An investigation of casein and whey protein interactions during rennet gelation to effectively incorporate whey proteins into cheddar cheese matrices

by

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Abstract

Cheddar cheese is formed by enzyme-induced coagulation of milk proteins, known as 'rennet gelation'. During rennet gelation of milk, only the casein proteins are coagulated while the whey proteins are expulsed from the coagulum. This results in ~ 20% of the protein being lost during cheese production. Although whey proteins are nutritionally valuable and can be converted to commercial products such as powder, they are often disposed of as a waste. Incorporating them back into cheese is an attractive in-situ method of utilising the whey protein released during cheese making. It also offers the opportunity to produce cheese with elevated protein and nutritional properties.

Concentrating native milk proteins using membrane filtration and denaturing whey proteins using heat are conventionally used to increase cheese yields and reduce protein loss. However, the impact of formulating cheese milk with altered whey protein contents and functionality on the rennet gelation stage is not yet fully understood. This knowledge is vital to producing cheese curds with acceptable coagulation times and curd properties. Therefore, this thesis aimed to deepen our understanding of the influence of whey proteins on the rennet gelation process. Interactions between whey proteins (in native and denatured forms), the rennet enzyme, and coagulating para-casein micelle particles were investigated experimentally.

Previous studies report that denaturing whey proteins in the presence of caseins impairs the coagulation of para-casein micelle particles during rennet gelation. However, less is known about the impact of whey proteins in their native form, which is relevant to the use of cheese milk with a protein composition altered using membrane filtration. Therefore, the first phase of this study aimed to investigate the effect of native whey proteins on rennet gelation kinetics. Cheese milks with a wide range of whey protein:casein ratios (with standardised casein concentrations) were formulated using native protein concentrate powders produced by membrane filtration. Oscillatory rheometry and casein macropeptide release measurements during rennet gelation demonstrated that native whey protein impaired enzymatic hydrolysis and significantly delayed the subsequent aggregation of para-casein micelle particles. These observations were independent of changes in the ionic balance or the viscosity of the different milk systems. Binding between whey protein and casein micelles or whey protein and rennet was not observed by dynamic light scattering particle size measurements or native poly-acrylamide gel electrophoresis.

While there was no evidence of binding between native whey proteins (~ 5nm) and casein micelles (~ 200 nm) that could be responsible for the impaired rennet gelation, it was instead proposed that whey proteins passively occupy the gaps in the 'κ-casein hairy layer' on the casein micelle surface, which arise as a result of enzyme hydrolysis of the κ-casein. The whey proteins thereby provide a steric hindrance to rennet reaching the casein micelle surface and a barrier to intimate contact between destabilised casein micelles leading to slower gelation.

Incorporating denatured whey protein aggregates into cheese gels has been previously proposed as a means to improve the overall cheese yield. However, the potential of modifying whey protein aggregate properties to mitigate the impaired rennet gelation caused by native whey proteins, has not yet been properly studied. Therefore, in the second phase of the study, protein aggregates with a wide range of sizes were produced by heat and power ultrasound. The effects of size and hydrophobicity differences in the whey protein aggregates produced by heat and heat coupled with ultrasound were investigated in relation to the kinetics of rennet gelation and protein retention in model non-fat cheddar cheeses. Rheological measurements showed that sufficiently large, denatured whey protein aggregates could avoid impairment of rennet gelation caused by native whey proteins, irrespective of changes in the soluble calcium concentration or the surface hydrophobicity of the aggregates. Whey protein aggregates formed by the combined heat and ultrasound treatment were more hydrophobic than the larger heat-treated aggregates and were better retained in the cheese. However, inclusion of sufficiently large aggregates in cheeses milks conferred an openness to the cheese microstructure, and showed promise in terms of improving the otherwise rigid non-fat cheese microstructure.

In the third phase of the study, the potential of power ultrasound to generate protein-stabilised water-in-oil-in-water double emulsions containing encapsulated whey protein was investigated as a means of incorporating whey proteins into cheese. Ultrasound was successfully applied to form whey protein-enriched water-in-oil-in-water double emulsions using minimal amounts of food-grade emulsifiers. These emulsions had a markedly higher rate of protein encapsulation than previously reported studies. The size distributions and protein encapsulation of the double emulsions could be tailored by manipulating the emulsion formulation and ultrasonic emulsification parameters. Whey protein-rich double emulsions were successfully incorporated into cooked curds formed by rennet gelation, without impairing gelation kinetics, to increase the retention of whey proteins that are otherwise lost during syneresis.

In this body of work, fundamental understanding of the role of native and denatured whey proteins during rennet gelation was developed. The specific interactions among whey proteins,

casein micelles and the rennet enzyme during the different stages of rennet gelation and cheese making were systematically studied. Further, power ultrasound was successfully used to formulate whey enriched double emulsions that can markedly improve the whey protein retention in cheeses. This understanding can be useful to implementing strategies to better incorporate whey protein into cheese matrices.

Declaration

This is to certify that:

i) The thesis comprises of my original work towards the PhD;

ii) Due acknowledgement has been given in the text to all related parties and other materials used;

iii) The thesis is fewer than 100,000 words in length, excluding tables, maps, bibliographies and appendices.

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Leong, TSH, Ong, L, **Gamlath, CJ**, Gras, S, Ashokkumar, M, Martin GJO 2020, "Formation of Cheddar cheese analogues using canola oil and ultrasonication – a comparison between single and double emulsion systems", *International Dairy Journal*, pp. 104683.

Li, W, **Gamlath, CJ**, Pathak, R, Martin, GJ, & Ashokkumar, M 2019, "Ultrasound–The Physical and Chemical Effects Integral to Food Processing", *Reference Module in Food Science, Elsevier*.

Munir, M, Nadeem, M, Qureshi, TM, Leong, TS, **Gamlath, CJ**, Martin, GJ, & Ashokkumar, M 2019, "Effects of high pressure, microwave and ultrasound processing on proteins and enzyme activity in dairy systems—A review", *Innovative Food Science & Emerging Technologies*, pp.102192

Leong, TSH, Walter, V, **Gamlath, CJ**, Yang, M, Martin GJO & Ashokkumar, M 2018, "Functionalised dairy streams: Tailoring protein functionality using sonication and heating", *Ultrasonics Sonochemistry*, 48, pp 499-508.

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Abbreviations

CCP	Colloidal calcium phosphate
CLSM	Confocal laser scanning microscopy
CMP	Casein macro peptide
CN	Casein
CNEM	Casein stabilized emulsion
DDT	Dichlorodiphenyltrichloroethane
DE	Double emulsion
DS	Diluted skim milk
EDTA	Ethylenediaminetetraacetic acid
α-LA	Alpha lactalbumin
β-LG	Beta lactoglobulin
BSA	Bovine serum albumin
GSH	Glutathione
Ig	Immunoglobulin
HT	Heat treated
LAB	Lactic acid bacteria
MF	Microfiltration
MFWP	Membrane filtered whey protein
PER	Protein efficiency ratio
P:F	Protein-to-fat ratio
PGPR	Polyglycerol polyricinoleate
RCT	Rennet clotting time
RP-HPLC	Reverse phase high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Single emulsion
SMUF	Simulated milk ultrafiltrate
SNF	Solid non-fat
T_d	Denaturation temperature
TGase	Transglutaminase
UF	Ultrafiltration

US	Ultrasound
WP	Whey protein
WPC	Whey protein concentrate
WPEM	Whey protein stabilized emulsion
WPI	Whey protein isolate

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Chapter 1

1. Introduction

Cheese is a broad group of nutritionally rich dairy products formed by coagulating milk. Cheeses are popular due to their long shelf life, diversity and versatility and, can be consumed as a dessert, a food ingredient or a part of a main meal with or without cooking (Fox et al., 2017a). There is a strong global demand for cheese, with production accounting for approximately 35% of the total mass of dairy products (~19×10^6 tonnes of cheese) (IDF (2012)).

Milk in its natural form is a stable aqueous dispersion of proteins, lactose and emulsified fat. During cheese production, casein micelles (50-200 nm) that account for 80% of the total milk protein are destabilised and coagulated to form a strong gel network that entraps large fat globules (~ 5 μm). Smaller whey proteins (~ 20% of milk proteins) and lactose are expulsed from the coagulum along with much of water, in a process known as 'syneresis'(Fox et al., 2017b). Whey proteins expulsed during syneresis have unique nutritional functionalities. Compared to caseins that coagulate in the acidic environment of the human stomach, whey proteins remain soluble and can travel faster from the stomach to the small intestine (Mahé et al., 1995; Walzem et al., 2002) enabling faster digestion and absorption. Further, they have higher proportions of branched amino acids (leucine, isoleucine, and valine) (Kimball & Jefferson, 2002; Walzem et al., 2002) and sulphur containing amino acids (cystine, methionine) (Witard et al., 2013). While branched amino acids participate in the translation–initiation pathway of protein synthesis in the body (Kimball & Jefferson, 2002; Walzem et al., 2002), the sulfhydryl group (SH) of cysteine is a rate limiting substrate for glutathione (GSH) synthesis, which controls the injurious effects of oxidative by-products of cellular metabolism in the body (Bounous, 2000; Zarogoulidis et al., 2015). Due to these nutritional benefits, whey proteins are recovered and converted into food products and ingredients such as whey powder (Gonzalez-Martınez et al., 2002), edible films and coatings (Kurek et al., 2014; Miller et al., 1998), oleogels (de Vries, Gomez, et al., 2017; de Vries et al., 2018; de Vries, Wesseling, et al., 2017) and emulsifiers (Sanmartín et al., 2018). However, due to resource and technical limitations within cheese manufacturing plants, only about 50% of the whey-rich aqueous streams are further processed for value addition, with the remainder disposed of as waste (Baldasso et al., 2011; Mollea et al., 2013).

An in-situ method of harnessing the nutritional functionalities of whey protein during cheese making would be to incorporate them into the cheese. Membrane technology is widely studied in this regard (Maubois, 2002; Mistry & Maubois, 2017; Pouliot, 2008), where it is used to concentrate milk proteins prior to cheese making. Studies performed to date on cheese production from milk with modified protein contents using membrane filtration have reported modified cheese yields and protein retention (Agrawal & Hassan, 2007; Chromik et al., 2010; Guinee et al., 2006; Mistry & Maubois, 2017; Neocleous et al., 2002). However, there is still a limited understanding of the effect of whey proteins on the milk coagulation process, and how this can be affected by altering the whey protein content separately to that of the casein. Developing this understanding requires an experimental approach that can account for other changes in the complex milk microenvironment (pH, soluble calcium content, viscosity) caused by changes in cheese milk formulation. This is the basis for Chapter 3 of this thesis.

Alternatively, whey protein rich streams expulsed during syneresis can be thermally denatured and added back into cheese milk to improve the retention of whey protein in the coagulum. However, addition of whey proteins increases the resulting cheese's moisture content and reduces fat retention, making it difficult to understand whether changes in the cheese properties are caused by whey protein retention alone. Studies conducted to date have incorporated denatured whey protein streams with relatively low concentrations (<10% w/w) into cheese milks (Baldwin et al., 1986; Banks & Muir, 1985; Brown & Ernstrom, 1982; Punidadas et al., 1999). While adding higher amounts of dilute whey streams during cheese milk formulation leads to prolonged clotting times due to the dilution of casein, thermally denaturing concentrated whey protein streams forms larger whey protein aggregates and gels (Alting et al., 2003; Baldwin et al., 1986; Giroux et al., 2015) that are difficult to process during cheese making. Power ultrasound processing is an emerging technology that enables particle size reduction in particulate slurries and gels (Jambrak et al., 2011; O'Sullivan et al., 2014; Yanjun et al., 2014; Zisu et al., 2011). It could therefore be a useful tool to formulate adequately concentrated cheese milks containing whey protein particulates of controlled size and properties. In order to effectively utilise ultrasound-assisted whey protein aggregate formation during cheese making, the kinetics of milk coagulation and the properties of final cheeses as affected by the altered functional properties of whey aggregates need to be studied in detail. Investigations to develop this understanding is the basis for chapter 4 of the thesis.

In contrast to directly incorporating whey protein in cheese milk, an aqueous whey protein phase could first be stably encapsulated inside fat/oil droplets that are then dispersed in the

cheese milk. This approach is based on ‘double emulsion technology’, which although widely considered for various food applications, has not yet been studied in terms of whey inclusion in cheese. However, there are several technical challenges to forming a stable whey protein filled water-in-oil-in water emulsion. These include leakage of the internal aqueous phase, Ostwald ripening, and the flocculation and coalescence of internal and external droplets (Garti, 1997) during cheese making. Emulsion stability is typically conferred by the addition of large amounts of hydrophilic and lipophilic emulsifiers (Matsumoto et al., 1976) that have prevented the widespread use of double emulsions in food industry. However, as whey proteins are amphiphilic and surface active, there is a potential to form stable water-in-oil-in-water double emulsions encapsulating concentrated whey proteins in the internal aqueous phase, while reducing the requirement for non-dairy emulsifiers. If high degrees of whey protein encapsulation could be achieved, these double emulsions could be used to replace milk fat in cheese milks, to form whey protein incorporated cheeses with significantly enhanced nutritional value. Understanding how double emulsion formulations can be tailored to achieve high whey protein encapsulation yields and produce whey protein-enriched cheese is the focus of Chapter 5 of the present thesis.

In summary, each of the individual chapters of this thesis has a specific focus. Chapter 2 provides details of the significance of cheese as a dairy product, fundamentals of cheese production from bovine milk and the current scientific understanding of the colloidal interactions that occur during cheese making. It further reports recent developments in strategies used to incorporate whey proteins into cheese matrices and highlights the research gaps addressed in the thesis. The experimental work conducted during the Ph.D. study are detailed in Chapters 3 to 5. Each chapter contains an introduction and a detailed materials and methods section before the discussion of the key findings. Conclusions drawn from the entire Ph.D. study are brought together in chapter 6 and directions to future studies are recommended. Finally, the appendices include a compilation of experiments conducted during the research study that are not directly related to the focus of the thesis.

References

Agrawal, P., & Hassan, A. (2007). Ultrafiltered milk reduces bitterness in reduced-fat Cheddar cheese made with an exopolysaccharide-producing culture. *Journal of Dairy Science, 90*(7), 3110-3117.

Alting, A. C., Hamer, R. J., de Kruif, C. G., Paques, M., & Visschers, R. W. (2003). Number of thiol groups rather than the size of the aggregates determines the hardness of cold set whey protein gels. *Food Hydrocolloids, 17*(4), 469-479.

Baldasso, C., Barros, T., & Tessaro, I. (2011). Concentration and purification of whey proteins by ultrafiltration. *Desalination, 278*(1-3), 381-386.

Baldwin, K. A., Baer, R., Parsons, J., Seas, S., Spurgeon, K., & Torrey, G. (1986). Evaluation of yield and quality of Cheddar cheese manufactured from milk with added whey protein concentrate. *Journal of Dairy Science, 69*(10), 2543-2550.

Banks, J. M., & Muir, D. (1985). Effect of incorporation of denatured whey protein on the yield and quality of Cheddar cheese. *International journal of dairy technology, 38*(1), 27-32. doi:https://doi.org/10.1111/j.1471-0307.1985.tb00536.x

Bounous, G. (2000). Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. *Anticancer Research, 20*(6), 4785-4792.

Brown, R. J., & Ernstrom, C. A. (1982). Incorporation of ultrafiltration concentratedwhey solids into cheddar cheese for increased yield. *Journal of Dairy Science, 65*(12), 2391-2395. doi:https://doi.org/10.3168/jds.s0022-0302(82)82514-9

Chromik, C., Partschefeld, C., Jaros, D., Henle, T., & Rohm, H. (2010). Adjustment of vat milk treatment to optimize whey protein transfer into semi-hard cheese: a case study. *Journal of food engineering, 100*(3), 496-503.

de Vries, A., Gomez, Y. L., van der Linden, E., & Scholten, E. (2017). The effect of oil type on network formation by protein aggregates into oleogels. *Rsc Advances, 7*(19), 11803-11812.

de Vries, A., Jansen, D., van der Linden, E., & Scholten, E. (2018). Tuning the rheological properties of protein-based oleogels by water addition and heat treatment. *Food Hydrocolloids, 79*, 100-109.

de Vries, A., Wesseling, A., van der Linden, E., & Scholten, E. (2017). Protein oleogels from heat-set whey protein aggregates. *Journal of colloid and interface science, 486*, 75-83.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017a). Cheese: historical aspects. In *Fundamentals of Cheese Science* (pp. 1-10): Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017b). Enzymatic coagulation of milk. In *Fundamentals of cheese science* (pp. 185-229). New York: Springer.

Garti, N. (1997). Double emulsions—scope, limitations and new achievements. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 123*, 233-246.

Giroux, H. J., Lanouette, G., & Britten, M. (2015). Effect of whey protein aggregates of various sizes on the formation and properties of rennet-induced milk gels. *Food Hydrocolloids, 45*, 272-278.

Gonzalez-Martınez, C., Becerra, M., Cháfer, M., Albors, A., Carot, J., & Chiralt, A. (2002). Influence of substituting milk powder for whey powder on yoghurt quality. *Trends in Food Science & Technology, 13*(9-10), 334-340.

Guinee, T. P., O'Kennedy, B., & Kelly, P. (2006). Effect of milk protein standardization using different methods on the composition and yields of Cheddar cheese. *Journal of dairy science, 89*(2), 468-482.

IDF. (2012). *Bulletin 485*. Retrieved from Brussels:

Jambrak, A. R., Lelas, V., Krešić, G., Badanjak, M., Brnčić, S. R., Herceg, Z., . . . Grčić, I. (2011). Rheological, functional and thermo-physical properties of ultrasound treated whey proteins with addition of sucrose or milk powder. *Mljekarstvo, 61*(1)), 79-91.

Kimball, S. R., & Jefferson, L. S. (2002). Control of protein synthesis by amino acid availability. *Current Opinion in Clinical Nutrition & Metabolic Care, 5*(1), 63-67.

Kurek, M., Galus, S., & Debeaufort, F. (2014). Surface, mechanical and barrier properties of bio-based composite films based on chitosan and whey protein. *Food Packaging and Shelf Life, 1*(1), 56-67.

Mahé, S., Benamouzig, R., Gaudichon, C., Huneau, J., De, I. C., Rautureau, J., & Tomé, D. (1995). Nitrogen movements in the upper jejunum lumen in humans fed low amounts of casein or beta-lactoglobulin. *Gastroentérologie clinique et biologique, 19*(1), 20-26.

Matsumoto, S., Kita, Y., & Yonezawa, D. (1976). An attempt at preparing water-in-oil-in-water multiple-phase emulsions. *Journal of colloid and interface science, 57*(2), 353-361.

Maubois, J. L. (2002). Membrane microfiltration: a tool for a new approach in dairy technology. *Australian Journal of Dairy Technology, 57*(2), 92.

Miller, K., Upadhyaya, S., & Krochta, J. (1998). Permeability of d-limonene in whey protein films. *Journal of food science, 63*(2), 244-247.

Mistry, V. V., & Maubois, J. (2017). Chapter 27-Application of Membrane Separation Technology to Cheese Production. *Cheese: Chemistry, Physics and Microbiology (4ª Ed.). PLH McSweeney PF Fox, PD Cotter, DW Everett (Eds.). Elsevier Academic Press, Cambridge, EUA. Pp*, 677-697.

Mollea, C., Marmo, L., & Bosco, F. (2013). Valorisation of cheese whey, a by-product from the dairy industry. In *Food industry*: IntechOpen.

Neocleous, M., Barbano, D., & Rudan, M. (2002). Impact 9 of Low Concentration Factor Microfiltration on Milk Component Recovery and Cheddar Cheese Yield. *Journal of Dairy Science, 85*(10), 2415-2424.

O'Sullivan, J., Arellano, M., Pichot, R., & Norton, I. (2014). The effect of ultrasound treatment on the structural, physical and emulsifying properties of dairy proteins. *Food Hydrocolloids, 42*, 386-396.

Pouliot, Y. (2008). Membrane processes in dairy technology—From a simple idea to worldwide panacea. *International Dairy Journal, 18*(7), 735-740.

Punidadas, P., Feirtag, J., & Tung, M. (1999). Incorporating whey proteins into mozzarella cheese. *International journal of dairy technology, 52*(2), 51-55. doi:https://doi.org/10.1111/j.1471-0307.1999.tb02070.x

Sanmartín, B., Díaz, O., Rodríguez-Turienzo, L., & Cobos, A. (2018). Emulsion characteristics of salad dressings as affected by caprine whey protein concentrates. *International journal of food properties, 21*(1), 12-20.

Walzem, R., Dillard, C., & German, J. B. (2002). Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. *Critical reviews in food science and nutrition, 42*(4), 353-375.

Witard, O. C., Jackman, S. R., Breen, L., Smith, K., Selby, A., & Tipton, K. D. (2013). Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *The American journal of clinical nutrition, 99*(1), 86-95.

Yanjun, S., Jianhang, C., Shuwen, Z., Hongjuan, L., Jing, L., Lu, L., . . . Wupeng, G. (2014). Effect of power ultrasound pre-treatment on the physical and functional properties of reconstituted milk protein concentrate. *Journal of food engineering, 124*, 11-18.

Zarogoulidis, P., Tsakiridis, K., Karapantzou, C., Lampaki, S., Kioumis, I., Pitsiou, G., . . . Kesisis, G. (2015). Use of proteins as biomarkers and their role in carcinogenesis. *Journal of Cancer, 6*(1), 9.

Zisu, B., Lee, J., Chandrapala, J., Bhaskaracharya, R., Palmer, M., Kentish, S., & Ashokkumar, M. (2011). Effect of ultrasound on the physical and functional properties of reconstituted whey protein powders. *Journal of Dairy Research, 78*(2), 226-232.

Chapter 2

2. Background and literature review

2.1. An overview of bovine milk

Bovine milk is a stable colloidal dispersion of proteins and emulsified fat in a mineral- and lactose-rich aqueous serum. Proteins are present both as small globular proteins (~ 5 nm) and as large protein assemblies known as casein micelles (50-600 nm). Fat exists in the form of large globules (0.1-20 μm) emulsified by a native phospholipid-based membrane (Fox et al., 2017b). The aqueous medium, known as milk serum, includes lactose, various salts and vitamins. Cow milk composition is subject to seasonal and varietal variability; however, its typical composition is provided in table 2.1.

Table 2.1. Typical composition of cow milk. Adapted from Fox et al. (2017a)

Component	Quantity (g/L)
Total solids	127
Fat	37
Protein	34
Lactose	48
Ash	7
Salts	**Quantity (mg/L)**
Sodium	500
Potassium	1450
Chloride	1200
Sulphate	100
Phosphate	750
Citrate	1750
Calcium	1200
Magnesium	130

2.1.1. Structure and functions of casein

Milk proteins are broadly categorized into two types depending on their solubility at pH 4.6. Those that precipitate at this pH are collectively known as caseins and account for approximately 80% of total proteins (Fox et al., 2017b; Whitney, 1999).

Caseins are a group of phosphorylated (contain phosphate groups bound to serine residues in the peptide change) proteins that are subdivided into 4 categories as $\alpha_{s1,}\ \alpha_{s2}, \beta\ and$ κ depending on their electrophoretic mobility, and represent approximately 38,

10, 34 and 15% of the casein, respectively. They are relatively small proteins, with molecular masses of 20–25 kDa (Fox et al., 2017a).

α_{s1} and α_{s2} caseins have the highest degree of phosphorylation (8-13 mol PO_4 per mol of protein) followed closely by β-casein (5 mol PO_4 per mol of protein). When isolated, they precipitate in the presence of high calcium concentrations (>6 mM Ca^{2+} 30 ºC) (Fox et al., 2017a) as the negative charge of exposed phosphate groups are neutralised by Ca^{2+} ions in the solution, resulting in a complex called colloidal calcium phosphate (CCP) (Fox & McSweeny, 1998). Because of its lower phosphate content (1-3 mol PO_4 per mol of protein), κ-casein binds with cations only weakly and is not precipitated by them (Fox et al., 2017a).α_{s2} and κ-caseins contain cysteine (a sulphur-containing amino acid) that are linked by intermolecular disulphide bonds. α_{s2}-casein generally exist as disulphide-linked dimers, whereas multiple κ-casein molecules may be disulphide linked to form polymers (Fox et al., 2017a). The absence of cysteine in α_{s1}- and β-caseins increases the flexibility of these proteins (Fox et al., 2017a).

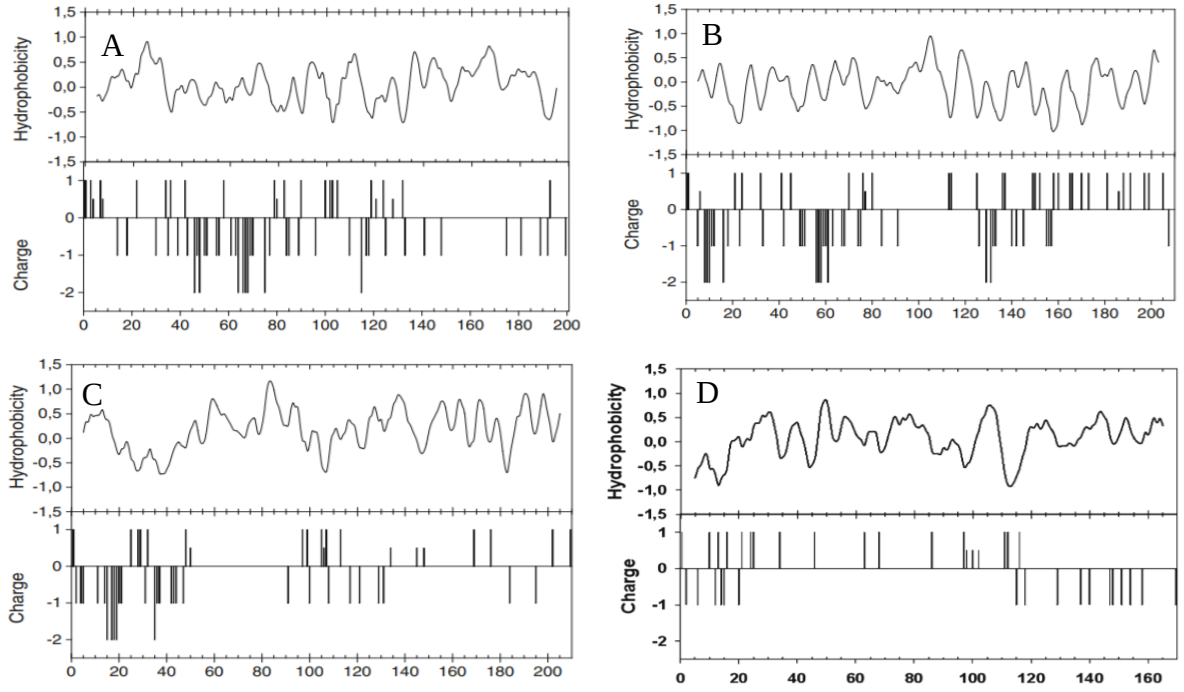
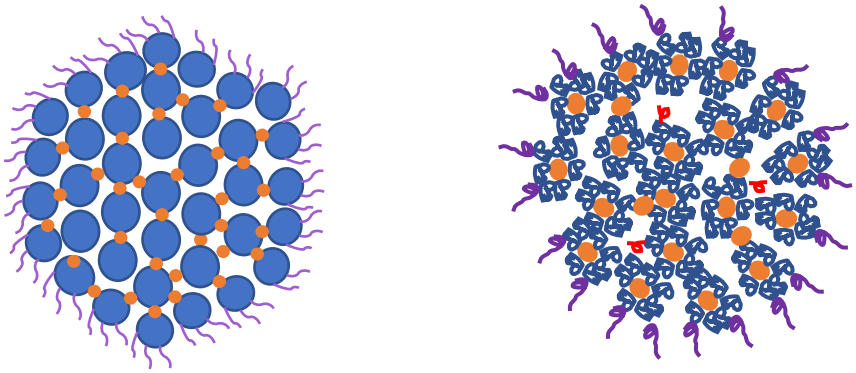


Figure 2.1: Distribution of the calculated hydrophobicity and charge of residues along the amino acid chain of α_{s1}(A), α_{s2} (B), β (C) *and* κ (D) caseins (adapted from Huppertz (2013)). Amino acids are numbered from the N terminus to the C terminus (left to right in the 'x' axes).

The hydrophobic, polar, and charged residues result in hydrophobic or hydrophilic regions in the peptide sequences of caseins (Figure. 2.1), giving the caseins strongly amphiphilic structures. A strongly hydrophobic character is observed towards the N-terminus (segment 21-110) of κ-casein, whereas the C terminal is highly hydrophilic (segment 110-120) (Figure.

2.1D). The interactions between hydrophilic and hydrophobic regions of caseins coupled with CCP formation, result in the formation of casein micelles (50-600 nm) (Walstra, 1990, 1999).

The casein micellar structure was first explained by Morr (1967) as a large micelle composed of smaller submicelles with an inner core of α_s-β casein complexes and an outer layer of α_s-κ casein complexes. Further work on this area (Payens, 1966; Schmidt, 1976; Slattery, 1976; Slattery & Evard, I973) led to an evolved sub-micellar model, which was widely accepted for many years (Figure 2.2A). The sub-micellar model hypothesised that casein micelles are made up of two types of smaller submicelles; one with hydrophobic α_s-β casein complexes and the other with α_s- κ casein complexes that have both hydrophobic and hydrophilic regions. The submicelles cluster in such a way so that the more hydrophobic α_s -β complexes form an inner core surrounded by a layer of κ-caseins, which protrude their hydrophilic C-terminal (Figure 2.1 D)ends out of the micelle surface as a so-called 'hairy layer'. While the hairy layer stabilises the micelle in the serum through electrostatic and steric interactions (Walstra, 1999), the micelle is held together by hydrophobic interactions (Horne, 1998) and CCP that forms bridges between phosphorylated caseins inside the micelle (Walstra, 1999).



A B

Figure 2.2. A: Schematic depiction of a casein submicelle model as adapted from Walstra (1999). Casein micelles are made up of α_s-β ( ) and α_s-κ submicelles ( ) with the hydrophilic κ -casein C-terminus ( ) protruding out of the micelle surface. CCP ( ) and hydrophobic interactions (not shown in the diagram) bind the submicelles together. B: Schematic representation of the casein micelle as adapted from Dalgleish (2011), incorporating calcium phosphate nanoclusters ( ) with their attached α_s and β caseins ( ) and the surface-located κ-casein ( ). Hydrophobically bound 'mobile' β-casein ( ) is present within the water cavities (blank spaces) inside the micelle. For clarity, the relative sizes of the individual components are not to scale.

However, recent studies with improved electron microscopic techniques, such as transmission electron microscopy (TEM) and cryo-TEM (McMahon & Oommen, 2008; Trejo et al., 2011),

have failed to show discrete sub-micelles, giving rise to an alternative to the sub-micellar model (Dalgleish, 2011). Rather, it has been suggested that phosphorylated α_{s1}-, α_{s2}-, β-casein, or their combinations stabilised by calcium phosphate nanoclusters, orient their hydrophobic domains outward, allowing hydrophobic interactions and binding with the non-polar regions of κ-caseins (McMahon & Oommen, 2008). While hydrophobic interactions are important in the aggregation of the calcium phosphate/protein nanoclusters, after they have been formed, other short-range interactions, such as calcium-bridging, hydrogen bond formation and van der Waals interactions occur, strengthening the micellar structure. Therefore, casein micelles remain largely intact even during cooling, whereas the hydrophobic interactions are weakened. β-caseins (with less phosphorylation) bind to the micelle mainly by hydrophobic interactions and are somewhat 'mobile' inside the micelle (Figure 2.2B). They are dissociated from the micelle during cooling (Dalgleish, 2011). Overall, the micelle interior can be visualised as a bi-continuous structure with calcium phosphate/casein nano clusters and water filled interconnected cavities (Trejo et al., 2011). Although present in relatively lower quantities compared to other salts (Table 2.1), calcium exist in equilibrium between the micelles and the milk serum. It is pivotal to casein micelle integrity, and dramatically affects cheese making (see section 2.4.5).

2.1.2. Structure of the whey proteins

Whey proteins remain soluble at pH 4.6 (Fox & McSweeny, 1998; Whitney, 1999), and are categorised into four types: α-lactalbumin (α-LA), β-lactoglobulin (β-LG), bovine serum albumin (BSA) and immunoglobulins (IG). These account for ~50%, 20%, 10% and 10% of the total whey protein, respectively (Fox et al., 2017a). They are relatively small (~5nm) and hydrophilic proteins with a native globular structure stabilised by intramolecular disulphide bonds (2, 4 and 17 in the case of β-LG, α-LA and BSA, respectively). In addition, β-LG and BSA each have one free thiol group (Fox et al., 2017a).

β-LG has a distinctly hydrophobic core in its native conformation, with low electron density regions in the secondary structure that provide extreme flexibility with respect to its native conformation (Jameson et al., 2002). A similar hydrophobic core is present in α-LA, albeit with numerous cation binding sites (Permyakov & Berliner, 2000). Treatments that destabilise native whey protein conformation and promote unfolding can induce hydrophobic interactions, and thiol-disulphide bridging or thiol thiol-thiol oxidation reactions between whey proteins in solution (Monahan et al., 1995). Such protein interactions occur during heating and thermal treatment, which is commonly utilised in cheese making to form whey protein-incorporated

cheese. For a detailed account of heat-induced whey protein denaturation and its applications during cheese making, the reader is directed to section 2.10. In the next section, the significance of cheese as a dairy product is introduced. Emphasis is given to cheese production process and the generation of whey as a by-product.

2.2. Cheese as a dairy product

2.2.1. Introduction

Cheese is an important dairy product believed to date back to Euphrates and Tigris civilizations when the nutritional value of milk from domesticated animals was identified and dairy products became an important component of human diet (Fox et al., 2017a). A diverse range of cheeses are now produced from the milk of various domesticated animals, predominantly the cow. Cheese is produced directly from milk, which in its natural form is a rich source of nutrients. While milk is susceptible to spoilage by pathogenic microorganisms due to the rich availability of nutritional components, conversion of milk into cheese, provides prolonged shelf life by reducing the water activity.

Although cheese has been produced for millennia, there is still much to be learned about its production. It is a complex food product, which is biochemically dynamic and undergoes changes in functionality, texture and flavour during manufacturing and storage. Cheese production follows a series of consecutive and concomitant biochemical changes such as protein coagulation, syneresis and fermentation which if controlled and balanced, leads to desirable textures, flavours and aromas (Fox et al., 2017a).

2.2.2. Nutritional properties and composition of cheese

From a nutritional perspective, cheese is rich in fat, protein, water and traces of carbohydrates and minerals. The composition varies between different types of cheeses, particularly with respect to the moisture content of soft and hard cheeses (Table 2.2). In all cheese types, the protein content is predominantly casein, as the caseins form the protein matrix, while the whey proteins are drained out from the curd with water. As caseins are less abundant in sulphur-containing amino acids than whey proteins, the nutritional value of cheese protein is lower than that of total milk proteins (Renner, 1993). Nonetheless, cheese proteins are 100% digestible in the human body. In addition, a significant amount of proteins is broken down to water soluble peptides by the cheese culture during cheese ripening (McSweeney, 2004; O'Brien & O'Connor, 2017), aiding digestibility. As the extent of proteolysis in cheese determines the

structure and the amino acid sequence of the peptides, the bioactivity of cheese varies depending on ripening time and the cheese culture used (Gupta et al., 2009).

Table 2.2. Composition of selected cheese types presented per 100 g. (Adapted from Holland et al. (1989) as cited in Callaghan (2017)).

Cheese type	Water (g)	Protein (g)	Fat (g)	Carbohydrate (g)	Na (mg)	Ca (mg)	Phosphate (mg)	Other minerals (mg)
Caerphilly	41.8	23.2	31.3	0.1	480	550	400	115
Camembert	50.7	20.9	23.7	Tr [b]	650	350	310	123.9
Cheddar (normal)	36	25.5	34.4	0.1	670	720	490	104.6
Cheddar (reduced fat)	47.1	31.5	15	Tr [b]	670	840	620	152
Cheshire	40.6	24	31.4	0.1	550	560	400	109.6
Cottage cheese	79.1	13.8	3.9	2.1	380	73	160	98.7
Cream cheese	45.5	3.1	47.4	Tr [b]	300	98	100	170.6
Danish blue	45.3	20.1	29.6	Tr [b]	1260	500	370	118.2
Edam	43.8	26	25.4	Tr [b]	1020	770	530	138.6
Emmental	35.7	28.7	29.7	Tr [b]	45	970	590	128.7
Feta	56.5	15.6	20.2	1.5	1440	360	280	116.1
Gouda	40.1	24	31	Tr [b]	910	740	490	130.9
Gruyère	35	27.2	33.3	Tr [b]	670	950	610	138.6
Mozzarella	49.8	25.1	21	Tr [b]	610	590	420	103.7
Parmesan	18.4	39.4	32.7	Tr [b]	1090	1200	810	161.4
Ricotta	72.1	9.4	11	2	100	240	170	124.7
Roquefort	41.3	19.7	32.9	Tr [b]	1670	530	400	126
Stilton	38.6	22.7	35.5	0.1	930	320	310	152.8

[a] The values indicated are the total of K, Mg, Fe and Zn contents in cheese. For a detailed mineral compositions in cheeses, readers are directed to Callaghan (2017) or Holland et al. (1989)
[b] Tr – Trace

Caseins are highly phosphorylated and upon proteolysis, form casein phosphopeptides (Dupas et al., 2009; Gagnaire et al., 2001; Lund & Ardö, 2004) that have antioxidant (Kitts, 2005) and immunostimulatory properties (Kitts & Nakamura, 2006). They also can influence calcium absorption in the small intestine (Meisel & FitzGerald, 2003). Other bioactive peptides formed during cheese ripening and gastric digestion have been found to confer antihypertensive (Sieber et al., 2010) effects. A comprehensive summary of the bioactivity of different types of cheeses is available in López-Expósito et al. (2012).

Milk fat provides the distinctive mouthfeel, aroma and flavour of cheese. Approximately 90% of milk fat is retained in cheese curd along with fat-soluble vitamins in milk (Callaghan, 2017). However, dietary guidelines now recommend reducing milk fat consumption, due to the presence of short chain saturated fatty acids that elevate the cholesterol content in blood (NHS, 2018; O'Brien & O'Connor, 2017). Therefore, there is increasing interest in producing low-fat cheeses (Moatsou et al., 2019) or cheese analogues with fat alternatives (Fox et al., 2017f) to reduce the unsaturated fatty acid content, while maintaining the desirable sensory attributes. Few existing strategies include, replacing milk fat with denatured whey proteins (Hinrichs, 2001) or emulsified alternative fats/oils (Fox et al., 2017f; Tsujii & Shirotani, 2015). Incorporation of double emulsions with an encapsulated aqueous phase inside the oil droplets into cheese milk, is an emerging technology that has potential to produce protein-rich, low-fat cheese products with desirable attributes (Giroux et al., 2013; Leong et al., 2020).

Most water-soluble carbohydrates, vitamins, and minerals are expulsed from the cheese matrix along with water, during cheese manufacture. Lactose is found only in trace quantities in cheese and is generally fermented into lactic acid during cheese ripening. Hence ripened cheeses can be safely consumed by lactose intolerant people that are deficient in β-galactosidase, which is involved in lactose digestion (Callaghan, 2017). Although most water-soluble minerals are lost during syneresis (expulsion of the aqueous phase), retained caseins have bound calcium in the form of CCP (see section 2.1.1.). Further, cheese production involves the addition of salt (sodium chloride) as a preservative and for flavouring (Fox et al., 2017g). Therefore, cheese is identified as a dietary source rich in sodium and bioavailable calcium.

2.3. Current state of cheese production

In addition to its nutritional value, cheese is popular due to its versatility and variety, being used as a dessert, a food ingredient or a part of a main meal with or without cooking (Fox et al., 2017a). Cheese production accounted for approximately 35% of the total mass of dairy products (~19×10^6 tonnes of cheese) in 2012, having grown at an average rate of 4% per year during the past 30 years (IDF (2012)). Australia alone produced 381,000 tonnes of cheese in 2018-2019, utilising more than a third of Australian milk. In the same financial year, Australia exported 166,000 tonnes of cheese, worth approximately $1 billion (DA, 2019).

Ongoing improvements to modern cheese production include the mechanisation and automation of processes, increasing cheese yields and the effective utilisation of all milk constituents. Membrane processing of milk and whey has provided excellent opportunities to increase cheese yields and minimise wastage of whey, but only if cheese milk formulations and production processes are modified to utilise all constituents in milk during cheese making (Johnson, 2017). This is a challenge that requires both the application of fundamental knowledge of protein chemistry, enzymology and colloidal interactions relevant to cheese manufacture, and revising of food regulations and standards (such as CODEX standards (CODEX, 2018, 2019) to allow commercialisation of cheeses with modified ingredients and compositions.

2.4. Cheddar cheese making process

Cheddar is one of the most popular cheeses globally (Hamdy et al., 2017; Lee et al., 2016; Ulpathakumbura et al., 2016). It is a semi-hard cheese, consisting of a casein-rich protein matrix, imbedded fat droplets, and moisture. It is formed by enzymatic destabilisation of milk to form a coagulum. The coagulum is subsequently allowed to undergo ripening, during which enzymatic and microbial proteolysis and lipolysis form desirable textures, aromas and flavour. The interactions of milk proteins occurring during cheese production are discussed in this section.

2.4.1. An overview

Prior to cheese making, cheese milks are standardised to a consistent protein-to-fat ratio. Then a cheese culture containing lactic acid bacteria is added to develop acidity. Milk is then coagulated by proteolysis followed by dehydration of the coagulum by cutting, agitation, cheddaring and salting. Curd granules are then formed into a characteristic cheddar shape by pressing. The fresh cheese is then ripened to develop flavour (Figure 2.3).

2.4.2. Standardisation and pasteurisation

The fat, protein and calcium content of milk, along with its pH, influence the composition of a final cheese. A protein-to-fat (P:F) ratio of 0.8-1, which is close to the natural composition of milk, is typically maintained during cheddar cheese making (Guinee et al., 2007). Cheese milk is pasteurised to destroy pathogenic and undesirable microorganisms that may cause defects or variability in cheese flavour and/or texture. However, the cheese flavour is more intense when it is made from raw milk than pasteurised milk, as indigenous lactic acid bacteria (LAB) that contribute positively to cheese flavour, are killed by pasteurisation, Further, indigenous

enzymes such as lipase, which contribute positively to cheese ripening, are also inactivated by pasteurisation. Therefore a sub-pasteurisation temperature (e.g. 68–70 °C) may be used for cheese milk and temperature treatments >72 °C for 15 s should be avoided (Fox et al., 2017d).

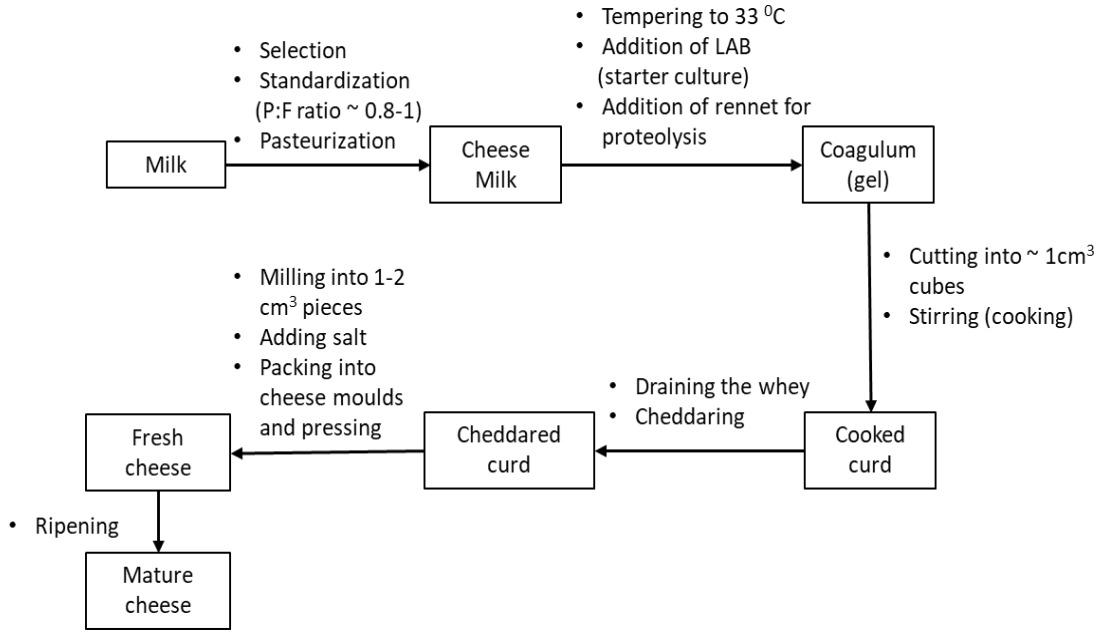


Figure 2.3: Cheddar cheese making process flow (adapted from Guinee et al. (2007), Ong et al. (2011), Ong et al. (2012) and Fox et al. (2017d)).

2.4.3. Acidification

After the milk has been standardised and pasteurised, it is transferred to vats and converted to a cheese curd by a process involving acidification, coagulation and dehydration. Acidification is generally achieved by the in-situ fermentation of lactose to of lactic acid. Initially, indigenous LAB was relied upon for this task, however their natural variability resulted in variable acidification rates and hence variable cheese quality (Fox et al., 2017d). Therefore, cultures of LAB that contain *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* are now commonly used (Guinee et al., 2007; Ong et al., 2011, 2012). The reduction in pH results in the solubilisation of CCP (Van Hooydonk (1986) as cited in Fox et al. (2017g)) with a concomitant dissociation of micellar casein (especially β-casein) (Dalgleish & Law, 1988). CCP becomes fully soluble at pH ~4.8-5.3 at room temperature (Van Hooydonk (1986) as cited in Fox et al. (2017g); Dalgleish and Law (1989)), and close to this pH range (~pH= 5.3-5.6) a maximum dissociation of the casein micelle is also reported. However, a further reduction in the pH (<5.3) leads to aggregation of caseins into increasingly more ordered structures until reaching its isoelectric point at pH~4.6 (Dalgleish & Law, 1988).

