk at temperatures between 20 and 120°C. *Journal of Agricultural and Food Chemistry, 46*, *2299–2305*.

Anema, S. G., & Klostermeyer, H. (1997). Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100°C. *Journal of Agricultural and Food Chemistry, 45*, *1108–1115*.

Anema, S. G., & Li, Y. (2015). Reassociation of dissociated caseins upon acidification of heated pH-adjusted skim milk. *Food Chemistry, 174*, *339–347*.

Aprianita, A., Donkor, O. N., Moate, P. J., Williams, S. R. O., Auldist, M. J., Greenwood, S., Hannah, M. C., Wales, W. J., & Vasiljevic, T. (2014). Effects of dietary cottonseed oil and tannin supplements on protein and fatty acid composition of bovine milk. *Journal of Dairy Research, 81*, *183–192*.

Awad, S. (2007). Effect of sodium chloride and pH on the rennet coagulation and gel firmness. *LWT-Food Science and Technology, 40*, *220–224*.

Bogahawaththa, D., Chandrapala, J., & Vasiljevic, T. (2017). Thermal denaturation of bovine immunoglobulin G and its association with other whey proteins. *Food Hydrocolloids, 72*, 350–357.

Bogahawaththa, D., Chandrapala, J., & Vasiljevic, T. (2019). Thermal denaturation of bovine β-lactoglobulin in different protein mixtures in relation to antigenicity. *International Dairy Journal, 91*, *89–97*.

Calvo, M. M., Law, A. J. R., & Leaver, J. (1995). Heat-induced interactions between serum-albumin, immunoglobulin, and kappa-casein inhibit the primary phase of renneting. *Journal of Agricultural and Food Chemistry, 43*, *2823–2827*.

Choi, J., Horne, D. S., & Lucey, J. A. (2007). Effect of insoluble calcium concentration on rennet coagulation properties of milk. *Journal of Dairy Science, 90*, *2612–2623*.

Choi, J., Horne, D. S., & Lucey, J. A. (2015). Effect of insoluble calcium concentration on endogenous syneresis rate in rennet-coagulated bovine milk. *Journal of Dairy Science, 98*, *5955–5966*.

Dalgleish, D. G. (1983). Coagulation of renneted bovine casein micelles: Dependence on temperature, calcium ion concentration and ionic strength. *Journal of Dairy Research, 50*, *331–340*.

Dalgleish, D. G., & Law, A. J. R. (1988). pH-Induced dissociation of bovine casein micelles. I. Analysis of liberated caseins. *Journal of Dairy Research, 55*, 529–538.

Daniloski, D., McCarthy, N. A., Markoska, T., Auldist, M. J., & Vasiljevic, T. (2022). Conformational and physicochemical characteristics of bovine skim milk obtained from cows with different genetic variants of β-casein. *Food Hydrocolloids, 124*, Article 107186.

Daviau, C., Famelart, M., Pierre, A., Goudédranche, H., & Maubois, J. (2000). Rennet coagulation of skim milk and curd drainage: Effect of pH, casein concentration, ionic strength and heat treatment. *Lait, 80*, *397–415*.

De Kruif, C. G. (1999). Casein micelle interactions. *International Dairy Journal, 9*, *183–188*.

Dissanayake, M., & Vasiljevic, T. (2009). Functional properties of whey proteins affected by heat treatment and hydrodynamic high-pressure shearing. *Journal of Dairy Science, 92*, *1387–1397*.

Famelart, M. H., Le Graet, Y., & Raulot, K. (1999). Casein micelle dispersions into water, NaCl and $CaCl_2$: Physicochemical characteristics of micelles and rennet coagulation. *International Dairy Journal, 9*, *293–297*.

Farkye, N. Y. (2017). Acid-heat coagulated cheeses (General aspects). In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.) (4th ed.,,*Cheese: Chemistry, physics and microbiology* (4th ed.,, Vol. 1, pp. 1103–1115). London: Chapman & Hall.