2.4.4. Coagulation

Acid-induced isoelectric precipitation of casein micelles is used as the only coagulation technique in the manufacture of fresh cheese types such as Cottage, Quarg, Feta-type cheese from UF-concentrated milk, and Mozzarella. Commercially, direct acidification using lactic acid, HCl, or gluconic acid-δ-lactone is also used in place of LAB-assisted fermentation (Fox et al., 2017d). However, all ripened cheeses, including cheddar, are produced by rennet coagulation, where milk clotting proteases (rennet) are used to hydrolyse and destabilise the casein micelles. Gelation is conducted at a temperature and pH (achieved by the lactic acid production by LAB) where the protease is most active. Rennet gelation is a critical step in cheddar cheese making and provides the basis for this thesis. Therefore, it is reviewed in detail in the next section.

2.4.5. Rennet gelation

As previously described in section 2.1.1, casein micelles are stably suspended in the milk serum due to the electrostatic and steric repulsion created by the κ-casein hairy layer (Walstra, 1999). Chymosin (an enzyme secreted in the calf intestine) is a key component of rennet that hydrolyses the hairy κ-casein layer (Figure 2.4a) by cleaving the peptide chain at the phenylalanine (105)-methionine (106) peptide bond to produce para κ-casein (residues 1-105) and casein macro peptide (CMP) (residues 106-169). CMP, having the hydrophilic C-terminal of κ-casein, solubilises into the aqueous phase while para κ-casein, having the hydrophobic N-terminal, remains attached to the micelle by hydrophobic interactions (Figure 2.4b). As the electrostatic and steric repulsion between the micelles is lost, the cleaved hydrophobic casein micelles aggregate to form clusters (Figure 2.4c). Aggregation initiates (also known as the onset of gelation) only after the majority (~80%) of the stabilising κ-casein has been cleaved (Chaplin & Green, 1980), as any un-cleaved hydrophilic 'κ –casein hairs' continue to cause electrostatic repulsion between partially hydrolysed micelles (Carlson et al., 1987; Darling & Dickson, 1979; Hyslop, 2003). The hydrolysis of κ-casein was initially assumed to be a first order enzymatic reaction, which was later demonstrated experimentally (Walstra, 1990).

The aggregation of the casein micelles is followed by the rearrangement of the gel, where the coagulum encloses the aqueous phase and water-soluble components into its network (Figure 2.4d). However, this coagulum is cut and agitated during cheese production to drain out the aqueous phase with dissolved whey protein in it. The expulsed aqueous stream is commonly referred to as 'cheese whey'.

From the standpoint of cheese production, the kinetics of rennet gelation and the strength of the final rennet gels are important. Longer gelling times prolong the cheese production process while softer gels cause high fat and casein loss with the whey. In general, there is an inverse relationship between the rennet clotting time and the gel strength; meaning, factors that prolong the gelling time tend to also decrease the gel strength. The pH of milk, coagulation temperature, and the soluble calcium concentration in milk are key factors that affect the rennet coagulating properties of milk (Fox et al., 2017d). However, it should be noted that these three parameters are interlinked, and their individual effect in relation to rennet gelation is difficult to study in isolation.

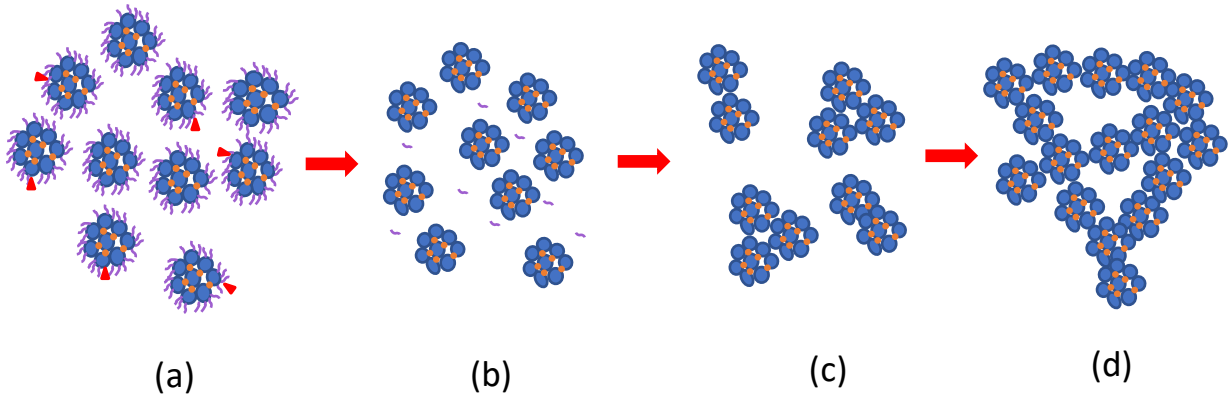


Figure 2.4: (a) Chymosin ( ) when added to a system with casein micelles ( ), (b) cleaves the κ – casein hairs ( ) that solubilise into the aqueous phase leaving hydrophobic casein micelles, (c) the hydrophobic micelles aggregate, (d) the aggregates rearrange to form a gel network.

Effect of pH

Casein micelles have a lower charge (hence a lower inter-micelle electrostatic repulsion) below the normal pH of milk (pH=6.6) (Darling & Dickson, 1979), due the protonation of carboxylic acid groups of aspartic acid (Asp) and glutamic acid (Glu) residues (Liu & Guo, 2008). At acidic pH's (pH ~4.8-5.3) CCP solubilizes to ionic calcium at room temperature (Van Hooydonk (1986) as cited in Fox et al. (2017g), Dalgleish and Law (1989)), while alkalisation of milk (pH >7) leads to a reduction in ionic calcium (Sinaga et al., 2017), which precipitates into the casein micelles as CCP. The lower electrostatic repulsion, due to the increased amount of ionic calcium that neutralises the charge on the casein micelle, leads to faster casein micelle aggregation and a shorter onset of gelation time at acidic pH (Esteves et al., 2003; Sinaga et al., 2017).

Effect of soluble calcium

Calcium in milk exists in an equilibrium between colloidal calcium phosphate bound to casein micelles and ionic calcium in the milk serum. The addition of calcium chloride to milk is known to decrease coagulation time, create firmer gels, and increase curd yield (Fox et al., 2017c; Sandra et al., 2012). This is because soluble calcium binds to the negatively charged calcium-sensitive residues in the casein micelle, and reduces the net repulsion between them (Dalgleish, 1983, 1984). Calcium bridging between the aggregates strengthens the gel structure during the rearrangement of the initial coagulum (Van Hooydonk et al., 1986).

However, excessive addition of calcium chloride (>10 mM) can negatively affect rennet gelation, as ionic calcium increases the positive charge on the casein micelle, resulting electrostatic repulsion (Fox et al., 2017c; Udabage et al., 2001). Nonetheless, addition soluble calcium to milk does not affect the hydrolysis of the casein micelle, and only helps bridging the cleaved micelles (Sandra et al., 2012).

Effect of gelation temperature

Chymosin activity is highest when the temperature is close to 30 ºC. While investigating a narrow range of gelation temperatures (20-40 ºC), Park et al. (1996) reported that the rate of enzyme hydrolysis (as measured by the rate of CMP release) was faster at 30 ºC than at 20 ºC. The overall rate of rennet gelation also increased significantly from 20 ºC to 30 ºC, however the improvement was less significant at a further increase to 40 ºC. At very low temperatures (~10 ºC), enzymatic hydrolysis of κ-casein does occur, however the aggregation of the cleaved micelles is much slower (Bansal et al., 2007). It is also reported that hydrolysis of β-casein, which is loosely bound to the micelle (see section 2.1.1.), also occurs at 10 ºC, after the completion of κ-casein hydrolysis. Although the mechanism is not clearly established, aggregation of casein micelles is impaired when the hydrolysis of β-casein is inhibited at low temperatures (Bansal et al., 2007).

From a cheddar cheese making perspective, rennet coagulation is typically carried out at temperatures of ~ 30-34 ºC and at the natural pH milk (pH 6.6) (Guinee et al., 2007; Ong et al., 2011, 2012), where a sufficiently fast rate of gelation is achieved.

2.4.6. Post-coagulation steps

During cheddar manufacture, the coagulum is cut into ~1 cm^3 pieces and stirred while the temperature is increased, to enable the expulsion of water from the curd (syneresis; see section 2.5). Higher cutting intensities and stirring speeds cause significant curd and fat losses (Everard et al., 2008), which should be avoided while maintaining an acceptable moisture expulsion.

The cooked curd is then drained using perforated screens, and the curd is 'cheddared'. Cheddaring involves piling of curd particles, cutting into blocks, followed by regular stacking and turning of the blocks. This characteristic step allows acidity development to progress (with the pH decreasing from ~ 6.1 to 5.4) thus solubilising CCP and fusing the curd granules. The curd blocks are milled into small pieces and dry-salted. Salt dissolves in moisture on the surface of the curd chips during the "mellowing" period that follows. The curds are then transferred to cheese moulds and pressed overnight at up to 200 kNm^{-2}. Pressed cheeses are matured at 6–10 °C for a period ranging from 3-4 months to 2 years, depending on the maturity desired (Fox et al., 2017e).

2.5. Whey generation during cheese syneresis

As described previously, cheese protein is predominantly casein, which is coagulated by enzymatic hydrolysis and/or isoelectric precipitation (see sections 2.4.4 and 2.4.5). Water soluble constituents including lactose and whey proteins, which remain soluble in the presence of casein-curdling enzymes and at low pH, are expulsed from the cheese curd with water. The expulsion of water and other water-soluble components from the curd, collectively known as 'syneresis', typically results in the expulsion of between 65-90% of the milk mass. Much of the variation depends on the cheese type and the composition of cheese milk (Table 2.3). Worldwide whey production was estimated to be around 180-190 x 10^6 tons/year in 2011, out of which only ~50% was processed for value addition, with the rest being disposed as waste (Baldasso et al., 2011; Mollea et al., 2013). As a waste, whey treatment is problematic due to its acidic pH and high organic load, which can reach a chemical oxygen demand of 100,000 mg $O_2\,L^{-1}$ (Yorgun et al., 2008).

Yadav et al. (2015) comprehensively discussed several physico-chemical and biotechnological methods used to convert cheese whey into value-added products such as whey powder, lactose derivatives, biogas, electricity and other protein products. Ultrafiltration can be used to concentrate lactose (~70% of whey solids) and whey proteins (~ 10% of whey solids and 20% of milk proteins) in whey. Lactose can then be recovered from the permeate by crystallization (Chen et al., 2018; de Souza et al., 2010; Sánchez-García et al., 2018; Sánchez-García et al., 2019) and the concentrated whey proteins in the retentate can be spray dried to produce whey protein concentrate powder.

Table 2.3. Gross production and compositions of different types of cheeses whey

Cheese type	Drained whey % *	pH of whey	Composition of whey						Reference
			Total solids%	Fat %	Protein %	Lactose%	Calcium (mg/mL)	Potassium (mg/mL)	
Cottage cheese	83.7	6.9		0.03	0.89				**Klei et al. (1998)**
	85.4								**Fedrick and Houlihan (1981)**
		4.5	6.23	0.38	0.66	4.80	0.77	1.82	**Nguyen et al. (2003)**
		4.4			0.38**	1.99	0.69	0.95	**Basak et al. (2018)**
			6.4		0.43	4.11		1.54	**Peri and Dunkley (1971)**
Cheddar	89.1-89.4				0.75***				**Leong et al. (2020)**
	89.8-90.6								**Lucey and Kelly (1994)**
	90. 4 -91.2			0.22-0.32 (SW) 0.95-1.86 (PW)					**Sapru et al. (1997)**
		5.41	6.55	0.25	0.83	4.95	0.24	0.94	**Ahmed et al. (2015)**
		6.0-6.3	5.9-7.2 (SW)	0.18-0.22 (SW)					**Karagül-Yüceer et al. (2003)**
							0.37(SW)	1.23 (SW)	**Wong et al. (1978)**
Parmesan	92.62		6.7 (DW) 8.59 (PW)	0.23 (DW) 0.61(PW)	0.85 (DW) 0.96 (PW)	4.81 (DW) 4.54(PW)			**Govindasamy-Lucey et al. (2004)**
	91.1-92.1			0.44	0.94		0.39		**Malacarne et al. (2006)**
Mozzarella	90.8-93.41[a]			0.07-0.53[a]	0.07-.11[a]				**Rudan et al. (1999)**
	92.55-92.66		6.6	0.07	0.94-0.89		0.05		**Metzger et al. (2000)**
		5.33	6.51	0.24	0.81	4.93	0.24	0.96	**Ahmed et al. (2015)**
		6.19-6.40	5.7-11.5	0.06-1.8	0.01-1.00		0.01-0.14	0.14-0.50	**Gernigon et al. (2010)**
				0.79	0.72				**Rektor and Vatai (2004)**
Cream cheese	64								**Jeon et al. (2012)**
Feta	~67-74								**Pappas et al. (1996)**
			7.87	0.39	1.61	5.33			**Anifantakis (1991)**
		6.3-4.8							**Kandarakis et al. (2001)**

*kg of whey produced per 100 kg of cheese milk, calculated as "100 - % Cheese yield" (Cheese yields were not adjusted for a constant moisture content).

** Total N x 6.38 , *** Data unpublished, [a] Variable with fat content in cheese milk (0.35-3.21%)

SW=sweet whey, DW -Drain whey, PW- Pressed whe

2.6. Whey protein as a functional dietary supplement

With the emergence of functional foods, there has been an increasing demand for whey proteins for use as food ingredient (Patel, 2015). Whey proteins have been a popular food supplement for years due to their health and nutritional benefits. Three key differences between whey proteins and caseins provide them with unique nutritional functionalities.

2.6.1. Amount of branched amino acids

The amino acid profile of whey proteins is more nutritionally valuable than that of caseins, having a higher proportion of branched-chain amino acids (leucine, isoleucine, and valine) that participate in the translation–initiation pathway of protein synthesis in the body (Kimball & Jefferson, 2002; Walzem et al., 2002). This is reflected in the higher protein efficiency ratio (PER) (a measure of the weight gain of young animals/humans per gram of protein consumed) of whey proteins (3.2) compared to caseins (2.6) (Walzem et al., 2002).

2.6.2. Solubility in low pH

In contrast to whey proteins that remain soluble in the stomach's acidic conditions, caseins in liquid milk coagulate upon reaching their isoelectric point of pH 4.6. (He & Giuseppin, 2014; Walzem et al., 2002). Therefore, whey proteins can travel faster from the stomach to the small intestine (Mahé et al., 1995; Walzem et al., 2002) and can be digested and absorbed at higher rates than casein (Pennings et al., 2011). As a result, they are a preferred protein supplement for postprandial muscle protein accretion in elderly men (Pennings et al., 2011; Pennings et al., 2012) and resistance-trained young men (Witard et al., 2013).

2.6.3. Presence of sulphur containing amino acids

The presence of proportionately more sulphur containing amino acids (cystine, methionine) in whey protein than casein (Witard et al., 2013) is also beneficial nutritionally. The sulfhydryl group (SH) of cysteine is a rate limiting substrate for glutathione (GSH) synthesis, which controls the injurious effects of oxidative by-products of cellular metabolism in the body (Bounous, 2000; Zarogoulidis et al., 2015). Dietary provision of mixed or individual whey proteins (e.g. α-lactalbumin and BSA) is believed to enhance the production of GSH in lymphocytes, leading to improved immune responses (Bounous et al., 1988). Study of the immunoenhancement by whey proteins has revealed a broader protective biological function with regard to tumour susceptibility. For example, slightly higher levels of GSH in the colon obtained by whey protein feeding was associated with a lower tumour burden (Enker & Jacobitz, 1976), while a diet formulated with whey proteins reduced the development of anthracene-induced mammary tumours (Hakkak et al., 2000). Although the reason for the antitumor activity of whey protein is not fully elucidated yet, increased GSH synthesis is believed to have an anti-tumour effect via stimulation of immune GSH pathway (Bounous, 2000).

Apart from GSH synthesis, partially denatured whey proteins and peptides produced from whey protein hydrolysis are believed to intervene in metabolic activities of cancer cells and

supress their growth. A multitude of studies have observed reduction on cancer cell growth in vitro, with a variety of mechanisms having been proposed to explain these observations, such as: inhibition of enzymes such as β-1,4-galactosyltransferases secreted by cancer cells by α-lactalbumin (Chandrasekaran et al., 2001; Roy et al., 2016; Teixeira et al., 2019), ease of permeation of partially denatured and stabilised α-lactalbumin into cancer cell chromosomes to trigger DNA fragmentation (Brinkmann et al., 2011; Teixeira et al., 2019), and pepsin mediated conversion of lactoferrin in whey protein into lactoferricin, which is a cytotoxic for cancer cells (Eliassen et al., 2006), during gastronomical digestion (Kuwata et al., 2001; Teixeira et al., 2019). However, to date, whey protein intake-induced anti-cancer effects are indiscernible in clinical studies (Teixeira et al., 2019).

Recent case studies on unique health and nutritional benefits of whey proteins and its derivatives, excluding applications in cancer therapy are listed in Table 2.4. These benefits have paved the pathway for whey protein and its derivatives to be consumed as products of their own or as a functional food ingredient; as discussed in the next section.

Table 2.4. Reported health and nutritional benefits of whey proteins, excluding applications in cancer therapy.

Whey protein derivative	Functionality	Health or nutritional benefit	Reference
Whey protein isolate, hydrolysate	Presence of branched amino acids	Increase in lean body mass, recover muscle damage in healthy people or cancer patients	(Dillon et al., 2012); Joy et al. (2013); (Lollo et al., 2014; Martin et al., 2013; Teixeira et al., 2019)
Pressure treated whey	Increase in the intercellular GHS level	Reduction in inflammation in cystic fibrosis patients	(Lands et al., 2010)
Peptides liberated from whey protein hydrolysis	Radical neutralization and elevating GSH activity	Protection of lung cells and prostate cells from oxidative damage.	(Kent et al., 2003; Kong et al., 2012)
Whey protein concentrate, Diet	Increased GHS synthesis	Lower tumour burden	(Attaallah et al., 2012; Hakkak et al., 2000)

formulated with whey proteins			
Whey proteins	Faster digestion, presence of branched amino acids	Muscle growth in elderly and resistance trained men	(Pennings et al., 2011; Pennings et al., 2012)

2.7. Whey proteins in food

2.7.1. Whey powder

Cheese whey is largely concentrated, and spray dried to produce a variety whey protein powders (e.g. whey protein concentrate, isolate or hydrolysate). Their characteristics differ according to the processing techniques applied before spray drying, such as demineralisation, lactose removal, hydrolysis or straightforward drying (Gonzalez-Martınez et al., 2002; Sinha et al., 2007). Whey powders containing lactose, when used as a food ingredient, provides sweetness in dry blends and browning in bakery products (Gonzalez-Martınez et al., 2002). Reduced-lactose whey protein concentrate and hydrolysate powders are popular dietary protein supplements with faster digestibility is reported in hydrolysates (Sinha et al., 2007).

2.7.2. Edible films and coatings

Whey protein edible films and coatings can be formed by heat denaturation followed by evaporation of water from thinly spread whey protein solutions. They have good mechanical properties at low humidity (Kurek et al., 2014) and act as barriers for gases and aromatic compounds (Kurek et al., 2014; Miller et al., 1998). However, they provide only moderate resistance to water due to the hydrophilic nature of proteins. Lipid-incorporated whey protein films are more hydrophobic and have improved resistance to moisture (Galus & Kadzińska, 2016).

2.7.3. As an emulsifier in food suspensions

Due to the amphiphilic nature of whey proteins (discussed in detail in section 2.11.1), they can migrate into oil-water interfaces and stabilise non-polar droplets in a polar bulk phase such as water. Therefore whey proteins are commonly used to stabilise edible dispersions of oil and water, such as salad dressings (Sanmartín, Díaz, et al., 2018; Sanmartín, Díaz Rubio, et al., 2018). The whey protein layer around an oil droplet can further be unfolded and crosslinked using chemical or physical denaturation methods such as glutaraldehyde cross-linking (Heelan

& Corrigan, 1998), thermal denaturation during spray drying (Picot & Lacroix, 2004) and dichlorodiphenyltrichloroethane- (DDT) assisted denaturation followed by application of ultrasound (Vong et al., 2014). Oil soluble nutraceutical compounds can be encapsulated in whey protein stabilised/coated oil droplets to prevent degradation when subjected to extreme thermal and pH conditions during food processing. This also enables stable release during gastronomical digestion (Diarrassouba et al., 2015)

2.7.4. Oleogels

Edible gels formed with a continuous non-polar liquid phase are of increasing interest in food applications (Co & Marangoni, 2012; Patel et al., 2014) as a solid-fat substitute with a lower saturated fatty acid content (Pehlivanoğlu et al., 2018). Although whey proteins are hydrophilic in their native form, when denatured, they can be successfully transferred into a continuous liquid oil phase using a sequence of solvent exchange steps that include centrifugation and evaporation of polar solvents. Gel formation is induced by centrifuging the final dispersion of whey proteins-in-oil to form a solid-like pellet (de Vries, Gomez, et al., 2017; de Vries et al., 2018; de Vries, Wesseling, et al., 2017). The structural characteristic of whey protein oleogels can be tuned as required, by changing the oil type to alter the polarity of the continuous phase (de Vries, Gomez, et al., 2017) and by adding water and heating to form capillary bridges between whey proteins inside the bulk oil phase (de Vries et al., 2018).

2.7.5. Yoghurt

Yogurts are produced by pH-induced aggregation of casein micelles in milk, which results from adding a starter culture that converts lactose to lactic acid. Denatured whey proteins are included in the casein gel to provide a finer, stronger network that reduces syneresis. Whey proteins can be added to yoghurt milks in the form of commercially available concentrates (Sodini et al., 2005) or denatured aggregates (Sodini et al., 2006; Torres et al., 2011). Alternatively, whey protein-fortified yoghurt milks are heat treated above denaturation temperature of whey proteins to induce casein-whey protein cross linking (discussed in detail in section 2.10.2) before inoculation with yoghurt culture (Labropoulos et al., 1981; Mottar et al., 1989).

2.7.6. Cheese

Whey protein is a major component of Ricotta, a traditional cheese produced by the heat denaturation (90 ºC) and acid precipitation (using citric acid) of whey proteins (mainly α-lactalbumin and β-lactoglobulin). It is a high-moisture, unripened cheese with a relatively high

pH (~6) that has a limited shelf-life due to its higher susceptibility to microbial contamination (Martins et al., 2010; Rubel et al., 2019). Aside from Ricotta, denatured and aggregated whey proteins can be added to casein-based cheese milk (discussed in detail in section 2.10.5) in an effort to retain them in various types of hard and semi-hard ripened cheeses, which have a longer shelf-life (Hinrichs, 2001).

As cheddar is one of the most consumed cheeses globally (Hamdy et al., 2017; Ulpathakumbura et al., 2016), there have been efforts to incorporate them into cheddar matrices (Haque et al., 2007; Stankey et al., 2017), including attempts to enhance retention by altering the size of the whey proteins (Stankey et al., 2017). These attempts are discussed in detail in the next four sections (section 2.8-2.11) with specific consideration given to casein-whey protein interactions during each processing pathway.

2.8. Current pathways to incorporate whey proteins into cheese

Different pathways used to incorporate whey proteins into cheese are depicted in Figure 2.5. Whey proteins can be incorporated into cheese either in their native form or denatured form. Microfiltration (MF) and ultrafiltration (UF) of cheese milk (discussed in detail in sections 2.9) concentrate native proteins in milk and can be used to facilitate cheese milk standardisation. To increase whey protein recovery into cheese, cheese milk can be heat treated to induce whey protein denaturation and cross-linking with casein (discussed in detail in sections 2.10.2, 2.10.3). Alternatively, whey or concentrated whey streams can be heat treated and reincorporated into cheese milk (sections 2.10.4 and 2.10.5) to incorporate whey proteins back into cheeses.

High hydrostatic pressure treatment of milk (Huppertz et al., 2004a; San Martín-González et al., 2007; Voigt et al., 2010) and transglutaminase treatment of milk (Aaltonen et al., 2014; Cadavid et al., 2020; D'Alessandro et al., 2019; Domagała et al., 2016; Pierro et al., 2010) are being studied as alternative non-thermal methods that can be used to help incorporate whey proteins in cheese. Another potential approach is using whey proteins as an emulsifier (see section 2.11) in cheese-analogues produced with non-dairy fats or oils (Tsujii & Shirotani, 2015; Ye et al., 2009), which can also include added nutraceutical compounds (Fernandes et al., 2017; Manios et al., 2017). The following section briefly describes the use of hydrostatic pressure and transglutaminase treatment of milk to incorporate whey proteins into cheese. Detailed reviews of the use of whey proteins in their native, heat-denatured and emulsified forms during cheese making are provided separately (sections 2.9-2.11), given their particular relevance to the present thesis.

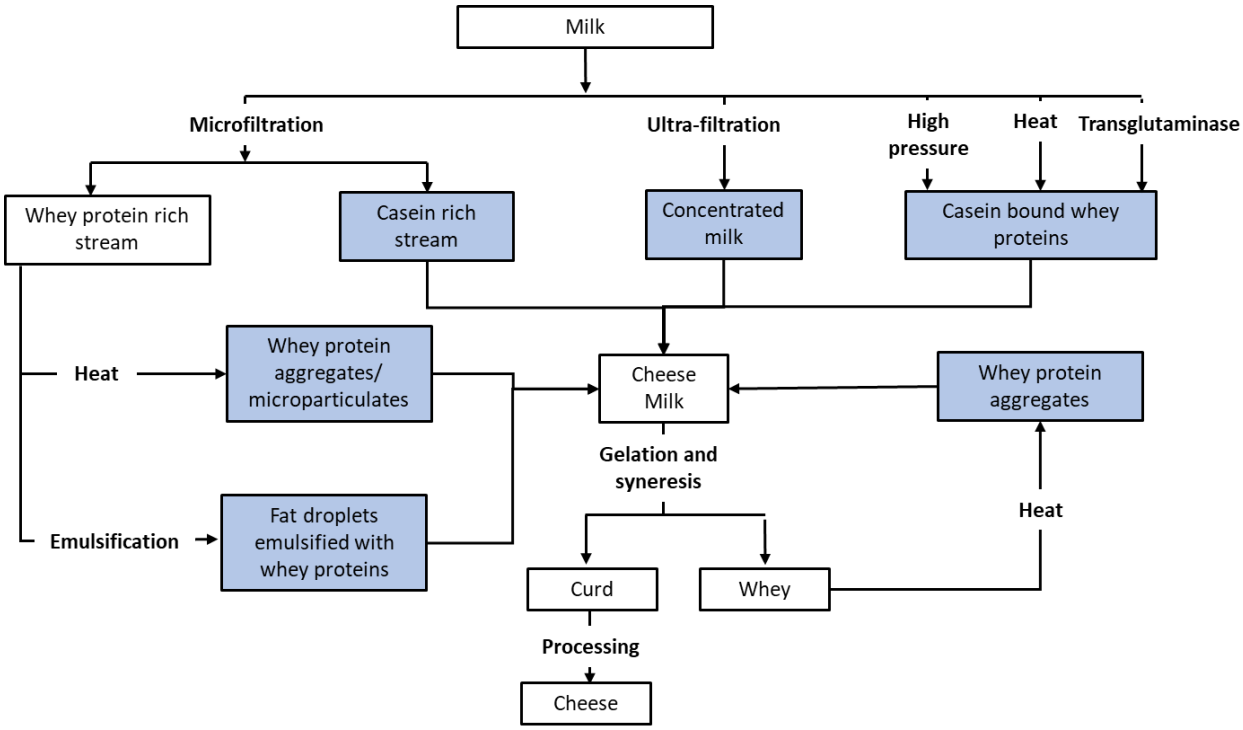


Figure 2.5: A representation of different pathways used to incorporate whey proteins into cheese.

2.8.1. High hydrostatic pressure treatment

Whey proteins denature at pressures above 400 MPa (Huppertz et al., 2004b; Lopez-Fandino et al., 1996) and a considerable amount of β-LG associates with casein micelles (Needs et al., 2000). When used for cheese making, pressure-treated milk increases cheese yield and reduces protein expulsion with whey (Huppertz et al., 2004b; Voigt et al., 2010). Details of the composition and the nutritional value of cheeses made by pressure-treated milk are provided in Chawla et al. (2011) and Santiago-López et al. (2018).

2.8.2. Transglutaminase treatment

Microbial transglutaminase (TGase) catalyses acyl transfer reactions between a γ-carboxy-amide group of protein-bound glutamine (acyl donor) and ε-amino group of lysine residues (acyl acceptor), resulting in protein polymerisation (Jaros et al., 2006). Caseins are a preferred substrate for TGase, as the active sites in native whey proteins are less accessible to the enzyme due to their compact globular structure (DeJong & Koppelman, 2002). Denaturing whey proteins by physical (e.g. heat) or chemical (e.g. adding ethanol or DDT) means improves the enzyme's access to active sites, facilitating whey protein-casein crosslinking (DeJong & Koppelman, 2002). Cheeses made with TGase-treated milk are reported to have higher yields and protein contents due to covalently incorporated whey proteins in the coagulum (Cozzolino

et al., 2003; Pierro et al., 2010). For a comprehensive review of recent advances in the application of TGase in cheese, see Gharibzahedi et al. (2018).

2.9. Incorporating native whey proteins into cheese

As previously described in section 2.5, native whey proteins in cheese milk do not actively participate in casein aggregation during rennet gelation and are lost during syneresis. Conventionally, heat denaturation of proteins in cheese milk streams is used to better incorporate whey protein into cheeses. Although heat-induced changes in whey proteins are well-documented, in typical cheese processing, milk does not undergo extensive heat treatment to cause whey protein denaturation. Nonetheless, there are still opportunities to explore how the functional properties of denatured proteins can be modified to improve the efficiency of cheese making and cheese quality.

Membrane filtration can be used to collectively concentrate all proteins in milk (using ultrafiltration) or to separate whey proteins from caseins based on their size (using microfiltration) (Maubois, 2002; Mistry & Maubois, 2017; Pouliot, 2008). This allows the formulation of cheese milk with native casein and whey proteins present at increased casein:whey ratios (CN:WP), which can increase the cheese yield and cut down the processing costs.

2.9.1. Casein-native whey protein interactions: impact on rennet gelation

To date, there have been only a few studies (Lelievre et al., 1990; Marziali & Ng-Kwai-Hang, 1986) investigating the effect of native whey proteins on the kinetics rennet gelation. Lelievre and colleagues observed an increased clotting time with added whey protein isolate (WPI), followed by an inhibition of α_{s1}-casein hydrolysis during cheese maturation; a process associated with flavour development. Although it was hypothesized that the rennet inhibition by a high molecular weight protein present in the WPI (similar to alpha-2-macroglobulin in blood serum) lead to delayed gelation, such a protein was not identified experimentally in their study.

It has been well documented that the increase in overall native protein concentration achieved by ultrafiltration of milk reduces the rennet clotting time (RCT) or the onset of gelation (Dalgleish, 1980), improves the rate of aggregation, and results in firmer gels at shorter gelling times (Garnot, 1988; Garnot et al., 1982; Mistry & Maubois, 2017; Sandra et al., 2011; Upreti et al., 2011). These changes are primarily due to the increase in casein concentration. RCT is the total time required by casein micelles to have undergone sufficient enzyme hydrolysis, to reduce the electrostatic and steric repulsions enough to allow them to interact with each other

via Brownian motion and to aggregate to a measurement extent (Dalgleish, 1980). An increase in the total protein concentration by ultrafiltration, increases the concentration of casein micelle particles, increasing their frequency of collision and reducing the RCT. Similarly, as casein aggregation is a diffusion-controlled process, an increase in protein content leads to a sharp increase in the subsequent rate of aggregation of rennetted micelles (Mistry & Maubois, 2017). The increased casein-casein interactions ultimately lead to a stronger protein matrix (Chamberland et al., 2019).

Whey protein-depleted, concentrated casein streams prepared by microfiltration are also used for cheese making (Papadatos et al., 2003a). They also undergo more rapid rennet gelation than regular milk (Maubois et al., 2001; Mistry & Maubois, 2017) due to increased casein-casein interactions. However, the effect of different concentrations of native whey proteins on the rennet gelation process have yet to be studied in isolation from changes in casein concentration, meaning that their influence is not yet understood.

2.9.2. Casein-native whey protein interactions: impact on cheese attributes

Cheese milk prepared by ultrafiltration (UF) has an increased protein concentration which results in higher curd yields (kg of curd/kg of cheese milk). However, at mild concentration factors, cheese yield and protein recovery are increased only marginally, whereas a significant increase in protein recovery is observed when the CN:WP ratio is higher (Table 2.5, Masotti et al. (2017)).

At higher concentration factors (>6x), UF cheese milk approaches the final solids contents of soft cheeses. At such high concentrations, more whey protein can remain entrapped in the stronger casein coagulum, reducing the extent of syneresis (Masotti et al., 2017). Other research has suggested that concentrated UF milk, when used during hard or semi hard cheese making, delays ripening due to the higher concentration of minerals retained in cheese and the inhibition of flavour-developing enzymes by native whey proteins (Mistry & Maubois, 2017).

Cheese milk produced via microfiltration that have an a higher than normal casein:whey protein content demonstrate lower protein loss during cheese making than regular cheese milk. However, the overall protein loss (combined losses during filtration and syneresis) is not altered (Chromik et al., 2010; Neocleous et al., 2002). When highly concentrated microfiltration retentates are used as cheese milk, it becomes increasingly difficult to cut the stronger coagulum and the cheeses lose their ‘openness’. Therefore the cheese making protocol has to be appropriately altered (Masotti et al., 2017). One such alteration is heat-treating the concentrated milk protein streams to induce casein-whey protein interactions (see section

2.10.3), which restores the 'openness' in cheese microstructure (Kethireddipalli & Hill, 2015; Masotti et al., 2017) and further improves whey protein retention (Chromik et al. 2010).

Table 2.5: Summary of studies on the use of membrane filtration for cheese milk formulation.

Cheese type	Membrane technology	Cheese milk composition	Remarks on cheese yield and composition	Remarks on cheese properties	Reference
Reduced fat cheddar	UF	CF=1.2x CN:F =1.35 CN% ~80%*	Yield = N/R Total protein content or the % water soluble N in cheese did not increase compared to the control with the same CN: fat ratio	Better flavour attributes compared to the control made with skim with same CN: fat ratio	(Agrawal & Hassan, 2007)
Low-fat Camembert type cheese	UF	CF = 6x CN~80%*	Yield = NR	Defects in cheese surface structure	(Hannon et al., 2009)
Cheddar	UF	CF = 1.1, 1.2 P:F ratio= 0.96 CN% = 79%	Faster curd formation. Reduction in moisture. Increase in cheese yield. No significant increase in the protein recovery in cheese.	NR	(Guinee et al., 2006)
Swiss	UF	CF~1.2 CN:F=0.87 CN%=78%* (Higher than the CN content in control)	Increase in nitrogen recovery in cheese.	Acceptable ripening and sensory attributes.	(Govindasamy-Lucey et al., 2011)
Gouda	MF followed by pasteurization	CF=1.5x, 2x P:F = 2:1 CN%=~84%, 89%*	No significant increase in protein recovery when MF alone was used. Increase in protein recovery when whey proteins are denatured by pasteurisation.	Acceptable slicing and sensory properties in all systems studied.	(Chromik et al., 2010)
Cheddar	MF	CF= 1.26, 1.51, 1.82 CN: fat ratio = 0.68* CN% ~ 84%, 86%, 88%*	Decrease in cheese moisture with increase in CF. MF did not change the overall cheese yield when compared against the original milk quantity.	NR	(Neocleous et al., 2002)

CF= concentration factor

NR = Not reported

CN% = Casein content as a proportion of total protein

CN:F = Casein:Fat ratio

P:F = Protein: fat ratio

*Calculated from presented data

2.9.3. Knowledge gaps in the understanding of casein-native whey protein interactions during rennet gelation

Membrane technology is a widely studied technique to help incorporate native whey proteins into cheese (Maubois, 2002; Mistry & Maubois, 2017; Pouliot, 2008). The studies performed to date have largely reported changes in final cheese yields and other cheese attributes in relation to cheese milk formulated with modified protein content and CN:WP ratios using membrane filtration (Table 2.5). In comparison, there is still a limited understanding of the effect of whey proteins on the rennet gelation process, and how this can be affected by altered CN:WP protein ratios. Developing this understanding requires an experimental approach that can account for other changes in the complex milk microenvironment (pH, soluble calcium content, viscosity) that can be altered when cheese milk is formulated with varying native CN:WP protein ratios. One previous study (Lelievre et al., 1990) revealed that native whey proteins impaired the rate of overall rennet gelation. However, the role of whey proteins on the enzyme hydrolysis and the aggregation phases of rennet gelation along with the impact on milk microenvironment was not fully elucidated. As the rennet gelation step accounts for a significant time period during the cheese making process, there is a need to understand the mechanisms by which native whey proteins interact with casein micelles and rennet enzyme in order to best utilise membrane technology for improved cheese making.

2.10. Incorporating denatured whey proteins into cheese

2.10.1. Thermal denaturation of whey proteins

Whey proteins have native globular structures that are subject to thermal denaturation. Whey proteins unfold their native tertiary confirmation, when subjected to temperatures above their denaturation temperature (T_d). This results in the exposure of reactive groups such as hydrophobic residues (Moro et al., 2001) and thiol groups (Monahan et al., 1995) buried in the globular core to the surrounding environment. The resulting 'hydrophobic effects' leads to the aggregation of whey proteins (Gosal & Ross-Murphy, 2000). The exposed thiol and disulphide groups interact with similar groups in caseins (κ-casein), whey proteins (α-LG,β-LG and BSA) or both and undergo thiol-disulphide interchange or thiol-thiol oxidation reactions to form covalently bonded aggregated polymeric complexes (Dissanayake et al., 2013; Monahan et al., 1995; Vasbinder et al., 2004). At higher protein concentrations, these aggregates interact to form a gel. In general terms, both whey protein unfolding, and the subsequent aggregation are collectively considered as whey protein denaturation.

As whey protein denaturation is an endothermic process T_d is generally evaluated by differential scanning calorimetry, with the protein heated at a constant heating rate (ºC/min). The temperature at the maximum heat of absorption is considered as the temperature of conformational transition, or the denaturation temperature (Rüegg et al., 1977). T_d is different for individual whey proteins differ depending on environmental properties that influence the tertiary structure (Table 2.6). Under conditions similar to native milk (e.g. pH~6-7, in a mineral buffer), BSA has a lower denaturation temperature than α-LA and β-LG (shaded rows in Table 2.6). In contrast, Ig undergoes a two-stage denaturation between 60-67 ºC and 70-74 ºC at pH 8.1 (Vermeer & Norde, 2000).

However, the extent of protein denaturation (percentage of whey proteins that undergo thermal unfolding and aggregation) is only partly determined by the protein structure. Differences in the initial concentrations of individual whey proteins in milk and the heating rate also play a role. This is because, unfolded proteins have to intimately interact with each other for hydrophobic and thiol mediated aggregates to form. For example, thermal treatment of milk at 80 ºC at its natural pH (pH~6.5) for 5 minutes, denatures >90% of BSA and Ig, while only a ~70% and 40% α-LA and β-LG (which are higher in concentration) are denatured (Law & Leaver, 2000). However, higher temperature, prolonged heat treatments (e.g. >80 ºC for 30 min) are reported to cause a significantly high extent of denaturation (>75%) of all the whey proteins in milk (Larson & Rolleri, 1955), possibly due to the provision of sufficient kinetic energy and interaction time for unfolded proteins.

Studies on the effect of heating rate on the extent of whey protein denaturation, aggregation and gel formation reveal that above the denaturation temperature, slow heating rates result in lower aggregation and gelation temperatures than it would at a faster heating rate (De Wit, 1990; Li et al., 2006). This is due to the sufficient time available for unfolded proteins to interact with each other (Li et al., 2006).

Whey protein denaturation is important in manufacturing whey protein-rich cheese in 2 ways:

1. Whey proteins, when denatured in the presence of caseins, bind to the casein micelle via disulphide bridging and can be actively incorporated into the casein matrix during rennet gelation. These will be discussed in detail in sections 2.10.2 and 2.10.3 of the thesis.
2. Whey-proteins, when denatured in the absence of casein, interact with each other via Brownian motion and form whey protein aggregates via hydrophobic and thiol interactions. These aggregates are larger than native whey proteins and therefore better

retained in the casein matrix during syneresis. These will be discussed in detail in sections 2.10.4, 2.1.5 of the thesis.

Table 2.6. Denaturation temperatures (T_d) (as measured by calorimetric methods) of different whey proteins reported in literature. SMUF= Simulated milk ultrafiltrate.

Protein	System parameters	T_d (^{0}C)	Reference
β-LG	0.8-3 mg/mL protein, pH =2, 0 M Urea, 0.1M KCl/HCl	78	(Griko & Privalov, 1992)
	0.8-3 mg/mL protein, pH =2, 2 M Urea, 0.1M KCl/HCl	70.8	
	0.8-3 mg/mL protein, pH =2, 4.4 M Urea, 0.1M KCl/HCl	56	
	0.8-3 mg/mL protein, pH =2, 0 M Urea, 0.1M NaH_2PO_4	85	
	0.8-3 mg/mL protein, pH =2, 2 M Urea, 0.1M NaH_2PO_4	81.3	
	0.8-3 mg/mL protein, pH =2, 4.4 M Urea, 0.1M NaH_2PO_4	68.6	
	0.8-3 mg/mL protein, pH =2, 0 M Urea, 0.2M NaH_2PO_4	91	
	0.8-3 mg/mL protein, pH =2, 2 M Urea, 0.2M NaH_2PO_4	84	
	0.8-3 mg/mL protein, pH =2, 4.4 M Urea, 0.2M NaH_2PO_4	76	
	0.8-3 mg/mL protein, 3.4 M Urea	-3	
	0.8-3 mg/mL protein, 4 M Urea	4	
	0.8-3 mg/mL protein, 4.4M Urea	6.3	
	10-40mg/mL protein, pH-8.5, 70mM Phosphate buffer	~64-65.5	(Qi et al., 1995)
	<10 mg/mL or >40 mg/mL protein, pH-8.5, 70mM Phosphate buffer	~66-70	
	10-40mg/mL protein, pH-6.5, 70mM Phosphate buffer	72-74.5	
	<10 mg/mL protein, pH-6.5, 70mM Phosphate buffer	74.5-77.5	
	>40 mg/mL protein, pH-6.5, 70mM Phosphate buffer	74-75	
	3.5-7 mM protein. pH=6.6, 0.2M Phosphate buffer	~78-79.7	(Gotham et al., 1992)
	1 mg/mL protein, pH 6.6 SMUF	78.2	
	1 mg/mL protein, pH 6.6 SMUF with iodate	71.2	
	1 mg/mL protein, pH 2.5 SMUF	83	
	1 mg/mL protein, pH 2.5 SMUF with iodate	80	
α-LA	0.3-0.7 mg/mL protein, 1mM $CaCl_2$, pH 3-6	~11-65	(Griko et al., 1994)
	0.3-0.7 mg/mL protein, 1mM $CaCl_2$, pH 6-7	~69	
	0.3-0.7 mg/mL protein, 1mM $CaCl_2$, pH 7-9	~65-69	
BSA	pH 6.6 SMUF	68.3	(Gotham et al., 1992)
	pH 6.6 SMUF with iodate	67.1	
	2-5 mg/mL (globulin free), pH 5.5 water	68-70	(Michnik, 2003)
	2-5 mg/mL (globulin and fatty acid free), pH 5.5 water	55.9-57.3	
	20 mg/mL, 0.01M phosphate buffer, 0.15M NaCl, pH 7-8	~60-64	(Giancola et al., 1997)
	20 mg/mL, 0.01M phosphate buffer, 0.15M NaCl, pH 6	~67	
Ig	6 mg/mL protein, 10 mM phosphate buffer, pH 8.1 Has a two-stage denaturation (Higher enthalpy at lower temperature and vice-versa)	~60-67 and 70-74	(Vermeer & Norde, 2000)

2.10.2. Whey protein denaturation in the presence of caseins: the impact on rennet gelation

As previously discussed, thermal denaturation of whey proteins in the presence of casein, results in complex formation among β-LG, α-LA and κ-casein (Donato & Guyomarc'h, 2009; Jang & Swaisgood, 1990; Mleko & Foegeding, 2000; Mulvihill & Donovan, 1987). The association of whey proteins (β-LG in particular) with casein is pH-dependent, with a higher degree of association observed in acidic conditions (Anema & Klostermeyer, 1997; Vasbinder & De Kruif, 2003). Approximately 80% of β-LG and 35% of α-LA were found to associate with casein micelles at pH 6.35 when heat treated at 80 ºC for 10 min (Vasbinder & De Kruif, 2003). Although, casein-whey protein complexes are formed on the micelle surface in acidic conditions (where the net surface charge of the casein micelles is lower and the micellar structure is more compact), in alkaline conditions (where the net charge on the casein micelles is higher and the micellar structure is swelled) such complexes are found in the serum phase (Vasbinder et al., 2003).

Heat induced casein-whey protein (CN-WP) interactions were found to increase the gel strength and decrease the clotting time during acid coagulation of casein (Cho et al., 1999; Lucey et al., 1999; Vasbinder & De Kruif, 2003). However, such interactions have also been found to delay rennet gelation (Singh et al., 1988; Singh & Waungana, 2001; Vasbinder & De Kruif, 2003). Although it was initially believed that disulphide bridging impaired κ -casein hydrolysis, a more recent study has shown that heat treatment delays the aggregation of the renneted casein micelles (para-casein micelles) rather than κ-casein hydrolysis (Vasbinder et al., 2003). A plausible explanation for this could be the formation of a whey protein coating on the casein micelle surface (Vasbinder et al., 2003) that increases the size (Anema & Li, 2003), reduce the speed of diffusion, and changes the functional properties of the casein micelle surface. Heat treatment of milk in alkaline conditions to induce casein-whey protein complexes in the serum (rather than on the micellar surface) can minimize impairment of rennet gelation (Guyomarc'h, 2006; Masotti et al., 2017).

Results that contradict the observations of impaired rennet gelation induced by heat treatment of milk are also reported in literature. Meza-Nieto et al. (2007) reported that supplementation of reconstituted skim milk powder with whey protein prior to heating at 65 °C for 30 min resulted in firmer rennet gels (Meza-Nieto et al., 2007). Although the degree of heat treatment used was shown not to result in extensive attachment of denatured whey protein to the casein micelles, the increased gel strength was attributed to the cross-linking between the casein

micelles by whey proteins (Meza-Nieto et al., 2007). A more likely explanation, however, is that the addition of whey powder simply increased the soluble calcium levels, which were not standardised, and are otherwise lacking in reconstituted skim milk powder (Martin et al., 2007), thereby limiting the rate of rennet gelation (Martin et al., 2008).

2.10.3. Whey protein denaturation in the presence of caseins: the impact on cheese attributes

Heat induced association of whey proteins and casein micelles is used to effectively incorporate whey proteins into cheese at the expense of longer gelation times (Hougaard et al., 2010; Masotti et al., 2017). The increased yield (Atasoy et al., 2008) is also partly due to the water binding ability of the whey proteins that reduces overall syneresis (Hougaard et al., 2010; Makhal et al., 2015; Rynne et al., 2004). High moisture makes the cheese curds brittle, less firm and less stretchable (Hougaard et al., 2010; Rynne et al., 2004), which can be advantageous when smoother textures are desired. Alternatively, concentration of cheese milk prior to heat treatment by micro-filtration (Chromik et al., 2010; Schreiber, 2000), or the addition of calcium chloride after heat treatment (Makhal et al., 2015) makes coagulation faster, increases the whey protein retention in cheese, while maintaining a firmer texture.

A summary of recent studies of cheese attributes as affected by the heat treatment of cheese milk is provided in table 2.7.

Table 2.7: Recent studies on thermal treatment of cheese milk and it effect on cheese properties.

Cheese type	Heat treatment conditions	Cheese milk composition	Remarks on cheese yield and composition	Remarks cheese properties	Reference
Urfa	65 °C for 20 min 72 °C for 5 min	Protein ~ 3.6% P:F~0.86*	Increase in the yield with thermal treatment. Increase in the soluble protein content with higher heat treatment.	Higher temperature treatment adversely affected sensory properties of cheese	(Atasoy et al., 2008)
Havarti type	72 °C, 100 °C and 120 °C for 2s	P:F~0.86*	Yield: N/R Impaired rennet gelation with the increase in the treatment temperature. Increase in moisture content Increase in the α-la and β-lg content in cheese	Lower strain at fracture “Short’/brittle texture Whiter in colour	(Hougaard et al., 2010)
Half-fat cheddar	77 °C, 82 °C and 87 °C for 26s	Protein 3.36% P:F=2.27	Yield = N/R Increase in moisture content Decrease in the protein content	Lower stress at fracture and flowability with increase in temperature	(Rynne et al., 2004)

Cottage cheese	85, 90 and 95 °C for 5 min with added $CaCl_2$	Skim milk with 8.4% SNF	Increase in the yield, total protein, moisture and whey protein content in cheese	A fragile texture at higher temperature treatments alone. Addition of calcium restored the firm texture	(Makhal et al., 2015)
Gouda	MF with 72 ^{0}C for 15s	CF: 1,1.5, 2 P:F = 2:1	Increase in the protein and β-lg content	Satisfactory sensory quality and slicing behaviour	(Chromik et al., 2010)

P:F = Protein: Fat ratio

SNF=Solid non-fat

*Calculated using data presented for experimental and control cheeses

2.10.4. Whey protein denaturation in the absence of caseins: the impact on rennet gelation

Another approach to incorporate whey proteins into cheese is denaturing them in the absence of casein to form sufficiently large whey protein aggregates via hydrophobic and disulphide bridges. These aggregates could potentially remain trapped in the casein matrix during syneresis. As discussed in section 2.10.1, high-temperature long-term heat treatments enable complete denaturation and aggregation of whey proteins. In order to avoid dilution of casein in cheese milk, the added whey protein streams need to be concentrated. However, heat treatment of concentrated whey protein streams leads to increased whey protein-whey protein interactions and the formation of larger aggregates and gels (Table 2.8) that cannot be homogeneously distributed during cheese milk formulation. Therefore, concentrated whey protein slurries after heat treatment are disintegrated into so-called microparticulates (~1 µm) using high-pressure homogenisation (Iordache & Jelen, 2003; Suárez et al., 2016) during cheese milk formulation. However a prior mechanical stirring step is required to improve the flowability of viscous, non-Newtonian streams of concentrated heat-denatured whey proteins, during valve homogenisation (Iordache & Jelen, 2003; Suárez et al., 2016). Alternatively, ultrasound assisted particle size reduction (see section 2.12.1) has the advantage of directly processing such streams.

Detailed studies of the impact of aggregated whey proteins on the kinetics of rennet gelation are limited. Perreault, Morin, et al. (2017) and Giroux et al. (2015) observed that aggregates of denatured whey proteins impaired the rate of rennet gelation; smaller aggregates more so than larger ones. Giroux et al. (2015) hypothesised that smaller aggregates (~41 nm), which are increasingly hydrophobic, adsorb onto hydrophobic hydrolysed casein micelles and restrict casein-casein interactions during aggregations. Contrastingly, Perreault, Morin, et al. (2017)

observed that the soluble fraction of denatured whey proteins that were less hydrophobic than larger aggregates, impaired rennet gelation more than larger aggregates (~8µm); suggesting that there is another mechanism apart from surface hydrophobicity that leads to the impaired rennet gelation.

Table 2.8. Change in whey protein aggregate size and heat-set gel strength as a function of protein concentration at constant temperature-time treatment combinations.

Temperature (^{0}C)	Time (min)	Protein concentration (mg/mL)	Aggregate size (um)	Reference
68.5	24 h	30	0.92	(Alting et al., 2003)
		45	0.122	
		60	0.301	
		75	0.305	
		90	0.322	
80	15 min	50	0.041	(Giroux et al., 2015)
		100	0.069	
		150	0.146	
		200	0.299	
Temperature (°C)	Time (min)	Protein concentration (mg/mL)	Gel strength (G')	Reference
90*	20 min	~28	~6.3	(Puyol et al., 2001)
		~44	~158	
		~111	~6310	

*Includes gradual heating followed by holding at 90 °C for 20 min.

2.10.5. Whey protein denaturation in the absence of caseins: the impact on cheese attributes

While there are limited studies investigating the effect of denatured whey protein aggregates on the kinetics of rennet gelation, the effects of denatured whey proteins on the composition, textural and sensory properties of cheese have been more widely studied.

Whey protein aggregates have been incorporated into cheese milk to investigate changes in the sensory attributes and composition of cheese (Banks & Muir, 1985; Brown & Ernstrom, 1982; Punidadas et al., 1999). Although cheese yield increased, there was significant fat loss (Baldwin et al., 1986; Brown & Ernstrom, 1982; Punidadas et al., 1999) unless the whey protein stream itself contained fat that interacted with proteins during heating and so could be

retained in the cheese matrix (Banks & Muir, 1985). Further, the observed increases in cheese yields were largely accompanied by increases in moisture (Banks & Muir, 1985; Brown & Ernstrom, 1982; Punidadas et al., 1999), owing to the water binding ability of denatured whey proteins (Hinrichs, 2001) and/or the casein content (Banks & Muir, 1985) in cheese.

Reports of increased yields in conjunction with increased whey protein retention are limited in the literature. This is possibly due to the whey protein aggregates being too small to be retained within the cheese matrix. Although aggregate size was not reported, these studies employed dilute whey protein streams (Banks & Muir, 1985; Brown & Ernstrom, 1982; Punidadas et al., 1999) and/or mild heat treatments (Brown & Ernstrom, 1982), which typically result in relatively small aggregates after heat treatment (Roefs & De Kruif, 1994).

More recent studies aimed at incorporating denatured whey proteins into cheese have involved 'microparticulation' technology, in which concentrated whey protein streams produced by membrane technology are subjected to severe heat treatment to form larger aggregates that are then disintegrated into smaller sizes (~1 µm) using high pressure homogenisation (Suárez et al., 2016). Microparticulate-incorporated cheeses have a higher moisture content (Di Cagno et al., 2014; McMahon et al., 1996; Perreault, Rémillard, et al., 2017), commonly attributed to the higher moisture binding ability of denatured whey proteins (Hinrichs, 2001). However, higher moisture contents have been reported even without significant increases in the protein content of (Di Cagno et al., 2014) or retention (Perreault, Rémillard, et al., 2017) in cheese. The higher moisture content provides a softer texture to otherwise firm semi-hard cheeses, making microparticulated whey protein a popular fat replacer (McMahon et al., 1996; Schenkel et al., 2013; Stankey et al., 2017; Sturaro et al., 2015). Nonetheless, the extent of whey protein retention in microparticulate-incorporated cheese is questionable. While Perreault, Rémillard, et al. (2017) and Di Cagno et al. (2014) reported a reduction in the protein retention and content in cheese, Stankey et al. (2017), Schenkel et al. (2013) and Sturaro et al. (2015) reported that the protein recovery was not changed. These variations could be attributed to the differences in the size of the microparticulates used, cheese milk formulations and the cheese making processes used. Detailed outcomes of cheese attributes as affected by incorporating whey protein aggregates into cheese milk are represented in table 2.9.

With increased interest in incorporating whey protein particulates in cheese matrices to alter their composition and texture, a comprehensive study that bridges the knowledge of the influence of physico-chemical properties denatured whey protein on gelation and subsequent

changes in cheese attributes is of interest in the context of optimising the 'macroparticulation' technology.

Table 2.9: A comparison of cheese attributes as affected by incorporating whey protein aggregates into cheese milk.

Cheese type	Heat treatment conditions and composition of the whey stream	Cheese milk characteristics	Remarks on cheese yield and composition	Remarks on cheese properties	Reference
Cheddar	Cheddar whey concentrated to 4.3-7.1% protein. 75 ºC for 30 min.	P:F~N/R. CN%=N/R. WP size: N/R	4.1% increase in cheese yield. 5.1% increase in moisture content. No significant increase in protein content. 1% decrease in fat content.	No colour or body defects Increase in acidity	(Brown & Ernstrom, 1982)
Cheddar	95 ºC for 20 min. 0.9% w/w protein	P:F~N/R. CN%=N/R. WP size: N/R	Increase in yield, moisture, fat and casein content in cheese with the increase in the levels of added whey	Development of slight off flavours	(Banks & Muir, 1985)
Mozzarella	95 ^{0}C for 5-7 min followed by homogenization. Mozzarella cheese whey.	P:F~N/R. CN%=N/R. 5%, 10% added WP. WP size: N/R. (Large variation in the extent of WP denaturation)	12.8% increase in cheese yield with a further (~18%) increase with homogenization*. 12.7% increase in moisture content*. 10.5%, 2.5% decreases in protein content. 18.4%, 15.6% decreases in fat content*.	Smooth meltability in all cheeses. All sensory properties were on the commercially available levels.	(Punidadas et al., 1999)
Cheddar	70 ºC for 15 minutes. 5.25% or 8.25% protein. Large variation in the extent of denaturation	P:F~N/R. CN%=N/R. 5%, 10% added WP. WP size: N/R (Large variation in the extent of WP denaturation).	~6.5% increase in yield when cheese milk had ~10% WP aggregates denatured at a higher WP concentration*. 2.7%-14.2% increases in moisture in all cheeses with added WP*. 2.2% and 4.6% increases in the protein content in cheeses with 10% added WP*. Fat loss in systems with added WP*.	Pasty, sticky texture of cheese with added WP.	(Baldwin et al., 1986)

Low fat cheddar	Commercially available microparticulate powder produced by heating at 85–90 °C	P:F~4.9-5*. CN%~81%* WP size: N/R 0.15%, 0.35%, 0.5% of added whey	No change in nitrogen recovery. No change in solids recovery. Reduction in fat recovery with increasing levels of added whey (4.1% reduction with 0.5% of added whey)*. 6.5% increase in actual cheese yield with 0.5% of added whey*. Improved yield was due to the water binding ability of denatured whey proteins.	Softer but less meltable. No noticeable sensory off flavours	(Stankey et al., 2017)
Reduced fat Gouda	Commercially available microparticulate concentrate (Simplesse) (53% protein)	P:F= 1.1, 2,10 CN%: NR WP size: N/R	No change in moisture, protein and fat contents in full fat, reduced fat and low fat cheeses with 1% w/w of microparticulate concentrate. Yield: N/R	Increased meltability.	(Schenkel et al., 2013)
Low fat Mozzarella	Commercially available microparticulate concentrate (Simplesse)	P:F= NR CN%: NR 0.6g Simplesse per kg of standardized low fat mozzarella cheese milk. WP size: 0.5-1 µm	~4.3% increase in moisture content.* Yield, protein, fat contents: N/R.	No change in the openness of cheese. Better meltability. Protein microparticulates were too small to create large enough gaps or form large serum channels in the casein structure of mozzarella cheese.	(McMahon et al., 1996)
Low-fat Caciotta-type cheese		Fat: 0.3% CN%: NR 0.5 wt/vol Microparticulate whey per semi skim cheese milk. WP size: N/R	~11% increase in yield* ~ 12% reduction in protein content* No change in fat content ~7.2% increase in moisture*	Increase in the free amino acids during ripening. Acceptable sensory attributes	(Di Cagno et al., 2014)

Caciotta cheese		P:F= 0.8, 0.9, 1 CN%: NR 2%, 3%, 4% MWP added to cheese milk WP size: N/R	No change in yield, fat, protein or moisture recovery in cheese at p=0.05	N/R	(Sturaro et al., 2015)
Not specified	Spray dried denatured whey protein concentrate	P:F= 0.8, 0.9, 1 CN%: NR 0.25%, 0.5%, 0.75% MWP added to cheese milk WP size: 6μm	>0.5% reduction in protein retention*. No change in fat retention 5.3% - 14% increase in moisture content*. 4.6%-14.8 increase in cheese yield*	Presence of whey protein aggregates were visualized using microscopy	(Perreault, Rémillard, et al., 2017)

*Numerical values calculated from data presented in each publication

P:F = protein:fat ratio

CN% = casein content as a percentage of total protein

W = whey protein aggregates

2.10.6. Knowledge gaps in the understanding of casein-denatured whey protein aggregate interactions during rennet gelation and cheese making

Studies conducted to date on incorporating aggregated whey protein in cheese milk have largely reported changes in terms of cheese yield, composition and texture (Table 2.9). Limited research has been conducted to investigate the effect of whey protein aggregates on the kinetics of rennet gelation, and has suggested contradictory mechanisms. Giroux et al. (2015) suggested that whey protein aggregates participate in hydrophobic interactions with cleaved casein micelles and impair the rate of rennet gelation, however more recent works by Perreault, Morin, et al. (2017) observed that the less hydrophobic soluble fractions of denatured whey proteins impairs rennet gelation more than the more hydrophobic sedimentable aggregates, suggesting that there is another mechanism other than just surface hydrophobicity that leads to an impaired rennet gelation.

Studies conducted to date have incorporated relatively low amounts of denatured whey proteins (<10%) into cheese milk containing fat. However, addition of whey proteins is reported to cause an increase in cheese moisture content and fat loss during cheese making (Table 2.9), making it difficult to attribute changes in the cheese properties to whey protein retention alone. One reason for not using higher whey protein contents during cheese milk formulation may be

the dilution of casein content in cheese milk by the addition of dilute whey protein streams, which leads to prolonged clotting times.

However, thermally denatured concentrated whey protein streams form larger whey protein aggregates and gels that are difficult to further process (Table 2.7). Power ultrasound processing is an emerging technology that enables particle size reduction in slurries and gels. It can also be a useful tool to formulate cheese milk containing whey protein particulates of controlled size using thermal and power ultrasound treatments and properties to investigate the mechanism by which denatured whey proteins interact with casein micelles during rennet gelation and cheese formation.

2.11. Incorporating whey proteins as an emulsifier during cheese making.

Milk fat is a key structural component in cheese and is present in milk as an emulsion, with the droplets' surface stabilised by a phospholipid-rich membrane. Milk fat provides the characteristic buttery aroma of cheese and is high in saturated fatty acids (Leong et al., 2019). Milk fat is increasingly replaced by alternatives such as sunflower oil, canola oil or olive oil that are less expensive as well as rich in healthier poly-unsaturated fatty acids (including ω-3 and ω-6) (Chouinard et al., 1998; Ramos et al., 2009). In addition, hydrophobic nutraceuticals are added to cheese milk to form 'functional' cheese (Boivin-Piché et al., 2016; Crevier et al., 2017; Fernandes et al., 2017; Manios et al., 2017). These oils and other hydrophobic ingredients need to be emulsified to ensure proper dispersion in the cheese milk.

2.11.1. Emulsifying properties of whey proteins

Emulsification is the process of creating a stable dispersion of two immiscible phases (one hydrophobic and one hydrophilic); typically, water and oil. Emulsions can be created by applying high-energy shear to an oil-water mixture to form droplets of the dispersed phase in the bulk phase. Addition of an emulsifier, which is an amphiphilic molecule that can adsorb at the oil-water interface, is necessary to reduce the interfacial tension of the emulsion droplets and to create steric and electrostatic repulsion to prevent coalescent between the dispersed droplets (Falkeborg & Guo, 2015; Kentish et al., 2008).

Milk proteins are amphiphilic biopolymers that can be used to stabilise food emulsions. Hydrophobic residues and regions on the surface of a protein can participate in hydrophobic interactions with the surface of oil droplets (Yamauchi et al., 1980).

The structure of adsorbed layers of different milk proteins can be differentiated owing to the disordered flexible structure of caseins and the compact globular structure of whey proteins

(see sections 2.1.1 and 2.1.2 for the structure of milk proteins). When adsorbed, caseins may form an entangled monolayer of flexible chains, with some hydrophobic segments in contact with the oil surface and others protruding into the aqueous medium. In contrast, interfacial monolayers formed by compact globular whey proteins can be described as two-dimensional assemblies of deformable close packed particles. However, at lower surface coverages, whey proteins may undergo partial unfolding (Dickinson, 2001).

The selective adsorption of different whey proteins onto an oil-water interface relies on the relative abundance of exposed hydrophobic groups. Therefore, pH-dependent conformational changes in whey proteins lead to changes in the relative absorbance of whey proteins. The maximum relative abundance of α-LA, β-LG and BSA on the oil-water interface occur at pH 3-5, 7-9 and 5-7 respectively (Yamauchi et al., 1980). Interfacial layers composing of β-LG are rather dense and thin (2–3 nm at neutral pH) (Atkinson et al., 1995). Immediately after adsorption, they exist as a monolayer of adsorbed particles that gradually unfold, strengthening the monolayer over time with non-chemical interactions and gradual formation of disulphide bonds (observed after 72 hrs) (Dickinson & Matsumura, 1991).

2.11.2. Interactions between whey protein-stabilised fat and casein micelles during rennet gelation

It has been shown that whey protein-stabilised fat droplets act as a filler, but do not directly interact with casein micelles during rennet gelation. They are mobile through the casein matrix even after a macroscopic gel is formed (Gaygadzhiev et al., 2011). However, faster gelation (Gaygadzhiev, Corredig, et al., 2009; Gaygadzhiev et al., 2011; Gaygadzhiev, Hill, et al., 2009) and finer casein micelle aggregate structures (Gaygadzhiev, Corredig, et al., 2009) have been reported when the fraction of emulsified fat present in the cheese milk is increased. This was attributed to the decrease in the "free" space available for casein micelles to form a gel network, as the oil fraction increases (Gaygadzhiev, Corredig, et al., 2009).

Due to the very small amount of whey protein required to emulsify fat droplets (e.g. 3% whey protein concentrate in the aqueous phase is required to form an emulsion with 20% oil (Kaltsa et al., 2013)), incorporating whey proteins as an emulsifier alone during cheese milk formulation may not substantially improve the whey protein content cheese. Therefore, this thesis will investigate the possibility of encapsulating concentrated aqueous solutions of whey proteins inside oil droplets that are then emulsified using whey proteins in a process known as double emulsification (discussed in detail in subsequent 2.12.3 section). This would enable

effective utilisation of whey proteins as an emulsifier, while simultaneously improving the whey protein content of the resulting cheese.

Ultrasound emulsification is an emerging technology that utilises the physical effects of ultrasound to form stable water in oil in double emulsions (Leong et al., 2020; Leong, Zhou, et al., 2017; Leong, Zhou, et al., 2018). The following section will review the applications of power ultrasound in dairy industry. Specific consideration is given to ultrasound-assisted protein aggregate size reduction and ultrasonic emulsification that are of interest in the present study

2.12. Power ultrasound as an emerging technology in dairy industry

Power ultrasound is an emerging technology in dairy industry with potential applications in cheddar cheese production. Sound waves of frequency beyond the upper limit of human hearing, generally from 20 kHz to several MHz, are referred to as ultrasound. Ultrasound (US) is broadly classified into low-frequency US (20 to 100 kHz), intermediate frequency US (100 kHz to 1 MHz) and high-frequency US (1 to 10 MHz). When ultrasound is applied to a liquid medium, alternating cycles of positive and negative pressure occur in the medium. When the instantaneous negative pressure exceeds the ambient pressure, the solubility of dissolved gases in the liquid is severely reduced, leading to air bubble generation and oscillation (Li et al., 2019). This phenomenon is referred to as ‘acoustic cavitation’. ‘Acoustic bubbles’ thus formed continue to oscillate and grow in compression and rarefaction cycles of the sound wave, and collapse adiabatically upon reaching their critical size (Leong et al., 2011) (Figure 2.6).

Cavitation bubbles collapse at a timescale of milliseconds (Barber & Putterman, 1991), and create sudden area of localised high fluid velocity, temperature and pressure changes (e.g. a few hundred meters per second, a several thousand K, and a thousands of atmospheres of pressure). As a result, physical effects such as shear, shockwaves, localised heating-cooling and micro-jetting occur in the liquid medium (Ashokkumar, 2010). In addition, the extreme thermal environments generated close to the collapsing bubble leads to the generation of a variety of chemical reactions via the generation of highly reactive radicals (Ashokkumar, 2010; Ashokkumar & Mason, 2000). The physical effects of US are prominent at low frequencies (20-40 kHz), while the chemical effects are more prominent at high frequencies (300-500 kHz) (Ashokkumar & Mason, 2007). Low-frequency high-intensity ultrasound generates strong shear and mechanical forces, therefore frequencies in the range 20–40 kHz and applied power intensities >10 W/cm^2 are widely used for industrial applications requiring shear and mixing (Mason & Peters, 2002).

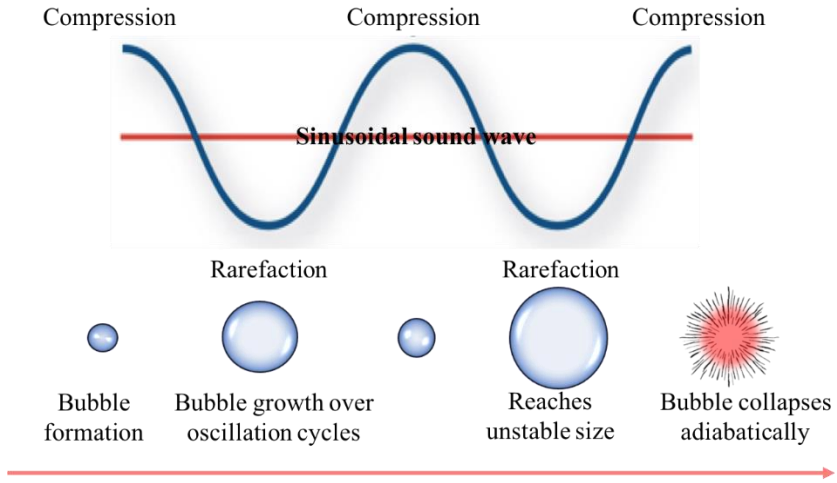


Figure 2.6: Acoustic bubbles oscillate and grow during compression and rarefaction cycles of the sound wave and collapse adiabatically (Reproduced with permission from Li et al. (2019), copyright Elsevier).

Micro jetting and shear are important physical effects of acoustic cavitation used in food processing (Li et al., 2019). In the presence of a solid, immiscible liquid or gas interface in the medium, acoustic bubbles undergo asymmetric collapse, forming microjets that penetrate inside and puncture the interface with high speed and pressure (Figure 2.7) (Li & Ashokkumar, 2018). Further, strong shear forces that accompany cavitation, force particles and molecules to collide at a greater force. These phenomena commonly cause aggregate/droplet fragmentation, emulsification (Shanmugam and Ashokkumar (2014); Leong et al. (2009)) and particulation: Monroy et al. (2018); Deshpande and Walsh (2018)), morphological changes in food components (Ibrahim et al., 2015; Sato, 2001) or particle/bubble detachment from surfaces (e.g. for membrane cleaning: Muthukumaran et al. (2006)). These effects can be exploited during food processing, for instance where modification at liquid-solid or liquid-gas interfaces are required.

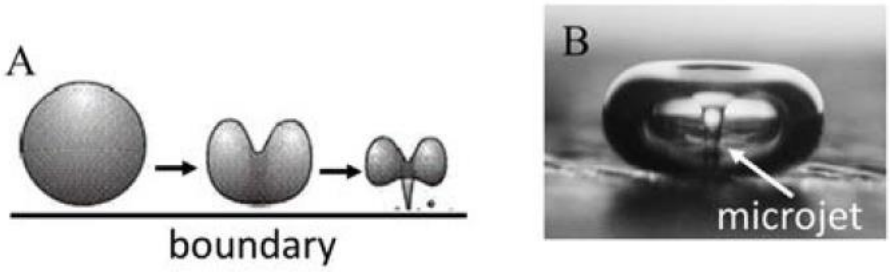


Figure 2.7:A schematic representation (A) and an image (B) of asymmetric bubble collapse and microjet formation near an interface (Li & Ashokkumar, 2018).

2.12.1. Ultrasound-assisted particle size reduction in dairy systems

Particle collision and abrasion resulting from the physical effects of ultrasound can break aggregates resulting from weaker intermolecular forces such as hydrophobic and van der Waals forces (Tian et al., 2004). Protein aggregates that rely on such intermolecular forces can be disintegrated by high-power sonication, providing ultrasound the ability to homogenise protein suspensions (Munir et al., 2019). For instance, Zisu et al. (2011) sonicated reconstituted whey protein concentrate (WPC) solutions (5% w/w) at 20 kHz and 0.52 W/mL, which reduced the mean particle size from 200 nm to ~145 nm within 5 minutes. In another study, O'Sullivan et al. (2014) reduced the aggregate size of reconstituted whey protein isolate (WPI) suspensions by 50% by applying 20 kHz ultrasound at 0.78 W/mL for 2 min. Similarly, aggregate size reductions have been reported with milk protein concentrate solutions (20 kHz, 300W electric power) (Yanjun et al., 2014), sodium caseinate solutions (0.78 W/mL, 20 kHz) (O'Sullivan et al., 2014) and denatured casein-whey protein mixtures (ultrasound conditions - 20 kHz, 0.26 W/mL for 1 min) (Leong, Walter, et al., 2018). Jambrak et al. (2014) observed that ultrasonication (20 kHz, 0.49‑0.54 W/mL or 43-48 W/cm^2) using a probe with a narrow but intense cavitation region, had a more profound effect on the aggregate size of WPC than bath sonication (40 kHz, 1 W/cm^2). However, information presented by Jambrak et al. (2014) on applied sonication energy and bulk power intensity are not adequate to directly compare the effect of localised intensity conveyed by different emitting geometries (narrower ultrasound emitting region in horn vs. broader ultrasound emitting region in the bath) on the extent of aggregate size reduction. Ultrasound-assisted particle homogenisation can be beneficial, as it can lead to increased solubility and decreased viscosity in dairy systems (Shen, Shao, & Guo, 2017; Zisu et al., 2011).

In addition to the size reduction of protein aggregates, US has been reported to impart changes in casein micelle structure and whey protein conformation (Munir et al., 2019). While working with reconstituted micellar casein powder solutions over a wide pH range (pH 6-12), Madadlou et al. (2009) reported a particle size reduction after sonication at 35 kHz, 0.020-0.067 W/mL power for 6 h. Nguyen and Anema (2010) suggested that casein micelles could be dissociated by US as they observed a reduction in the particle size of skim milk during sonication (22.5 kHz, ~2.8W/mL up to 10 min). However, Chandrapala et al. (2012) argued that the reduced particle size in reconstituted micellar solutions and skim milk is the result of aggregate dissociation and fat droplet breakage, which can have a significant effect on particle size measurement even when present in small amounts in skim milk. They further confirmed that

ultrasound (20 kHz, 0.52W/mL) had no effect on casein micelle integrity in US treatments of up to 1 h. Nonetheless, in alkaline pH, when the casein micelle is enlarged due to electrostatic repulsion, sonicating (20 kHz 0.32 W/mL power, 15 minutes) skim milk was shown to compromise casein micelle integrity (Liu et al., 2014). Although US (20 kHz) causes changes in the secondary structure of milk proteins (Pathak et al., 2020; Silva et al., 2018), the amino acid composition remains unchanged even after prolonged sonication (Pathak et al., 2020).

2.12.2. Ultrasonic emulsification

As previously introduced in section 2.11.1, emulsification is the process of forming a stable dispersion of two immiscible phases. The addition of an emulsifier is necessary to reduce the surface tension on the emulsion droplet interface and to create steric and/or electrostatic repulsion between the dispersed droplets (Falkeborg & Guo, 2015; Kentish et al., 2008; Li et al., 2019). High-pressure homogenisation and rotor-stator mixing devices are most commonly used for emulsion preparation at present. However, ultrasonic emulsification is gaining popularity due to its ability to produce very fine emulsions and the high degree of control.

Ultrasound emulsification proceeds in two stages of physical phenomena (Kentish & Feng, 2014; Kentish et al., 2008; Li et al., 2019). First, the ultrasound field produces propagating waves in the oil-water interface. These waves subsequently become unstable and, in the case of a typical oil-in water emulsion, the oil phase erupts into the continuous aqueous phase, forming moderately large droplets. Secondly, the cavitation-induced shear and turbulence break the existing droplets to a submicron size (Li & Fogler, 1978). The first stage is only relevant when the two phases are un mixed before emulsification, however, the second stage can be used reduce the droplet size in pre-formed coarse emulsions (Leong, Juliano, et al., 2017).

Dairy based emulsions formed using ultrasound have enhanced stability due to the structural changes in milk proteins caused by cavitation (Kaltsa et al., 2013; Shanmugam & Ashokkumar, 2014). For example, food-based oil-in-water emulsions made with flax seed oil and skim milk using ultrasound remained stable for a period of 7 days without the addition of an external emulsifying agent (Shanmugam & Ashokkumar, 2014). Similarly, stable (up to 10 days) submicron-sized olive oil-in-water emulsions have been formed with ultrasound using WPI as an emulsifier, however in the presence of hydrocolloids to mitigate depletion flocculation (Kaltsa et al., 2013).

2.12.3. Production of double emulsions using ultrasound

A recent advancement in emulsification in food applications is the formation of double emulsions (Leong, Zhou, et al., 2017; Leong, Zhou, et al., 2018). A double emulsion contains an inner emulsion of the opposite phase inside the primary emulsion droplet (Leong, Zhou, et al., 2017). Water-in-oil-in-water (w/o/w) double emulsions can be produced by first preparing a stable water-in-oil emulsion (W/O) with a high proportion of the aqueous phase. This single emulsion can be then emulsified into an aqueous phase (e.g. skim milk) to form a water-in-oil-in-water (w/o/w) double emulsion. Recent works by (Leong, Zhou, et al., 2017; Leong, Zhou, et al., 2018) have demonstrated ultrasound's ability to formulate water-in-oil-in-water double emulsions that can encapsulate skim milk inside emulsified oil droplets. Such double emulsions can be produced with an apparent volume fraction of emulsion droplets similar to a regular single emulsion with a higher oil content. This can enable the formation of products, such as cheese, with a reduced fat content, which mimics the microstructure and textural properties of full-fat products (Leong et al., 2019; Lobato-Calleros et al., 2006; Lobato-Calleros et al., 2008). In addition, double emulsions can be included in cheese milk formulations to produce cheeses augmented with water soluble nutritional components (Giroux et al., 2013). These applications are discussed in detail in the next section.

2.12.4. Potential for the formation of whey protein filled double emulsions to be incorporated into cheeses

Double emulsions are increasingly included in cheese milk formulation to produce cheeses enriched with water soluble nutritional components (Giroux et al., 2013) or to reduce the fat content (Leong et al., 2019; Lobato-Calleros et al., 2006; Lobato-Calleros et al., 2008). However, there are several challenges to forming a stable all-dairy double emulsion, which have prevented their widespread use in the food industry. These challenges include leakage of the internal aqueous phase, Ostwald ripening, and the flocculation and coalescence of internal and external droplets during storage (Garti, 1997; Leong, Zhou, et al., 2017). The internal droplets have to be sufficiently small to be encapsulated within the larger outer droplets, however, larger outer droplets (~20-100 μm) tend to cream, flocculate and coalesce (Garti, 1997). Therefore the stability is conferred by the addition of large amounts of hydrophilic and lipophilic emulsifiers (Matsumoto et al., 1976).

Recent works by Leong, Zhou, et al. (2017) demonstrated that ultrasonic emulsification could be used to form skim milk-in-oil-in-skim milk double emulsions using skim milk, sunflower (containing 10-20% w/w Span 80 as a lipophilic emulsifier) with no added hydrophilic

emulsifiers, achieving encapsulation yields of ~35%. The proteins in the skim milk emulsify the oil droplets in the outer aqueous phase. They further demonstrated that ultrasound power (hence cavitation intensity) could be manipulated to change the size distribution of the external droplets. Entrapment was more prevalent in larger droplets and reduced with the reduction in size of the external droplets. However, the stability of the internal aqueous droplets was suboptimal even with 20% w/w Span 80, coalescing into a continuous aqueous phase and becoming dispersed into the outer bulk aqueous medium. Further work by Leong, Zhou, et al. (2018) replaced Span 80 with a food-grade emulsifiers (1% Polyglycerol polyricinoleate - PGPR and 5% lecithin in the oil phase), while achieving an encapsulation yield of ~50%. The increased encapsulation with added lecithin was attributed to highly viscous gel-like interactions that occurred between the internal aqueous droplets.

These skim milk-in-oil-in-skim milk double emulsions were later incorporated into cheese milk to form cheese analogues with reduced fat contents (Leong et al., 2020). Although double emulsion-filled cheeses did not have a higher protein content compared to the control full-fat cheese, the problem of excessive cheese hardness generally observed in reduced-fat cheeses could be partly mitigated, as the double emulsion droplets with an internal aqueous phase could occupy a higher apparent volume fraction in cheese compared to a single emulsion with the same fat content (Leong et al., 2020). One reason for the unchanged protein content of the double emulsion cheeses, even with skim milk encapsulated inside the emulsion droplets, is the relatively low protein concentration of skim milk (~3.5%) combined with the limited aqueous phase encapsulation yield of 15-20%.

Alternatively, there is potential to exploit the amphiphilic properties of whey proteins to form stable water-in-oil-in-water double emulsions encapsulating concentrated whey proteins in the internal aqueous phase, while reducing the requirement for non-dairy lipophilic emulsifiers. If high degrees of whey protein encapsulation could be achieved, these double emulsions could be used in replacement of milk fat to form whey protein incorporated cheddar curds and cheese with significantly enhanced protein content. Incorporating whey proteins into cheese curds in the form of an encapsulated aqueous phase to increase the protein content of cheese has not yet been reported in the literature. In the following sections, the potential to form whey encapsulated double emulsions using novel power ultrasound assisted strategies is reviewed.

2.13. Knowledge gaps to be addressed in this thesis

As reviewed above, most of the existing studies on the incorporation of whey proteins into cheese matrices have investigated the use of membrane filtration technologies and thermal

treatment of milk protein streams. These studies have reported changes in cheese yields, and textural and sensory attributes of cheese as affected by the addition of whey protein. However, there is limited understanding on how added whey proteins alters the kinetics of casein aggregation and gel formation during the rennet coagulation phase of cheese making. Rennet gelation is a critical stage during cheese making, as:

(a) Rennet gelation is a time-consuming stage of cheese making;
(b) The strength of the rennet coagulum determines the extent of protein/fat loss during the post-gelation processing of cheese;
(c) Rennet gelation involves the addition of the chymosin enzyme, which is expensive and not generally recoverable in the process.

A comprehensive understanding on the mechanisms by which added whey protein interacts with casein micelles during rennet gelation would enable optimised formulation of cheese milk to reduce gelation times and obtain a sufficiently firm coagulum with reduced enzyme additions. Therefore, a key aim of this thesis was to broaden the understanding of casein micelle-whey protein interactions during rennet induced milk coagulation. This aim was fulfilled in Chapters 3 and 4.

Chapter 3: Investigating the role of native whey proteins on the rennet gelation of bovine milk

Membrane filtration is used to concentrate milk proteins in cheese milk (Nelson & Barbano, 2005) to increase the volumetric productivity of the cheese vats. While ultrafiltration concentrates all of the milk proteins, microfiltration separates larger casein micelles from the smaller whey proteins (Maubois, 2002; Papadatos et al., 2003b; Zulewska et al., 2009). Microfiltration retentates enable the formulation of cheese milk with an increased whey protein content. It was previously reported that addition of native whey proteins to milk impairs the rate of rennet gelation, however, the specific impacts on enzyme hydrolysis and casein micelle aggregation in systems with controlled microenvironments have not yet been studied in detail.

This gap is addressed in Chapter 3 by formulating cheese milks with a broad range of casein:whey protein ratios, using membrane filtered native casein and whey protein concentrates. The specific impacts of whey protein on enzyme hydrolysis and casein aggregation are each studied using a combination of oscillatory rheometry coupled with casein macropeptide release measurements during rennet gelation, while taking the changes in the ionic balance and viscosity in milk environment into account. The aim was to propose a

mechanism for the observed changes in the kinetics of rennet gelation and to specify which whey proteins interact with the casein micelles and rennet enzymes.

Chapter 4: Investigating the role of native whey proteins on the rennet gelation of bovine milk

In the current literature, denatured whey proteins have been incorporated into cheese milks at relatively low concentrations. When denatured whey protein is included in cheese milk, the resulting cheese can have higher yields and softer textures, however at the cost of significant fat loss. Concentrated whey proteins could be included into cheese milk without significant dilution of caseins, however, heat denaturation of whey protein at higher concentrations results in the formation of large aggregates and gels that cannot be homogeneously distributed in cheese milk.

Chapter 4 addresses this challenge by using thermal treatment coupled with power ultrasound to form differently sized whey protein aggregates from concentrated whey streams. As Chapter 3 will reveal, native whey proteins impair the rennet gelation and prolong clotting times. The potential of denatured whey protein aggregates to mitigate the impaired rennet gelation caused by native whey proteins are investigated in Chapter 4, taking into account the surface properties of aggregates and the changes in the ionic balance of the reconstituted milks. A mechanism behind the reported changes is also proposed and validated using commercially available cheese whey and skim milk. The whey protein aggregates are then incorporated into model non-fat cheddar cheese systems to reveal the changes in cheese yields and microstructure, as affected by casein-denatured whey protein interactions alone.

A novel approach to incorporate whey proteins into cheese matrices is then investigated in Chapter 5, with the aim of entrapping high concentrations of whey protein the oil droplets, using so-called double emulsions.

Chapter 5: Formation of rennet gel matrices fortified with ultrasonically generated whey protein-enriched water-in-oil-in-water double emulsions

Ultrasonic emulsification can produce small droplets that can enhance the stability of dairy based emulsions. Water-in-oil-in-water double emulsions encapsulating an internal aqueous phase inside the oil droplets can be formed using ultrasound emulsification. The use of double emulsions has so far been reported to reduce the fat content or to incorporate water soluble nutraceuticals into cheese. Stabilising the internal aqueous phase of dairy-based double emulsions has been shown to be challenging due to the requirement for large amount of

lipophilic non-dairy emulsifiers. It has been demonstrated recently that an internal skim milk phase containing hydrocolloids as viscosity modifiers could greatly reduce the non-dairy emulsifiers used to stabilise the internal aqueous phase.

Whey proteins have good emulsification properties. Chapter 5 investigates the potential of using ultrasound to generate stable water-in-oil-in-water double emulsions with concentrated solutions of whey proteins as the internal aqueous phase to reduce the requirement for non-dairy lipophilic emulsifiers and to enhance the protein content of cheese. Ultrasonic power is manipulated to control the size distribution and to achieve sufficiently high whey protein encapsulation yields, previously not reported in the literature. Double emulsion droplets that mimic the size distribution of native whey protein globules are to be incorporated into cheese milks to produce whey protein enriched curd matrices.

2.14. References

Aaltonen, T., Huumonen, I., & Myllärinen, P. (2014). Controlled transglutaminase treatment in Edam cheese-making. *International Dairy Journal, 38*(2), 179-182.

Agrawal, P., & Hassan, A. (2007). Ultrafiltered milk reduces bitterness in reduced-fat Cheddar cheese made with an exopolysaccharide-producing culture. *Journal of Dairy Science, 90*(7), 3110-3117.

Ahmed, S., Sharif, M. K., Butt, M. S., & Nawaz, H. (2015). Physicochemical and amino acid profiling of cheddar, mozzarella and paneer whey. *Biological Sciences-PJSIR, 58*(3), 155-158.

Alting, A. C., Hamer, R. J., de Kruif, C. G., Paques, M., & Visschers, R. W. (2003). Number of thiol groups rather than the size of the aggregates determines the hardness of cold set whey protein gels. *Food Hydrocolloids, 17*(4), 469-479.

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*(4), 1108-1115.

Anema, S. G., & Li, Y. (2003). Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. *Journal of Dairy Research, 70*(1), 73-83.

Anifantakis, E. (1991). Traditional feta cheese. *Feta and related cheeses*, 49-69.

Ashokkumar, M. (2010). *Theoretical and experimental sonochemistry involving inorganic systems*: Springer Science & Business Media.

Ashokkumar, M., & Mason, T. (2007). Sonochemistry, Kirk-Othmer encylcopedia of chemical technology. In: John Wiley & Sons New York.

Ashokkumar, M., & Mason, T. J. (2000). Sonochemistry. *Kirk-Othmer Encyclopedia of Chemical Technology*.

Atasoy, A. F., Yetişmeyen, A., Türkoğlu, H., & Özer, B. (2008). Effects of heat treatment and starter culture on the properties of traditional Urfa cheeses (a white-brined Turkish cheese) produced from bovine milk. *Food Control, 19*(3), 278-285.

Atkinson, P. J., Dickinson, E., Horne, D. S., & Richardson, R. M. (1995). Neutron reflectivity of adsorbed β-casein and β-lactoglobulin at the air/water interface. *Journal of the Chemical Society, Faraday Transactions, 91*(17), 2847-2854.

Attaallah, W., Yılmaz, A. M., Erdoğan, N., Yalçın, A. S., & Aktan, A. Ö. (2012). Whey protein versus whey protein hydrolyzate for the protection of azoxymethane and dextran sodium sulfate induced colonic tumors in rats. *Pathology & Oncology Research, 18*(4), 817-822.

Baldasso, C., Barros, T., & Tessaro, I. (2011). Concentration and purification of whey proteins by ultrafiltration. *Desalination, 278*(1-3), 381-386.

Baldwin, K. A., Baer, R., Parsons, J., Seas, S., Spurgeon, K., & Torrey, G. (1986). Evaluation of yield and quality of Cheddar cheese manufactured from milk with added whey protein concentrate. *Journal of Dairy Science, 69*(10), 2543-2550.

Banks, J. M., & Muir, D. (1985). Effect of incorporation of denatured whey protein on the yield and quality of Cheddar cheese. *International journal of dairy technology, 38*(1), 27-32. doi:https://doi.org/10.1111/j.1471-0307.1985.tb00536.x

Bansal, N., Fox, P. F., & McSweeney, P. L. (2007). Aggregation of rennet-altered casein micelles at low temperatures. *Journal of agricultural and food chemistry, 55*(8), 3120-3126.

Barber, B. P., & Putterman, S. J. (1991). Observation of synchronous picosecond sonoluminescence. *Nature, 352*(6333), 318.

Basak, B., Fatima, A., Jeon, B.-H., Ganguly, A., Chatterjee, P. K., & Dey, A. (2018). Process kinetic studies of biohydrogen production by co-fermentation of fruit-vegetable wastes and cottage cheese whey. *Energy for sustainable development, 47*, 39-52.

Boivin-Piché, J., Vuillemard, J.-C., & St-Gelais, D. (2016). Vitamin D-fortified Cheddar type cheese produced from concentrated milk. *Journal of Dairy Science, 99*(6), 4140-4145.

Bounous, G. (2000). Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. *Anticancer Research, 20*(6), 4785-4792.

Bounous, G., Kongshavn, P., & Gold, P. (1988). The immunoenhancing property of dietary whey protein concentrate. *Clin Invest Med, 11*(4), 271-278.

Brinkmann, C., Thiel, S., Larsen, M., Petersen, T., Jensenius, J. C., & Heegaard, C. (2011). Preparation and comparison of cytotoxic complexes formed between oleic acid and either bovine or human α-lactalbumin. *Journal of Dairy Science, 94*(5), 2159-2170.

Brown, R. J., & Ernstrom, C. A. (1982). Incorporation of ultrafiltration concentratedwhey solids into cheddar cheese for increased yield. *Journal of Dairy Science, 65*(12), 2391-2395. doi:https://doi.org/10.3168/jds.s0022-0302(82)82514-9

Cadavid, A. M., Bohigas, L., Toldrà, M., Carretero, C., Parés, D., & Saguer, E. (2020). Improving quark-type cheese yield and quality by treating semi-skimmed cow milk with microbial transglutaminase. *LWT*, 109756.

Callaghan, Y. O. C., TP; O'Brien, NM. (2017). Nutritional Aspects of Cheese. In P. G. Fox, T P; Cogan, TM; McSweeney, PLH (Ed.), *Fundamentals of cheese science* (2 ed., pp. 715-729). Neew York: Springer.