Forsbäck, L., Lindmark-Månsson, H., Svennersten-Sjaunja, K., Bach Larsen, L., & Andrén, A. (2011). Effect of storage and separation of milk at udder quarter level on milk composition, proteolysis, and coagulation properties in relation to somatic cell count. *Journal of Dairy Science, 94*, *5341–5349*.

Fox, P. F. (2003). The major constituents of milk. In G. Smit (Ed.), *Dairy Processing: Improving quality* (pp. 5–41). Cambridge, England: Woodhead publishing.

Gaygadzhiev, Z., Massel, V., Alexander, M., & Corredig, M. (2012). Addition of sodium caseinate to skim milk inhibits rennet-induced aggregation of casein micelles. *Food Hydrocolloids, 26*, *405–411*.

Grewal, M. K., Vasiljevic, T., & Huppertz, T. (2021). Influence of calcium and magnesium on the secondary structure in solutions of individual caseins and binary casein mixtures. *International Dairy Journal, 112*, *1–9*.

Griffin, M. C. A., Lyster, R. L. J., & Price, J. C. (1988). The disaggregation of calcium-depleted casein micelles. *European Journal of Biochemistry, 174*, *339–343*.

Guinee, T. P., & Fox, P. F. (2017). Salt in cheese: physical, chemical and biological aspects (General aspects). In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.) (4th ed.,,*Cheese: chemistry, physics and microbiology* (4th ed.,, Vol. 1, pp. 317–375). London: Chapman & Hall.

Gülseren, I., Alexander, M., & Corredig, M. (2010). Probing the colloidal properties of skim milk using acoustic and electroacoustic spectroscopy. Effect of concentration, heating and acidification. *Journal of Colloid and Interface Science, 351*, *493–500*.

Horne, D.S., & Lucey, J.A. (2017). Rennet-induced coagulation of milk. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter & D. W. Everett (Eds.), Cheese: Chemistry, physics and microbiology, Vol. 1. General aspects (4th ed.) (pp. 821–842). London: Chapman & Hall.

Huppertz, T.. (2013). Chemistry of the caseins. In McSweeney, P.L.H., & Fox, P.F. (Eds.), Advanced dairy chemistry: Vol. 1A: Proteins: Basic aspects, (4th ed.) (pp. 135–160). Boston, MA: Springer.

Huppertz, T., & Fox, P. F. (2006). Effect of NaCl on some physico-chemical properties of concentrated bovine milk. *International Dairy Journal, 16*, *1142–1148*.

Huppertz, T., & de Kruif, C. G. (2007). Rennet-induced coagulation of enzymatically cross-linked casein micelles. *International Dairy Journal, 17*, *442–447*.

Huppertz, T., Fox, P., & Kelly, A. (2018). The caseins: Structure, stability, and functionality. In R. Y. Yada (Ed.), *Proteins in Food processing (2nd ed.*.,, pp. 49–92). United Kingdom: Elsevier.

Kannan, A., & Jenness, R. (1961). Relation of milk serum proteins and milk salts to the effects of heat treatment on rennet clotting. *Journal of Dairy Science, 44*, *808–822*.

Kelly, A. L., Huppertz, T., & Sheehan, J. J. (2008). Pre-treatment of cheese milk: Principles and developments. *Dairy Science and Technology, 88*, *549–572*.

Kethireddipalli, P., Hill, A. R., & Dalgleish, D. G. (2011). Interaction between casein micelles and whey protein/k-casein complexes during renneting of heat-treated reconstituted skim milk powder and casein micelle/serum mixtures. *Journal of Agricultural and Food Chemistry, 59*, *1442–1448*.

Lauzin, A., Pouliot, Y., & Britten, M. (2020). Understanding the differences in cheese-making properties between reverse osmosis and ultrafiltration concentrates. *Journal of Dairy Science, 103*, *201–209*.

Law, A. J. R., & Leaver, J. (1998). Effects of acidification and storage of milk on dissociation of bovine casein micelles. *Journal of Agricultural and Food Chemistry, 46*, 5008–5016.