Carlson, A., Hill, C. G., Jr., & Olson, N. F. (1987). Kinetics of milk coagulation: I. The kinetics of kappa casein hydrolysis in the presence of enzyme deactivation. *Biotechnol Bioeng, 29*(5), 582-589. doi:10.1002/bit.260290507

Chamberland, J., Mercier-Bouchard, D., Dussault-Chouinard, I., Benoit, S., Doyen, A., Britten, M., & Pouliot, Y. (2019). On the Use of Ultrafiltration or Microfiltration Polymeric Spiral-Wound Membranes for Cheesemilk Standardization: Impact on Process Efficiency. *Foods, 8*(6), 198. doi:https://doi.org/10.3390/foods8060198

Chandrapala, J., Martin, G., Zisu, B., Kentish, S., & Ashokkumar, M. (2012). The effect of ultrasound on casein micelle integrity. *Journal of Dairy Science, 95*(12), 6882-6890.

Chandrasekaran, E., Chawda, R., Piskorz, C., Locke, R. D., Ta, A., Sharad, G., . . . Matta, K. L. (2001). Human ovarian cancer, lymphoma spleen, and bovine milk GlcNAc: β1, 4Gal/GalNAc transferases: two molecular species in ovarian tumor and induction of GalNAcβ1, 4Glc synthesis by α-lactalbumin. *Carbohydrate research, 334*(2), 105-118.

Chaplin, B., & Green, M. L. (1980). Determination of the proportion of κ-casein hydrolysed by rennet on coagulation of skim-milk. *J Dairy Res, 47*, 351-358.

Chawla, R., Patil, G. R., & Singh, A. K. (2011). High hydrostatic pressure technology in dairy processing: a review. *Journal of Food Science and Technology, 48*(3), 260-268.

Chen, G., Talebi, S., Gras, S., Weeks, M., & Kentish, S. (2018). A review of salty waste stream management in the Australian dairy industry. *Journal of environmental management, 224*, 406-413.

Cho, Y., Lucey, J., & Singh, H. (1999). Rheological properties of acid milk gels as affected by the nature of the fat globule surface material and heat treatment of milk. *International Dairy Journal, 9*(8), 537-545.

Chouinard, P., Girard, V., & Brisson, G. (1998). Fatty acid profile and physical properties of milk fat from cows fed calcium salts of fatty acids with varying unsaturation. *Journal of Dairy Science, 81*(2), 471-481.

Chromik, C., Partschefeld, C., Jaros, D., Henle, T., & Rohm, H. (2010). Adjustment of vat milk treatment to optimize whey protein transfer into semi-hard cheese: a case study. *Journal of food engineering, 100*(3), 496-503.

Co, E. D., & Marangoni, A. G. (2012). Organogels: an alternative edible oil-structuring method. *Journal of the American Oil Chemists' Society, 89*(5), 749-780.

CODEX. (2018). General Standard for Cheese. In (Vol. CXS 208-1999): Codex Alimentarius.

CODEX. (2019). Standard for Cheddar. In (Vol. CXS 263-1966): Codex Alimentarius.

Cozzolino, A., Di Pierro, P., Mariniello, L., Sorrentino, A., Masi, P., & Porta, R. (2003). Incorporation of whey proteins into cheese curd by using transglutaminase. *Biotechnology and applied biochemistry, 38*(3), 289-295.

Crevier, B., Bélanger, G., Vuillemard, J.-C., & St-Gelais, D. (2017). Production of cottage cheese fortified with vitamin D. *Journal of Dairy Science, 100*(7), 5212-5216.

D'Alessandro, A., Martemucci, G., Loizzo, P., & Faccia, M. (2019). Production of cheese from donkey milk as influenced by addition of transglutaminase. *Journal of Dairy Science, 102*(12), 10867-10876.

DA. (2019). *Information on the production, sales, and trends for Australian manufactured cheeses.* Retrieved from https://www.dairyaustralia.com.au/industry/production-and-sales/cheese

Dalgleish, D. G. (1980). Effect of milk concentration on the rennet coagulation time. *Journal of Dairy Research, 47*(2), 231-235.

Dalgleish, D. G. (1983). Coagulation of renneted bovine casein micelles: dependence on temperature, calcium ion concentration and ionic strength. *Journal of Dairy Research, 50*(3), 331-340.

Dalgleish, D. G. (1984). Measurement of electrophoretic mobilities and zeta-potentials of particles from milk using laser Doppler electrophoresis. *Journal of Dairy Research, 51*(3), 425-438.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles—review and possible improvements. *Soft matter, 7*(6), 2265-2272.

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*(4), 529-538.

Dalgleish, D. G., & Law, A. J. R. (1989). pH-induced dissociation of bovine casein micelles. II. Mineral solubilization and its relation to casein release. *Journal of Dairy Research, 56*(5), 727-735.

Darling, D. F., & Dickson, J. (1979). The determination of the zeta potential of casein micelles. *Journal of Dairy Research, 46*(2), 329-332.

de Souza, R. R., Bergamasco, R., da Costa, S. C., Feng, X., Faria, S. H. B., & Gimenes, M. L. (2010). Recovery and purification of lactose from whey. *Chemical Engineering and Processing: Process Intensification, 49*(11), 1137-1143.

de Vries, A., Gomez, Y. L., van der Linden, E., & Scholten, E. (2017). The effect of oil type on network formation by protein aggregates into oleogels. *Rsc Advances, 7*(19), 11803-11812.

de Vries, A., Jansen, D., van der Linden, E., & Scholten, E. (2018). Tuning the rheological properties of protein-based oleogels by water addition and heat treatment. *Food Hydrocolloids, 79*, 100-109.

de Vries, A., Wesseling, A., van der Linden, E., & Scholten, E. (2017). Protein oleogels from heat-set whey protein aggregates. *Journal of colloid and interface science, 486*, 75-83.

De Wit, J. (1990). Thermal stability and functionality of whey proteins. *Journal of Dairy Science, 73*(12), 3602-3612.

DeJong, G., & Koppelman, S. (2002). Transglutaminase catalyzed reactions: impact on food applications. *Journal of Food Science, 67*(8), 2798-2806.

Deshpande, V. K., & Walsh, M. K. (2018). Effect of sonication on the viscosity of reconstituted skim milk powder and milk protein concentrate as influenced by solids concentration, temperature and sonication. *International Dairy Journal, 78*, 122-129.

Di Cagno, R., De Pasquale, I., De Angelis, M., Buchin, S., Rizzello, C., & Gobbetti, M. (2014). Use of microparticulated whey protein concentrate, exopolysaccharide-producing *Streptococcus thermophilus*, and adjunct cultures for making low-fat Italian Caciotta-type cheese. *Journal of Dairy Science, 97*(1), 72-84. doi:https://doi.org/10.3168/jds.2013-7078

Diarrassouba, F., Garrait, G., Remondetto, G., Alvarez, P., Beyssac, E., & Subirade, M. (2015). Food protein-based microspheres for increased uptake of vitamin D3. *Food Chemistry, 173*, 1066-1072.

Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B: Biointerfaces, 20*(3), 197-210.

Dickinson, E., & Matsumura, Y. (1991). Time-dependent polymerization of β-lactoglobulin through disulphide bonds at the oil-water interface in emulsions. *International journal of biological macromolecules, 13*(1), 26-30.

Dillon, E. L., Basra, G., Horstman, A. M., Casperson, S. L., Randolph, K. M., Durham, W. J., . . . Hatch, S. S. (2012). Cancer cachexia and anabolic interventions: a case report. *Journal of cachexia, sarcopenia and muscle, 3*(4), 253-263.

Dissanayake, M., Ramchandran, L., Donkor, O., & Vasiljevic, T. (2013). Denaturation of whey proteins as a function of heat, pH and protein concentration. *International Dairy Journal, 31*(2), 93-99.

Domagała, J., Najgebauer-Lejko, D., Wieteska-Śliwa, I., Sady, M., Wszołek, M., Bonczar, G., & Filipczak-Fiutak, M. (2016). Influence of milk protein cross-linking by transglutaminase on the rennet coagulation time and the gel properties. *Journal of the Science of Food and Agriculture, 96*(10), 3500-3507.

Donato, L., & Guyomarc'h, F. (2009). Formation and properties of the whey protein/k-casein complexes in heated skim milk–A review. *Dairy Science and Technology, 89*(1), 3-29.

Dupas, C., Adt, I., Cottaz, A., Boutrou, R., Molle, D., Jardin, J., . . . Degraeve, P. (2009). A chromatographic procedure for semi-quantitative evaluation of caseinphosphopeptides in cheese. *Dairy science & technology, 89*(6), 519-529.

Eliassen, L. T., Berge, G., Leknessund, A., Wikman, M., Lindin, I., Løkke, C., . . . Kogner, P. (2006). The antimicrobial peptide, lactoferricin B, is cytotoxic to neuroblastoma cells in vitro and inhibits xenograft growth in vivo. *International Journal of Cancer, 119*(3), 493-500.

Enker, W. E., & Jacobitz, J. L. (1976). Experimental carcinoma of the colon induced by 1, 2-dimethylhydrazine-diHCl: value as a model of human disease. *Journal of Surgical Research, 21*(4), 291-299.

Esteves, C., Lucey, J., Wang, T., & Pires, E. (2003). Effect of pH on the gelation properties of skim milk gels made from plant coagulants and chymosin. *Journal of Dairy Science, 86*(8), 2558-2567.

Everard, C., O'Callaghan, D., Mateo, M., O'Donnell, C., Castillo, M., & Payne, F. (2008). Effects of cutting intensity and stirring speed on syneresis and curd losses during cheese manufacture. *Journal of Dairy Science, 91*(7), 2575-2582.

Falkeborg, M., & Guo, Z. (2015). Dodecenyl succinylated alginate (DSA) as a novel dual-function emulsifier for improved fish oil-in-water emulsions. *Food Hydrocolloids, 46*, 10-18.

Fedrick, I., & Houlihan, D. (1981). The effect of lactose hydrolysis on the yield of cottage cheese curd. *Australian Journal of Dairy Technology, 36*(3), 104.

Fernandes, R. V. d. B., Guimarães, I. C., Ferreira, C. L. R., Botrel, D. A., Borges, S. V., & de Souza, A. U. (2017). Microencapsulated rosemary (Rosmarinus officinalis) essential oil as a biopreservative in minas frescal cheese. *Journal of food processing and preservation, 41*(1), e12759.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017a). Cheese: historical aspects. In *Fundamentals of Cheese Science* (pp. 1-10): Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017b). Chemistry of Milk Constituents. In *Fundamentals of Cheese Science* (pp. 19-44). New York: Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017c). Enzymatic coagulation of milk. In *Fundamentals of cheese science* (pp. 185-229). New York: Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017d). Overview of Cheese Manufacture. In *Fundamentals of Cheese Science*. New York: Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017e). Principal Families of Cheese. In *Fundamentls of cheese science*. New york: Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017f). Processed cheese and substitute/imitation cheese products. In *Fundamentals of cheese science* (pp. 589-627): Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017g). Salting of cheese curd. In P. F. G. Fox, T P; Cogan, TM; McSweeney, PLH (Ed.), *Fundamentals of Cheese Science* (pp. 251-275). New York: Springer.

Fox, P. F., & McSweeny, P. L. H. (1998). Milk Proteins. In *Dairy Chemistry and Biochemistry* (pp. 146-187). London: Blackie Academic & Professional.

Gagnaire, V., Mollé, D., Herrouin, M., & Léonil, J. (2001). Peptides identified during Emmental cheese ripening: origin and proteolytic systems involved. *Journal of agricultural and food chemistry, 49*(9), 4402-4413.

Galus, S., & Kadzińska, J. (2016). Whey protein edible films modified with almond and walnut oils. *Food Hydrocolloids, 52*, 78-86.

Garnot, P. (1988). Influence of milk concentration by UF on enzymatic coagulation. *Federaion Internationale de Laiterie*.

Garnot, P., Rank, T., & Olson, N. (1982). Influence of protein and fat contents of ultrafiltered milk on rheological properties of gels formed by chymosin. *Journal of Dairy Science, 65*(12), 2267-2273.

Garti, N. (1997). Double emulsions—scope, limitations and new achievements. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 123*, 233-246.

Gaygadzhiev, Z., Corredig, M., & Alexander, M. (2009). The impact of the concentration of casein micelles and whey protein-stabilized fat globules on the rennet-induced gelation of milk. *Colloids and Surfaces B: Biointerfaces, 68*(2), 154-162.

Gaygadzhiev, Z., Corredig, M., & Alexander, M. (2011). Rennet coagulation properties of milk in the presence of oil droplets stabilised by a combination of sodium caseinate and whey protein isolate. *Dairy science & technology, 91*(6), 719-737.

Gaygadzhiev, Z., Hill, A., & Corredig, M. (2009). Influence of the emulsion droplet type on the rheological characteristics and microstructure of rennet gels from reconstituted milk. *Journal of Dairy Research, 76*(3), 349-355.

Gernigon, G., Schuck, P., & Jeantet, R. (2010). Processing of Mozzarella cheese wheys and stretchwaters: A preliminary review. *Dairy science & technology, 90*(1), 27-46.

Gharibzahedi, S. M. T., Koubaa, M., Barba, F. J., Greiner, R., George, S., & Roohinejad, S. (2018). Recent advances in the application of microbial transglutaminase crosslinking in cheese and ice cream products: A review. *International journal of biological macromolecules, 107*, 2364-2374.

Giancola, C., De Sena, C., Fessas, D., Graziano, G., & Barone, G. (1997). DSC studies on bovine serum albumin denaturation Effects of ionic strength and SDS concentration. *International journal of biological macromolecules, 20*(3), 193-204.

Giroux, H. J., Constantineau, S., Fustier, P., Champagne, C. P., St-Gelais, D., Lacroix, M., & Britten, M. (2013). Cheese fortification using water-in-oil-in-water double emulsions as carrier for water soluble nutrients. *International Dairy Journal, 29*(2), 107-114.

Giroux, H. J., Lanouette, G., & Britten, M. (2015). Effect of whey protein aggregates of various sizes on the formation and properties of rennet-induced milk gels. *Food Hydrocolloids, 45*, 272-278.

Gonzalez-Martınez, C., Becerra, M., Cháfer, M., Albors, A., Carot, J., & Chiralt, A. (2002). Influence of substituting milk powder for whey powder on yoghurt quality. *Trends in Food Science & Technology, 13*(9-10), 334-340.

Gosal, W. S., & Ross-Murphy, S. B. (2000). Globular protein gelation. *Current Opinion in Colloid & Interface Science, 5*(3-4), 188-194.

Gotham, S. M., Fryer, P. J., & Pritchard, A. M. (1992). β-lactoglobulin denaturation and aggregation reactions and fouling deposit formation: a DSC study. *International journal of food science & technology, 27*(3), 313-327.

Govindasamy-Lucey, S., Jaeggi, J., Bostley, A., Johnson, M., & Lucey, J. (2004). Standardization of milk using cold ultrafiltration retentates for the manufacture of Parmesan cheese. *Journal of Dairy Science, 87*(9), 2789-2799.

Govindasamy-Lucey, S., Jaeggi, J. J., Martinelli, C., Johnson, M., & Lucey, J. (2011). Standardization of milk using cold ultrafiltration retentates for the manufacture of Swiss cheese: Effect of altering coagulation conditions on yield and cheese quality. *Journal of dairy science, 94*(6), 2719-2730.

Griko, Y. V., Freire, E., & Privalov, P. L. (1994). Energetics of the. alpha.-Lactalbumin States: A Calorimetric and Statistical Thermodynamic Study. *Biochemistry, 33*(7), 1889-1899.

Griko, Y. V., & Privalov, P. (1992). Calorimetric study of the heat and cold denaturation of. beta.-lactoglobulin. *Biochemistry, 31*(37), 8810-8815.

Guinee, T. P., Mulholland, E., Kelly, J., & Callaghan, D. (2007). Effect of protein-to-fat ratio of milk on the composition, manufacturing efficiency, and yield of Cheddar cheese. *Journal of dairy science, 90*(1), 110-123.

Guinee, T. P., O'Kennedy, B., & Kelly, P. (2006). Effect of milk protein standardization using different methods on the composition and yields of Cheddar cheese. *Journal of dairy science, 89*(2), 468-482.

Gupta, A., Mann, B., Kumar, R., & Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. *International journal of dairy technology, 62*(3), 339-347.

Guyomarc'h, F. (2006). Formation of heat-induced protein aggregates in milk as a means to recover the whey protein fraction in cheese manufacture, and potential of heat-treating milk at alkaline pH values in order to keep its rennet coagulation properties. A review. *Le Lait, 86*(1), 1-20.

Hakkak, R., Korourian, S., Shelnutt, S. R., Lensing, S., Ronis, M. J., & Badger, T. M. (2000). Diets containing whey proteins or soy protein isolate protect against 7, 12-dimethylbenz (a) anthracene-induced mammary tumors in female rats. *Cancer Epidemiology and Prevention Biomarkers, 9*(1), 113-117.

Hamdy, S. M., Mahmoud, H. S., & Abbas, K. A.-H. (2017). Research Article Improving the Quality of Ultrafiltered Ras Cheese using Mature Cheddar Cheese Slurry.

Hannon, J. A., Lortal, S., Tissier, J.-P., & Famelart, M.-H. (2009). Limited ripening of low-fat UF-cheese due to CaPO 4 barrier? *Dairy science & technology, 89*(6), 555-568.

Haque, Z. U., Kuecuekoener, E., & Aryana, K. J. (2007). Influence of fat-replacing ingredients on process and age induced soluble nitrogen content and ultrastructure of lowfat Cheddar cheese. *Food science and technology research, 13*(4), 338-344.

He, T., & Giuseppin, M. L. (2014). Slow and fast dietary proteins differentially modulate postprandial metabolism. *International journal of food sciences and nutrition, 65*(3), 386-390.

Heelan, B., & Corrigan, O. (1998). Preparation and evaluation of microspheres prepared from whey protein isolate. *Journal of Microencapsulation, 15*(1), 93-105.

Hinrichs, J. (2001). Incorporation of whey proteins in cheese. *International Dairy Journal, 11*(4), 495-503.

Holland, B., Unwin, I., & Buss, D. (1989). *Milk products and eggs. Fourth supplement to McCance and Widdowson's the composition of foods*: Royal Socity of Chemistry.

Horne, D. S. (1998). Casein Interactions: Casting Light on the Black Boxes, the Structure in Dairy Products. *Int. Dairy Journal, 8*, 171-177.

Hougaard, A. B., Ardö, Y., & Ipsen, R. (2010). Cheese made from instant infusion pasteurized milk: Rennet coagulation, cheese composition, texture and ripening. *International Dairy Journal, 20*(7), 449-458.

Huppertz, T. (2013). Chemistry of the Caseins. In P. L. H. McSweeney & P. F. Fox (Eds.), *Advanced Dairy Chemistry* (Vol. 1A: Proteins: Basic Aspects, 4th Edition). New York: Springer.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004a). Effects of high pressure treatment on the yield of cheese curd from bovine milk. *Innovative food science & emerging technologies, 5*(1), 1-8.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004b). Plasmin activity and proteolysis in high pressure-treated bovine milk. *Le Lait, 84*(3), 297-304.

Hyslop, D. B. (2003). Enzymatic coagulation of milk. In *Advanced Dairy Chemistry—1 Proteins* (pp. 839-878): Springer.

Ibrahim, I. K., Hussin, S. M., & Al-Obaidi, Y. (2015). Extraction of cellulose nano crystalline from cotton by ultrasonic and its morphological and structural characterization. *Int. J. Mater. Chem. Phys., 1*, 99-109.

IDF. (2012). *Bulletin 485*. Retrieved from Brussels:

Iordache, M., & Jelen, P. (2003). High pressure microfluidization treatment of heat denatured whey proteins for improved functionality. *Innovative Food Science & Emerging Technologies, 4*(4), 367-376.

Jambrak, A. R., Mason, T. J., Lelas, V., Paniwnyk, L., & Herceg, Z. (2014). Effect of ultrasound treatment on particle size and molecular weight of whey proteins. *Journal of food engineering, 121*, 15-23.

Jameson, G. B., Adams, J. J., & Creamer, L. K. (2002). Flexibility, functionality and hydrophobicity of bovine β-lactoglobulin. *International Dairy Journal, 12*(4), 319-329.

Jang, H. D., & Swaisgood, H. E. (1990). Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *Journal of Dairy Science, 73*(4), 900-904.

Jaros, D., Partschefeld, C., Henle, T., & Rohm, H. (2006). Transglutaminase in dairy products: chemistry, physics, applications. *Journal of texture studies, 37*(2), 113-155.

Jeon, S.-S., Lee, S.-J., Ganesan, P., & Kwak, H.-S. (2012). Comparative study of flavor, texture, and sensory in cream cheese and cholesterol-removed cream cheese. *Food Science and Biotechnology, 21*(1), 159-165.

Johnson, M. (2017). A 100-Year Review: Cheese production and quality. *Journal of Dairy Science, 100*(12), 9952-9965.

Joy, J. M., Lowery, R. P., Wilson, J. M., Purpura, M., De Souza, E. O., Wilson, S. M., . . . Jäger, R. (2013). The effects of 8 weeks of whey or rice protein supplementation on body composition and exercise performance. *Nutrition journal, 12*(1), 86.

Kaltsa, O., Michon, C., Yanniotis, S., & Mandala, I. (2013). Ultrasonic energy input influence on the production of sub-micron o/w emulsions containing whey protein and common stabilizers. *Ultrasonics sonochemistry, 20*(3), 881-891.

Kandarakis, I., Moatsou, G., Georgala, A., Kaminarides, S., & Anifantakis, E. (2001). Effect of draining temperature on the biochemical characteristics of Feta cheese. *Food Chemistry, 72*(3), 369-378.

Karagül-Yüceer, Y., Drake, M., & Cadwallader, K. R. (2003). Aroma-active components of liquid cheddar whey. *Journal of food science, 68*(4), 1215-1219.

Kent, K., Harper, W., & Bomser, J. (2003). Effect of whey protein isolate on intracellular glutathione and oxidant-induced cell death in human prostate epithelial cells. *Toxicology in vitro, 17*(1), 27-33.

Kentish, S., & Feng, H. (2014). Applications of power ultrasound in food processing. *Annual review of food science and technology, 5*, 263-284.

Kentish, S., Wooster, T., Ashokkumar, M., Balachandran, S., Mawson, R., & Simons, L. (2008). The use of ultrasonics for nanoemulsion preparation. *Innovative Food Science & Emerging Technologies, 9*(2), 170-175.

Kethireddipalli, P., & Hill, A. R. (2015). Rennet coagulation and cheesemaking properties of thermally processed milk: Overview and recent developments. *Journal of agricultural and food chemistry, 63*(43), 9389-9403.

Kimball, S. R., & Jefferson, L. S. (2002). Control of protein synthesis by amino acid availability. *Current Opinion in Clinical Nutrition & Metabolic Care, 5*(1), 63-67.

Kitts, D. (2005). Antioxidant properties of casein-phosphopeptides. *Trends in Food Science & Technology, 16*(12), 549-554.

Kitts, D., & Nakamura, S. (2006). Calcium-enriched casein phosphopeptide stimulates release of IL-6 cytokine in human epithelial intestinal cell line. *Journal of Dairy Research, 73*(1), 44-48.

Klei, L., Yun, J., Sapru, A., Lynch, J., Barbano, D., Sears, P., & Galton, D. (1998). Effects of milk somatic cell count on cottage cheese yield and quality. *Journal of Dairy Science, 81*(5), 1205-1213.

Kong, B., Peng, X., Xiong, Y. L., & Zhao, X. (2012). Protection of lung fibroblast MRC-5 cells against hydrogen peroxide-induced oxidative damage by 0.1–2.8 kDa antioxidative peptides isolated from whey protein hydrolysate. *Food Chemistry, 135*(2), 540-547.

Kurek, M., Galus, S., & Debeaufort, F. (2014). Surface, mechanical and barrier properties of bio-based composite films based on chitosan and whey protein. *Food Packaging and Shelf Life, 1*(1), 56-67.

Kuwata, H., Yamauchi, K., Teraguchi, S., Ushida, Y., Shimokawa, Y., Toida, T., & Hayasawa, H. (2001). Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats. *The Journal of nutrition, 131*(8), 2121-2127.

Labropoulos, A., Palmer, J., & Lopez, A. (1981). Whey protein denaturation of UHT processed milk and its effect on rheology of yogurt. *Journal of texture studies, 12*(3), 365-374.

Lands, L., Iskandar, M., Beaudoin, N., Meehan, B., Dauletbaev, N., & Berthiuame, Y. (2010). Dietary supplementation with pressurized whey in patients with cystic fibrosis. *Journal of medicinal food, 13*(1), 77-82.

Larson, B., & Rolleri, G. (1955). Heat denaturation of the specific serum proteins in milk. *Journal of Dairy Science, 38*(4), 351-360.

Law, A. J., & Leaver, J. (2000). Effect of pH on the thermal denaturation of whey proteins in milk. *Journal of agricultural and food chemistry, 48*(3), 672-679.

Lee, N.-K., Jeewanthi, R., Park, E.-H., & Paik, H.-D. (2016). Physicochemical and antioxidant properties of Cheddar-type cheese fortified with Inula britannica extract. *Journal of dairy science, 99*(1), 83-88.

Lelievre, J., Creamer, L., & Tate, K. (1990). Inhibition of calf vell and microbial rennet action by whey protein concentrate. *Milchwissenschaft, 45*(2), 71-75.

Leong, T. S. H., Ashokkumar, M., & Kentish, S. (2011). The fundamentals of power ultrasound-A review.

Leong, T. S. H., Juliano, P., & Knoerzer, K. (2017). Advances in Ultrasonic and Megasonic Processing of Foods. *Food Engineering Reviews, 9*(3), 237-256.

Leong, T. S. H., Ong, L., Gamlath, C. J., Gras, S. L., Ashokkumar, M., & Martin, G. J. O. (2019). *Formation of cheddar cheese analogues using canola oil and ultrasonication – a comparison between single and double emulsion systems*

Leong, T. S. H., Ong, L., Gamlath, C. J., Gras, S. L., Ashokkumar, M., & Martin, G. J. O. (2020). Formation of cheddar cheese analogues using canola oil and ultrasonication – a comparison between single and double emulsion systems *International Dairy Journal*, 104683.

Leong, T. S. H., Walter, V., Gamlath, C. J., Yang, M., Martin, G. J., & Ashokkumar, M. (2018). Functionalised dairy streams: Tailoring protein functionality using sonication and heating. *Ultrasonics sonochemistry, 48*, 499-508.

Leong, T. S. H., Wooster, T., Kentish, S., & Ashokkumar, M. (2009). Minimising oil droplet size using ultrasonic emulsification. *Ultrasonics sonochemistry, 16*(6), 721-727.

Leong, T. S. H., Zhou, M., Kukan, N., Ashokkumar, M., & Martin, G. J. (2017). Preparation of water-in-oil-in-water emulsions by low frequency ultrasound using skim milk and sunflower oil. *Food Hydrocolloids, 63*, 685-695.

Leong, T. S. H., Zhou, M., Zhou, D., Ashokkumar, M., & Martin, G. J. (2018). The formation of double emulsions in skim milk using minimal food-grade emulsifiers–A comparison between ultrasonic and high pressure homogenisation efficiencies. *Journal of food engineering, 219*, 81-92.

Li, J., Eleya, M. O., & Gunasekaran, S. (2006). Gelation of whey protein and xanthan mixture: Effect of heating rate on rheological properties. *Food hydrocolloids, 20*(5), 678-686.

Li, M., & Fogler, H. (1978). Acoustic emulsification. Part 2. Breakup of the large primary oil droplets in a water medium. *Journal of Fluid Mechanics, 88*(3), 513-528.

Li, W., & Ashokkumar, M. (2018). Introduction to Ultrasound and Sonochemistry. *The Electrochemical Society Interface, 27*(3), 43-46.

Li, W., Gamlath, C. J., Pathak, R., Martin, G. J., & Ashokkumar, M. (2019). Ultrasound–The Physical and Chemical Effects Integral to Food Processing.

Liu, Y., & Guo, R. (2008). pH-dependent structures and properties of casein micelles. *Biophysical Chemistry, 136*(2-3), 67-73.

Liu, Z., Juliano, P., Williams, R. P., Niere, J., & Augustin, M. A. (2014). Ultrasound improves the renneting properties of milk. *Ultrasonics sonochemistry, 21*(6), 2131-2137.

Lobato-Calleros, C., Rodriguez, E., Sandoval-Castilla, O., Vernon-Carter, E., & Alvarez-Ramirez, J. (2006). Reduced-fat white fresh cheese-like products obtained from W1/O/W2 multiple emulsions: Viscoelastic and high-resolution image analyses. *Food Research International, 39*(6), 678-685.

Lobato-Calleros, C., Sosa-Pérez, A., Rodríguez-Tafoya, J., Sandoval-Castilla, O., Pérez-Alonso, C., & Vernon-Carter, E. (2008). Structural and textural characteristics of reduced-fat cheese-like products made from W1/O/W2 emulsions and skim milk. *LWT-Food Science and Technology, 41*(10), 1847-1856.

Lollo, P., Amaya-Farfan, J., Faria, I., Salgado, J., Chacon-Mikahil, M., Cruz, A., . . . Arruda, M. (2014). Hydrolysed whey protein reduces muscle damage markers in Brazilian elite soccer players compared with whey protein and maltodextrin. A twelve-week in-championship intervention. *International Dairy Journal, 34*(1), 19-24.

López-Expósito, I., Amigo, L., & Recio, I. (2012). A mini-review on health and nutritional aspects of cheese with a focus on bioactive peptides. *Dairy science & technology, 92*(5), 419-438.

Lopez-Fandino, R., Carrascosa, A., & Olano, A. (1996). The effects of high pressure on whey protein denaturation and cheese-making properties of raw milk. *Journal of Dairy Science, 79*(6), 929-936.

Lucey, J. A., & Kelly, J. (1994). Cheese yield. *International journal of dairy technology, 47*(1), 1-14.

Lucey, J. A., Munro, P. A., & Singh, H. (1999). Effects of heat treatment and whey protein addition on the rheological properties and structure of acid skim milk gels. *International Dairy Journal, 9*(3), 275-279.

Lund, M., & Ardö, Y. (2004). Purification and identification of water-soluble phosphopeptides from cheese using Fe (III) affinity chromatography and mass spectrometry. *Journal of agricultural and food chemistry, 52*(21), 6616-6622.

Madadlou, A., Mousavi, M. E., Emam-djomeh, Z., Ehsani, M., & Sheehan, D. (2009). Sonodisruption of re-assembled casein micelles at different pH values. *Ultrasonics sonochemistry, 16*(5), 644-648.

Mahé, S., Benamouzig, R., Gaudichon, C., Huneau, J., De, I. C., Rautureau, J., & Tomé, D. (1995). Nitrogen movements in the upper jejunum lumen in humans fed low amounts of casein or beta-lactoglobulin. *Gastroentérologie clinique et biologique, 19*(1), 20-26.

Makhal, S., Kanawjia, S., & Giri, A. (2015). Role of calcium chloride and heat treatment singly and in combination on improvement of the yield of direct acidified Cottage cheese. *Journal of food science and technology, 52*(1), 535-541.

Malacarne, M., Summer, A., Fossa, E., Formaggioni, P., Franceschi, P., Pecorari, M., & Mariani, P. (2006). Composition, coagulation properties and Parmigiano-Reggiano cheese yield of Italian Brown and Italian Friesian herd milks. *Journal of Dairy Research, 73*(2), 171-177.

Manios, Y., Moschonis, G., Mavrogianni, C., van den Heuvel, E., Singh-Povel, C. M., Kiely, M., & Cashman, K. D. (2017). Reduced-fat Gouda-type cheese enriched with vitamin D 3 effectively prevents vitamin D deficiency during winter months in postmenopausal women in Greece. *European journal of nutrition, 56*(7), 2367-2377.

Martin, G. J. O., Williams, R. P. W., Choong, C., Lee, B., & Dunstan, D. E. (2008). Comparison of rennet gelation using raw and reconstituted skim milk. *International Dairy Journal, 18*, 1077-1080.

Martin, G. J. O., Williams, R. P. W., & Dunstan, D. E. (2007). Comparison of casein micelles in raw and reconstituted skim milk. *Journal of Dairy Science, 90*, 4543-4551.

Martin, V., Ratel, S., Siracusa, J., Le Ruyet, P., Savary-Auzeloux, I., Combaret, L., . . . Dardevet, D. (2013). Whey proteins are more efficient than casein in the recovery of muscle functional properties following a casting induced muscle atrophy. *PloS one, 8*(9), e75408.

Martins, J. T., Cerqueira, M. A., Souza, B. W., Carmo Avides, M. d., & Vicente, A. n. A. (2010). Shelf life extension of ricotta cheese using coatings of galactomannans from nonconventional sources incorporating nisin against Listeria monocytogenes. *Journal of agricultural and food chemistry, 58*(3), 1884-1891.

Marziali, A., & Ng-Kwai-Hang, K. (1986). Effects of milk composition and genetic polymorphism on coagulation properties of milk. *Journal of Dairy Science, 69*(7), 1793-1798.

Mason, T. J., & Peters, D. (2002). *Practical sonochemistry: Power ultrasound uses and applications*: Woodhead Publishing.

Masotti, F., Cattaneo, S., Stuknytė, M., & De Noni, I. (2017). Technological tools to include whey proteins in cheese: Current status and perspectives. *Trends in Food Science & Technology, 64*, 102-114.

Matsumoto, S., Kita, Y., & Yonezawa, D. (1976). An attempt at preparing water-in-oil-in-water multiple-phase emulsions. *Journal of colloid and interface science, 57*(2), 353-361.

Maubois, J. L. (2002). Membrane microfiltration: a tool for a new approach in dairy technology. *Australian Journal of Dairy Technology, 57*(2), 92.

Maubois, J. L., Fauquant, J., Famelart, M. H., & Caussin, F. (2001). Milk microfiltrate, a convenient starting material for fractionation of whey proteins and derivatives. *Proteins, 96*, 46.

McMahon, D. J., Alleyne, M., Fife, R., & Oberg, C. (1996). Use of fat replacers in low fat Mozzarella cheese. *Journal of Dairy Science, 79*(11), 1911-1921. doi:https://doi.org/10.3168/jds.s0022-0302(96)76560-8

McMahon, D. J., & Oommen, B. (2008). Supramolecular structure of the casein micelle. *Journal of Dairy Science, 91*(5), 1709-1721.

McSweeney, P. L. (2004). Biochemistry of cheese ripening. *International journal of dairy technology, 57*(2-3), 127-144.

Meisel, H., & FitzGerald, R. J. (2003). Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. *Current pharmaceutical design, 9*(16), 1289-1296.

Metzger, L., Barbano, D., Rudan, M., & Kindstedt, P. (2000). Effect of milk preacidification on low fat Mozzarella cheese. I. Composition and yield. *Journal of Dairy Science, 83*(4), 648-658.

Meza-Nieto, M. A., Vallejo-Cordoba, B., González-Córdova, A. F., Félix, L., & Goycoolea, F. M. (2007). Effect of β-Lactoglobulin A and B Whey Protein Variants on the Rennet-Induced Gelation of Skim Milk Gels in a Model Reconstituted Skim Milk System. *Journal of Dairy Science, 90*(2), 582–593.

Michnik, A. (2003). Thermal stability of bovine serum albumin DSC study. *Journal of thermal analysis and calorimetry, 71*(2), 509-519.

Miller, K., Upadhyaya, S., & Krochta, J. (1998). Permeability of d-limonene in whey protein films. *Journal of food science, 63*(2), 244-247.

Mistry, V. V., & Maubois, J. (2017). Chapter 27-Application of Membrane Separation Technology to Cheese Production. *Cheese: Chemistry, Physics and Microbiology (4ª Ed.). PLH McSweeney PF Fox, PD Cotter, DW Everett (Eds.). Elsevier Academic Press, Cambridge, EUA. Pp*, 677-697.

Mleko, S., & Foegeding, E. (2000). Physical properties of rennet casein gels and processed cheese analogs containing whey proteins. *Milchwissenschaft, 55*(9), 513-516.

Moatsou, G., Zoidou, E., Choundala, E., Koutsaris, K., Kopsia, O., Thergiaki, K., & Sakkas, L. (2019). Development of Reduced-Fat, Reduced-Sodium Semi-Hard Sheep Milk Cheese. *Foods, 8*(6), 204.

Mollea, C., Marmo, L., & Bosco, F. (2013). Valorisation of cheese whey, a by-product from the dairy industry. In *Food industry*: IntechOpen.

Monahan, F. J., German, J. B., & Kinsella, J. E. (1995). Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *Journal of Agricultural and Food Chemistry, 43*(1), 46-52.

Monroy, Y., Rivero, S., & García, M. A. (2018). Microstructural and techno-functional properties of cassava starch modified by ultrasound. *Ultrasonics sonochemistry, 42*, 795-804.

Moro, A., Gatti, C., & Delorenzi, N. (2001). Hydrophobicity of whey protein concentrates measured by fluorescence quenching and its relation with surface functional properties. *Journal of agricultural and food chemistry, 49*(10), 4784-4789.

Morr, C. V. (1967). Effect of oxalate and urea upon ultracentrifugation properties of raw and heated skim skim milk casein micelles. *I. Dairy Sci, 50*, 1744-1750.

Mottar, J., Bassier, A., Joniau, M., & Baert, J. (1989). Effect of heat-induced association of whey proteins and casein micelles on yogurt texture. *Journal of Dairy Science, 72*(9), 2247-2256.

Mulvihill, D., & Donovan, M. (1987). Whey proteins and their thermal denaturation-a review. *Irish Journal of Food Science and Technology, 11*(1), 43-75.

Munir, M., Nadeem, M., Qureshi, T. M., Leong, T. S., Gamlath, C. J., Martin, G. J., & Ashokkumar, M. (2019). Effects of high pressure, microwave and ultrasound processing on proteins and enzyme activity in dairy systems—A review. *Innovative food science & emerging technologies*, 102192.

Muthukumaran, S., Kentish, S. E., Stevens, G. W., & Ashokkumar, M. (2006). Application of ultrasound in membrane separation processes: a review. *Reviews in chemical engineering, 22*(3), 155-194.

Needs, E. C., Capellas, M., Bland, A. P., Manoj, P., Macdougal, D., & Paul, G. (2000). Comparison of heat and pressure treatments of skim milk, fortified with whey protein concentrate, for set yogurt preparation: effects on milk proteins and gel structure. *Journal of Dairy Research, 67*(3), 329-348.

Nelson, B., & Barbano, D. (2005). A microfiltration process to maximize removal of serum proteins from skim milk before cheese making. *Journal of Dairy Science, 88*(5), 1891-1900.

Neocleous, M., Barbano, D., & Rudan, M. (2002). Impact 9 of Low Concentration Factor Microfiltration on Milk Component Recovery and Cheddar Cheese Yield. *Journal of Dairy Science, 85*(10), 2415-2424.

Nguyen, M., Reynolds, N., & Vigneswaran, S. (2003). By-product recovery from cottage cheese production by nanofiltration. *Journal of Cleaner production, 11*(7), 803-807.

Nguyen, N. H., & Anema, S. G. (2010). Effect of ultrasonication on the properties of skim milk used in the formation of acid gels. *Innovative Food Science & Emerging Technologies, 11*(4), 616-622.

NHS. (2018). Dairy and alternatives in your diet. Retrieved from https://www.nhs.uk/live-well/eat-well/milk-and-dairy-nutrition/

O'Sullivan, J., Arellano, M., Pichot, R., & Norton, I. (2014). The effect of ultrasound treatment on the structural, physical and emulsifying properties of dairy proteins. *Food Hydrocolloids, 42*, 386-396.

O'Brien, N. M., & O'Connor, T. P. (2017). Nutritional Aspects of Cheese. In *Cheese* (pp. 603-611): Elsevier.

Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. (2011). Microstructure of milk gel and cheese curd observed using cryo scanning electron microscopy and confocal microscopy. *LWT-Food Science and Technology, 44*(5), 1291-1302.

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*(1), 119-130.

Papadatos, A., Neocleous, M., Berger, A., & Barbano, D. (2003a). Economic feasibility evaluation of microfiltration of milk prior to cheesemaking. *Journal of Dairy Science, 86*(5), 1564-1577.

Papadatos, A., Neocleous, M., Berger, A. M., & Barbano, D. M. (2003b). Economic Feasibility Evaluation of Microfiltration of Milk Prior to Cheesemaking. *J. Dairy Sci., 86*, 1564–1577.

Pappas, C., Kondyli, E., Voutsinas, L., & Mallatou, H. (1996). Effects of starter level, draining time and aging on the physicochemical, organoleptic and rheological properties of feta cheese. *International journal of dairy technology, 49*(3), 73-78.

Park, S. Y., Niki, R., & Nakumara, K. (1996). Rheological Behavior of Casein Micelles and Reconstituted Skim Milk Gels: The Effect of Temperature on Gelation Induced by Rennet. *Food Science and Technology International, Tokyo, 2*(2), 103-107.

Patel, A. R., Rajarethinem, P. S., Gręd owska, A., Turhan, O., Lesaffer, A., De Vos, W. H., . . . Dewettinck, K. (2014). Edible applications of shellac oleogels: spreads, chocolate paste and cakes. *Food & function, 5*(4), 645-652.

Patel, S. (2015). Functional food relevance of whey protein: A review of recent findings and scopes ahead. *Journal of Functional Foods, 19*, 308-319.

Pathak, R., Leong, T. S. H., Martin, G. J., & Ashokkumar, M. (2020). Amino acid and secondary structure integrity of sonicated milk proteins. *Australian Journal of Chemistry*, 170-179.

Payens, T. A. J. (1966). Association of Caseins and their Possible Relation to Structure of the Micelle1. *Journal of Dairy Science yr:1966 vol:49 iss:11 pg:1317 -1324, 49*, 1317 -1324.

Pehlivanoğlu, H., Demirci, M., Toker, O. S., Konar, N., Karasu, S., & Sagdic, O. (2018). Oleogels, a promising structured oil for decreasing saturated fatty acid concentrations: Production and food-based applications. *Critical reviews in food science and nutrition, 58*(8), 1330-1341.

Pennings, B., Boirie, Y., Senden, J. M., Gijsen, A. P., Kuipers, H., & van Loon, L. J. (2011). Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *The American journal of clinical nutrition, 93*(5), 997-1005.

Pennings, B., Groen, B., de Lange, A., Gijsen, A. P., Zorenc, A. H., Senden, J. M., & Van Loon, L. J. (2012). Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. *American Journal of Physiology-Endocrinology and Metabolism, 302*(8), E992-E999.

Peri, C., & Dunkley, W. (1971). Reverse osmosis of cottage cheese whey. 1. Influence of composition of the feed. *Journal of food science, 36*(1), 25-30.

Permyakov, E. A., & Berliner, L. J. (2000). α-Lactalbumin: structure and function. *FEBS letters, 473*(3), 269-274.

Perreault, V., Morin, P., Pouliot, Y., & Britten, M. (2017). Effect of denatured whey protein concentrate and its fractions on rennet-induced milk gels. *International Dairy Journal, 64*, 48-55. doi:https://doi.org/10.1016/j.idairyj.2016.09.005

Perreault, V., Rémillard, N., Chabot, D., Morin, P., Pouliot, Y., & Britten, M. (2017). Effect of denatured whey protein concentrate and its fractions on cheese composition and rheological properties. *Journal of Dairy Science, 100*(7), 5139-5152. doi:https://doi.org/10.3168/jds.2016-12473

Picot, A., & Lacroix, C. (2004). Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. *International Dairy Journal, 14*(6), 505-515.

Pierro, P. D., Mariniello, L., Sorrentino, A., Giosafatto, C. V. L., Chianese, L., & Porta, R. (2010). Transglutaminase-induced chemical and rheological properties of cheese. *Food biotechnology, 24*(2), 107-120.

Pouliot, Y. (2008). Membrane processes in dairy technology—From a simple idea to worldwide panacea. *International Dairy Journal, 18*(7), 735-740.

Punidadas, P., Feirtag, J., & Tung, M. (1999). Incorporating whey proteins into mozzarella cheese. *International journal of dairy technology, 52*(2), 51-55. doi:https://doi.org/10.1111/j.1471-0307.1999.tb02070.x

Puyol, P., Perez, M., & Horne, D. (2001). Heat-induced gelation of whey protein isolates (WPI): effect of NaCl and protein concentration. *Food Hydrocolloids, 15*(3), 233-237.

Qi, X. L., Brownlow, S., Holt, C., & Sellers, P. (1995). Thermal denaturation of β-lactoglobulin: effect of protein concentration at pH 6.75 and 8.05. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1248*(1), 43-49.

Ramos, M. J., Fernández, C. M., Casas, A., Rodríguez, L., & Pérez, Á. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource technology, 100*(1), 261-268.

Rektor, A., & Vatai, G. (2004). Membrane filtration of Mozzarella whey. *Desalination, 162*, 279-286.

Renner, E. (1993). Nutritional aspects of cheese. In *Cheese: chemistry, physics and microbiology* (pp. 557-579): Springer.

Roefs, S. P., & De Kruif, K. G. (1994). A model for the denaturation and aggregation of β-lactoglobulin. *European Journal of Biochemistry, 226*(3), 883-889. doi:https://doi.org/10.1111/j.1432-1033.1994.00883.x

Roy, S. S., Mukherjee, S., Ballard, B. R., & Das, S. K. (2016). Protection against dimethylbenz [a] anthracene-induced breast cancer in female rats by α-lactalbumin. *International journal of cancer and oncology, 3*(1), 1.

Rubel, I. A., Iraporda, C., Gallo, A., Manrique, G. D., & Genovese, D. B. (2019). Spreadable ricotta cheese with hydrocolloids: Effect on physicochemical and rheological properties. *International Dairy Journal, 94*, 7-15.

Rudan, M. A., Barbano, D. M., Yun, J. J., & Kindstedt, P. S. (1999). Effect of fat reduction on chemical composition, proteolysis, functionality, and yield of Mozzarella cheese. *Journal of Dairy Science, 82*(4), 661-672.

Rüegg, M., Moor, U., & Blanc, B. (1977). A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. *Journal of Dairy Research, 44*(3), 509-520.

Rynne, N. M., Beresford, T. P., Kelly, A. L., & Guinee, T. P. (2004). Effect of milk pasteurization temperature and in situ whey protein denaturation on the composition, texture and heat-induced functionality of half-fat Cheddar cheese. *International Dairy Journal, 14*(11), 989-1001.

San Martín-González, M., Rodríguez, J., Gurram, S., Clark, S., Swanson, B., & Barbosa-Cánovas, G. (2007). Yield, composition and rheological characteristics of cheddar cheese made with high pressure processed milk. *LWT-Food Science and Technology, 40*(4), 697-705.

Sánchez-García, Y. I., Bhangu, S. K., Ashokkumar, M., & Gutiérrez-Méndez, N. (2018). Sonocrystallization of lactose from whey. *Technological Approaches for Novel Applications in Dairy Processing*, 51.

Sánchez-García, Y. I., Gutiérrez-Méndez, N., Orozco-Mena, R. E., Ramos-Sánchez, V. H., & Leal-Ramos, M. Y. (2019). Individual and combined effect of pH and whey proteins on lactose crystallization. *Food Research International, 116*, 455-461.

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*(4), 951-956.

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*(1), 75-82.

Sanmartín, B., Díaz, O., Rodríguez-Turienzo, L., & Cobos, A. (2018). Emulsion characteristics of salad dressings as affected by caprine whey protein concentrates. *International journal of food properties, 21*(1), 12-20.

Sanmartín, B., Díaz Rubio, O., Rodríguez Turienzo, L., & Cobos García, Á. (2018). Emulsion characteristics of salad dressings as affected by caprine whey protein concentrates.

Santiago-López, L., Aguilar-Toalá, J. E., Hernández-Mendoza, A., Vallejo-Cordoba, B., Liceaga, A. M., & González-Córdova, A. F. (2018). Invited review: Bioactive compounds produced during cheese ripening and health effects associated with aged cheese consumption. *Journal of Dairy Science, 101*(5), 3742-3757.

Sapru, A., Barbano, D. M., Yun, J. J., Klei, L. R., Oltenacu, P. A., & Bandler, D. K. (1997). Cheddar cheese: influence of milking frequency and stage of lactation on composition and yield. *Journal of Dairy Science, 80*(3), 437-446.

Sato, K. (2001). Crystallization behaviour of fats and lipids—a review. *Chemical Engineering Science, 56*(7), 2255-2265.

Schenkel, P., Samudrala, R., & Hinrichs, J. (2013). The effect of adding whey protein particles as inert filler on thermophysical properties of fat-reduced semihard cheese type Gouda. *International journal of dairy technology, 66*(2), 220-230. doi:https://doi.org/10.1111/1471-0307.12036

Schmidt, D. G. (1976). Association of caseins and casein micelle structure. In *Developments in Dairy Chemistry*1* (Vol. 9, pp. 61-86). London: Elsevier Applied Science.

Schreiber, R. (2000). Funktionalitat und Labgelbildung hocherhitzter Caseinmicellsuspensionen-Modellierung von Prozessparametern zur sicheren Kaseherstellung. *FORTSCHRITT BERICHTE-VDI REIHE 3 VERFAHRENSTECHNIK*, All-All.

Shanmugam, A., & Ashokkumar, M. (2014). Ultrasonic preparation of stable flax seed oil emulsions in dairy systems–physicochemical characterization. *Food Hydrocolloids, 39*, 151-162.

Sieber, R., Bütikofer, U., Egger, C., Portmann, R., Walther, B., & Wechsler, D. (2010). ACE-inhibitory activity and ACE-inhibiting peptides in different cheese varieties. *Dairy science & technology, 90*(1), 47-73.

Silva, M., Zisu, B., & Chandrapala, J. (2018). Influence of low-frequency ultrasound on the physico-chemical and structural characteristics of milk systems with varying casein to whey protein ratios. *Ultrasonics sonochemistry, 49*, 268-276.

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*(1), 179-197.

Singh, H., Shalabi, S. I., Fox, P. F., Flynn, A., & Barry, A. (1988). Rennet coagulation of heated milk: influence of pH adjustment before or after heating. *Journal of Dairy Research, 55*(2), 205-215.

Singh, H., & Waungana, A. (2001). Influence of heat treatment of milk on cheesemaking properties. *International Dairy Journal, 11*(4-7), 543-551. doi:https://doi.org/10.1016/s0958-6946(01)00085-1

Sinha, R., Radha, C., Prakash, J., & Kaul, P. (2007). Whey protein hydrolysate: Functional properties, nutritional quality and utilization in beverage formulation. *Food Chemistry, 101*(4), 1484-1491.

Slattery, C. W. (1976). Casein Micelle Structure; An Examination of Models: Review. *Journal of Dairy Science, 59*, 1547–1556.

Slattery, C. W., & Evard, R. (I973). A model for the formation and structure of casein micelles from subunits of variable composition. *Biochimica et Biophysica Acta, 317*, 529-538.

Sodini, I., Mattas, J., & Tong, P. S. (2006). Influence of pH and heat treatment of whey on the functional properties of whey protein concentrates in yoghurt. *International Dairy Journal, 16*(12), 1464-1469.

Sodini, I., Montella, J., & Tong, P. S. (2005). Physical properties of yogurt fortified with various commercial whey protein concentrates. *Journal of the Science of Food and Agriculture, 85*(5), 853-859.

Stankey, J. A., Lu, Y., Abdalla, A., Govindasamy-Lucey, S., Jaeggi, J. J., Ø Mikkelsen, B., . . . Andersen, C. B. (2017). Low-fat Cheddar cheese made using microparticulated whey proteins: Effect on yield and cheese quality. *International journal of dairy technology, 70*(4), 481-491.

Sturaro, A., De Marchi, M., Zorzi, E., & Cassandro, M. (2015). Effect of microparticulated whey protein concentration and protein-to-fat ratio on Caciotta cheese yield and composition. *International Dairy Journal, 48*, 46-52. doi:https://doi.org/10.1016/j.idairyj.2015.02.003

Suárez, A., Fernández, L., Balbarie, P., Iglesias, J. R., & Riera, F. A. (2016). Predicting the properties of the whey protein microparticles produced by heat and mechanical treatments. *European Food Research and Technology, 242*(8), 1211-1220.

Teixeira, F. J., Santos, H. O., Howell, S. L., & Pimentel, G. D. (2019). Whey protein in cancer therapy: A narrative review. *Pharmacological research*.

Tian, Z. M., Wan, M. X., Wang, S. P., & Kang, J. Q. (2004). Effects of ultrasound and additives on the function and structure of trypsin. *Ultrasonics sonochemistry, 11*(6), 399-404.

Torres, I. C., Janhøj, T., Mikkelsen, B. Ø., & Ipsen, R. (2011). Effect of microparticulated whey protein with varying content of denatured protein on the rheological and sensory characteristics of low-fat yoghurt. *International Dairy Journal, 21*(9), 645-655.

Trejo, R., Dokland, T., Jurat-Fuentes, J., & Harte, F. (2011). Cryo-transmission electron tomography of native casein micelles from bovine milk. *Journal of Dairy Science, 94*(12), 5770-5775.

Tsujii, S., & Shirotani, N. (2015). Cheese-like food article. In: Google Patents.

Udabage, P., McKinnon, I., & Augustin, M. (2001). Effects of mineral salts and calcium chelating agents on the gelation of renneted skim milk. *Journal of Dairy Science, 84*(7), 1569-1575.

Ulpathakumbura, C., Ranadheera, C. S., Senavirathne, N., Jayawardene, L., Prasanna, P., & Vidanarachchi, J. K. (2016). Effect of biopreservatives on microbial, physico-chemical and sensory properties of Cheddar cheese. *Food bioscience, 13*, 21-25.

Upreti, P., Mistry, V. V., & Acharya, M. R. (2011). Characterization of rennet coagulation of milk concentrated by vacuum condensing and ultrafiltration. *Dairy science & technology, 91*(3), 383-395.

Van Hooydonk, A. (1986). pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 1. Effect of acidification on physico-chemical properties. *Netherlands Milk and Dairy Journal, 40*, 281-296.

Van Hooydonk, A., Hagedoorn, H., & Boerrigter, I. (1986). The effect of various cations on the renneting of milk. *Netherlands Milk and Dairy Journal (Netherlands)*.

Vasbinder, A. J., & De Kruif, C. G. (2003). Casein–whey protein interactions in heated milk: the influence of pH. *International Dairy Journal, 13*(8), 669-677.

Vasbinder, A. J., Rollema, H. S., & De Kruif, C. G. (2003). Impaired rennetability of heated milk; study of enzymatic hydrolysis and gelation kinetics. *Journal of Dairy Science, 86*(5), 1548-1555.

Vasbinder, A. J., van de Velde, F., & de Kruif, C. G. (2004). Gelation of casein-whey protein mixtures. *Journal of Dairy Science, 87*(5), 1167-1176.

Vermeer, A. W., & Norde, W. (2000). The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. *Biophysical journal, 78*(1), 394-404.

Voigt, D. D., Donaghy, J. A., Patterson, M. F., Stephan, S., & Kelly, A. L. (2010). Manufacture of Cheddar cheese from high-pressure-treated whole milk. *Innovative food science & emerging technologies, 11*(4), 574-579.

Vong, F., Son, Y., Bhuiyan, S., Zhou, M., Cavalieri, F., & Ashokkumar, M. (2014). A comparison of the physical properties of ultrasonically synthesized lysozyme-and BSA-shelled microbubbles. *Ultrasonics sonochemistry, 21*(1), 23-28.

Walstra, P. (1990). On the Stability of Casein Micelles1. *Journal of Dairy Science, 73*, 1965-1979.

Walstra, P. (1999). Casein sub-micelles: do they exist. *International Dairy Journal, 9*, 189}192.

Walzem, R., Dillard, C., & German, J. B. (2002). Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking. *Critical reviews in food science and nutrition, 42*(4), 353-375.

Whitney, R. M. (1999). Proteins of Milk. In N. P. Wong, R. Jenness, M. Keeney, & E. H. Marth (Eds.), *Findamentals of Dairy Chemistry* (pp. 81-107). Maryland: Aspen Publication.

Witard, O. C., Jackman, S. R., Breen, L., Smith, K., Selby, A., & Tipton, K. D. (2013). Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *The American journal of clinical nutrition, 99*(1), 86-95.

Wong, N., LaCroix, D., & McDonough, F. (1978). Minerals in whey and whey fractions. *Journal of Dairy Science, 61*(12), 1700-1703.

Yadav, J. S. S., Yan, S., Pilli, S., Kumar, L., Tyagi, R. D., & Surampalli, R. Y. (2015). Cheese whey: A potential resource to transform into bioprotein, functional/nutritional proteins and bioactive peptides. *Biotechnology Advances, 33*(6), 756-774.

Yamauchi, K., Shimizu, M., & Kamiya, T. (1980). Emulsifying properties of whey protein. *Journal of food science, 45*(5), 1237-1242.

Yanjun, S., Jianhang, C., Shuwen, Z., Hongjuan, L., Jing, L., Lu, L., . . . Wupeng, G. (2014). Effect of power ultrasound pre-treatment on the physical and functional properties of reconstituted milk protein concentrate. *Journal of food engineering, 124*, 11-18.

Ye, A., Cui, J., Taneja, A., Zhu, X., & Singh, H. (2009). Evaluation of processed cheese fortified with fish oil emulsion. *Food Research International, 42*(8), 1093-1098.

Yorgun, M., Balcioglu, I. A., & Saygin, O. (2008). Performance comparison of ultrafiltration, nanofiltration and reverse osmosis on whey treatment. *Desalination, 229*(1-3), 204-216.

Zarogoulidis, P., Tsakiridis, K., Karapantzou, C., Lampaki, S., Kioumis, I., Pitsiou, G., . . . Kesisis, G. (2015). Use of proteins as biomarkers and their role in carcinogenesis. *Journal of Cancer, 6*(1), 9.

Zisu, B., Lee, J., Chandrapala, J., Bhaskaracharya, R., Palmer, M., Kentish, S., & Ashokkumar, M. (2011). Effect of ultrasound on the physical and functional properties of reconstituted whey protein powders. *Journal of Dairy Research, 78*(2), 226-232.

Zulewska, J., Newbold, M., & Barbano, D. M. (2009). Efficiency of serum protein removal from skim milk with ceramic and polymeric membranes at 50°C1. *J. Dairy Sci, 92*, 1361–1377.

Chapter 3

3. Investigating the role of native whey proteins in the rennet gelation of bovine milk

The aim of this chapter is to develop fundamental understanding on how native whey proteins interact with casein micelles during rennet induced hydrolysis and aggregation of casein micelles. This work further discusses the mechanism by which native whey proteins affects the kinetics of rennet gelation and demonstrates how whey protein depleted streams produced from membrane filtration can undergo rapid gelation, compared to normal cheese milk.

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2.1 Introduction

Rennet gelation is a key process in cheese making that transforms liquid milk to a coagulum. Much research has been undertaken to understand the mechanisms of rennet gelation (Guinee & Wilkinson, 1992). This has facilitated improvement of cheese production processes to manufacture better quality cheese at a lower cost. More recently, the advent of membrane filtration technology has enabled control over the protein composition of the milk used for cheese making (Maubois, 2002). While the overall mechanisms of rennet gelation are well established (Lucey, 2002), a more detailed understanding of the mechanisms in relation to protein composition is required to take full advantage of membrane technology in the manufacture of renneted cheese.

Milk is a complex system, comprising of an emulsion of fat globules, a colloidal suspension of two distinct types of proteins (caseins & whey proteins), and an aqueous solution of lactose and various minerals (Jenness, 1999). Whereas, whey proteins are soluble in water, caseins (CN) are present in milk in the form of large colloidal aggregates (50-200 nm in diameter) known as casein micelles. The surface of casein micelles is rich in κ-casein, which has a hydrophilic section that protrudes outwards like a 'hairy layer', stabilising the micelles through electrostatic and steric repulsion (Walstra, 1999). Rennet gelation destabilises the casein micelles by selectively cleaving the 'hairy layer' using a proteolytic enzyme called 'chymosin' that is found in rennet (Vasbinder et al., 2003). As this enzymatic reaction progresses, the cleaved C-terminal of the κ-CN known as casein macro peptide (CMP) solubilises in the milk serum. The casein micelles become progressively more hydrophobic allowing them to aggregate to form a coagulum (Fox et al., 2017; Vasbinder et al., 2003; Walstra, 1990).

Casein micelles are central to the rennet gelation process and the rate of coagulation increases with increasing casein concentration (Fox et al., 2017). Ultrafiltration can be used to concentrate milk proteins in cheese milk (Nelson & Barbano, 2005), which increases the rate of gelation as well as the volumetric productivity of the cheese vats. Whereas UF concentrates all of the milk proteins, microfiltration can separate larger casein micelles from the smaller whey proteins (Maubois, 2002; Papadatos et al., 2003; Zulewska et al., 2009). This enables the production of cheese milks from MF retentates with increased casein:whey protein (CN:WP) ratios.

Although the overall mechanisms of rennet gelation are well established, the role of whey proteins has yet to be fully elucidated. As separate soluble proteins, the whey proteins are not directly involved in the rennet gelation process. However, when combined with heat treatment above the denaturation temperature (~65-70 °C), whey proteins have been found to impair rennet gelation leading to elongated gelation time and weaker gels (Vasbinder et al., 2003) . This behaviour was attributed to the formation disulphide bridging between thiol groups of the κ-CN and denatured β-lactoglobulin (β-LG), the most abundant of the whey proteins (Jang & Swaisgood, 1990). Although it was initially thought that this disulphide bridging blocked the κ-casein hairs from being cleaved (Hinrichs, 2001), a more recent study has shown that heat treatment has a more significant effect on the aggregation of the renneted casein micelles (para-casein micelles) than on the actual κ-casein hydrolysis (Vasbinder et al., 2003). In a contrasting study, supplementation of reconstituted skim milk powder with whey protein prior to heating at 65 °C for 30 min and subsequent rennet gelation was found to result in firmer gels (Meza-Nieto et al., 2007). Although this degree of heat treatment was shown not to result in extensive attachment of denatured whey protein to the casein micelles, the increased gel strength was attributed to the cross-linking between the casein micelles by whey proteins (Meza-Nieto et al., 2007). A more likely explanation, however, is that the addition of whey powder simply increased the soluble calcium levels, which were not standardised and are otherwise lacking in reconstituted skim milk powder (Martin et al., 2007), thereby limiting the rate of rennet gelation (Martin et al., 2008).

In cheese production, milk does not typically undergo severe enough heat treatment for whey proteins to be denatured. Therefore, it is the absence of native whey protein in MF retentates that is of interest from a cheese making perspective. However, to date there have been only a few studies (Lelievre et al., 1990) investigating the effect of native whey proteins on the rennet gelation process focusing mainly on the overall gelation properties of milk systems. Lelievre and colleagues observed an increased clotting time with added WPI and an inhibition of α_{s1}-casein hydrolysis, which develops flavour during the cheese maturation stage. However, the effects native whey proteins have on the enzyme hydrolysis of κ-CN and the aggregation phases post-renneting have not yet been distinguished clearly. Therefore, in this study we investigate for the first time the effect of native whey protein on the kinetics of the enzymatic hydrolysis and the aggregation phases of rennet gelation. This was done by comparing the gelation of milks prepared with a wide range of WP:CN ratios (with a constant CN

concentration) using casein concentrate and whey protein concentrate (WPC) powders produced through microfiltration.

Establishing the role of native whey protein on the kinetics and mechanisms of rennet gelation will enable the cheese industry to optimise the formulation of cheese milks with different WP:CN ratios to maximise productivity and reduce the total production cost. Further, a detailed kinetic study of rennet gelation will provide new insights, especially into the secondary aggregation stage of casein micelles that is less understood to date.

3.2. Materials and Methods

3.2.1. *Production of casein and whey powders*

All casein and whey protein solutions were made from reconstituted powders that were isolated using a microfiltration process. The microfiltration consisted of a 3-stage batch concentration process adapted from Nelson and Barbano (Nelson & Barbano, 2005), designed to isolate casein from whey proteins in skim milk. ISOFLUX^{TM} microfiltration membranes with a nominal pore size of ~ 0.15 µm were operated in a membrane filtration pilot plant (GEA, 2012 Model Type R). The microfiltration process was operated with a nominal permeate flux of between 60-90 kg/hour, feed pressure of 1 bar, and recirculation pressure between 2.5-2.9 bar.

700 kg of pasteurized skim milk was processed through the microfiltration pilot plant in stage 1. Casein was concentrated in the retentate as whey proteins were selectively removed in the permeate. Retentate was bled from the holding tank at a rate of ~ 30 kg/hour. Approximately 3X protein concentration of the retentate occurred at the completion of each stage of processing. Prior to stage 2 and 3 diafiltration, a volume of filtered water was added to the retentate to return it to ~1X protein concentration. Temperature was maintained at 55 °C throughout the process.

The permeate (ideal whey stream) was collected and separately concentrated using a multistage ultrafiltration (UF) process. The UF was operated at a nominal permeate flux of between 240-300 L/hour, feed pressure of 3 bar, and recirculation pressure of between 4.0-4.1 bar. Approximately 20X protein concentration was achieved by the UF process. 2 stages of diafiltration with 90 kg of water were performed. Temperature was maintained at 10 °C throughout the ultrafiltration process.

The concentrated liquid retentate and permeate streams were collected and spray dried (GEA-Niro, Mobile Minor). Liquid was fed into the spray dryer via a rotary atomizer set with an outlet temperature of 80 °C. Water was evaporated at a rate of ~2 kg/hour.

3.2.2. *Compositional analysis of casein and whey powder*

Protein analysis of powders were performed based on the Dumas Combustion method using a LECO Trumac NCS analyser (LECO Corporation, Michigan, USA) following a method that complies with the standard ISO 1489. Samples of powder (~ 0.1 g) were weighed onto a ceramic boat and dried in the oven overnight (104 °C). The boats were then loaded into the LECO analyser and combusted at a temperature of 1100 °C within the furnace. Protein values were determined from the measured nitrogen % by multiplying with the ratio 6.38 (Mariotti et al., 2008). The protein compositions of the casein powder and native whey protein powder were 84.9% w/w and 76.3% w/w respectively.

The proportion of casein and whey protein were also assessed using the LECO, using reconstituted liquid samples after casein and/or whey proteins were precipitated by acid to determine the non-casein nitrogen and non-protein nitrogen. The protein in the casein powder was determined to be 97.0% w/w casein and 3.0% w/w whey protein. The protein in the native whey protein powder was determined to be 1.5% w/w casein and 98.5% w/w whey protein.

3.2.3. *Standardised cheese milks*

Batches of concentrated casein (10% w/w) and whey protein (10% w/w and 20% w/w) were prepared by reconstituting ultrafiltered casein and WPC powders (preparation method is provided in the supplementary data). Sodium azide (Chem-Supply, assay: 99%) was added to each solution at 0.02% w/w to inhibit microbial action and stored at 4 °C. Four milk systems were prepared by mixing the concentrated solutions to achieve WP:CN ratios of 0.03:1, 0.25:1 (the ratio in natural milk), 1:1 and 4:1 w/w while keeping the casein concentration constant at 0.0264 g/g to match the casein content of skim milk. Calcium chloride (Chem-Supply, assay: 93%) was added to each milk system to achieve a final concentration of 4.7 mM. The pH (measured from Mettler Toledo Education Line pH meter) of every milk system was maintained between 6.6 and 6.8. Replicate samples made from concentrated casein and whey protein batches were tested during each experiment. The number of replicates of each experiment are denoted in the caption of figures illustrated in the results and discussion section.

3.2.4. Determination of gelation kinetics by rheometry

Samples (20 mL) of each milk system were brought to 31 °C in a water bath and renneted with 0.2 mL of 3.5 IMCU/mL rennet solution (prepared from 200 IMCU/mL Chymax Plus FPC, Cheeselinks, Australia, batch numbers 3233770 & 3241446). The rate of gelation was determined by measuring the storage modulus (G') as a function of time using an AR-G2 rheometer (TA Instruments) with a controlled shear strain of 2.5% at 1 Hz using the standard concentric cylinder (996284) tool at 31 °C for 1 hr. The onset of gelation was defined as the time at which G' first exceeded >0.2 Pa (after normalising the G' at t = 0 to -0.2 Pa).

3.2.5. Soluble calcium measurement

Each milk system, before and after the addition of calcium chloride, was ultracentrifuged at 100,000 x g using a Beckman Coulter ultracentrifuge unit (Model: Optima L-100 XP with type 70 Ti rotor) for 1 hr at 4 °C. The supernatant was immediately separated and filtered through 0.45 µm syringe filters. The filtrate was acid precipitated with 100% w/v trichloro acetic acid (BDH AnalR, assay: 99.5%) (1.2 mL per 0.5 mL sample) and diluted to 10 mL with deionized and filtered water. The samples were then centrifuged at 839 g for 5 minutes using a Thermofisher Hereus Megafuge 8 bench top centrifuge with HIGHConic III fixed angle rotor and the supernatant was filtered using a 0.45 µm syringe filters. 0.3 mL of the filtrate was diluted with 4.7 mL of deionized and filtered water and the soluble calcium was measured with inductive coupled plasma (ICP) optical emission spectrometer (Varian 720-ES with SPS 3 auto sampler, wavelength: 315.887 nm, power: 1 kW, plasma flow: 15 L/min, auxiliary flow: 1.5 L/min, nebulizer flow: 0.75 L/min, pump rate: 15 rpm).

3.2.6. Particle size measurement

Freshly prepared samples of each milk system were diluted 2000 times with deionized and filtered water and analysed under the 'milk protein size analysis' option (particle refractive index: 1.45, particle absorption 0.001, viscosity of water 0.8872 cP, refractive index of water 1.33, measurement angle: 173° backscatter) of a Malvern Zetasizer (Nano ZS) using disposable cells (PTS0012) at 25 °C.

3.2.7. Viscosity measurement

The viscosity of each milk system was measured with the same rheometer described above using a flow procedure with an increasing strain rate (0-500 s^{-1}) at 31 °C using a 40 mm 2° steel cone (988134). The gradient of the linear shear stress vs strain rate graph was recorded as the viscosity.

3.2.8. Enzymatic kinetics

The kinetics of the hydrolysis of κ-casein by rennet were tracked by measuring the change in the casein macro peptide (CMP) concentration in the serum using a RP-HPLC (SHIMADZU controller SCL-10AVP, oven CTO-10ASVP, pump LC-10ATVP, auto injector SIL-10ADVP, diode array detector APDM10AVP) with eluents A (Water: Acetonitrile: Tri-fluoro acetic acid 900:100:1) and B (Water: Acetonitrile: Tri fluoro acetic acid 100:900:1). The gradient was started with 15% of B and increased to 28% over 13 min, 32% over 22 min, 70% over 3 min, kept at 70% for 5 min and returned to the starting condition. A C18 column (Jupiter 5 µm, 300 A^0, 250 x 4.6 mm) was used and the flow rate was 0.8 mL at 30 °C (Vasbinder et al., 2003). Prepared milk samples were renneted as described above and 1.4 mL of 10% w/v trichloro acetic acid was added to 2 mL of renneted milk at the desired time intervals to inhibit rennet action and precipitate the caseins. The samples were immediately refrigerated to 4 °C. The stored samples were centrifuged at 2717 g in a Thermofisher Hereus Megafuge 8 benchtop centrifuge with HIGHConic III fixed angle rotor for 20 minutes and the supernatants filtered using 0.45 µm syringe filters. 0.2 mL of the permeate was diluted in 2 mL of a 70:30 mixture of A and B and used for analysis. Preliminary analyses were carried out to identify the peak given by the CMP by renneting a sample of WP:CN 0.03:1 for 3 hr and comparing the chromatogram with an un-renneted sample pre-treated identically.

3.3. Results and Discussion

3.3.1. Overall kinetics of gelation

Rennet induced gelation of milk proceeds in three overlapping stages: i) hydrolysis of κ-casein, ii) interaction between para-casein micelles, and iii) the formation of a cross-linked gel (Carlson & Hill Jr, 1987; Hyslop, 2003). Rheology was used in this study to compare the overall rate of milk gelation as a function of different ratios of casein and whey protein. Figure 3.1(a) illustrates the change in G' of each milk system (at 31 °C) with time after the addition

of rennet. The abrupt increase in G' indicates the onset of gelation and is the characteristic behaviour of a gelling system under kinetic control (Meza-Nieto et al., 2007). A comparison between the four milk systems show that the rate of gelation decreased with increasing whey protein concentration. At or below the native WP:CN ratio (i.e.0.25:1 and 0.03:1) the onset of gelation did not change significantly (Figure 3.1c). However, at higher WP:CN ratios (1:1 and 4:1) the increase in whey protein concentration significantly delayed the onset of gelation. The value of G' reached after 60 min decreased with increasing whey protein across the range of WP:CN ratios tested. (Figure 3.1b). These results show that native whey proteins can inhibit the rennet gelation process. Subsequent experiments were performed to determine which stages of the gelation process were impaired, and to help reveal the inhibitory mechanisms of the native whey proteins.

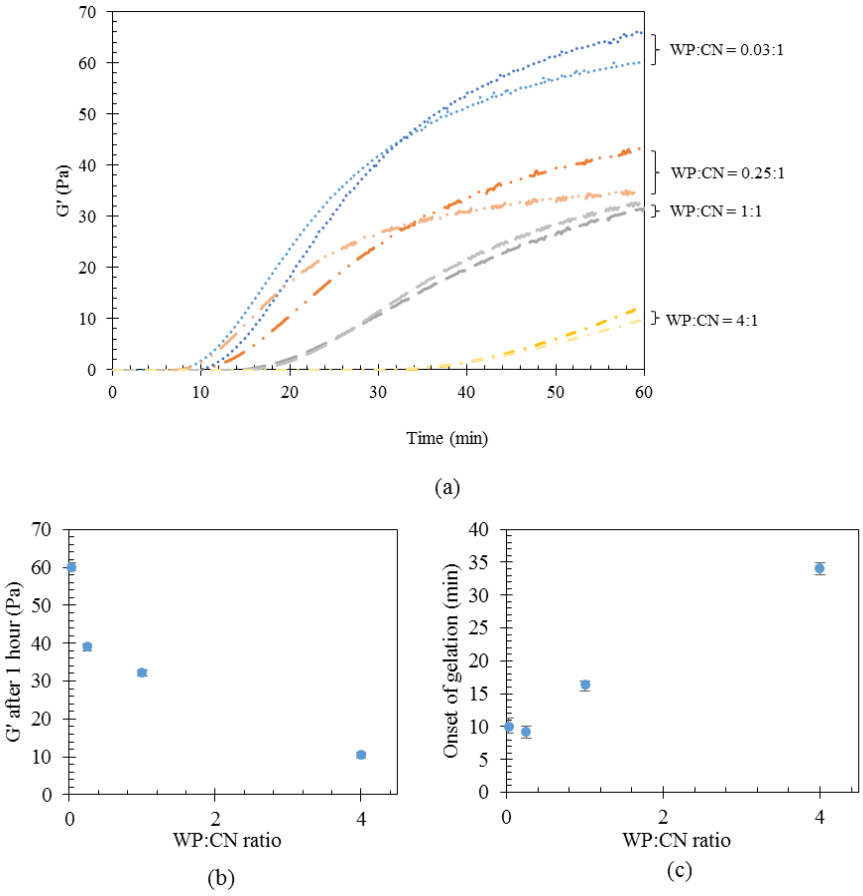


Figure 3.1.: (a) G' as a function of time after addition of rennet for milk systems with WP:CN ratios of 0.03:1 (·····), 0.25:1 (—·—) , 1:1 (– – –) and 1:4 (— · —).(b) Gel strength after 1 hr as a function of WP:CN ratio in each milk system (c) Onset of gelation as a function of the WP:CN ratio in each milk system.. The error bars indicate the standard deviation of measurements of duplicate experiments.

3.3.2. Rate of κ-casein hydrolysis

To investigate whether the presence of native whey protein affects the rate of κ-CN hydrolysis, experiments were conducted in which the concentration of CMP in the serum was measured at different time intervals after addition of rennet (Figure 3.2a). When the systems with WP:CN ratios of 0.03:1 and 4:1 are compared, a decreased rate of hydrolysis is observed in the milk system with the higher concentration of whey protein. This is consistent with the results of Lelièvre et al. (Lelievre et al., 1990) and further with the observed delay in the onset of gelation (Figure 3.1c). While impaired gelation due to whey addition was previously attributed to enzymatic inhibition by Lelièvre and colleagues (Lelievre et al., 1990), adequate data were not presented to conclude that the κ-CN hydrolysis was impaired. The results from the current study (Figure 3.2a) confirm that κ-CN hydrolysis was impaired, which increased the time taken to sufficiently reduce the electrostatic and steric repulsion between the micelles.

The reduction in the rate of κ–CN hydrolysis caused by the presence of native whey proteins could be attributed to one of two mechanisms: i) a reduction in the diffusivity of the chymosin molecule, or ii) enzymatic inhibition. According to the Stokes-Einstein equation, the diffusivity of a molecule in an ideal system is inversely proportional to the viscosity of the medium (Young et al., 1980). Experiments were performed to determine whether the change in the viscosity (leading to a reduction in diffusivity) caused by the presence of whey protein contributed to the observed delay in the onset of gelation. For this, the viscosities of the milk systems were measured (Figure 3.3a) and rennet gelation experiments were conducted on milk systems prepared with the same casein concentration as skim milk but substituting xanthan gum for the whey protein to match the viscosities of the original milk systems. The results show that the onset of gelation is not affected by the presence of xanthan gum (Figure 3.3b), indicating that the increased viscosity resulting from the addition of whey protein is not responsible for the reduced rate of κ-CN hydrolysis. This could be explained by the fact that the viscosity of the actual aqueous medium through which the enzymes diffuse is not actually increased by the presence of whey proteins (or xanthan gum), and that the change in bulk viscosity does not significantly affect the diffusivity of the small chymosin molecules.

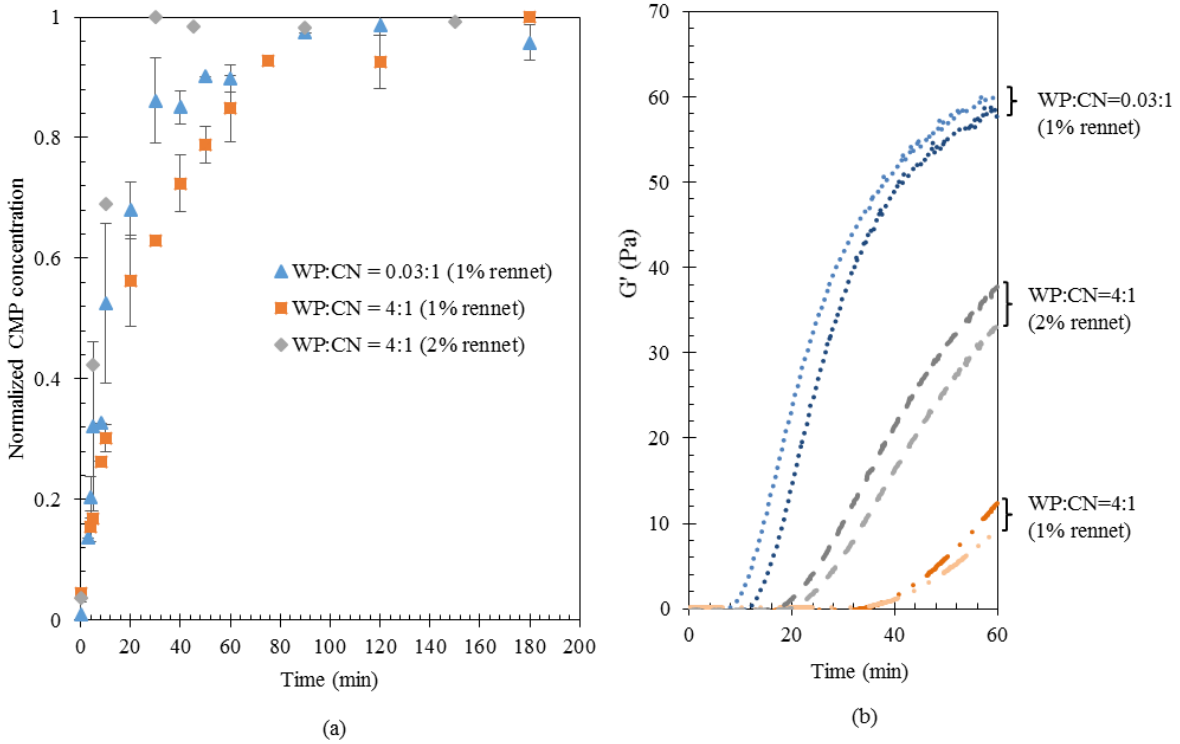


Figure 3.2: (a) The kinetics of κ-casein hydrolysis based on measurements of casein macro peptide (CMP) concentration (normalised to the maximum CMP concentration in each trial) with time, in milk systems with WP:CN ratios of 0.03:1 with 1% rennet (▲), 4:1 with 1% rennet (■), and 4:1 with 2% rennet (◆). The error bars indicate the standard deviation of measurements of quadruplicate experiments for the WP:CN 0.03:1 (1% rennet) system and triplicate experiments for the 4:1 (1% rennet) system. (b) G' as a function of time after addition of rennet in milk systems with WP:CN ratios of 0.03:1 with 1% rennet (.....), 4:1 with 1% rennet (- - - - -), and 4:1 with 2% rennet (—··—··). Duplicate experiments were performed as shown for each condition.

As the change in viscosity could not account for the delayed onset of gelation, the observed decrease in the rate of hydrolysis must instead indicate enzymatic inhibition. Enzyme inhibition mechanisms are broadly classified as either competitive, uncompetitive or non-competitive. Competitive inhibition would mean that one or more of the native whey proteins competes with κ-CN for the chymosin active site. For uncompetitive inhibition, the inhibitory protein(s) binds exclusively to the enzyme-substrate (chymosin-κ-CN) complex. In a non-competitive reaction, the inhibitor binds to both the substrate and the enzyme-substrate complex (Bisswanger, 2002). Conceptually it seems plausible that any of the mechanisms could occur, and it is not clear yet which of the native whey proteins is responsible.

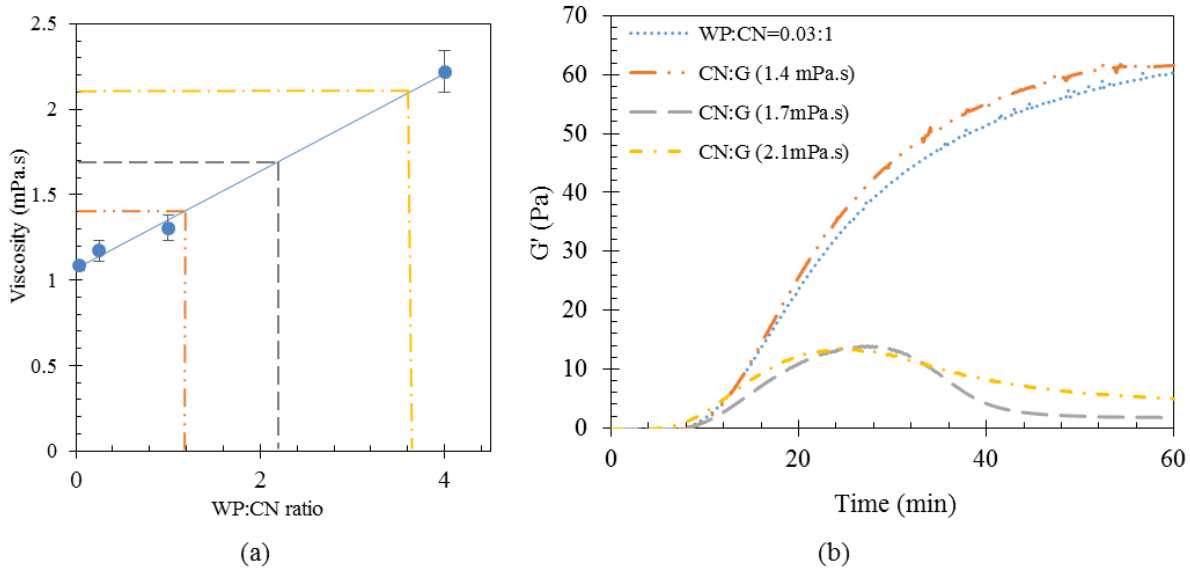


Figure 3.3: (a) Change in the viscosity of each milk system with the increase in the concentration of whey protein. (b) The gelation behaviour observed in casein-xanthan gum (CN:G) mixtures with viscosities 1.4 mPa.s (— · · —), 1.7 mPa.s (- - -) and 2.1Pa.s(— · —) compared with the original milk system with WP:CN = 0.03:1 (·····). The corresponding WP:CN ratios of milk systems with the similar viscosities to gum systems are represented with matching line styles in figure (a).The error bars indicate the standard deviation of duplicate measurements.

3.3.3. Aggregation of para-casein micelles

The observed enzymatic inhibition may not be the only cause for the delayed onset of gelation and the subsequent decrease in the rate of gelation. To clarify whether the impaired hydrolysis could fully account for impaired gelation, an experiment was carried out in which twice the amount of rennet (2%) was added to the WP:CN 4:1 system. This was done to achieve rapid κ-CN hydrolysis in the presence of whey protein, and to test if this could overcome the previously observed delay in the onset of gelation. The results show that even though faster κ-CN hydrolysis occurred in the high-rennet/high WP (2% rennet WP:CN 4:1) system (Figure 3.2a), the onset of gelation occurred later than that of the low rennet/low WP (1% rennet WP:CN 0.03:1) system (Figure 3.2b). This shows that in addition to inhibiting the action of the chymosin, the whey protein impaired the aggregation of the para-casein micelles.

Aggregation of para-casein micelles relies on two factors: i) diffusion through the medium via Brownian motion so that particles can approach each other; and ii) reaction of the particles so that they can fuse together. The first factor is governed by the concentration (affecting the probability of particles coming to the same location) and the diffusivity of the particles

(affected by the particle size and medium viscosity)(Carlson et al., 1987). Although the presence of the whey proteins was found not to affect the diffusivity of the chymosin enzyme sufficiently to affect an appreciable change in the rate of κ-CN hydrolysis, it is possible that the diffusivity of the much larger (10-50x the diameter) casein micelles may be affected, as they will 'see' the protein suspension (rather than the aqueous solution) as the continuous phase through which they must move. However, the experiments using xanthan gum in place of whey protein to increase the viscosity (Figure 3.3b) suggest that this is not the case, at least with respect to the onset of gelation, which was not affected. While the experiments performed with xanthan gum at viscosities of 1.7 mPa.s and 2.1 mPa.s (equivalent to WP:CN ratios of ~2.3:1 and ~3.6:1 showed some unusual behaviour, the experiment performed at a viscosity of 1.4 mPa.s (equivalent to a WP:CN ratio of ~1.2:1) showed no observable difference in the onset or rate of gelation compared to the control (WP:CN 0.03:1) (Figure 3.3b). This is in contrast to the significant delays in the onset and reductions in the rates of gelation observable for the samples containing whey protein (as opposed to xanthan gum) (Figure 3.1). Therefore, the presence of whey protein must inhibit the reaction of the para-casein micelles, rather than the particle diffusion. This is further confirmed by dynamic light scattering measurements, which showed that the particle size (and therefore the diffusivity of the particles, which is what is actually measured), was not significantly altered (analysed by one way ANOVA with $\alpha = 0.01$ or 99% confidence interval) (MS excel extract is provided in supplementary data) by the presence of whey protein (Table 3.1).

Table 3.1: The particle size of casein in the milk systems with different WP:CN ratios. The standard deviation of measurements in triplicate samples is indicated with (±).

Whey Protein:Casein ratio	Z- average diameter (nm)
0.03:1	139.3 ± 2.9
0.25:1	140.7 ± 2.9
1:1	133.6 ± 3.0
4:1	133.3 ± 2.7

Aggregation of colloidal particles, such as para-casein micelles, requires overcoming the energy barrier presented by electrostatic and steric repulsion. The electrostatic repulsion is due to the repulsion of like charges (in this case negative) on the surface of the casein micelles. In

some systems, the strength of electrostatic repulsions can be gauged by measurement of the ζ-potential. This was attempted, but unfortunately reliably comparable measurements could not be obtained across the range of WP:CN ratios because the presence of whey protein in the medium has a potential to alter the diffusivity of the casein micelles under an electric field, hence the dynamic light scattering measurement. Nonetheless, further understanding can be gained by considering the fundamentals of the electrostatic repulsive forces, which are predominantly affected by three factors: the pH (which will affect the net surface charge of the ionic proteins), the ionic strength (which reduces the depth of the electrical double layer), and the presence of charge-neutralising calcium ions. As the pH was kept consistent between the experiments, this is not an explanatory factor. The addition of WPC resulted in an increase in the ionic strength as a function of increasing WP:CN ratio. As this should in fact decrease the electrostatic repulsion, it cannot explain the observed impairment in aggregation that occurred with increasing WP:CN.

The final factor contributing electrostatic stabilisation is the soluble calcium concentration that is known to increase the rate of rennet gelation by neutralising the net-negative surface charge (Dalgleish, 1983, 1984). It is believed that the hydrolysis of κ-casein causes bare patches on the casein micelles exposing negatively charged amino acid residues (Sandra et al., 2012) that continue to repel the para-casein micelles leading to elongated gelation times. The Ca^{2+} ions in the milk serum can reduce the overall charge of such residues and being divalent, help bridge two negatively charged micelles. Higher concentrations of soluble calcium thereby enable closer approach and faster aggregation (Dalgleish, 1984; Hooydonk et al., 1986; Martin et al., 2008). Previous studies (Hooydonk et al., 1986; Udabage et al., 2001) have shown that the increase in Ca^{2+} by addition of $CaCl_2$ (up to 50 mmol/kg = 2000 ppm) in milk results in faster gelation.

In our experiments, calcium chloride was added (4.7 mM) to each milk system to ensure sufficient calcium was available for gelation so as to minimise this effect. To investigate the effect of the addition of whey protein isolate, the soluble calcium in each milk system was measured before and after the addition of calcium chloride (Figure 3.4). The results show a clear increase in the soluble calcium as a function of increasing WP:CN ratio. This trend is expected as the WPC powder contains a total calcium concentration of 4.1 mg/g. Although the presence of additional calcium should result in firmer gels and faster gelation, the opposite was observed in this study, confirming that calcium is not responsible for the observed impairment

in gelation resulting from an increase in whey protein. To further confirm this conclusion, two additional experiments were carried out (data not shown). In the first, WP:CN 0.03:1 systems with increased soluble calcium concentrations up to 1015 ppm showed a faster aggregation than the WP:CN 0.03:1 systems with only 300 ppm. In the other experiment, WP:CN 4:1 systems without any addition of $CaCl_2$ showed no gelation for 1 hour. This observation makes it possible to conclude that even though soluble calcium helps to neutralise the electrostatic repulsion in casein micelles, the presence of whey protein has a more significant negative impact in the gelation behaviour observed in this study.