Leaver, J., Law, A. J. R., Horne, D. S., & Banks, J. M. (1995). Influence of heating regime and pH on the primary phase of renneting of whole milk. *International Dairy Journal, 5*, *129–140*.

Lefebvre-cases, E., Gastaldi, E., Vidal, V., Marchesseau, S., Lagaude, A., Cuq, J.-L., & De la Fuente, B. T. (1998). Identification of interactions among casein gels using dissociating chemical agents. *Journal of Dairy Science, 81*, *932–938*.

Le Graët, Y., & Gaucheron, F. (1999). pH-induced solubilisation of minerals from casein micelles: Influence of casein concentration and ionic strength. *Journal of Dairy Research, 66*, 215–224.

Liu, X. T., Zhang, H., Wang, F., Luo, J., Guo, H. Y., & Ren, F. Z. (2014). Rheological and structural properties of differently acidified and renneted milk gels. *Journal of Dairy Science, 97*, *3292–3299*.

Lucey, J. A. (2002). Formation and physical properties of milk protein gels. *Journal of Dairy Science, 85*, *281–294*.

Lucey, J. A. (2017). Formation, structural properties, and rheology of acid-coagulated milk gels (General aspects). In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.) (4th ed.,,*Cheese: chemistry, physics and microbiology* (4th ed.,, Vol. 1, pp. 363–419). London: Chapman & Hall.

McSweeney, P. L. H. (2007). Acidification. In P. L. H. McSweeney (Ed.), *Cheese problems solved* (pp. 34–49). Cambridge, England: Woodhead Publishing.

Okigbo, L. M., Richardson, G. H., Brown, R. J., & Ernstrom, C. A. (1985). Casein composition of cow's milk of different chymosin coagulation properties. *Journal of Dairy Science, 68*. *4887–1892*.

Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. (2012). The effect of pH at renneting on the microstructure, composition and texture of Cheddar cheese. *Food Research International, 48*, *119–130*.

Paludetti, L. F., Kelly, A. L., & Gleeson, D. (2020). Effect of thermoresistant protease of Pseudomonas fluorescens on rennet coagulation properties and proteolysis of milk. *Journal of Dairy Science, 103*, *4043–4055*.

Payens, T.A. J., & Both, P.. (1980). Enzymatic clotting process. IV. The chymosin-triggered clotting of para-κ-casein. In Blank, M. (Ed.), Bioelectrochemistry: Ions, Surfaces, Membranes (Advances in Chemistry series) (pp. 129–141). Washington, DC: American Chemical Society.

Ramet, J. P., El-Mayda, E., & Weber, F. (1983). Influence of salting of reconstituted milk on curdling by rennet. *Journal of Texture Studies, 14*, *11–19*.

Sandra, S., & Corredig, M. (2013). Rennet induced gelation of reconstituted milk protein concentrates: The role of calcium and soluble proteins during reconstitution. *International Dairy Journal, 29*, *68–74*.

Sandra, S., Ho, M., Alexander, M., & Corredig, M. (2012). Effect of soluble calcium on the renneting properties of casein micelles as measured by rheology and diffusing wave spectroscopy. *Journal of Dairy Science, 95*, *75–82*.

Schiffer, S., Scheidler, E., Kiefer, T., & Kulozik, U. (2021). Effect of temperature, added calcium and pH on the equilibrium of caseins between micellar state and milk serum. *Foods, 10*, *822*.

Sinaga, H., Bansal, N., & Bhandari, B. (2017). Effects of milk pH alteration on casein micelle size and gelation properties of milk. *International Journal of Food Properties, 20*, *179–197*.

Strange, E. D., van Hekken, D. L., & Holsinger, V. H. (1994). Effect of sodium chloride on the solubility of caseins. *Journal of Dairy Science, 77*, *1216–1222*.

Van Hooydonk, A. C. M., Hagedoorn, H. G., & Boerrigter, I. J. (1986b). pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 2. Effect of pH on renneting of milk. *Netherlands Milking and Dairy Journal, 40*, *297–313*.