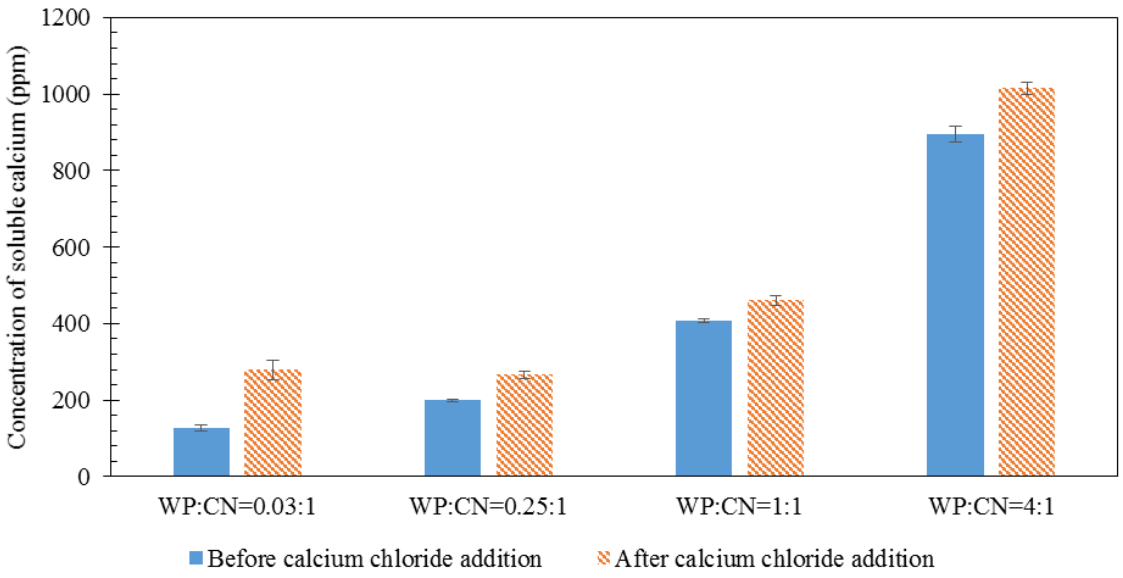
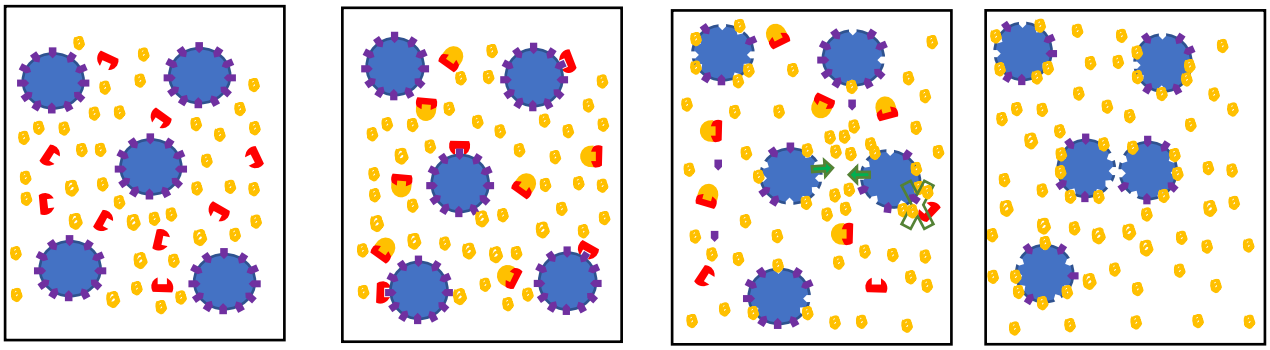


Figure 3.4: Soluble calcium in each milk system before (▬) and after (▨) the addition of calcium chloride. The error bars indicate the standard deviation of measurements of duplicate samples.

To summarise, when chymosin is added to a system containing casein micelles and native whey proteins (Figure 3.5a), the enzyme is inhibited resulting in slower hydrolysis (Figure 3.5b). We have also shown that neither the inhibition of the enzymatic hydrolysis, nor an increase in viscosity, nor a reduction in the electrostatic repulsion can fully account for the observed decrease in the rate of gelation in the presence of native whey protein. We therefore propose that this leaves only physical or steric hindrance as the remaining explanatory mechanism. While there is no evidence to suggest that the native whey proteins become permanently attached to the para-casein micelles, they are the right size to occupy the gaps in the 'hairy layer' that arise as the κ-CN is cleaved from the micelles. In this way, the native whey proteins

can provide a physical barrier to intimate contact between cleaved para-casein micelles, partially substituting for the reduction in steric repulsion caused by removal of the κ-CN 'hairy layer' (Figure 3.5c). As hard particles, they would represent a strong physical barrier for the aggregation of cleaved micelles (Figure 3.5d), in comparison to the increased viscosity resulting from the addition of the more fluid xanthan gum. A related observation that is consistent with this idea, is that in both WP:CN 4:1 systems with 1% and 2% rennet, the onset of gelation occurred after approximately 70% of the total CMP was released, while in the CN:WP 1:0.03 system, the onset occurred after around 50% of the CMP was released (Figure 3.2a). Increasing concentrations of whey protein can more effectively shield collisions between para-casein micelles thereby increasing the amount of κ-CN that must be removed before gelation can occur.



(a) (b) (c) (d)

Figure 3.5: Schematic representation of the proposed mechanisms for the contribution of native whey proteins to the decreased rate of rennet gelation. (a) Chymosin ( ) is added to a system with casein micelles ( ) and whey proteins ( ). (b) Whey proteins inhibit ( ) the chymosin action and decrease the rate of hydrolysis of κ-CN. (c) Whey proteins occupy the gaps in the 'hairy layer' ( ) that arise as the κ-CN is cleaved from the micelles providing a steric hindrance for chymosin ( ) to reach the remaining κ-CN hairs. This reduces the rate at which attractions ( ) between the casein micelles overcome the repulsive forces. (d) Whey proteins cause an additional steric hindrance to the intimate contact between cleaved micelles resulting in slower aggregation.

3.4. Conclusions

This chapter demonstrated that native whey protein inhibits κ-CN hydrolysis by chymosin and also impairs the rate of aggregation of the para-casein micelles. While the higher concentrations of whey protein examined in the this study were not practically relevant in a commercial setting (WP:CN of 1:1 and 4:1), they enabled clear trends to be observed that helped reveal the underlying mechanisms of the inhibitory roles of whey protein during rennet gelation of milk.

The results confirm that the above inhibitory effects are not caused by the associated changes in the mineral balance or viscosity, but rather directly by the native whey proteins themselves. More studies are required to determine the exact mechanism of enzyme inhibition and which of the whey proteins are responsible. Subsequent attempts were made to identify individual whey proteins responsible for chymosin inhibition and impairment of casein aggregation. These results are reported in section 7.1.

The milk that was tested with a greatly reduced whey protein content (WP:CN of 0.03:1) is of practical relevance to the use of MF retentates in cheese making. The results show that the rate of gelation is increased when native whey protein is not present, which would translate to more efficient and productive cheese vats. Alternatively, as the gelation rate is improved in casein rich streams, conventional rennetting times in cheese production could be achieved using a reduced amount of rennet. This reduces the requirement for clotting enzymes that are not recoverable in the process. Further investigations into the effect of the removal of whey proteins on curd microstructure would be beneficial to demonstrating the potential of using casein retentate from MF in the manufacture of renneted cheese.

3.5. References

Bisswanger, H. (2002). *Enzyme Kinetics-Principles and Methods* Weinheim: WILEY-VCH.

Carlson, A., Hill, C. G., & Olson, N. F. (1987). Kinetics of milk coagulation: II. Kinetics of the secondary phase: Micelle flocculation. *Biotechnology and Bioengineering, 29*(5), 590-600.

Carlson, A., & Hill Jr, C. G. (1987). Kinetics of Milk Coagulation: 1. The Kinetics of Kappa Casein Hydrolysis in the Presence of Enzyme Deactivation. *Biotechnology and Bioengineering*, 582-589.

Dalgleish, D. G. (1983). Coagulation of renneted bovine casein micelles: dependence on temperature, calcium ion concentration and ionic strength. *Journal of Dairy Research, 50*(3), 331-340.

Dalgleish, D. G. (1984). Measurement of electrophoretic mobilities and zeta-potentials of particles from milk using laser Doppler electrophoresis. *Journal of Dairy Research, 51*(3), 425-438.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. (2017). Enzymatic coagulation of milk. In *Fundamentals of cheese science* (pp. 185-229): Springer.

Guinee, T. P., & Wilkinson, M. (1992). Rennet coagulation and coagulants in cheese manufacture. *International Journal of Dairy Technology, 45*(4), 94-104.

Hinrichs, J. (2001). Incorporation of whey proteins in cheese. *International Dairy Journal, 11*(4), 495-503.

Hooydonk, A. C. M. v., Hagedoorn, H. G., & Boerrigter, I. J. (1986). The effect of various cations on the renneting of milk. *Neth. Milk, 0*, 369–390.

Hyslop, D. B. (2003). Enzymatic coagulation of milk. In *Advanced Dairy Chemistry—1 Proteins* (pp. 839-878): Springer.

Jang, H. D., & Swaisgood, H. E. (1990). Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *J. Dairy Sci, 73*, 900–904.

Jenness, R. (1999). Composition of Milk. In N. P. Wong (Ed.), *Fundamentals of Dairy Chemistry* (pp. 1-38). Gaithersburg, Maryland: Aspen Publishers, Inc.

Lelievre, J., Creamer, L., & Tate, K. (1990). Inhibition of calf vell and microbial rennet action by whey protein concentrate. *Milchwissenschaft, 45*(2), 71-75.

Lucey, J. A. (2002). Formation and physical properties of milk protein gels. *Journal of dairy science, 85*(2), 281-294.

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting nitrogen into protein—beyond 6.25 and Jones' factors. *Critical reviews in food science and nutrition, 48*(2), 177-184.

Martin, G. J. O., Williams, R. P. W., Choong, C., Lee, B., & Dunstan, D. E. (2008). Comparison of rennet gelation using raw and reconstituted skim milk. *International Dairy Journal, 18*, 1077-1080.

Martin, G. J. O., Williams, R. P. W., & Dunstan, D. E. (2007). Comparison of casein micelles in raw and reconstituted skim milk. *Journal of Dairy Science, 90*, 4543-4551.

Maubois, J. L. (2002). Membrane microfiltration: a tool for a new approach in dairy technology. *Australian Journal of Dairy Technology, 57*(2), 92.

Meza-Nieto, M. A., Vallejo-Cordoba, B., González-Córdova, A. F., Félix, L., & Goycoolea, F. M. (2007). Effect of β-Lactoglobulin A and B Whey Protein Variants on the Rennet-Induced Gelation of Skim Milk Gels in a Model Reconstituted Skim Milk System. *Journal of Dairy Science, 90*(2), 582–593.

Nelson, B., & Barbano, D. (2005). A microfiltration process to maximize removal of serum proteins from skim milk before cheese making. *Journal of Dairy Science, 88*(5), 1891-1900.

Papadatos, A., Neocleous, M., Berger, A. M., & Barbano, D. M. (2003). Economic Feasibility Evaluation of Microfiltration of Milk Prior to Cheesemaking. *J. Dairy Sci., 86*, 1564–1577.

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*(1), 75-82.

Udabage, P., McKinnon, I. R., & Augustin, M. A. (2001). Effects of mineral salts and calcium-chelating agents on the gelation of renneted skim milk. *J. Dairy Sci, 84*, 1569–1575.

Vasbinder, A. J., Rollema, H., & De Kruif, C. (2003). Impaired rennetability of heated milk; study of enzymatic hydrolysis and gelation kinetics. *Journal of dairy science, 86*(5), 1548-1555.

Walstra, P. (1990). On the Stability of Casein Micelles1. *Journal of Dairy Science, 73*, 1965-1979.

Walstra, P. (1999). Casein sub-micelles: do they exist? *International Dairy Journal, 9*(3), 189-192.

Young, M., Carroad, P., & Bell, R. (1980). Estimation of diffusion coefficients of proteins. *Biotechnology and Bioengineering, 22*(5), 947-955.

Zulewska, J., Newbold, M., & Barbano, D. M. (2009). Efficiency of serum protein removal from skim milk with ceramic and polymeric membranes at 50°C1. *J. Dairy Sci, 92*, 1361–1377.

Chapter 4

4. Incorporating whey protein aggregates produced with heat and ultrasound treatment into rennet gels and model non-fat cheese systems

This chapter investigates the effect of size and surface hydrophobicity of native and denatured whey protein aggregates prepared by thermal and ultrasound treatments on the kinetics of rennet gelation and protein retention in model non-fat cheese systems. The influence of the upstream method of whey protein separation (i.e: membrane filtration or cheese syneresis) on protein properties before and after thermal denaturation are also studied. A mechanism for the changes in the kinetics of gelation, microstructure and protein retention is proposed.

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4.1 Introduction

Rennet gelation converts liquid milk into a coagulum during the production of many types of cheeses (Guinee & Wilkinson, 1992). Milk in its natural form is a stable colloidal suspension comprised of emulsified fat, minerals, proteins (casein and whey proteins) and lactose. Casein is present in milk as large colloidal aggregates (50-200 nm) known as casein micelles, which consist of a hydrophobic core rich in αs_1, αs_2 and β-caseins covered by a hydrophilic κ-casein-rich 'hairy' layer that provides electrostatic and steric stability (Jenness, 1999; Walstra, 1999). During rennet gelation, the chymosin enzyme present in rennet (a secretion from the calf intestine) cleaves the κ-casein hairy layer, which destabilizes the casein micelle, facilitating aggregation and subsequent gel formation through hydrophobic interactions (Fox et al., 2017; Vasbinder, Rollema, et al., 2003). Contrastingly, the globular whey proteins, which are small (5-10 nm) (Fox et al., 2000; Rüegg et al., 1977) compared to cleaved casein micelles, drain out from the gel structure. This process, known as "syneresis", results in the loss of approximately 20% of the total protein in milk during cheese making (Hinrichs, 2001). Fat globules are larger (~0.1-15 μm in diameter) are mostly retained in the coagulum, providing cheese its distinctive creaminess (Logan et al., 2015).

Previous attempts to incorporate whey proteins with the aim of producing high-protein cheeses (Baldwin et al., 1986; Banks & Muir, 1985; Brown & Ernstrom, 1982; Chamberland, Benoit, et al., 2019; Chamberland, Mercier-Bouchard, et al., 2019; Hinrichs, 2001; Ismail et al., 2011; Lo & Bastian, 1998; Mead & Roupas, 2001; Punidadas et al., 1999; Stankey et al., 2017) used two approaches: (i) ultrafiltration to increase the protein concentration in the cheese milk and (ii) heat denaturation of whey proteins to facilitate casein-whey protein or whey protein-whey protein crosslinking. The effective concentration of native proteins (both casein and whey proteins) in cheese milk using ultrafiltration has been researched extensively since 1969 and varying quantities of increased cheese yields and protein retentions are reported depending on the concentration of the ultrafiltrate used and the type of cheese (Mistry & Maubois, 2017). Most recently Chamberland, Benoit, et al. (2019) and Chamberland, Mercier-Bouchard, et al. (2019) also reported increased cheese yields and protein recovery (Chamberland, Benoit, et al., 2019; Chamberland, Mercier-Bouchard, et al., 2019) when cheese milks were formulated with ultrafiltration retentates, as compared to whole milk or traditionally standardized cheese milk due to the expulsion of lower volumes of whey, leading to a lower loss of whey proteins (Chamberland, Mercier-Bouchard, et al., 2019).

At elevated temperatures (>65 ºC), whey proteins denature and can then form intermolecular disulphide bridges between free thiol groups present in some of the casein and whey proteins

(e.g. α-lactalbumin, β-lactoglobulin and κ-casein) (Monahan et al., 1995; Vasbinder, Alting, et al., 2003). The application of heat to denature and attach whey proteins to micellar κ-casein has been used to reduce the syneresis of yoghurt and cream cheese, which are made by acid gelation (Kethireddipalli et al., 2010; Lucey et al., 1998). However, this approach impairs rennet gelation, by coating the casein micelles with denatured whey proteins (Singh & Waungana, 2001; Vasbinder, Rollema, et al., 2003). Further, while an increase in the moisture content of cheddar cheese made from heat-treated milk has been recorded (Lo & Bastian, 1998), there was no increase in whey protein retention.

Alternatively, heat denaturation of casein-depleted whey protein streams can form whey protein aggregates while avoiding whey protein-casein interactions. Banks and Muir (1985) attempted to incorporate such heat-treated and acid-precipitated whey concentrate (produced by heating 0.9% w/w whey protein streams at 95 ºC for 20 minutes and precipitating at pH 6 to achieve final protein and fat contents of 6.6% w/w and 4.5% w/w, respectively) into cheese. Although the cheese yield increased by 9%, the increase was mainly attributed to the retention of fat-protein complexes (Banks & Muir, 1985). In another study, Baldwin et al. (1986) attempted to incorporate partially denatured (~78-80%, with no reported aggregation) whey proteins, produced from the heat treatment (70 ºC for 15 minutes) of whey protein concentrates, into cheddar cheese. Although they reported a 3.4% increase in the total nitrogen content in cheese, the final cheese yield was not significantly improved due to fat loss resulting from a weaker gel matrix. In a similar study, Brown and Ernstrom (1982), incorporated heat-denatured whey protein (75 ºC for 30 min) into cheese milk. Although the cheese yield increased by 4%, protein content was not affected. Similar increases in overall cheese yields are reported for mozzarella cheese made by incorporating acidified denatured whey proteins (Punidadas et al., 1999), however with no quantification of protein retention.

Earlier studies to incorporate denatured whey proteins into cheese mostly investigated changes in the sensory attributes and the composition of cheeses (Banks & Muir, 1985; Brown & Ernstrom, 1982; Punidadas et al., 1999) rather than attempting to optimise whey protein aggregate properties to increase whey protein retention. Further, the observed increases in cheese yields were largely followed by increases in moisture (Banks & Muir, 1985; Brown & Ernstrom, 1982; Punidadas et al., 1999), fat (Banks & Muir, 1985) and/or casein content (Banks & Muir, 1985) in cheese, rather than whey protein retention. This may be due to the production of whey protein aggregates that were too small to be retained within the cheese matrix. Although aggregate size was not reported, these studies used dilute whey protein streams (Banks & Muir, 1985; Brown & Ernstrom, 1982; Punidadas et al., 1999), and/or mild heat

treatments (Brown & Ernstrom, 1982), which typically result in relatively small aggregates after heat treatment (Roefs & De Kruif, 1994).

The advent of membrane technology has enabled the production of concentrated whey protein streams and powders, that upon heat treatment, can produce larger whey protein aggregates, which can then be disintegrated into so-called microparticulates (~1 μm) using high-pressure homogenisation (Suárez et al., 2016). The effect of incorporating microparticulated whey protein in cheese as a fat replacer has been of increasing interesting in the past decade. However, the extent of whey protein aggregate retention is still under debate. For example, Sturaro et al. (2015) found` that increasing the whey protein-to-fat ratio decreased the yield of low-fat Caciotta cheese. Contrastingly, Stankey et al. (2017) and Perreault, Rémillard, et al. (2017) reported that incorporation of microparticulated whey protein in low fat cheddar cheeses and semi hard cheeses could increase cheese yield, however much of this was an increased moisture content resulting from the enhanced water binding capacity of the denatured whey proteins. Similar increases in moisture content have also been reported for whey incorporated into Caciotta cheese (Di Cagno et al., 2014).

Regardless of the effect on yield, it has been shown that incorporation of microparticulated whey proteins can improve the texture of otherwise firm low-fat cheese curds (Schenkel et al., 2013; Stankey et al., 2017). Schenkel et al. (2013) hypothesised that the presence of inert whey protein particulates in the casein network could lead to fewer casein crosslinks and therefore an improved (softer) texture. However, both Schenkel et al. (2013) and Stankey et al. (2017) reported that the protein retention in the cheeses was not significantly improved. Another similar study by McMahon et al. (1996) concluded that the whey protein microparticulates used as a fat replacer were too small (0.5-1 μm) to create large enough gaps in the casein structure of mozzarella cheese.

With increased interest in incorporating whey protein particulates in cheese matrices to alter their composition and texture, studies are now aimed at understanding the effect of denatured whey protein aggregates on kinetics of rennet gelation and establishing mechanisms behind textural changes. Perreault, Morin, et al. (2017) et al recently reported that both sediment able (~ 8 μm) and soluble protein fractions in denatured whey protein concentrates can negatively impact the rate of rennet gelation. A complementary study by Giroux et al. (2015) proposed that denatured whey proteins participate in hydrophobic interactions with casein micelles and impair rennet gelation. However, the relative effect of the properties of native whey proteins

and denatured whey proteins of different sizes, on the kinetics of rennet gelation and cheese microstructure is yet to be fully understood.

Ultrasound (US) processing can be used to alter the functional properties of protein suspensions (Ashokkumar et al., 2009; Gordon & Pilosof, 2010; Leong et al., 2018). When ultrasound (sound with a frequency > 20 kHz) is applied to a liquid, minuscule air bubbles in the medium can expand and then collapse violently during acoustic rarefaction and compression cycles. Particularly during low-frequency high-power sonication, strong physical forces such as shear, shock waves and microjets form as a result of acoustic cavitation, which can cause particles in suspension to collide at sufficient force to result in disintegration (Mason et al., 2011). Therefore, low-frequency high-power ultrasound can effectively be used to reduce the size of large whey protein aggregates at processing times as short as 5 seconds (Ashokkumar et al., 2009; Gordon & Pilosof, 2010; Leong et al., 2018). This offers a means of controlling the size of whey protein aggregates when formulating cheese milks, by reducing larger heat-induced aggregates to the desired size range. Compared to conventionally used high pressure valve homogenization, which require a prior mechanical stirring step to improve the flowability of viscous, non-Newtonian streams of concentrated heat-denatured whey proteins (Suárez et al., 2016), US assisted whey protein size reduction has the advantage of directly processing such streams.

The effect of aggregate properties on the initial rennet gelation, aggregate, and the mechanism behind cheese microstructural changes are of interest in the context of optimising this technology. Therefore, in this study, denatured whey protein aggregates of different sizes were produced using heat treatment followed by ultrasound, and the effect of aggregate size on rennet gelation kinetics investigated. In addition, the retention of whey protein aggregates incorporated into model non-fat cheeses (prepared using a cheddar cheese making protocol), was quantified and investigated in relation to observed microstructural changes.

4.2 Materials and Methods

4.2.1 Milk protein concentrate powders

Two types of whey protein concentrates powders were used as the source of whey protein: a membrane-filtered whey protein (MFWP) and a commercially available whey protein concentrate (WPC) from cheese whey ("Select whey protein concentrate" purchased from Professional Whey, Erina, Australia). Pasteurized skim milk ("Pauls Skinny Milk", produced by Lactalis Australia, South Brisbane, Australia) was used as the casein source.

MFWP was produced using a previously reported method (Gamlath et al., 2018). The casein pH 4.5 composition of the skim milk as well as MFWP and WPC powders was determined (in at least duplicates) as described in section 4.2.2. The MFWP was 75.2 ± 0.9% w/w whey protein and 1.1 ± 0.9% w/w casein (total protein content of 76.3 ± 0.5% w/w), WPC was ~ 71.8 ± 0.8 w/w whey protein and ~ 1.2 ± 0.3 w/w casein (total protein content of 73.0 ± 0.1% w/w), and the skim milk was 2.67 ± 0.03 % w/w casein and 0.56 ± 0.05 % whey protein (total protein content of 3.23 ± 0.05% w/w).

Unless otherwise specified, WPC and MFWP were dissolved in distilled and deionized water, at respective powder concentrations of 10% w/w and 20% w/w. The solutions were stirred for 2 hours at room temperature before each experiment.

4.2.2 Protein quantification

Protein analysis of WPC, MFWP,skim milk, curds and whey was performed based on the Dumas combustion method using a LECO Trumac CNS analyser (LECO Corporation, Michigan, USA) using a method that complies with the ISO 1489 standard. . Samples of milk (~ 2mL) milk protein powder (~ 0.1 g), cheese curds (~ 0.2 g), or whey (~ 2mL) were weighed onto a ceramic boat and dried in the oven overnight (104 °C). The boats were then loaded into the LECO analyser and combusted at a temperature of 1100 °C within the furnace. Protein values were determined from the measured nitrogen % by multiplying with the ratio 6.38 (Mariotti, Tomé, & Mirand, 2008).

Protein retention in curds were calculated using the following equation.

$$Protein\ retention = \frac{Protein\ content\ of\ curd\ in\ \%\frac{w}{w} \times Mass\ of\ curd}{Protein\ content\ of\ cheese\ milk\ in\ \%\frac{w}{w} \times Mass\ of\ cheese\ milk} \times 100$$

Casein and whey fractions of skim milk, MFWP and WPC were determined using a previously reported method (Shahani & Sommer, 1951) with a few modifications. For pH 4.6 precipitable nitrogen determination, 20 mL of milk or 10% w/w whey protein solutions were mixed with 10 mL of distilled and deionized water. The samples were heated to 40 ºC in a water bath and acidified with 1 mL of 10% w/w hydrochloric acid. Water was added to make a total volume of 40 and the sample was cooled to room temperature. pH of the sample was adjusted to 4.6-4.7 using a 1M hydrochloric acid or sodium hydroxide solution. The precipitated proteins were removed by centrifugation (839 g for 10 min) followed by filtration through 0.22 μm syringe filters. ~ 2 mL of filtrate was loaded into a nickel lined ceramic boat and dried overnight (104°C) before pH 4.6 soluble nitrogen determination using the LECO analyser. In MFWP,

and skim milk, where there is no whey protein denaturation, pH 4.6 soluble nitrogen content indicated the non-casein nitrogen content. The percentage of casein in the pH 4.6 precipitate of WPC (where denatured whey proteins are present) was quantified using SDS PAGE (Figure 7.6 in Appendix 1) and was used to calculate the casein content.

For non-protein nitrogen determination, 10 mL of milk or 10% w/w whey protein solution were mixed with 10 mL of 30% w/v trichloroacetic acid. Precipitated proteins were removed by centrifugation (839 g for 10 min) followed by filtration through a 0.22 μm syringe filter. ~ 2 mL of filtrate was loaded into a nickel lined ceramic boat and dried overnight (104 °C) before non-protein nitrogen determination using the LECO analyser. pH 4.6 soluble nitrogen content, non-protein nitrogen content and the percentage of caseins in the pH 4.6 precipitates were used to calculate the casein and whey protein nitrogen contents in each system. Protein values were then determined by multiplying the measured or calculated nitrogen percentages by a conversion factor of 6.38 (Mariotti et al., 2008)

4.2.3 Fat quantification

The fat content of MFWP, WPC and centrifuged WPC dispersion were measured according to the Bligh and Dyer method (Manirakiza et al., 2001). 10 g of 10% w/w MFWP, 10% w/w WPC or centrifuged (at 3,842 x g for 20 min) 10% w/w WPC were added to a falcon tube along with 10 mL chloroform and 20 mL of methanol. The sample was shaken vigorously for 2 min and another 10 mL of chloroform was added. The sample was then centrifuged at 3,842 x g for 10 mins and the bottom chloroform rich layer was separated using a Pasteur pipette. 20 mL of a 10% v/v methanol in chloroform mix was used for the secondary extraction. Pooled chloroform extracts were dried overnight at 64 ºC and the dry mass was measured for fat quantification. WPC powder had a measured fat content of 5.28 ± 0.25 % w/w. Fat content of MFWP powder was <0.1% w/w. Centrifuged 10% w/w WPC solutions had a fat content of 0.188 ± 0.032% w/w which translated to a 1.88 ± 0.32% w/w fraction of nano-sized fat droplets in WPC powder.

4.2.4. Heat and ultrasound treatment

Whey protein solutions used in rennet gelation trials and cheese trials were treated in different sample volumes. For rennet gelation trials, 25 mL of WPC (10% w/w) or MFWP (20% w/w) solutions were heated in a water bath at 85 ºC for 15 min and then immediately cooled by immersing in an ice bath. WPC systems used for cheese trials were heated for 15 min in batches of 50 mL, placed in a water bath at 95 ºC. All milk protein systems had similar temperature vs.

time profiles (Figure 7.3 in appendices) during heating. The heated solutions were then sonicated in batches of 25 mL, at 20 kHz frequency and 50% amplitude for 1 min using an 11 mm horn (Model 102C (CE), Branson Ultrasonics, St. Louis, USA). The measured temperatures of whey protein streams during the ultrasound treatments were below 45 ^{0}C. The power delivered was 33 W as determined by calorimetry.

4.2.5. Particle size measurement

The size of particles in freshly prepared 10% w/w reconstituted samples MFWP and WPC was measured by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Panalytical Ltd, Malvern, UK) at 25 °C. The MFWP and WPC solutions were diluted 1000 and 500 times respectively, with deionized and distilled water before analysis. The 'milk protein size analysis' setting was used (particle refractive index: 1.45, particle absorption 0.001, viscosity of water 0.8872 cP, refractive index of water 1.33, measurement angle: 173° backscatter). The WPC solution was also centrifuged at 3,842 x g for 20 min, and the top and middle layers of the supernatant measured.

The volume mean diameter of the whey protein aggregates in the heated and sonicated MFWP (20% w/w) and WPC (10% w/w) solutions was measured using a Malvern Mastersizer 2000 with a Hydro EV accessory (Malvern Panalytical Ltd, Malvern, UK). Distilled water was used as the diluent. A particle refractive index of 1.45 and an absorption of 0.001 were used for the measurement.

4.2.6. Rheological measurements during rennet gelation

Two sets of four milk systems were prepared by mixing MFWP (20% w/w) or WPC (10% w/w) with skim milk diluted using distilled and deionized water. The first set contained skim milk diluted with distilled and deionized water (DS), diluted skim milk with untreated MFWP (DS MFWP), diluted skim milk with heat-treated MFWP (DS HT MFWP) and diluted skim milk with heated and sonicated MFWP (DS HT US MFWP). All systems, excluding DS, consisted of a 1:1 casein-to-whey protein ratio while the casein concentration was constant at 2.1% w/w for all four systems. The second set contained four similar systems prepared with skim milk diluted with distilled and deionized water (DS), diluted skim milk with untreated WPC (DS WPC), diluted skim milk with heat-treated WPC (DS HT WPC) and diluted skim milk with heated and sonicated WPC (DS HT US WPC). The DS system in the second set had a casein concentration of 1.9% w/w. All systems excluding DS, consisted of a ~ 0.9:1 casein-to-whey protein ratio with a slightly higher casein concentration (~2.0 % w/w) compared to the DS system. However, the DS system in the second set, had a casein concentration of 1.9% w/w.

The measured pH of all milk systems was between 6.80 and 6.85. Samples (20 mL) of each milk system were equilibrated at 31 °C in a water bath for 1 hour and renneted with 0.2 mL of 3.5 IMCU/mL rennet solution (prepared from 200 IMCU/mL Chymax Plus FPC, Cheeselinks, Lara, Australia, batch numbers 3233770 & 3241446). The change in the storage modulus (G') as a function of time was measured using an AR-G2 rheometer (TA Instruments, New Castle, USA) with a controlled shear strain of 2.5% at 1 Hz using a concentric cylinder (996284) tool at 31 °C for 1 hr. The G' at t = 0 was normalised to -0.02 Pa in all measurements.

4.2.7. Soluble calcium

Each whey protein dispersion was ultracentrifuged at 100,000 x g using a Beckman Coulter ultracentrifuge unit (Model: Optima L-100 XP with type 70 Ti rotor) for 1 hr at 4 °C The separated supernatant was filtered through 0.45 µm syringe filters. 1.2 mL of 100% w/v Trichloro acetic acid (BDH AnalR, Dubai, UAE) was added per 0.5 mL sample and diluted to 10 mL with deionized and filtered water. The samples were then centrifuged at 839 g for 5 minutes using a Thermofisher Hereus Megafuge 8 bench top centrifuge with HIGHConic III fixed angle rotor. 0.3 mL of the filtered (using 0.45 µm syringe filters) supernatant was diluted with 4.7 mL of deionized and filtered water and the soluble calcium was measured with an inductive coupled plasma (ICP) optical emission spectrometer ,Varian 720-ES (Varian Inc. California, USA) with SPS 3 auto sampler, wavelength: 315.887 nm, power: 1 kW, plasma flow: 15 L/min, auxiliary flow: 1.5 L/min, nebulizer flow: 0.75 L/min, pump rate: 15 rpm.

4.2.8. Surface hydrophobicity

Protein surface hydrophobicity was determined using a 1-anilinonaphthalene-8-sulfonate (ANS) fluorometric assay (Alizadeh-Pasdar & Li-Chan, 2000). A stock solution of 8×10^{-3} M ANS (Sigma Aldrich, Missouri, USA) was prepared in 0.1 M, pH 7.4 phosphate buffer and stored in darkness at room temperature. Whey protein solutions were diluted in phosphate buffer to at least three concentrations between 0.001-0.025 % w/w. 20 µL of ANS solution was added to 4 mL of protein solution, wrapped in aluminium foil and vortexed for 15 min. The relative fluorescence intensity (normalised to the maximum measurable intensity of the equipment) of each sample and the dilution blanks was measured using excitation and emission wavelengths of 390 (slit width 5 mm) and 470 nm (slit width 5 mm).

In order to measure the effect of fat content on the surface hydrophobicity of whey protein systems three 10% w/w WPC solutions were prepared by mixing an untreated 10% w/w WPC solution (with 0.528% w/w fat) and a centrifuged 10% w/w WPC solution (0.188% w/w fat) to reach final fat contents of 0.528% w/w, 0.400% w/w and 0.250% w/w. A 10% w/w MFWP

system with a 0.260% w/w fat content was also prepared by adding anhydrous milk fat to 15mL of 10% WPC solution and sonicating at 7.4W for 3s. Diluted samples from each system were used for the surface hydrophobicity measurement.

Relative fluorescence intensity in response to protein concentration was determined by linear regression and used as an index for surface hydrophobicity. Absolute surface hydrophobicity values showed significant variability between batches of measurements carried out with freshly prepared buffer. However similar trends were observed with heat and heat plus ultrasound treatments. For consistency, we report hydrophobicity values measured using the same buffer throughout the manuscript.

4.2.9. Model cooked rennet gel and cheese systems

Cheese milks were formulated by blending 10% w/w WPC solutions (treated or otherwise) and distilled and deionized water into skim milk to achieve a total protein concentration of 3.66% w/w and a casein:whey protein ratio of ~ 1.9:1. Diluted skim milk (with distilled and deionized water) with a matching casein concentration was used as a control fat-free cheese milk. Cooked rennet gels were made from 100 g batches of these milks using a cheddar cheesemaking protocol adapted from Ong et al. (2012) and Ong et al. (2013). Cheese milk was transferred 120 mL sterilized containers and warmed to 31 °C in a water bath. Mesophilic starter culture (Cheeselinks, Lara, Australia) containing a mix of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* was added at a concentration of 4×10^{-3} % w/w. Milk was ripened until a pH drop of 0.1 units was achieved. Rennet (200 IMCU/mL, Chymax Plus FPC, Cheeselinks, Lara, Australia, batch number 3241446) was added to reach a final concentration of 10^{-2} % v/w. Gelation was allowed to proceed for 1.5 hours at 33 °C until a firm gel was formed, and the coagulum was cut into ~1 cm cubes. Cooking was done by shaking the mixture in an orbital shaker (60 rpm) at room temperature for 1 hour. Whey was then drained by straining for 40 minutes and the cooked curd and whey was stored separately overnight at 4 °C until compositional analysis.

Model cheeses were produced using 10 kg of cheese milk with similar composition to those used for rennet gel trials. After cutting the coagulum, cooking was done by stirring the mixture manually (at approximately 30-60 rpm) while increasing the temperature from 33 °C to 38 °C over ~ 45 min. The mixture was further cooked at 38 °C until the pH reached ~ 6.1 and the whey was then drained. Cheddaring of the cooked curd was performed until a pH of 5.5 was reached. The cheddared curd was then cut into ~5 cm pieces, mixed with salt at 2.5% weight of the curd mass, packed into a mould lined with a cheese cloth and pressed overnight at an

applied mass of 129 g/cm^2 cheese. Sweet whey from draining and cheddaring were pooled together and stored at 4 °C prior to compositional analysis. Pressed cheeses were vacuum packed and stored at 4 °C until further analysis.

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

SDS-PAGE was carried out using Bio-Rad (Bio-Rad Technologies, Gladesville, Australia) pre-cast 4-20% Criterion TGX 18 well gel cassettes in a Bio-Rad Criterion cell. Tris/glycine/SDS running buffer (Bio-Rad) was prepared on the day of running by diluting the buffer solution 10 times with distilled and deionised water. Whey samples were diluted to 15% v/v in deionised water and combined with Laemmli buffer (Bio-Rad) (with 5% v/v mercaptoethanol (Sigma Aldrich, Missouri, USA) at a volume ratio of 1:1. Samples were heated at 90 °C for 5 min to denature the proteins, and subsequently cooled to room temperature. Gel lanes were loaded with 20 µL of sample alongside an 'All blue' protein standard (Bio-Rad) and run for 43 min at 200 V. Gels were then removed from the cassettes, washed with distilled and deionised water, stained with Coomassie Biosafe stain for 60 min under gentle mixing, and de-stained under gentle mixing with distilled and deionised water for atleast 60 min. Stained gels were then loaded into a Bio-Safe Gel-Doc unit (Bio-Rad) for imaging.

4.2.11. Confocal laser scanning microscopy

Microstructural analysis of cheese and rennet gels, was performed by confocal microscopy. For rennet gels, skim milk and WPC were stained separately. 30 µL of Fluorescein sodium salt (Sigma Aldrich, Missouri, USA) (0.1% w/v in pH 8 distilled water) and 10 µL of Fast green (Sigma Aldrich, Missouri, USA) (0.2 % w/v in distilled and deionised water) were added to 100 mL of skim milk and 50 mL of WPC solution, respectively. While Fast Green participate in ionic interactions with basic amino acid residues in proteins (Tas et al., 1980), Fluorescein sodium salt interacts with the tryptophan (Barbero et al., 2009) residues. Since proteins in skim milk and WPC have both basic and tryptophan residues, unbound stains in skim milk and WPC solutions could result in cross staining once the milk systems were formulated. In order to remove unbound Fast Green and Fluorescein, stained skim milk and WPC solutions were transferred to dialysis tubes (7 kDa and 10 kDa, respectively), and dialysed for 2 days against unstained skim milk and WPC solutions, that were replaced with fresh material every 24 hours. Milk systems were formulated using stained skim milk and WPC to match the casein and whey protein concentrations used in the cheese trials, and rennetted accordingly. Rennetted milk samples were immediately transferred into a concave well in a microscope slide (BRAND®, 76x26 mm, concavity 15-18 mm and depth of 0.6 – 0.8 mm) and covered with a cover slip.

The microscope slides were incubated at 31 °C for 1 hour then removed and stored at room temperature until analysis.

For cheese samples, slices (~5 mm x 5 mm x 1 mm slices) were stained with 2 drops each of Fast green (Sigma Aldrich, Missouri, USA) (0.2 % w/v in distilled and deionised water) and Nile red (Sigma Aldrich, Missouri, USA) (0.1% w/v in ethanol) dyes and placed on a glass slide. Dyes were absorbed for ~ 3 min, excess dye was wiped off, and a cover slip applied (Leong et al., 2018).

A Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to image the gels with the laser lines set at 486 and 633 nm for excitation. The fluorescence emission was captured through a 60X oil immersion lens using 2 photo multiplier tubes set at 500-600 nm (Nile red and Fluorescein emission) and 655-755 nm (Fast green emission). The percentage of saturated pixels in the images was adjusted to 0.3% for uniformity.

4.2.12. Statistical analysis

For cheese/curd compositional analysis, results from duplicate or triplicate measurements were analysed with Minitab 17 (Minitab LLC, Pennsylvania, USA) using a one-way ANOVA (with a 95% confidence interval), followed by a Tukey's pairwise comparison.

4.3 Results and Discussion

4.3.1 Effect of aggregated microfiltered whey proteins on rennet gelation

In a previous study, native whey proteins were found to impair the rate of rennet gelation, and this effect was attributed in part to their small size (~ 5 nm) (Gamlath et al., 2018). Therefore, to study the effect of incorporating differently sized heat denatured whey protein aggregates on the kinetics of rennet gelation, denatured whey protein aggregates of two size ranges were first produced using heat treatment alone (HT) and heat treatment followed by sonication (HT US). To investigate the mechanism of rennet gelation in the presence of native and aggregated whey proteins, MFWP was used initially as the source of whey protein as it is produced with minimal processing and has low casein and fat content. Particle size measurements confirmed that prior to heat treatment, MFWP contained only native whey proteins, with a single peak at 5-7 nm (Figure 4.1a) that corresponds to globular native whey proteins (Laiho et al., 2015; Rosa et al., 2006). Upon heat treatment, denatured whey protein particulates with a broad size distribution between 60-600 µm were formed. However, upon sonication, the majority of the microparticles were disintegrated to smaller particles between 0.01 µm - 2 µm in size (Figure 4.1b). Heat-induced aggregation of whey proteins (Mulvihill & Donovan, 1987) and the ability

of low-frequency high-power US to break apart whey protein aggregates, is well-known (Wang & Wang, 2004). The results presented here are consistent with this knowledge.

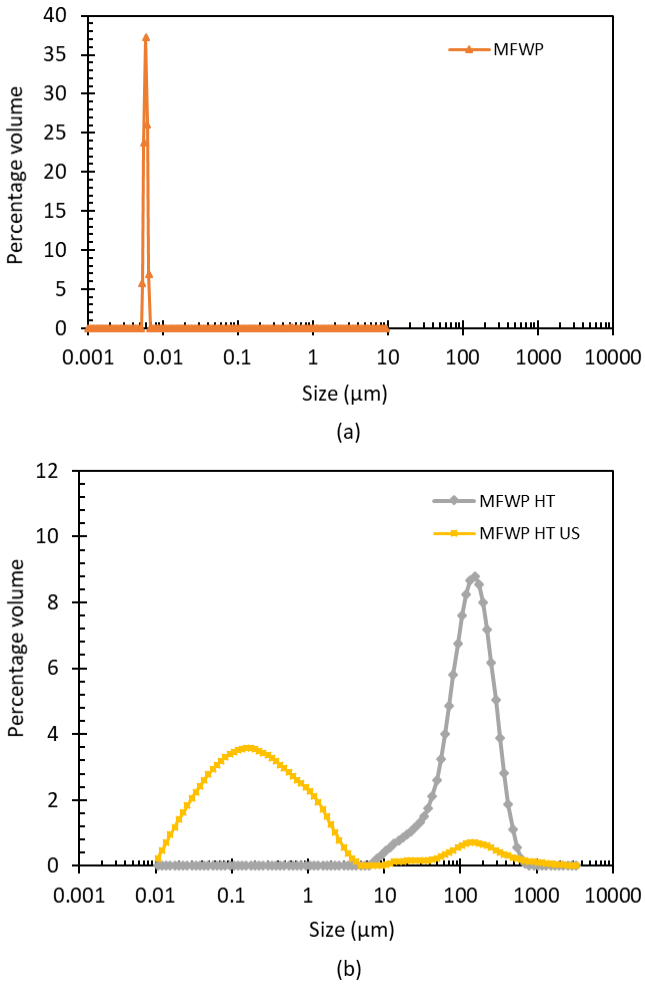


Figure 4.1: Particle size distribution of (a) untreated microfiltered whey protein (MFWP) (▲) as measured by dynamic light scattering (Malvern Zetasizer). Particle size distributions of (b) heat-treated MFWP (◆) and heat-treated and sonicated MFWP (■) measured by laser diffraction (Malvern Mastersizer). Two particle sizing methods were used to accurately measure across the wide particle size range (~ 5 nm to ~600 µm).

Next, milk systems were prepared for rennet gelation by mixing the treated whey protein solutions with diluted skim milk. The rennet gelation kinetics of four milk systems containing (i) diluted skim milk (DS), (ii) diluted skim milk with untreated/native MFWP (DS MFWP), (iii) diluted skim milk with heated MFWP (DS HT MFWP), and (iv) diluted skim milk with heated then sonicated MFWP (DS HT US MFWP) were analysed using oscillatory rheology. Figure 4.2 illustrates the change in the storage modulus (G') of each milk system (at 31 °C) with time after rennet addition. The rapid increase in G' indicates the onset of gelation which is characteristic of a gelling system under kinetic control (Meza-Nieto et al., 2007). Gelation

was fastest for diluted skim milk alone, and slowest when native MFWP was added to diluted skim milk (Figure 4.2). With MFWP, the rate of gelation was higher when the whey proteins were in the form of large heat-denatured aggregates than when the denatured whey protein aggregate size was reduced by sonication. However, the gelation rate of the DS HT US MFWP was still faster than that of the DS MFWP system.

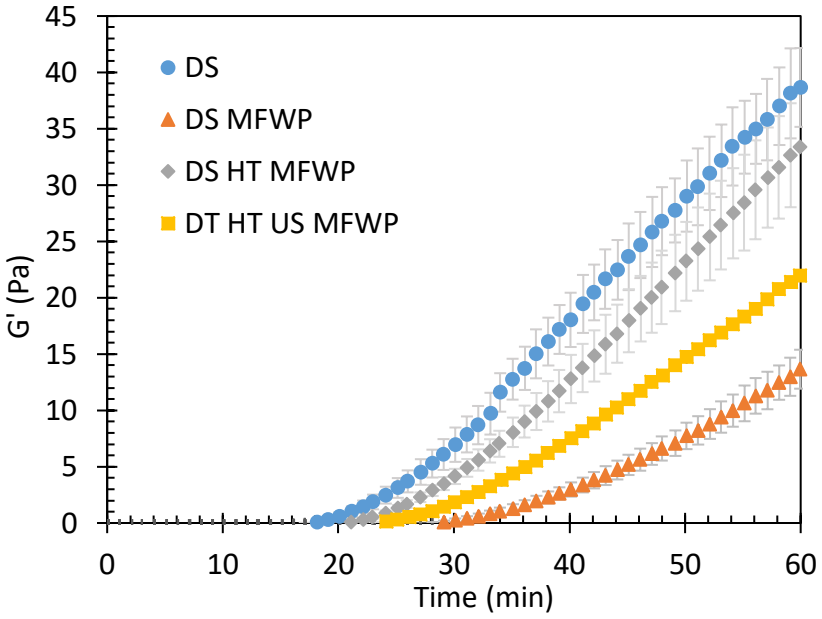


Figure 4.2: Kinetics of rennet gelation of milk systems made with diluted skim milk only (●), diluted skim milk with untreated/native MFWP (▲), diluted skim milk with heated MFWP (◆) and diluted skim milk with heated then sonicated MFWP (■). The error bars represent the standard deviation of measurements of duplicate samples.

The impaired gelation with added native MFWP agrees with our previous study in which native whey protein was shown to impair rennet gelation (Gamlath et al., 2018). The present results demonstrate that incorporating larger whey protein aggregates produced using heat denaturation can greatly reduce the impairment. It was hypothesised that the improved rate of gelation could be due to the larger whey protein aggregates no longer being able to occupy the spaces created in the cleaved casein micelle and therefore are not able to provide a steric barrier to the aggregation of renneted casein micelles. In addition, a reduction in the surface hydrophobicity of whey protein aggregates as the size increases could reduce interactions between cleaved casein micelles and whey protein aggregates during gelation. It is also possible that changes in the soluble calcium in the medium as a result of heat and US treatment could influence the gelation rate. The soluble calcium of untreated MFWP (Table 4.1) was similar (at a 95% CI) to its total calcium content of 888 ± 16 ppm, indicating that negligible

amounts of calcium were bound to proteins in the MFWP. When subjected to heat and US treatments the soluble calcium content in MFWP remained essentially unchanged. The soluble calcium in all milk systems after the addition of whey streams was also measured. Although there was a slight increase (statistically insignificant) in the soluble calcium content after MFWP addition, the soluble calcium content across MFWP, HT MFWP and HT US MFWP systems remained unchanged (data not shown). As the soluble calcium concentration was the same for the MFWP, HT MFWP and HT US MFWP systems, this should not be an explanatory factor in the observed differences in gelation kinetics.

Table 4.1: Soluble calcium and surface hydrophobicity of differently treated MFWP systems.

Whey protein system	Soluble calcium (ppm)	Surface hydrophobicity index
MFWP	883 ± 5^{a}	755 ± 3^{a}
MFWP HT	882 ± 8^{a}	3623 ± 172^{b}
MFWP HT US	863 ± 17^{a}	4782 ± 25^{c}

* The standard deviation of measurements of at least duplicates samples prepared from the same treatment tube are denoted alongside the mean value. The absolute value of the surface hydrophilicities changed significantly in trials done on different days with freshly prepared buffer, however, a similar increasing trend with HT and HT US treatments were observed.

** Significantly different values ($p < 0.05$) across a column are denoted using superscript letters.

The hydrophobicity of MFWP increased with heat treatment followed by a further increase after sonication (Table 4.1). Thermal denaturation of whey proteins is known to expose hydrophobic domains that are otherwise buried within the native globular structure (Giroux et al., 2015). Such hydrophobic regions interact to form larger whey protein aggregates which are later disintegrated by cavitation induced shear forces of US. Disintegrated aggregates have more exposed hydrophobic residues that are otherwise buried within larger aggregates.

A complementary study by Giroux et al. (2015) also reported improved rennet gelation when whey proteins were denatured at varying concentrations (5% w/w – 20% w/w) to form small aggregates with an average size ranging between 41 nm to 299 nm. In their experiments, the larger aggregates (299 nm) were found to be less hydrophobic than the smaller aggregates (41 nm). In contrast to the current study, in which the smaller aggregates were made by fragmenting large aggregates, the small and large aggregates in the study by Giroux et al. (2015) were made by heat denaturation at varying concentrations of protein. Their study concluded that the smaller, more hydrophobic aggregates could better interact with the hydrophobic renneted

casein micelles than the less hydrophobic larger aggregates, and more severely impair casein network formation. Contrastingly, Perreault, Morin, et al. (2017) reported that the less hydrophobic soluble protein fraction of denatured whey protein concentrates can impair rennet gelation more severely compared to the more hydrophobic sediment-able fraction that has a larger aggregate size (~ 8µm). Nonetheless, in our study, aggregate hydrophobicity cannot be directly correlated to either the size of the whey protein aggregates or to the rate of gelation. The smallest native MFWP (5-7 nm) had the lowest hydrophobicity (755 ± 3) followed by large HT MFWP (size: 60-600 µm, hydrophobicity index: 3623 ± 172 and medium sized HT US MFWP (size 0.01 µm - 2 µm, hydrophobicity index: 4782 ± 25). Therefore, irrespective of the hydrophobicity, it appears that the size of the whey protein/aggregate is the key factor in determining its effect on rennet gelation. While small (5-7 nm) native whey proteins can occupy the spaces created on renneted casein micelles (which is ~50-200 nm in diameter), larger heat-denatured aggregates (60-600 µm) and heat-denatured and sonicated aggregates (0.1-2 µm) are too large to be able to do so.

4.3.2 Effect of aggregates made from whey protein concentrate powder on rennet gelation

While MFWP was used here to understand the mechanism behind the effect of aggregating whey protein on rennet gelation, whey protein concentrates produced from cheese whey are more widely produced and of more industrial relevance. To investigate the effect of replacing MFWP with WPC on the kinetics of rennet gelation, cheese milks (with a casein:whey protein ratio of 0.9:1) were formulated by incorporating whey protein solutions into diluted skim milk. Four milk systems with diluted skim milk (DS), diluted skim milk with untreated WPC (DS WPC), diluted skim milk with heat-treated WPC (DS HT WPC) and diluted skim milk with heat-treated and sonicated WPC (DS HT US WPC) were analysed.

The particle size distributions of treated WPC samples were first determined (Figure 4.3a). Compared to the untreated MFWP solution that had a single peak at 5-7 nm, two peaks were observed in the untreated WPC solution at 50-90 nm and 150-450 nm. After centrifuging the WPC solution, the fat-rich top layer had a peak at 65-400 nm reflecting the presence of homogenised fat (Thiebaud et al., 2003) . The protein-rich middle layer had two peaks at 30-60 nm and 80-300 nm. Peak at 30-60 nm could possibly correspond to small whey proteins aggregates (Giroux et al., 2015) while the peak at 80-300 nm corresponds to submicron sized fat globules (Michalski et al., 2002) which was measured to be 1.88 ± 0.33 % w/w of powder, and relatively large whey protein aggregates and/or casein micelles (Glantz et al., 2010). Similar to MFWP, upon heat treatment, denatured whey protein particulates were formed in

the 10% w/w WPC solutions, as indicated in the particle size distribution (major peak between 4-250 µm). After sonication the majority of these particles were disintegrated into smaller particles <6 µm, with only a small amount of larger aggregates remaining (Figure 4.3b).

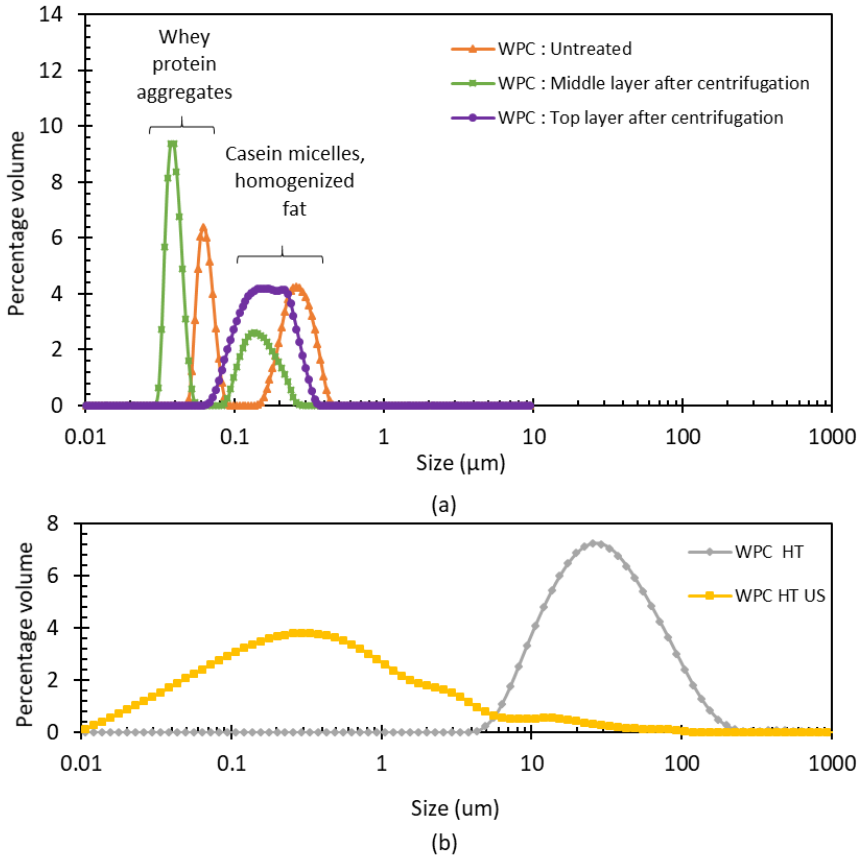


Figure 4.3: Particle size distributions of (a) untreated WPC (▲), the middle layer of centrifuged WPC (■) and the top layer of centrifuged WPC (●) as measured by dynamic light scattering (Malvern Zetasizer). Particle size distributions of (b) heat-treated WPC (◆) and heat-treated and sonicated WPC (■) measured by laser diffraction (Malvern Mastersizer). Two particle sizing methods were used to accurately measure across a wide particle size range (~30 nm to ~900 µm).

The trends in gelation kinetics with respect to whey protein aggregate size for the DS WPC, DS HT WPC and DS HT US WPC systems (Figure 4.4) was similar to that of the comparable systems made from MFWP (Figure 4.2). Again, improved gelation compared to the untreated WPC occurred after heat treatment (DS HT WPC). The gelation rate reverted to that of the untreated WPC when the WPC aggregate size was reduced using ultrasound (DS HT US WPC). This is consistent with proposed mechanism that increasing the size of the whey proteins by heat-induced aggregation prevents the whey proteins from associating with, and thereby impairing, the gelation of the renneted casein micelles. The storage moduli in all WPC systems were less than those in the equivalent MFWP system. This could be explained by the lower

overall casein concentration in the WPC systems. Also, in contrast to the native MFWP that impaired the rennet gelation of diluted skim milk, the addition of untreated WPC improved the rate of rennet gelation. As the pH of all milk systems were similar, the improved gelation could be either due to the slightly higher casein concentration in the WPC samples (2.0% w/w) compared to the DS (1.9 % w/w), or due to an increase in the soluble calcium with the addition of WPC. A separate analysis of soluble calcium measurement of milk systems showed that the impact of adding WPC streams into diluted skim milk was similar to adding MFWP streams (data not shown). Therefore, the only explanation for the improved rennet gelation in SD WPC sample would be the slightly higher casein concentration.

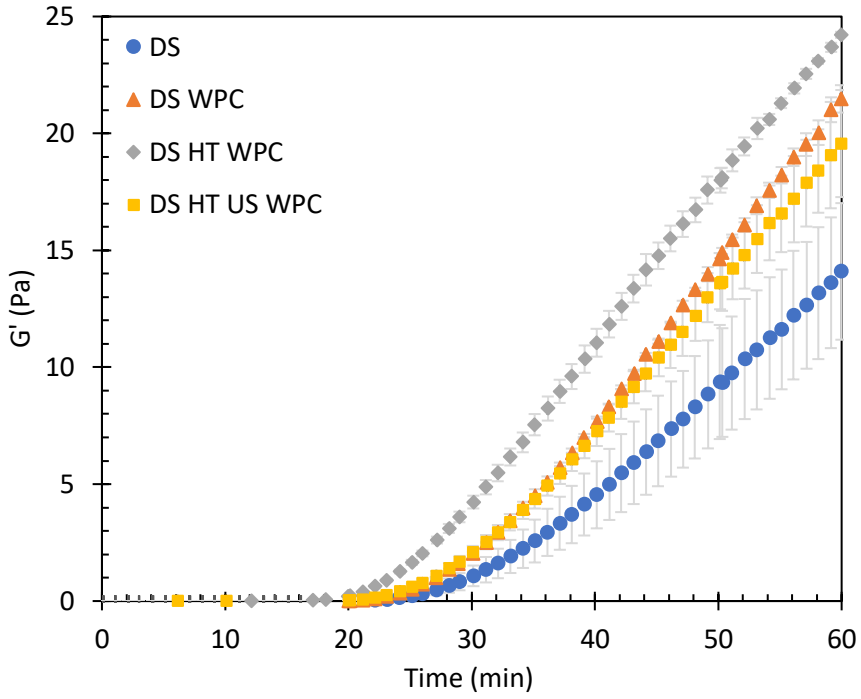


Figure 4.4: Kinetics of rennet gelation of milk systems made with diluted skim milk only (●), diluted skim milk with untreated WPC (▲), diluted skim milk with heated WPC (◆) and diluted skim milk with heated then sonicated WPC (■). The error bars represent the standard deviation of measurements of duplicate samples.

As with the MFWP systems, the hydrophobicity of the WPC systems increased with heat-treatment and further increased with subsequent ultrasound (Table 4.2). The relatively higher surface hydrophobicity of the untreated WPC compared to the untreated MFWP could be due to the presence of aggregates (with more exposed hydrophobic sites), as indicated in the size distribution data and residual fat in commercial WPC (5.28% w/w fat in WPC powder compared to <0.1% w/w in MFWP powder. In order to investigate whether residual fat in commercial WPC could fully account for the increase in hydrophobicity, a separate experiment

was carried out by formulating 10% WPC solutions with fat contents between 0.250% w/w and 0.528% w/w. Although we observed a linear reduction in surface hydrophobicity with the reduction in fat, extrapolation of data showed that fat cannot fully account for the increase in hydrophobicity (Figure 4.5). A 10% w/w MFWP system prepared by incorporating 0.260% w/w anhydrous milk fat that showed a significantly low surface hydrophobicity compared to the WPC systems with 0.25% w/w or 0.4% w/w fat (Figure 4.5) confirming that the increase in surface hydrophobicity in WPC is primarily due to the presence of denatured whey protein aggregates. Distinct to the WPC, heat treatment of the WPC was found to significantly reduce the soluble calcium content (Table 4.2). This can be attributed to the presence of casein in the WPC, as it is known that soluble calcium shifts to colloidal calcium associated with casein micelles upon heating (Zhang & Aoki, 1996). Sonication did not cause further changes in the calcium equilibrium. As equal amounts of whey protein dispersions were added to DS WPC, DS HT WPC and DS HT US WPC milk systems, the lower soluble calcium in HT WPC and HT US WPC system would translate to a lower soluble calcium content in DS HT WPC and DS HT US WPC milk systems compared to that of DS WPC system. Although a reduction in soluble calcium should impair the rate of rennet gelation (Martin et al., 2010), the opposite was observed in this study. This further confirms that the size of the whey proteins/aggregates is key in determining the rate of rennet gelation irrespective of the changes in the soluble calcium.

Table 4.2: Soluble calcium and surface hydrophobicity of differently treated WPC systems.

Whey protein system	Soluble calcium (ppm)	Surface hydrophobicity index
WPC	318 ± 1^{a}	3090 ± 100^{a}
WPC HT	231 ± 3^{b}	8891 ± 473^{b}
WPC HT US	233 ± 2^{b}	14590 ± 223^{c}

* The standard deviation of the measurements of at least duplicates samples prepared from the same treatment tube are denoted alongside the mean value. The absolute value of the surface hydrophilicities changed significantly in different days with freshly prepared buffer, however, a similar increasing trend with HT and HT US treatments were observed.

**Significantly different values ($p < 0.05$) across a column are denoted using superscript letters.

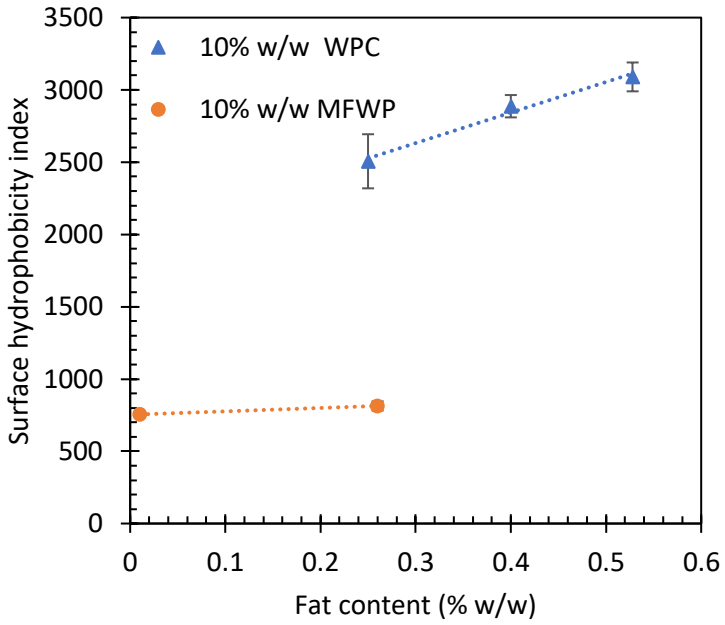


Figure 4.5: Surface hydrophobicity indices of 10% w/w WPC (▲) and MFWP (●) systems with varying fat contents.

4.3.3 Incorporation of whey protein aggregates into rennet gels

To complement the gelation kinetics study, insights into how whey proteins are incorporated into the gel matrix could be gained through confocal imaging of the gel microstructure. Despite the observed differences in the gelation rates and gel strengths of the differently treated WPC systems, the microstructure images (Figure 4.6) show entrapment of the whey proteins within the casein gel matrix of all the sample. However, the microstructure of the samples appeared to differ. In the gels with untreated WPC, the whey proteins were present as relatively small clusters spread evenly throughout the casein matrix. Whereas in the system with heated-treated WPC, the whey proteins existed as larger, separated clusters, while in the heated and sonicated system the clusters were smaller yet interconnected. Presumably, smaller more dispersed aggregate clusters should be more effective than larger more localised aggregates at reducing the number of casein-casein crosslinks by acting as an inert barrier inside the casein matrix. The higher number of casein-casein cross links that can form with larger aggregates could be the reason for the higher final gel strengths observed in the DS HT WPC system, compared to the DS WPC and DS HT US WPC systems (Figure 4.4).

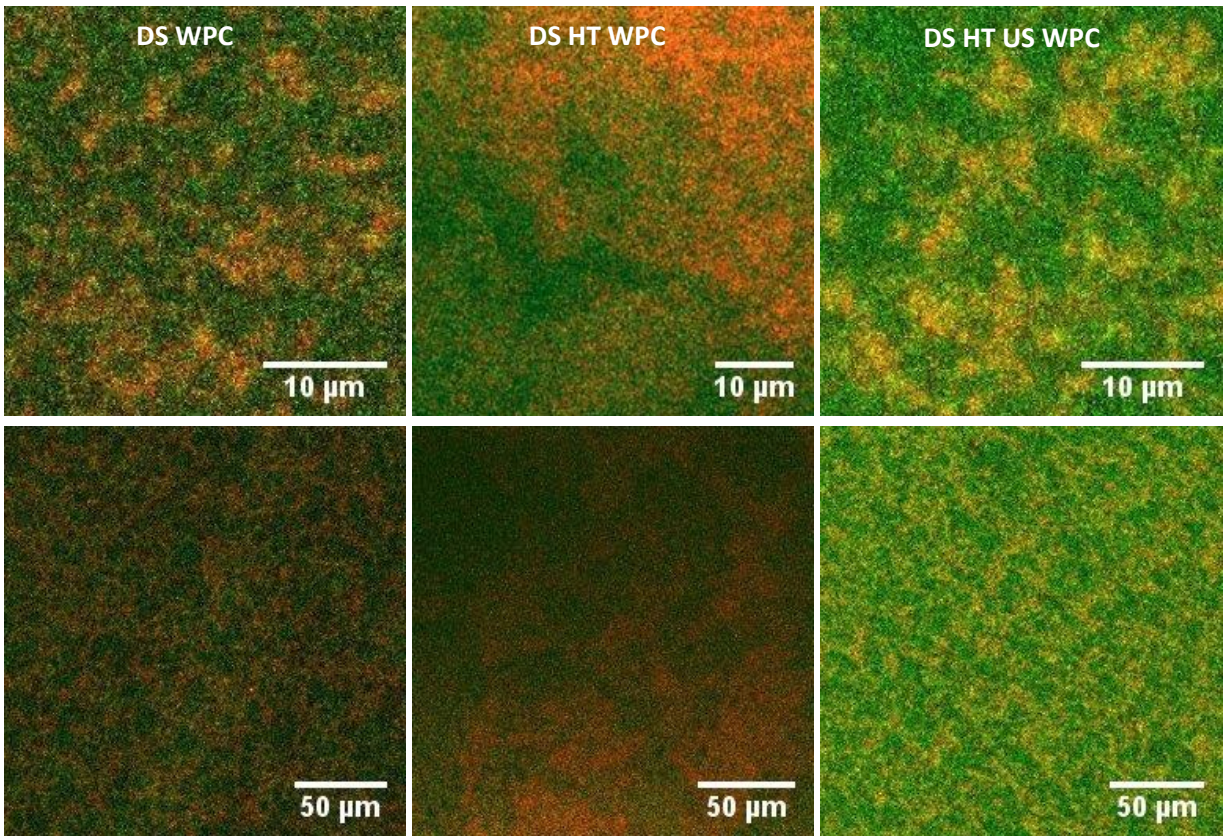


Figure 4.6: Microstructural images of rennet gels made from skim milk with added untreated (left), heat-treated (middle) and heated then sonicated (right) WPC. Skim milk and WPC were stained in green and red respectively. The yellow areas representing the overlay of green and red are representative of whey protein clusters in the casein (green) matrix. The images in the top and bottom rows are of different magnifications.

Although whey proteins are well incorporated into the initial gel matrix (Figure 4.6), their retention in the cheese despite multiple post-gelation processing steps such as cutting, cooking, cheddaring and pressing will depend on how strongly whey proteins are entrapped and bound within the casein matrix. Both aggregate size and surface hydrophobicity could be determining factor in whey protein retention. For example, inert fat droplets (droplets that are not coated in casein micelles and therefore do not interact with the casein matrix) are better retained in cheeses when they are similar in size to the pores created in the gel matrix (Logan et al., 2015). Although an increased hydrophobicity of whey protein aggregates (with heat and US treatments) did not appear to affect the rate of casein micelle aggregation during rennet gelation, it could nonetheless result in stronger interactions between the casein matrix and whey protein aggregates that could help retain them within the casein gel structure during post-coagulation processing. Therefore, to assess whether the size or hydrophobicity of the whey protein aggregates influence their retention in the rennet gel matrix, the protein retention in

cooked rennet gel systems made with DS, DS WPC, DS HT WPC and DS HT US WPC systems was measured (Table 4.3).

Table 4.3: Compositional analysis and protein retention in rennet gel curds and whey made from diluted skim milk (DS), diluted skim milk with untreated whey protein concentrate (DS WPC), diluted skim milk with heat-treated WPC (DS HT WPC) and diluted skim milk with heat-treated and sonicated WPC (DS HT US WPC).

Cheese milk system	Moisture content in cooked curd (% w/w)	Total solids in drained whey (% w/w)	Protein content in cooked curd (% w/w)	Protein content in drained whey (% w/w)	Mass of cooked curd (g)	Protein retention in cooked curd (% of input protein)	Protein loss in drained whey (% of input protein)
DS	83.5±0.3[a]	5.57±0.22[a]	10.6±0.4[a]	0.58±0.38[a]	22.7±0.1 [a]	83.5±3.1[a]	15.6±0.9
DS WPC	83.8±1.2 [a]	6.82±0.12[b]	10.2±0.3 [a]	1.47±0.13 [b]	25.1	69.7±1.9[b]	29.6±1.8 [b]
DS HT WPC	83.3±0.7 [a]	6.77±0.14[b]	11.5±0.8 [a]	1.35±0.10 [b]	23.6±2.1[a]	73.3±1.7[b]	30.0±3.4 [b]
DS HT US WPC	83.6±0.2 [a]	6.41±0.01[b]	11.0±0.1 [a]	1.11±0.06 [c]	26.8±0.0[a]	81.0±0.6[a]	22.2±1.2[c]

* The standard deviation of at least duplicate measurements of duplicate milk systems are denoted alongside the mean value except for DS WPC system. Please note that the DS WPC cooked curds were formed once and replicate samples were measured.

** Significantly different values ($p < 0.05$) across a column are denoted using superscript letters.

In conventional cheese making, most of the casein is retained in the curd as part of the cheese matrix, whereas almost all the whey protein is lost into the whey. Although the overall protein retention decreased in DS WPC system compared to DS system, it is worth noting the casein content in DS WPC cheese milk (as a percentage of total protein) was lower compared to DS cheese milk. Compared to DS WPC system, the protein retention increased for the heat-treated WPC system and increased further for the WPC that had been heated then ultrasound treated. This is reflected by 5.2% and 16.2% increases in protein retention, compared to the system with untreated WPC. The increased retention of the heat-treated WPC can be attributed to the increased size of the aggregates. The further increase in retention of the heated and ultrasound-treated WPC, which had somewhat smaller but more hydrophobic aggregates than the heat-treated WPC, suggests hydrophobicity can increase the retention of sufficiently large whey protein aggregates. While the majority of the whey proteins are expulsed during the cutting and cheddaring stage of cheese making, these results suggest that sufficiently large and hydrophobic whey protein aggregates can associate strongly enough with renneted casein micelles to facilitate retention

inside the coagulum. Somewhat relatedly Perreault, Rémillard, et al. (2017) also reported that protein retention in semi-hard cheeses (20-24% fat) are improved by adding the sediment-able fraction of denatured whey protein concentrates as compared to adding the soluble fraction. Ismail et al. (2011) reported an increase in the overall cheese yield when denatured whey protein aggregates were incorporated during mozzarella cheese making from buffalo milk containing fat <2%. However, this appears to be the first time an increase in whey protein retention has been reported for cheese milk containing negligible fat.

3.4 Retention of whey protein aggregates in model fat-free cheese

Cheddar cheese making involves post-cooking steps such as salting and pressing that can lead to further expulsion of loosely bound proteins from the cheese matrix. Therefore, model cheeses were made from 10 kg batches of the different WPC cheese milk systems to investigate whether the whey protein aggregates retained in the curds could be maintained through the post-cooking process.

As with the curds, compositional analysis of the cheeses showed an increase in protein retention with heat and heat-coupled US treatment (Table 4.4). The increase in protein retention is likely due to an increase in the retention of whey proteins. SDS-PAGE analysis of the sweet whey was performed that provided further evidence of whey protein retention in the DS HT US WPC cheese (Figure 4.7). Analysis of the cheese was not possible due to the difficulty of melting and solubilising the fat-free cheeses, even after EDTA addition. The intensities of α-lactalbumin, β-lactoglobulin and serum albumin bands of the sweet whey from the DS HT US WPC cheese were low compared to those from the DS WPC and DS HT WPC cheeses, suggesting that more whey protein was retained in the DS HT US WPC cheese (Figure 4.7). It should be noted that the low intensity of the whey protein bands in the DS sweet whey is due to the low initial whey protein concentration in the cheese milk, translating to a relatively low whey protein loss. Together the composition and SDS-PAGE data indicate that more whey protein was incorporated in cheese when both the aggregate size and the surface hydrophobicity of the whey proteins were increased by heat or heat coupled with ultrasound. Despite the small increase in whey protein retention with increased size and hydrophobicity of added WPC systems, protein retention in all WPC-enriched cheeses were significantly low compared to DS cheese, suggesting that retention of added whey protein is not as good as the retention of proteins in skim milk. Although the rate of para-casein aggregation improved when denatured whey protein aggregates were added to cheese milk instead of untreated whey proteins(Figure 4.4) , the high proportion of denatured whey protein used in this study (35% of total protein)

may not have allowed the formation of a homogeneous para-casein matrix and was responsible for the poor retention of added whey protein.

In traditionally made cheddar cheeses, fat globules trapped inside the protein matrix acts as a cheese structure breaker providing an 'openness' to its microstructure (Lobato-Calleros et al., 2007). Therefore, a common problem associated with low-fat cheese is the rigidity caused by a strongly interlinked protein structure. Due to the very low fat content (<0.02% w/w) in the milk formulation used in these experiments, the DS cheese (Figure 4.8a top two rows) and DS WPC cheese (Figure 4.8b top two rows) had a hard and gummy texture. However, despite the low-fat content, cheese made with incorporation of heated (Figure 4.8c top two rows) or heated and sonicated (Figure 4.8d top two rows) WPC were softer and closer to regular cheddar cheese in appearance.

Table 4.4: Compositional analysis of model cheeses.

Cheese type	Total solids in sweet whey (% w/w)	Moisture content in pressed curd (% w/w)	Total protein in sweet whey (% w/w)	Protein content in pressed curd (% w/w)	Protein retention in pressed curd (% of input protein)	Cheese Yield
DS	5.73±0.01[a]	54.3±0.3[a]	0.79±0.03[a]	40.78±0.24[a]	85.8±0.5[a]	6.0%
DS WPC	6.31±0.02[b]	53.2±0.3[a]	1.30±0.00[b]	40.89±0.27[a]	68.3±0.4[b]	6.1%
DS HT WPC	6.73±0.02[c]	58.6±0.5[b]	1.18±0.05[c]	36.34±0.04[b]	70.7±0.1[c]	6.1%
DS HT US WPC	6.36±0.02[b]	61.1±1.2[c]	1.06±0.01[d]	33.82±1.00[c]	72.7±2.1[c]	7.9%

*The standard deviation of the measurements of duplicate or triplicate samples are denoted alongside the mean value.

**Significantly different values ($p < 0.05$) across a column are denoted using superscript letters.

***Note that the total weight of curds and sweet whey used for protein retention and loss calculations were measured only once.

Confocal microscopy was used to investigate the role of the incorporated whey protein aggregates on the cheese microstructure (Figure 4.8, bottom rows). Cheese made from diluted skim milk and diluted skim milk with added untreated WPC showed a smoother more compact

cheese protein structure (represented in green) with small pore (represented in black) (Figure 4.8a bottom two rows). This is a more compact microstructures than is typical of cheese made from full cream milk (with ~3.4% fat) (Lobato-Calleros et al., 2007). With the addition of heat-treated WPC the microstructure was interrupted with larger pores and cracks (Figure 4.8c bottom two rows), and with heat-treated then sonicated WPC the structure was more granular (Figures 4.8d bottom two rows). Due to the very low fat content in cheese milk (<0.02% w/w) no distinguishable areas of fat were observed by the Nile Red channel (represented in red). The protein analysis indicated whey protein retention improved in DS HT WPC and DS HT US WPC cheeses. It is possible that the retained whey introduced weak spots and breaking points in the casein matrix introducing irregularities to the cheese.

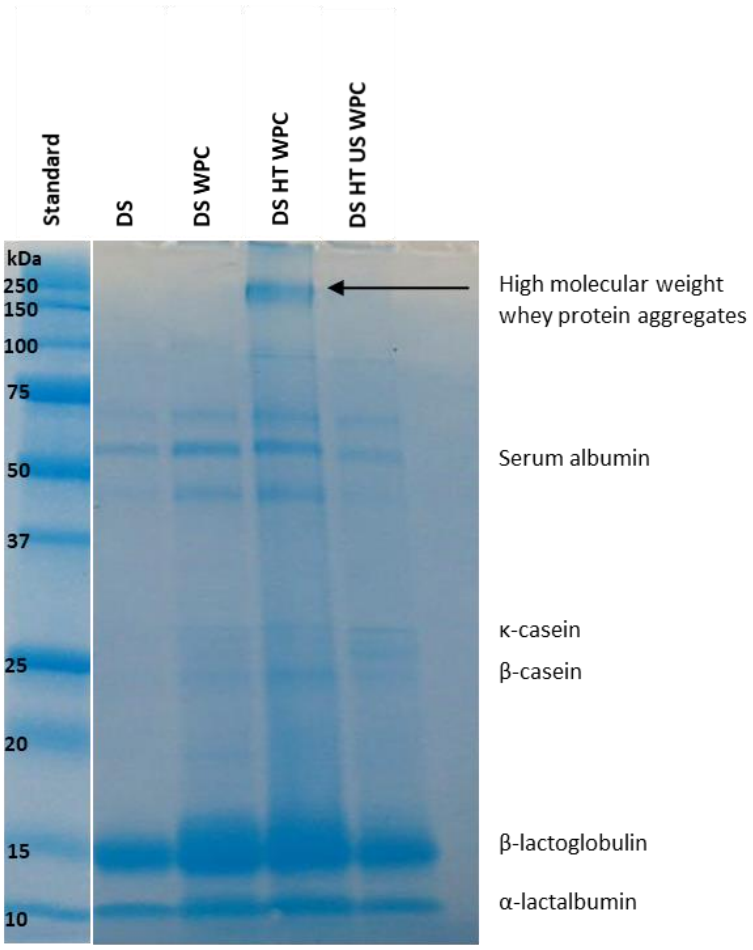
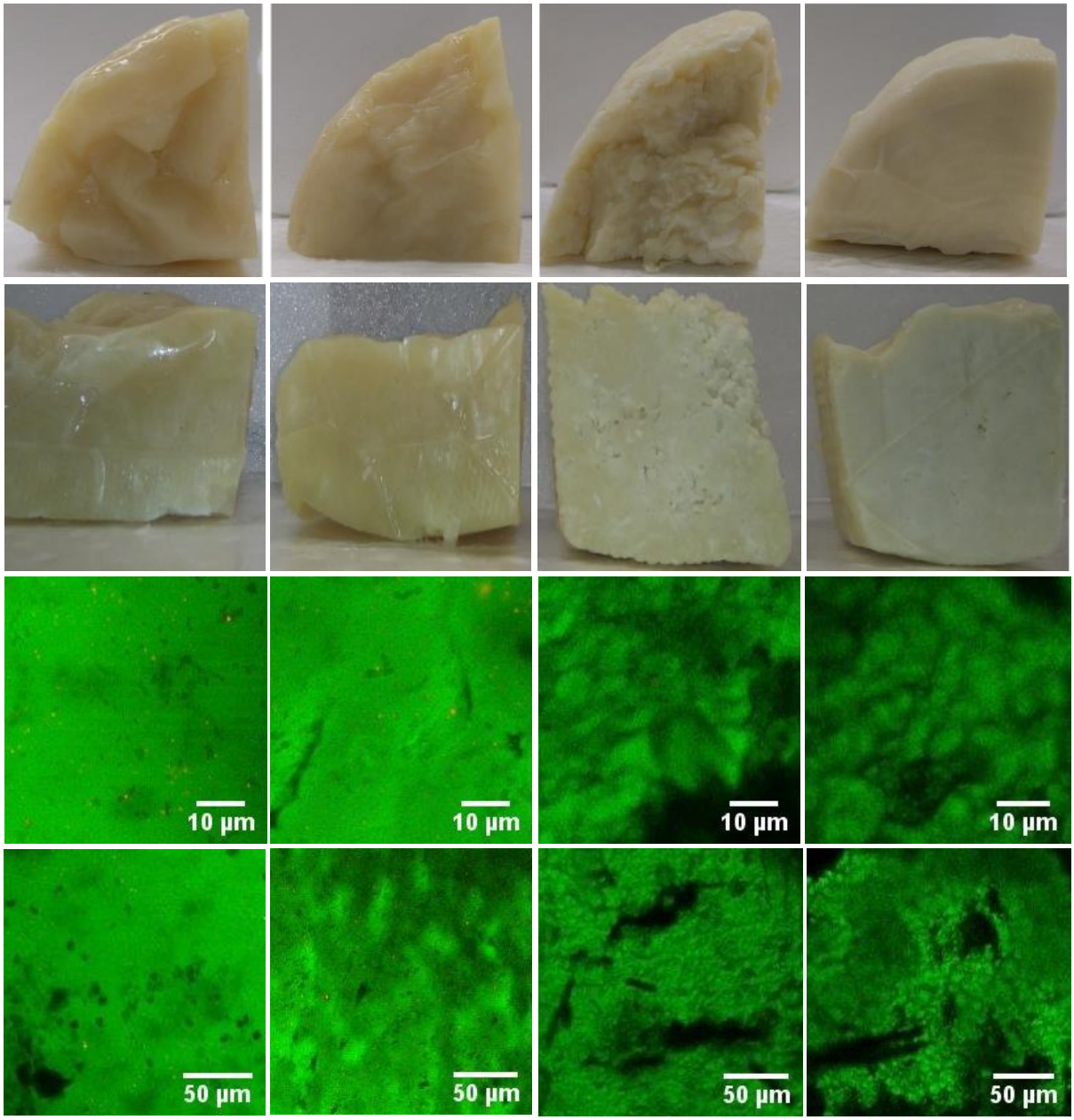


Figure 4.7: SDS-PAGE gel of sweet whey samples from cheeses made from diluted skim milk (DS), diluted skim milk with untreated whey protein concentrate (DS WPC), diluted skim milk with heat-treated WPC (DS HT WPC) and diluted skim milk with heat-treated and sonicated WPC (DS HT US WPC).

Denatured whey protein having a high moisture binding ability is known to increase the moisture content in cheese providing them a softer texture (Stankey et al., 2017) by hydrating

the protein matrix. (Hennelly et al., 2005) while working with acid-gelled cheeses reported that up to 50% w/w moisture content, moisture in cheese remains almost entirely bound to the protein matrix as water of hydration. They further reported that above 54% w/w moisture content, finely distributed water coalesces forming free water in the protein matrix. Although such 'limiting' moisture contents are not reported in literature for rennet cheeses, we observe dark 'pores' or serum pockets in in DS HT WPC (moisture contents 58.6%) and DS HT US WPC (moisture contents 61.1% w/w) cheese microstructure that are possibly formed by the coalescence of water.

In addition to the presence of pores, DS HT WPC cheeses and DS HT US WPC cheese has a granular or 'flaky 'protein' microstructure. We believe that the granular structure results from the disturbance to the casein matrix, caused by the inclusion of differently sized whey protein aggregates from the gel, which introduces weak spots and breaking points in the casein matrix.



(a) (b) (c) (d)

Figure 4.8: Visual appearance of the surface (top row) and interior (second row), and the microstructure (bottom two rows) of model cheeses made with (a) DS, (b) DS WPC, (c) DS HT WPC and (d) DS HT US WPC. Black areas in the microstructural images represent pores in the cheese while green areas represent the protein matrix.

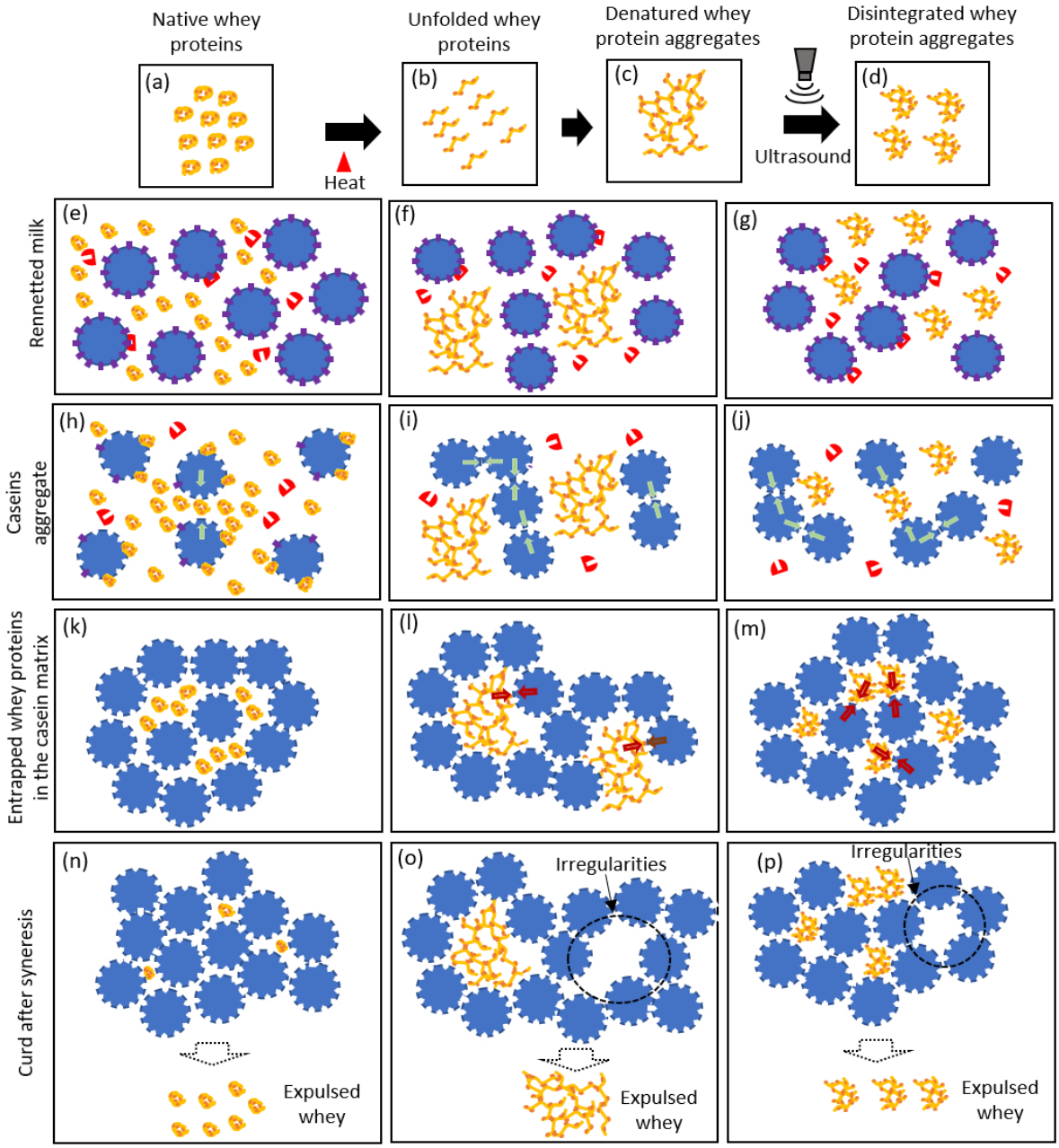


Figure 4.9: Proposed mechanism for the effect of whey protein aggregates on the rate of rennet gelation and the protein retention in cheese. Upon heat treatment, native whey proteins ( ) (a) unfold (b) and expose their buried hydrophobic residues ( ). Subsequently, denatured whey proteins aggregate due to hydrophobic interactions (c). Acoustic shear disintegrates large whey aggregates into smaller particles with more exposed hydrophobic residues (d). When whey proteins are added to a system containing casein micelles ( ) and rennet ( ) (e), (f), (g), native whey proteins occupy the spaces created on the micelle surface from the cleavage κ-casein impairing the enzyme hydrolysis process. They further provide a steric barrier to interactions between cleaved micelles ( ), reducing the rate of gelation (h). Sufficiently large heat-treated or heat- and ultrasound-treated whey protein aggregates are unable to interact with cleaved casein micelles, or obstruct aggregation and allows a stronger coagulum to be formed (i), (j). More hydrophobic aggregates can interact ( ) with the casein matrix more (l), (m) compared to native whey proteins (k), and are therefore less likely to be expulsed during whey syneresis.

A part of the smaller native whey proteins escapes the coagulum (n) during syneresis. Inclusion of larger whey protein aggregates facilitates a more granular cheese structure with irregularities (o), (p).

The present results suggest that during rennet gelation, incorporating heat-denatured (Figure 4.9c) and US-treated whey protein aggregates (Figure 4.9d) can reduce the impairment of rennet gelation caused by high concentrations of native whey proteins (Figure 4.9h). At the same whey protein concentration, incorporating larger aggregates that are less numerous results in less steric hindrance compared to smaller aggregates that are more numerous, leading to faster rennet gelation (Figure 4.9 i, j). During subsequent gelation large whey protein aggregates can become incorporated into the casein matrix, which is enhanced by their higher surface hydrophobicity (Figure 4.9 l, m) compared to native whey proteins. During post-gelation processing of the curd, a part of the whey protein is released from the casein matrix, however some hydrophobic aggregates can be retained due to hydrophobic interactions with the renneted casein. The inclusion of sufficiently large whey protein aggregates during cheese milk formulation provides an "openness" and irregularity to the microstructure and reduce the rigidity of the casein matrix in low-fat cheese (Figure 4.9 o, p). The present results suggest that during rennet gelation, incorporating heat-denatured (Figure 8c) and US-treated whey protein aggregates (Figure 4.9d) can reduce the impairment of rennet gelation caused by high concentrations of native whey proteins (Figure 4.9h). At the same whey protein concentration, incorporating larger aggregates that are less numerous results in less steric hindrance compared to smaller aggregates that are more numerous, leading to faster rennet gelation (Figure 4.9 i, j). During subsequent gelation large whey protein aggregates can become incorporated into the casein matrix, which is enhanced by their higher surface hydrophobicity (Figure 4.9 l, m) compared to native whey proteins. During post-gelation processing of the curd, a part of the whey protein is released from the casein matrix, however some hydrophobic aggregates can be retained due to hydrophobic interactions with the renneted casein. The inclusion of sufficiently large whey protein aggregates during cheese milk formulation provides an "openness" and irregularity to the microstructure and reduce the rigidity of the casein matrix in low-fat cheese (Figure 4.9 o, p).

4.4 Conclusions

This chapter demonstrated that sufficiently large whey protein aggregates formed using heat or heat plus ultrasound treatment on whey protein streams, could avoid impairment of rennet gelation caused by native whey protein in whey protein-rich cheese milks with a constant casein concentration. Further, these treatments can improve whey protein retention when incorporated into cheese production from whey protein- rich cheese milks. Irrespective of

changes in the soluble calcium in the systems, larger aggregates can reduce the steric barrier created by native whey proteins and smaller whey protein aggregates. Further, more hydrophobic aggregates can interact with the casein matrix, which reduces the expulsion of whey protein during syneresis. However, the inclusion of larger whey protein aggregates into cheese milk facilitates a more open cheese microstructure and mitigates the increased hardness that is observed in low fat cheeses. This chapter demonstrated the potential of using heat and ultrasound treatment to increase the retention of whey protein and improve the texture of low-fat cheese.

4.5. References

Alizadeh-Pasdar, N., & Li-Chan, E. C. (2000). Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. *Journal of Agricultural and Food Chemistry, 48*(2), 328-334. doi:https://doi.org/10.1021/jf990393p

Ashokkumar, M., Lee, J., Zisu, B., Bhaskarcharya, R., Palmer, M., & Kentish, S. (2009). Hot topic: Sonication increases the heat stability of whey proteins. *Journal of Dairy Science, 92*(11), 5353-5356. doi:https://doi.org/10.3168/jds.2009-2561

Baldwin, K. A., Baer, R., Parsons, J., Seas, S., Spurgeon, K., & Torrey, G. (1986). Evaluation of yield and quality of cheddar cheese manufactured frommilk with added whey protein concentrate. *Journal of Dairy Science, 69*(10), 2543-2550. doi:https://doi.org/10.3168/jds.s0022-0302(86)80699-3

Banks, J. M., & Muir, D. (1985). Effect of incorporation of denatured whey protein on the yield and quality of Cheddar cheese. *International journal of dairy technology, 38*(1), 27-32. doi:https://doi.org/10.1111/j.1471-0307.1985.tb00536.x

Barbero, N., Barni, E., Barolo, C., Quagliotto, P., Viscardi, G., Napione, L., . . . Bussolino, F. (2009). A study of the interaction between fluorescein sodium salt and bovine serum albumin by steady-state fluorescence. *Dyes and pigments, 80*(3), 307-313.