Van Hooydonk, A. C. M., Hagedoorn, H. G., & Boerrigter, I. J. (1986a). pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 1. Effect of acidification on physico-chemical properties. *Netherlands Milking and Dairy Journal, 40*, *281–296*.

Zhao, Z., & Corredig, M. (2015). Changes in the physico-chemical properties of casein micelles in the presence of sodium chloride in untreated and concentrated milk protein. *Dairy Science and Technology, 95*, *87–99*.

Zhao, Z., & Corredig, M. (2016). Influence of sodium chloride on the colloidal and rennet coagulation properties of concentrated casein micelle suspensions. *Journal of Dairy Science, 99*, *6036–6045*.

Chapter 8– General conclusions and future directions

8.1.Introduction

During cheese-making, cheese-milk may undergo a series of pre-treatments prior to coagulation with rennet. Cheese-milk pre-treatments are essential for quality control of both cheese and whey coproducts as they help to (1) improve the coagulation properties of milk, (2) ensure safety by controlling the microbial load in raw milk, (3) preserve the intrinsic quality of the specific cheese variety by mitigating seasonal variations (ensure product consistency), and (4) improve whey composition and (5) the overall cheese-making efficiency by maximising cheese yield. The last three goals (3 – 5) are achieved through modulating the partitioning of milk constituents between the curd and whey. Partitioning in the context of cheese-making refers to how milk constituents and RA are distributed between the curd (or the final cheese) and whey, that is the proportion retained into the curd/final cheese or lost in whey as a percentage of the initial amount in cheese-milk. Understanding the impact of different cheese-milk pre-treatment conditions on partitioning behaviour of proteins in particular and RA is, thus, important in order to optimise the cheese-making process.

8.2. The gap identified and research questions

Despite CN being the major protein in bovine milk and the most important milk protein for cheese-making, there is still a lack of research on how the partitioning of individual CNs during cheese-making change with variations in the physico-chemical properties of the milk systems due to natural factors or induced by processing. In addition,

the question why individual CNs (or other milk constituents) and/or RA partitioning during cheese-making are affected by variations in the physico-chemical properties of milk also needs to be answered.

8.3. Overall aim and scope

This work focuses on providing more insights particularly into variations in partitioning of individual CNs and calf RA during renneting of reconstituted skim milk as influenced by some common cheese-milk pre-treatments, that is adjusting/modifying milk composition (standardisation), and ionic strength and pH (pre-salting and pre-acidification). Changes in partitioning of Ca, another key influential constituent on the cheese-making properties of milk, was also assessed in order to better understand the observed partitioning behaviours of CNs. The partitioning of fat which plays a passive role during renneting but contributes more on cheese yield was discussed in the literature review only, and the same also goes for the extent of κ-CN hydrolysis by rennet that is required to induce milk coagulation (Chapter 2). An attempt was also made to partially answer the second question (why?) for the impact of skim milk concentration.

8.4. Major findings and conclusions for specific aims

Regarding RA partitioning, the first aim was to address the issue of subjectivity and time consumption of the existing milk clotting assay used for the determination of the residual RA in cheese and whey products. The second aim was to answer the question how does increasing the reconstituted skim milk concentration (3 – 9.4% protein) affects the concentration of residual calf RA in whey as well as its partitioning between the curd and whey? As indicated in Chapter 3, the first objective has been achieved by improving a former

spiking technique. The improvement included (a) determining an objective RCT (defined as the renneting time at G' = 1 Pa) by applying small amplitude oscillatory rheological measurement (instead of a visual clotting time), and (b) estimating an unknown RA in a spiked whey sample directly from a linear calibration curve of RCT against the inverse of standard RA in a serum phase of the same RSM used for whey preparation (instead of estimation based on pre-incubation time–visual clotting time relationship). The spike level can be any known RA (control) along the linear range of the calibration curve. Another advantage is that calibration curves can be created using any G' value along the enzymatic phase or tan δ (G"/G') = 1 since all led to same results. Surprisingly, the concentration of the residual RA (IMCU/mL) in rennet whey was the same as the initial concentration of RA (IMCU/mL) added to milk. This relationship helped to develop a simple model to estimate the RA retained into the curd or lost in whey, that is ((RA/mL of milk × total volume of whey×100)/total RA added to milk) or (100 – total RA in whey), respectively.

The consistency of the findings between this new approach and the common RP-HPLC method confirms that the approach is accurate, and also the model can be applied effectively to estimate RA partitioning during cheese-making if enzymes are not denatured by the processing conditions. Moreover, RA partitioning was found to correlate linearly with moisture and protein partitioning, as well as the total volume of whey removed, whereas the concentration of residual RA in whey and the initial RA added to milk (IMCU/mL) were the same regardless of reconstituted skim milk concentration and acidification (Chapter 4). On this basis, and since the findings are consistent with the previous study (Chapter 3), it can be concluded that RA partitioning is not driven by enzyme-CN associations. Instead, it is

proposed that CN-CN interactions could be the driving force, and the same hypothesis might be true for the partitioning of CNs.

On the partitioning of individual CNs, the specific objectives were to examine the impact of reconstituted skim milk total solids concentration (8 – 25%), and pre-salting (0 – 3 M, NaCl) and pre-acidification (pH 5 – 7) of reconstituted skim milk (~3.2% protein) on the percentage distribution of individual CNs between rennet curd and whey. The effect of variation in milk concentration was examined in Chapter 5 under both renneting only and renneting combined with slow acidification achieved using GDL, whereas the impacts of pre-salting and pre-acidification are discussed in Chapter 7. Two CNs only (α_{s2}-CN, κ-CN) were found in rennet whey at all milk concentration, acidification and pre-salting levels ≤ 0.9 M, NaCl, whereas higher pre-salting levels especially at pH 5.5 and 7 led to the loss of all CNs as well as para-κ-CN into rennet whey. Increasing reconstituted skim milk TS concentration significantly increases ($p < 0.05$) the partitioning of α_{s2}-CN and κ-CN into rennet curds. The same is also true for total CN, total protein and total Ca, and no effect of acidification was found except for total Ca. Chapter 6 indicates that there is an increase in hydrophobic interactions, restricted structural re-arrangements and limited syneresis in addition to more Ca binding with the rise in milk concentration, which explain the partitioning behaviour of α_{s2}-CN and κ-CN. As indicated in Chapter 7, the optimum pH for highly pre-salted milk was 6.

The overall conclusion of Chapter 5 is that increasing reconstituted skim milk TS positively affects αs_2 and κ- CNs only whereas αs_1 and β- CNs can be 100% retained into rennet curds regardless of milk concentration. The same conclusion also applies to the observed changes in the partitioning of individual CNs due to variation in pre-acidification

levels, but unlike increasing milk concentration, increasing acidification level (decreasing pH) below pH 6.5 has a negative impact on retentions of αs_2 and κ- CNs (Chapter 7). On the other hand, increasing the pre-salting levels (increasing NaCl concentration) reduces the retention of all CNs (Chapter 7).

8.5.Future directions

It is hoped that findings of the present research will be exploited by dairy industries for cheese yield and quality predictions, but as a limitation, all aspects of this topic were not covered. The gap is still wide for both questions stated above (how? and why?) as there are many other natural factors (including genetic, age, feeding and seasons) or cheese-milk pre-treatments (such as temperature (chilling, thermisation, full pasteurisation or UHT), raw versus concentrated milk systems, milk concentration methods (LCF–HCF- MF, NF, UF, RO or VC) and standardisation methods (CFR, individual CNs or lactose) that were not covered in the present research. Therefore, further studies investigating the impact of one or a combination of any of these factors on partitioning of individual CNs and RA during the manufacture of different specific cheese varieties especially at an industrial scale would be interesting. In addition, the role of specific CN interactions as the drivers of partitioning (of both milk constituents and RA) and changes in the gel structure during renneting of different milk systems under various renneting conditions is yet to be established in future studies. Studies on the effect of milk standardisation involving fat such as CN-to-fat ratio would be crucial in trying to improve both CN and fat retentions towards whey quality in full-fat cheese industries.