Brown, R. J., & Ernstrom, C. A. (1982). Incorporation of ultrafiltration concentratedwhey solids into cheddar cheese for increased yield. *Journal of Dairy Science, 65*(12), 2391-2395. doi:https://doi.org/10.3168/jds.s0022-0302(82)82514-9

Chamberland, J., Benoit, S., Harel-Oger, M., Pouliot, Y., Jeantet, R., & Garric, G. (2019). Comparing economic and environmental performance of three industrial cheesemaking processes through a predictive analysis. *Journal of Cleaner production, 239*, 118046. doi:https://doi.org/10.1016/j.jclepro.2019.118046

Chamberland, J., Mercier-Bouchard, D., Dussault-Chouinard, I., Benoit, S., Doyen, A., Britten, M., & Pouliot, Y. (2019). On the Use of Ultrafiltration or Microfiltration Polymeric Spiral-Wound Membranes for Cheesemilk Standardization: Impact on Process Efficiency. *Foods, 8*(6), 198. doi:https://doi.org/10.3390/foods8060198

Di Cagno, R., De Pasquale, I., De Angelis, M., Buchin, S., Rizzello, C., & Gobbetti, M. (2014). Use of microparticulated whey protein concentrate, exopolysaccharide-producing *Streptococcus thermophilus*, and adjunct cultures for making low-fat Italian Caciotta-type cheese. *Journal of Dairy Science, 97*(1), 72-84. doi:https://doi.org/10.3168/jds.2013-7078

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. (2017). Enzymatic coagulation of milk. In *Fundamentals of cheese science* (pp. 185-229): Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2000). *Fundamentals of cheese science*. Maryland: Aspen Publishers.

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

Giroux, H. J., Lanouette, G., & Britten, M. (2015). Effect of whey protein aggregates of various sizes on the formation and properties of rennet-induced milk gels. *Food Hydrocolloids, 45*, 272-278.

Glantz, M., Devold, T., Vegarud, G., Månsson, H. L., Stålhammar, H., & Paulsson, M. (2010). Importance of casein micelle size and milk composition for milk gelation. *Journal of dairy science, 93*(4), 1444-1451. doi:https://doi.org/10.3168/jds.2009-2856

Gordon, L., & Pilosof, A. M. (2010). Application of high-intensity ultrasounds to control the size of whey proteins particles. *Food Biophysics, 5*(3), 203-210. doi:https://doi.org/10.1007/s11483-010-9161-4

Guinee, T. P., & Wilkinson, M. (1992). Rennet coagulation and coagulants in cheese manufacture. *International Journal of Dairy Technology, 45*(4), 94-104.

Hennelly, P. J., Dunne, P. G., O'Sullivan, M., & O'Riordan, D. (2005). Increasing the moisture content of imitation cheese: effects on texture, rheology and microstructure. *European Food Research and Technology, 220*(3-4), 415-420.

Hinrichs, J. (2001). Incorporation of whey proteins in cheese. *International Dairy Journal, 11*(4), 495-503.

Ismail, M., Ammar, E. T., & El-Metwally, R. (2011). Improvement of low fat mozzarella cheese properties using denatured whey protein. *International Journal of Dairy Technology, 64*(2), 207-217. doi:https://doi.org/10.1111/j.1471-0307.2010.00654.x

Jenness, R. (1999). Composition of Milk. In N. P. Wong (Ed.), *Fundamentals of Dairy Chemistry* (pp. 1-38). Gaithersburg, Maryland: Aspen Publishers, Inc.

Kethireddipalli, P., Hill, A. R., & Dalgleish, D. G. (2010). Protein interactions in heat-treated milk and effect on rennet coagulation. *International Dairy Journal, 20*(12), 838-843. doi:https://doi.org/10.1016/j.idairyj.2010.06.003

Laiho, S., Ercili-Cura, D., Forssell, P., Myllärinen, P., & Partanen, R. (2015). The effect of dynamic heat treatments of native whey protein concentrate on its dispersion characteristics. *International Dairy Journal, 49*, 139-147. doi:https://doi.org/10.1016/j.idairyj.2015.05.002

Leong, T. S. H., Walter, V., Gamlath, C. J., Yang, M., Martin, G. J., & Ashokkumar, M. (2018). Functionalised dairy streams: Tailoring protein functionality using sonication and heating. *Ultrasonics sonochemistry, 48*(1), 499-508. doi:https://doi.org/10.1016/j.ultsonch.2018.07.010

Lo, C., & Bastian, E. (1998). Incorporation of native and denatured whey proteins into cheese curd for manufacture of reduced fat, Havarti-type cheese. *Journal of dairy science, 81*(1), 16-24. doi:https://doi.org/10.3168/jds.s0022-0302(98)75545-6

Lobato-Calleros, C., Reyes-Hernández, J., Beristain, C., Hornelas-Uribe, Y., Sanchez-Garcia, J., & Vernon-Carter, E. (2007). Microstructure and texture of white fresh cheese made with canola oil and whey protein concentrate in partial or total replacement of milk fat. *Food Research International, 40*(4), 529-537. doi:https://doi.org/10.1016/j.foodres.2006.10.011

Logan, A., Leis, A., Day, L., Øiseth, S. K., Puvanenthiran, A., & Augustin, M. A. (2015). Rennet gelation properties of milk: influence of natural variation in milk fat globule size and casein micelle size. *International Dairy Journal, 46*, 71-77. doi:https://doi.org/10.1016/j.idairyj.2014.08.005

Lucey, J. A., Tamehena, M., Singh, H., & Munro, P. A. (1998). Effect of interactions between denatured whey proteins and casein micelles on the formation and rheological properties of acid skim milk gels. *Journal of Dairy Research, 65*(4), 555-567. doi:https://doi.org/10.1017/s0022029998003057

Manirakiza, P., Covaci, A., & Schepens, P. (2001). Comparative study on total lipid determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and modified Bligh & Dyer extraction methods. *Journal of food composition and analysis, 14*(1), 93-100.

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting nitrogen into protein—beyond 6.25 and Jones' factors. *Critical reviews in food science and nutrition, 48*(2), 177-184.

Martin, G. J. O., Williams, R. P., & Dunstan, D. E. (2010). Effect of manufacture and reconstitution of milk protein concentrate powder on the size and rennet gelation behaviour of casein micelles. *International Dairy Journal, 20*(2), 128-131. doi:https://doi.org/10.1016/j.idairyj.2009.08.007

Mason, T. J., Cobley, A., Graves, J., & Morgan, D. (2011). New evidence for the inverse dependence of mechanical and chemical effects on the frequency of ultrasound. *Ultrasonics sonochemistry, 18*(1), 226-230. doi:https://doi.org/10.1016/j.ultsonch.2010.05.008

McMahon, D. J., Alleyne, M., Fife, R., & Oberg, C. (1996). Use of fat replacers in low fat Mozzarella cheese. *Journal of Dairy Science, 79*(11), 1911-1921. doi:https://doi.org/10.3168/jds.s0022-0302(96)76560-8

Mead, D., & Roupas, P. (2001). Effect of incorporation of denatured whey proteins on chemical composition and functionality of pizza cheese. *Australian Journal of Dairy Technology, 56*(1), 19.

Meza-Nieto, M. A., Vallejo-Cordoba, B., González-Córdova, A. F., Félix, L., & Goycoolea, F. M. (2007). Effect of β-Lactoglobulin A and B Whey Protein Variants on the Rennet-Induced Gelation of Skim Milk Gels in a Model Reconstituted Skim Milk System. *Journal of Dairy Science, 90*(2), 582–593.

Michalski, M.-C., Michel, F., & Geneste, C. (2002). Appearance of submicronic particles in the milk fat globule size distribution upon mechanical treatments. *Le Lait, 82*(2), 193-208.

Mistry, V. V., & Maubois, J.-L. (2017). Application of membrane separation technology to cheese production. In *Cheese* (pp. 677-697): Elsevier.

Monahan, F. J., German, J. B., & Kinsella, J. E. (1995). Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *Journal of Agricultural and Food Chemistry, 43*(1), 46-52.

Mulvihill, D., & Donovan, M. (1987). Whey proteins and their thermal denaturation-a review. *Irish Journal of Food Science and Technology, 11*(1), 43-75.

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*(1), 119-130.

Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. (2013). The effect of calcium chloride addition on the microstructure and composition of Cheddar cheese. *International Dairy Journal, 33*(2), 135-141. doi:https://doi.org/10.1016/j.idairyj.2013.03.002

Perreault, V., Morin, P., Pouliot, Y., & Britten, M. (2017). Effect of denatured whey protein concentrate and its fractions on rennet-induced milk gels. *International Dairy Journal, 64*, 48-55. doi:https://doi.org/10.1016/j.idairyj.2016.09.005

Perreault, V., Rémillard, N., Chabot, D., Morin, P., Pouliot, Y., & Britten, M. (2017). Effect of denatured whey protein concentrate and its fractions on cheese composition and rheological properties. *Journal of Dairy Science, 100*(7), 5139-5152. doi:https://doi.org/10.3168/jds.2016-12473

Punidadas, P., Feirtag, J., & Tung, M. (1999). Incorporating whey proteins into mozzarella cheese. *International journal of dairy technology, 52*(2), 51-55. doi:https://doi.org/10.1111/j.1471-0307.1999.tb02070.x

Roefs, S. P., & De Kruif, K. G. (1994). A model for the denaturation and aggregation of β-lactoglobulin. *European Journal of Biochemistry, 226*(3), 883-889. doi:https://doi.org/10.1111/j.1432-1033.1994.00883.x

Rosa, P., Sala, G., Van Vliet, T., & Van De Velde, F. (2006). Cold gelation of whey protein emulsions. *Journal of texture studies, 37*(5), 516-537. doi:https://doi.org/10.1111/j.1745-4603.2006.00066.x

Rüegg, M., Moor, U., & Blanc, B. (1977). A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. *Journal of Dairy Research, 44*(3), 509-520.

Schenkel, P., Samudrala, R., & Hinrichs, J. (2013). The effect of adding whey protein particles as inert filler on thermophysical properties of fat-reduced semihard cheese type Gouda. *International journal of dairy technology, 66*(2), 220-230. doi:https://doi.org/10.1111/1471-0307.12036

Shahani, K., & Sommer, H. (1951). The protein and non-protein nitrogen fractions in milk. I. Methods of analysis. *Journal of dairy science, 34*(10), 1003-1009.

Singh, H., & Waungana, A. (2001). Influence of heat treatment of milk on cheesemaking properties. *International Dairy Journal, 11*(4-7), 543-551. doi:https://doi.org/10.1016/s0958-6946(01)00085-1

Stankey, J. A., Lu, Y., Abdalla, A., Govindasamy-Lucey, S., Jaeggi, J. J., Ø Mikkelsen, B., . . . Andersen, C. B. (2017). Low-fat Cheddar cheese made using microparticulated whey proteins: Effect on yield and cheese quality. *International journal of dairy technology, 70*(4), 481-491.

Sturaro, A., De Marchi, M., Zorzi, E., & Cassandro, M. (2015). Effect of microparticulated whey protein concentration and protein-to-fat ratio on Caciotta cheese yield and composition. *International Dairy Journal, 48*, 46-52. doi:https://doi.org/10.1016/j.idairyj.2015.02.003

Suárez, A., Fernández, L., Balbarie, P., Iglesias, J. R., & Riera, F. A. (2016). Predicting the properties of the whey protein microparticles produced by heat and mechanical treatments. *European Food Research and Technology, 242*(8), 1211-1220.

Tas, J., Ploeg, M. v. d., Mitchell, J., & Cohn, N. (1980). Protein staining methods in quantitative cytochemistry. *Journal of microscopy, 119*(3), 295-311.

Thiebaud, M., Dumay, E., Picart, L., Guiraud, J., & Cheftel, J. (2003). High-pressure homogenisation of raw bovine milk. Effects on fat globule size distribution and microbial inactivation. *International Dairy Journal, 13*(6), 427-439. doi:https://doi.org/10.1016/s0958-6946(03)00051-7

Vasbinder, A. J., Alting, A. C., & de Kruif, K. G. (2003). Quantification of heat-induced casein–whey protein interactions in milk and its relation to gelation kinetics. *Colloids and Surfaces B: Biointerfaces, 31*(1-4), 115-123. doi:ttps://doi.org/10.1016/s0927-7765(03)00048-1

Vasbinder, A. J., Rollema, H., & De Kruif, C. (2003). Impaired rennetability of heated milk; study of enzymatic hydrolysis and gelation kinetics. *Journal of dairy science, 86*(5), 1548-1555.

Walstra, P. (1999). Casein sub-micelles: do they exist? *International Dairy Journal, 9*(3), 189-192. doi:https://doi.org/10.1016/s0958-6946(99)00059-x

Wang, L., & Wang, Y. J. (2004). Application of high-intensity ultrasound and surfactants in rice starch isolation. *Cereal chemistry, 81*(1), 140-144. doi:https://doi.org/10.1094/cchem.2004.81.1.140

Zhang, Z. P., & Aoki, T. (1996). Behaviour of calcium and phosphate in bovine casein micelles. *International Dairy Journal, 6*(8-9), 769-780. doi:https://doi.org/10.1016/0958-6946(96)00006-4

Chapter 5

5. Formation of rennet gel matrices fortified with ultrasonically generated whey protein enriched water-in-oil-in-water emulsions

This chapter investigates the formation of protein-enriched water-in-oil-in-water double emulsions with minimal non-dairy lipophilic emulsifiers, using whey protein and power ultrasound. It also demonstrates the successful encapsulation of higher concentrations of water-soluble proteins inside the oil droplets and in cheddar curd matrices without altering rennet gelation kinetics.

5.1. Introduction

The nutritional value of bovine milk and dairy food products is important to consumers. Milk protein includes casein and whey protein, both of which are a good source of nutritional protein (Barłowska et al., 2011). However during cheese production, water soluble whey proteins are drained out from the coagulum during syneresis (Heino et al., 2007), representing a loss of approximately 20% of the protein from the cheese. The protein in cheese whey retains much of its native nutritional and functional properties (Heino et al., 2007) and can be used to produce protein supplements (e.g. whey protein concentrate powder) or animal feed (Siso, 1996). While these are useful products, the former requires further processing and a certain minimum scale of production to be commercially viable, and the latter is of much lower value than the protein in cheese.

Attempts have been made to incorporate whey protein back into cheese, to make better use of the whey protein and to increase the protein content of the cheese. Difference technologies have been applied to cheese whey to facilitate incorporation of whey protein into cheese, including the use of membrane filtration to preconcentrate the protein, thermal denaturation to aggregate the protein, and the addition of spray-dried whey protein solids (Hinrichs, 2001). However, these technologies each have their limitations. The addition of membrane concentrated whey protein has been associated with the development of bitter flavours due to the retention of undesirable water soluble enzymes (Hinrichs, 2001; Puhan & Gallmann, 1981; Schkoda & Kessler, 1997). Incorporating thermally denatured whey proteins can alter the rate of coagulation (Kethireddipalli et al., 2010; Singh & Waungana, 2001; Vasbinder et al., 2003) and lead to curds with excessive moisture. The addition of whey protein powders can result in poor binding of the curd (Brown & Ernstrom, 1982; Hinrichs, 2001; Punidadas et al., 1999).

The inclusion of double emulsion into cheese is an emerging approach, which has so far been used to reduce the fat content(Felfoul et al., 2015; Leong et al., 2020; Lobato-Calleros et al., 2007; Lobato-Calleros et al., 2006; Lobato-Calleros et al., 2008) or to encapsulate nutrients into cheese(Giroux et al., 2013) .Put simply, a double emulsion is an emulsion in which the emulsion droplets contain an inner emulsion of the opposite phase (Leong, Zhou, et al., 2017). For incorporation of whey proteins into cheeses, it is proposed here that a water-in-oil-in water emulsion (W/O/W) can be prepared, where the inner aqueous phase contains a high concentration of whey proteins. Such as a double emulsion can be formulated as the cheese milk, enabling the whey protein to be entrapped in the fat globules of the cheese. The double emulsion can be produced by first preparing a stable water-in-oil emulsion (W/O) with a high

proportion of aqueous phase containing whey protein. This single emulsion can be then emulsified into skim milk to form a water-in-oil-in-water (w/o/w) double emulsion. If the double emulsion droplets have the same apparent volume fraction and size distribution of native milk fat globules (which solely comprise of fat), they could be used to make reduced-fat, high-protein cheeses that mimic the fat microstructure and cheese texture of full-fat cheese (Leong et al., 2020). In addition to reducing the fat content and increasing the protein content, the use of double emulsions allows the substitution of anhydrous milk fat (rich in saturated fatty acids (Chouinard et al., 1998; DiNicolantonio & O'Keefe, 2017)) with less expensive and potentially healthier fat alternatives such as sunflower, canola or olive oil, which are rich in poly-unsaturated fatty acids (including ω-3 and ω-6) (Chouinard et al., 1998; Ramos et al., 2009), further improving the nutritional value of cheese analogues.

Emulsions are inherently thermodynamically unstable, and forming stable double emulsions is challenging. The formation of producing a stable inner W/O emulsion is particularly challenging (Leong, Zhou, et al., 2017), typically relying on the addition of large amounts of surfactants to confer kinetic stability (Matsumoto et al., 1976). Emulsification can be achieved using high-shear devices such as high-pressure homogenisers (Stang et al., 2001), which can be applied to the formation of double emulsions (Leong et al., 2018). High-power low-frequency (~20 kHz) ultrasound can also be used for emulsification (Thompson & Doraiswamy, 1999), and being highly controllable, has been effectively applied to both the primary and secondary emulsification stages of double emulsion production (Leong, Zhou, et al., 2017; Leong et al., 2018).

Ultrasonic emulsification is governed by two mechanisms. First, the ultrasound field produces propagating waves on an oil-water interface, destabilising it, so that elements from one phase can break off to form new droplets in the continuous phase. This process leads to the formation of medium to large droplets. Secondly, the shear and turbulence resulting from cavitation (implosion of bubbles via sound wave driven oscillation) continues to break the existing droplets to submicron sizes (Leong, Juliano, et al., 2017; Li & Fogler, 1978). Power ultrasound has been successfully used to produce stable micron and submicron sized fat/oil droplets using milk proteins as the emulsifier (Kaltsa et al., 2013; Leong, Zhou, et al., 2017; Mahdi Jafari et al., 2006; Shanmugam & Ashokkumar, 2014). The ability of the strong physical effects of cavitation induce structural changes in milk proteins that enhance their emulsifying properties can reduce the need for non-dairy emulsifiers in dairy based applications (Shanmugam & Ashokkumar, 2014).

To date, dairy-based double emulsions have only been produced with low protein concentrations in the inner aqueous phase. The ability to encapsulate significant quantities of whey protein inside the internal aqueous phase of double emulsions has yet to be investigation. Leong and co-workers formed dairy-based W/O/W double emulsions using skim milk (with a total protein content of ~4%) as the inner aqueous phase, using food-grade surfactants to stabilise the droplets. This single emulsion was then emulsified into the secondary aqueous phase using skim milk proteins to stabilise the outer secondary emulsion (Leong, Zhou, et al., 2017). While this formulation resulted in stable double emulsions, it did not represent a significant encapsulation of whey proteins.

The aim of the study present in this chapter is develop the fundamental understanding needed to produce whey-protein enriched cheese via double emulsion encapsulation, by investigating the effect of ultrasonication and formulation parameters on droplet properties, encapsulation efficiency and stability. The first objective is to produce stable water-in-oil single emulsions using high loading fractions of concentrated whey protein solutions, sunflower oil and minimal amounts of the food-grade emulsifiers lecithin and polyglycerol polyricinoleate (PGPR). These protein-enriched single emulsions can be then loaded into an aqueous phase at a high ratio to form double emulsion enriched in whey protein. The second objective is to incorporate these double emulsions into cheese milk and to study their effect on protein coagulation and the protein retention in model cheddar curd matrices. By developing an understanding of how to successfully formulate and incorporate whey protein enriched double emulsions into cheese matrices, this study will help develop a technology to reintegrate whey streams into cheese production to produce nutritionally enhanced products.

5.2. Materials and Methods

5.2.1. Materials

Whey protein concentrate (WPC) powder (‘Select whey protein concentrate’ purchased from Professional Whey, Erina NSW, Australia) was used as the whey protein source for encapsulation into the inner emulsions and for stabilising the oil-in-water single and double emulsions. The protein content of the WPC powder was measured to be 73% w/w (1.2% w/w casein and 71.8% w/w whey protein) according to section 4.2.2. The level of denaturation in powders was ~5% of total whey proteins. Food-grade surfactants, lecithin and polyglycerol polyricinoleate (PGPR) were sourced from a confectionary company in Australia and used as the emulsifying agents in the primary water-in-oil emulsions. Sunflower oil (O) (Woolworths Home brand, Australia) was used as the oil phase in all experiments. Pasteurised and

homogenised skim milk (Paul's brand, Australia) with a fat content of <0.1% was used as the outer aqueous phase of the double emulsions. Sodium azide (Chem Supply, Australia) was added at ~0.03 wt % to the milk to limit microbial growth during storage. Rennet solutions for gelation trials were prepared by diluting 200 IMCU/mL Chymax Plus FPC rennet solution (batch number 3241446, Cheeselinks, Australia) in distilled and deionised water. A starter culture containing a mix of *Lactococcus lactis* subsp. lactis and *Lactococcus lactis* subsp. cremoris (Cheeselinks, Australia) was used for curd trials. Nile red (Sigma Aldrich) and Fast green (Sigma Aldrich) dyes were used in fluorescence and confocal microscopy analysis.

5.2.2 Formation of primary water-in-oil emulsions

During initial trials, 1% w/w lecithin and 4% w/w polyglycerol polyricinoleate (PGPR) were added to sunflower oil (O) and stirred at 40 °C until fully dissolved. The aqueous phase (W1) of the single emulsions was prepared by dissolving varying amounts of WPC powder to achieve final powder concentrations of 20%, 30%, or 40% w/w in distilled and deionised water. The viscosities of these systems were measured to be 6.8, 29.6 and 109.1 mPa.s in the increasing order of solid loading. These protein solutions also contained 2% w/w NaCl (Sigma Aldrich, Australia) as an entrapment marker (Leong, Zhou, et al., 2017). The solutions' densities were measured to be 1.05, 1.08 and 1.14 mg/mL in the increasing order of solid loading.

To produce water-in-oil primary emulsions (W1/O), protein solutions and oil were loaded into a sonication cell cooled via water circulation (at room temperature) at W1:O volume ratios of 3:7 or 4:6 and a total liquid volume of 25 mL. Emulsification was carried out for 3 minutes by placing the horn tip of a 20 kHz, 11 mm ultrasonic horn at the oil and water interface. The applied calorimetric power power intensity and energy density were 33.8 W, 1.35 W/mL and 243 J/mL respectively. The bulk temperature of the sample was maintained below 50 ºC in all emulsification trials.

Experiments were performed aimed at reducing the use of non-dairy emulsifiers, in which the PGPR concentration in the oil phase was varied (4% w/w, 3% w/w and 2% w/w) while maintaining a lecithin concentration of 1% w/w.

5.2.3 Double emulsion formation

The outer aqueous phase (W2) of the double emulsion was prepared by dissolving 5% w/w WPC powder in distilled and deionised water. Pre-formed W1/O emulsions were emulsified into the outer aqueous phase at ratios of 2:8, 3:7, and 4:6 to produce whey-protein enriched double emulsions. Emulsification was performed in the same sonication cell as 5.2.1 with room

temperature cooling water applied and total sample volumes of 25 mL. The samples were emulsified for 90 s at various calorimetric power levels (6.8 W 20.3 W and 33.8 W) by placing the 20 kHz 11 mm ultrasonic horn at the emulsion-water interface. The power intensities and energy densities corresponding to the different applied powers were 0.27 w/mL, 0.81 W/mL and 1.35 W/mL, and 24.5 J/mL, 73.1 J/mL and 121.7 J/mL, respectively.

5.2.4 Rennet gelation and kinetics

20 mL double emulsion cheese (DE cheese) milk and control cheese milks were formulated by mixing a double emulsion or a single emulsion with skim milk to reach a final fat and protein contents of 2.8% w/w and 4.3% w/w. The double emulsion had a W1:O ratio of 3:7 and a SE: W2 ratio of 4:6. Internal aqueous phase with 40% w/w WPC and 2% w/w sodium chloride and oil phase with 2% w/w sodium chloride, 1% lecithin and 3% PGPR were loaded to make 25 mL and sonicated at 33.8 W for 3 minutes . A 5% w/w WPC solution was used as the secondary aqueous phase and a 20.3 W power was applied for 90s (power density = 0.81 W/mL) for secondary emulsification. A single emulsion for control cheese milk was formulated by directly emulsifying oil into a 5% WPC solution at a O:W loading of 4:6 using a 20.3 W ultrasonic power applied for 90s (power density = 0.81 W/mL). Gelation kinetics were measured according to section 3.2.4

5.2.5 Formation of cooked curds

Double emulsion cheese (DE cheese) milk and control cheese milks were formulated by mixing a double emulsion or a single emulsion with skim milk to reach a final fat and protein contents of 4.2 ± 0.1% w/w and 4.4 ± 0.1% w/w (2.8% w/w casein 1.6% w/w whey protein). Similar methods were used for double and single emulsion formation as reported in section 5.2.4. A cheese milk with whey protein emulsified fat was used as a control, in order to maintain similar interactions between the fat globule membrane and the casein matrix that affects curd formation and fat loss.

Cooked curds were prepared according to a previously reported method (Cipolat-Gotet et al., 2016) with some modifications. 10g of cheese milk was loaded into a 50 mL yellow capped bottles tube and warmed to 31 ºC. Mesophilic starter culture containing a mix of *Lactococcus lactis* subsp. lactis and *Lactococcus lactis* subsp. cremoris was added at a concentration of 1 x 10^{-5} % w/w and ripened for 45 min. Rennet was added to reach a final concentration of 1 x 10^{-4} % w/w and gelled for 2 hours. The curds were then cut into ~ 1 cm^3 cubes using a spatula. Water bath temperature was set to 70 ºC allowing the sample temperature to gradually increase

to 56 ºC within 30 min. The curds were strained through a 1 mm x 1mm sieve for 30 mins. Final curd and whey masses were measured and stored overnight for compositional analysis.

5.2.6 Droplet size measurements

The droplet size of the initial W1/O single emulsion was estimated by analysing the images taken with optical microscope, using ImageJ software. In the software, a bandpass filter was used to enhance the edges of the emulsion droplets. An area size threshold of <0.1 μm^2 was applied to avoid noise being counted by the software. The area of each droplet was measured by the software and, by assuming that the droplets were spherical, the diameter determined. A volume-based particle size distribution was then derived, again assuming spherical droplets. Emulsion droplet size data collected from images taken from at least duplicate samples from the same emulsion were pooled together for plotting the size distribution. Each imaged had >150 counted droplets.

The particle size of the W1/O/W2 double emulsion droplets was measured using the Malvern Mastersizer 2000 according to section 4.2.5. The refractive index and absorption used by the software to determine the size of the droplets are 1.462 and 0.001 respectively.

5.2.7 Conductivity measurements

Sodium chloride added into the inner aqueous phase of the double emulsion was used as the entrapment marker, which aids to indicate the amount of inner aqueous phase that are able to be encapsulated in the double emulsion. The release of the inner phase increases the concentration of the salt in the outer phase, resulting an increase in conductivity and decrease in encapsulation yield. A series of standard solutions representing 0%, 25%, 50%, 75% and 100% of salt release were prepared in order to quantitatively relate the changes in conductivity to the release of salt from the emulsions. Standards for each specific W1/O/W2 emulsions formulations were prepared by including the same concentration of each component. The standards were prepared by sonication using the 20 kHz horn at 33.8 W power for 90 seconds that corresponded to a delivered energy density of 121.7 J/mL. Sonication at these conditions was sufficient to ensured complete homogenization of the fat droplets in the standards. Conductivity in all systems was measured within ~ 15 min of formation, using a k = 1.0 laboratory conductivity sensor (TPS, Australia) connected to TPS LabCHEM-Cond conductivity meter (TPS, Australia). The conductivity probe was calibrated using a 2.76 mS standard solution.

The measured conductivities of the emulsions were correlated to the percentage of salt release using the standard curves and encapsulation yield was calculated as the proportion of total salt that remained inside the oil droplets

$$Encapsulation\ yield\ \% = 100\% - \%\ salt\ release$$

Then, the proportion of encapsulated WPC in the inner aqueous phase as a percentage of the total double emulsion was calculated as:

$$Proportion\ of\ encpasulated\ WPC\ \left(\frac{g}{L} of\ DE\right) = 1000\ \times W1{:}O\ loading\ \% \times SE{:}W2\ loading\ \% \times WPC\ concentration\ in \frac{g}{g} \times Density\ of\ W1\ phase\left(\frac{g}{mL}\right) \times Encapsulation\ yield\%$$

5.2.8 Optical and fluorescence microscopy

An emulsion sample of approximately 3 μL was transferred onto a glass slide and covered with a cover slip. An optical microscope (Olympus, Japan) fitted with a 60× oil immersion optical lens was used to visualise the emulsions.

For fluorescence microscopy, fluorescent dye, Nile red was added to the oil phase of the double emulsion at a concentration ~ 0.02% w/w. The excitation source was a mercury lamp laser passed through a filter (U-MWIB3, Olympus, Japan) such that the excitation wavelength was in the range 460–495 nm. At this excitation range, Nile Red emits in the yellow (>565 nm) range of the visible spectrum. The same optical microscope was used to visualize fluorescence emission.

5.2.9 Confocal laser scanning microscopy

Nile red (1 mg/mL in ethanol) and Fast green (2mg/mL in water) were added to oil and aqueous phases of the emulsions to reach final concentrations of ~10^{-3} mg/ mL and ~20^{-3} mg/ mL respectively. Emulsions were made with stained oil and aqueous phases as described in sections 5.2.2 and 5.2.3. Emulsions were diluted 10 times using simulated milk ultrafiltrate (Martin et al., 2007) before imaging. Cooked curds were also prepared with stained oil and aqueous phases as described in section 5.2.5.

A thin slice from the cooked curd or ~ 5 μL of diluted emulsions were transferred to a glass slide and carefully covered with a cover slip. A confocal microscope (Leica SP5) was used to imaging with the laser lines set at 488 and 633 nm for excitation. The fluorescence emission

was captured through a 63× lens using 2 photo multiplier tubes set at 500–620 nm (Nile red emission) and 660–710 nm (Fast green emission).

5.2.10 Protein quantification

Protein analysis of all protein streams was performed based on the Dumas combustion method using a LECO Trumac CNS analyser (LECO Corporation, Michigan, USA) according to sections 4.2.2 .

Following formula were used for retention or loss calculations.

$$Protein\ retention\ in\ curd = \frac{Protein\ content\ of\ curd\ in\ \%\frac{w}{w} \times Mass\ of\ curd}{Protein\ content\ of\ cheese\ milk\ in\ \%\frac{w}{w} \times Mass\ of\ cheese\ milk} \times 100$$

$$Protein\ loss\ in\ whey = \frac{Protein\ content\ of\ whey\ in\ \%\frac{w}{w} \times Mass\ of\ whey}{Protein\ content\ of\ cheese\ milk\ in\ \%\frac{w}{w} \times Mass\ of\ cheese\ milk} \times 100$$

5.2.11 Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using Bio-Rad (Bio-Rad Technologies, Gladesville, Australia) pre-cast 4-20% Criterion TGX 18 well gel cassettes or Bio-Rad 16.5% Criterion tris-tricine gel cassettes in a Bio-Rad Criterion cell. For sample preparation, 22 mg of cooked curd or 143 µL of whey were mixed with respective volumes of 978 µL and 143 µL of distilled and deionised water. 10 µL of 0.4 M ethylenediaminetetraacetic acid ware added to the sample and mixed in an orbital shaker for 1 hr. SDS-PAGE of these samples were preformed using the protocol in section 4.2.10 using either a Bio-Rad 10x Tris/Glycine/SDS running buffer or a Bio-Rad Tris/Tricine/SDS running buffer that is compatible with the gel used

5.2.12 RP-HPLC

The relative abundance of β-lactoglobulin and α-lactalbumin in cooked cured were measure by a RP-HPLC (Agilent technologies, binary pump 1260, vial sampler 1260, degasser 1260 HiP, diode array detector 1290) with eluents A (Water: Acetonitrile: Tri-fluoro acetic acid 950:50:0.7) and B (Acetonitrile: Tri fluoro acetic acid 1000:0.5). The gradient was started with 0% of B which was maintained for the initial 5 minutes. Then it was increased to 31% over the next 9.8 min, to 40% over the next 17.1 min, to 100% over the next 6.7, kept at 100% for 3.7 min and returned to the starting condition within 2.7 min (Creusot & Gruppen, 2007). A C18 column (Jupiter 5 µm, 300 A^0, 250 x 4.6 mm) was used with a flow rate of 0.8 mL (at 40

°C) and injection volume 50 µL. 110 mg of curd or 714 µL of whey was mixed with 889 µL or 286 µL of distilled and deionized water. 10 µL of 0.4M Ethylenediaminetetraacetic acid (EDTA) was added to the mixture, shaken for 1 hr and centrifuged at 2717 g in a Thermofisher Hereus Megafuge 8 benchtop centrifuge with HIGHConic III fixed angle rotor for 20 minutes. The supernatants were further diluted (5 times in cooked curd samples and 10 times in whey samples) with eluent A and filtered by a 0.45 µm syringe filters before injection.

5.3 Results and Discussion

5.3.1 Formulation of whey-protein enriched water-in-oil primary emulsions

The size of the primary W1/O emulsion droplets in relation to that of the secondary W1/O/W2 double emulsion droplets is key to both encapsulation efficiency and double emulsion stability. The W1/O single emulsion (SE) droplets should be small in to avoid creaming or flocculation and to allow multiple inner emulsion droplets to be encapsulated within oil droplets of similar size to those in native (i.e. approximately 5-10 µm in diameter). As such, submicron-sized water droplets containing whey protein are targeted for the double emulsion formulation. To maximise the amount of whey protein encapsulated inside the double emulsion droplets, W1/O emulsions were formed i) using high aqueous phase loading rates of 30% or 40% v/v (i.e. aqueous phase-to-oil ratios of 3:7 or 4:6), and ii) with high concentrations of WPC in the inner aqueous phase (20%, 30% and 40% w/w) (preliminary trials showed that 40% w/w was the maximum possible WPC concentration that could be completely solubilised). The size of W1/O emulsion droplets that could be produced as function of both loading rate and whey protein concentration was investigated by optical microscopy and image analysis using ImageJ software (Figure 5.1).

At a W1:O ratio of 3:7, the emulsion with 20% WPC in the aqueous phase had the majority of the droplets in the desired submicron range (between about 0.3-1 µm) (Figure 5.1). At higher WPC concentrations (30% and 40%), the size distribution shifted slightly, with some droplets in the micron range (1-3 µm), likely too large for double emulsion encapsulation. The volumetric proportion of droplets >1 µm was 4.5%, 24.3% and 34.0% in the emulsions with 20%, 30% and 40% WPC, respectively.

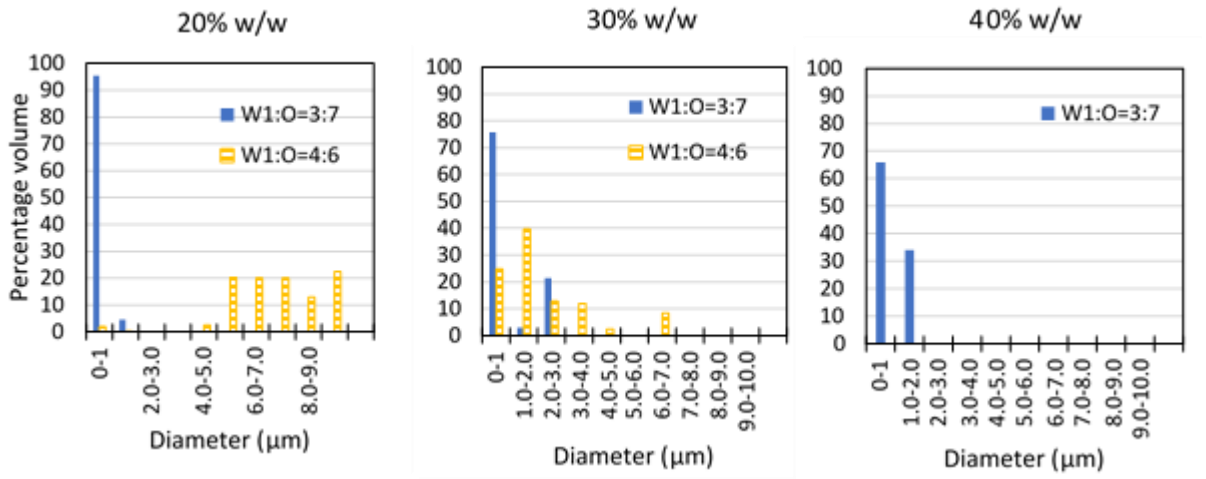


Figure 5.1: Droplet size distributions of the W1/O emulsions formed using 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions and sunflower oil at W1:O ratios of 3:7 and 4:6. Data collected from multiple images (at least 3) were pooled together to determine the size distributions. Note that there was significant variability in the size distribution of W1:O = 4:6 systems due to incomplete emulsification. Microscopic images representing each emulsion are provided in Figure 7.4 of appendices.

Numerous factors can affect the size of droplets resulting from ultrasonic emulsification, including the interfacial tension, the viscosities of the dispersed and bulk phases, the W:O ratio, and the intensity and duration of the applied sonication (Gaikwad & Pandit, 2008). As the type of oil, the W:O ratio, the emulsifier (lecithin and PGPR) concentration, and ultrasonic parameter were kept constant in this study, the increase in the droplet size is likely due to an increase in the dispersed phase viscosity at higher WPC concentrations (the viscosities of the 20%, 30% and 40% WPC solutions were measured to be 6.8, 29.6 and 109.1 mPa.s, respectively). Although the effect of aqueous phase viscosity on droplet size on ultrasonic emulsification has not been reported for water-in-oil emulsions, it has previously been shown for oil-in-water emulsions that an increase in the dispersed phase viscosity increases the droplet size (Gaikwad & Pandit, 2008). This was primarily attributed to an increase in cavitation threshold (resulting in lowering of cavitation activity) due to the increase in dispersed phase viscosity (Gaikwad & Pandit, 2008), which limits the extent of disintegration of the emulsion droplets.

When the W:O loading was increased to 4:6, proper emulsification could not be achieved at any of the WPC concentrations (Figure 5.1B). Although the aqueous phase could be seen to immediately mix into the oil phase upon ultrasonication of the 20% and 30% WPC solutions, it was visibly evident that the emulsification of the sample was irregular. The microscopic

images indicated that although sub-micron sized droplets were formed in some parts of the sample (Figure 7.4), the majority of the droplets were in the micron range (Figures 5.1, 7.4,). Further, there were gel-like regions that formed in the solutions. In the system with 40% WPC, the aqueous phase could not be mixed into the oil phase as a gel was rapidly formed in the region of the horn.

At higher fractions of aqueous phase, more cavitation may occur due to the presence of more dissolved air pockets for bubble nucleation, which in turn reduces the cavitation threshold pressure (Crum, 1982). This leads to less violent individual bubble collapse that may reduce the magnitude of the shear forces generated that may not be adequate to break the closely packed larger droplets (that occupy a higher volume fraction compared to a system with a lower W:O loading) that are formed as the aqueous phase is dispersed into the oil. Further, at higher water fractions, the overall lipophilic emulsifier concentration in the systems is lower; hence the ability to stabilise a large number of smaller droplets is limited. Additionally, as the bulk solution becomes less mobile due to droplet packing, the physical effects of cavitation may be restricted to areas experiencing higher rates of collision, leading to an increase in the localised temperature, denaturation and subsequent gelation of whey proteins.

5.3.2. Reducing the emulsifier concentration

At a W1:O ratio of 3:7 emulsions could be produced with the majority of the droplets in the desired submicron range using a PGPR and lecithin concentration in the oil phase of 4% w/w and 1% w/w, respectively. Attempts were then made to reduce the amount of emulsifier, to reduce the formulation cost. Here, the PGPR concentration was reduced from 4% w/w in the oil phase to 3% and 2% w/w while keeping the lecithin concentration constant at 1% w/w. With a PGPR concentration of 3%, the droplet size distributions (Figure 5.2) were quite comparable to those at 4% (Figure 5.1), with 3%, 25% and 44% of the dispersed phase volume occupied by droplets exceeding 1 μm in diameter, for the 20%, 30% and 40% WPC solutions, respectively. However, a further reduction in the PGPR concentration to 2% resulted in a dramatic change in the droplet size distributions, with micron-sized droplets occupying the majority of the volume (Figure 7.5). This suggested that the combination of 2% PGPR and 1% lecithin were not sufficiently to stabilise the large amounts of interfacial associated with sub-micron sized oil droplets created during ultrasonication. As a result of poor stabilisation smaller droplets coalescence into larger droplets. There was no apparent effect of the WPC concentration on the droplet size of systems containing 2% PGPR and 1% lecithin, presumably as hydrophilic whey protein molecules (Galus & Kadzińska, 2016) do not have a suitable

hydrophilic-lipophilic balance (HLB) to stabilise water-in-oil emulsions (Gülseren & Corredig, 2012). Attempts to create W/O emulsions without PGPR or lecithin failed (with a 20% w/w WPC solution and sunflower oil at a W1:O loading of 3:7), as the oil and aqueous phases separated soon after sonication. Emulsions formed with 3% PGPR and 1% lecithin were observed by microscopic images to be stable for at least after 3 days of storage in ambient conditions (data not shown). Therefore, these emulsifier concentrations were used to stabilise the internal aqueous phase during double emulsion formation.

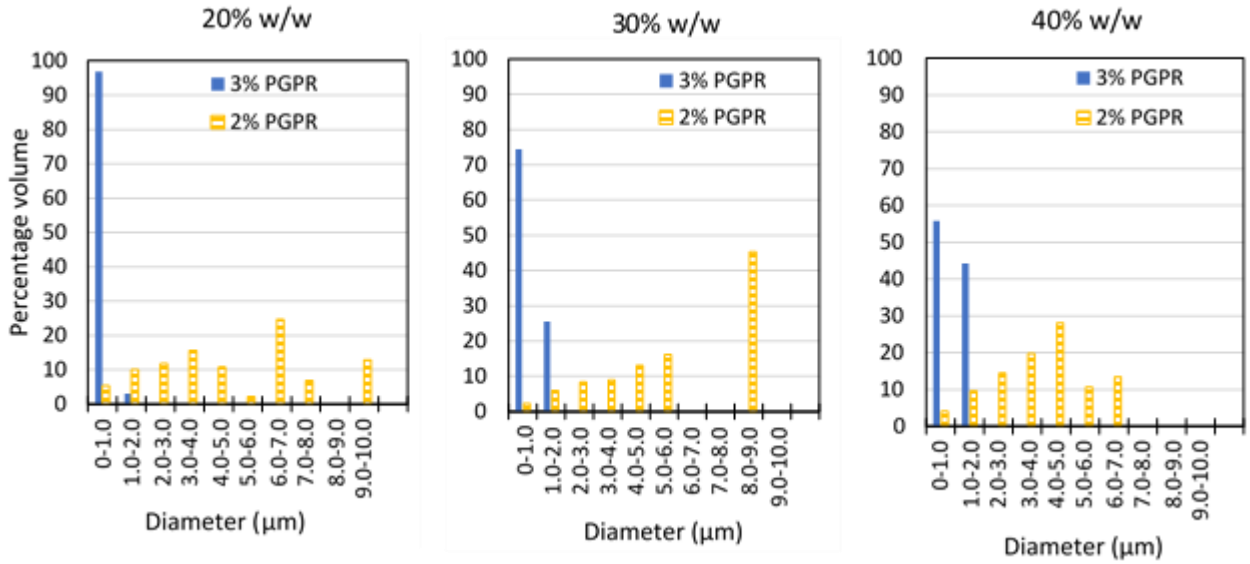


Figure 5.2: Droplet size distributions of W1/O emulsions formed using 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions and sunflower oil at W1:O ratios of 3:7, using 3% w/w and 2% w/w PGPR in the oil phase while maintaining a lecithin concentration of 1%. Data collected from multiple images were pooled together to determine the size distributions. Microscopic images representing each emulsion are provided in Figure 7.5 of appendices.

5.3.3. Double emulsion formation

To ensure successful whey protein encapsulation during double emulsion formation, multiple W1/O droplets should be encapsulated within a secondary oil droplet. The secondary (or outer) oil-in-water emulsions can be stabilised by milk proteins (Leong, Zhou, et al., 2017), rather than requiring low HLB surfactants such as lecithin and PGPR. For the second emulsification step, whey protein was used as the emulsifier, as it has proven successful in forming stable oil-in-water emulsions via high pressure homogenisation (Desrumaux & Marcand, 2002) ultrasonic emulsification in the presence of other stabilisers (Kaltsa et al., 2013). Although the freely available hydrophobic residues are less abundant in globular whey proteins compared to

casein proteins in milk, they readily adsorb to an oil-water interface and form stable emulsions (Leman et al., 1989). Further, whey proteins have unique nutritional properties (see section 2.6) that make them an attractive emulsifier for food applications. As the minimum required lipophilic emulsifier concentration to produce the submicron size W1/O droplets was used, the amount of free emulsifier in the oil phase is low, meaning the secondary oil droplets are likely to be emulsified predominantly by the whey protein in the secondary, outer aqueous phase.

In addition to the emulsion formulation and the size of the internal droplets, the ultrasonication parameters are critical to the secondary emulsification stage of double emulsion production. The intensity and duration of ultrasonication must be balanced to ensure oil droplets of the desired size are produced, without breaking and releasing the water-in-oil emulsion. To investigate the effect of ultrasonic parameters on the encapsulation efficiency, double emulsions were formed with W1/O:W2 loadings of 2:8, 3:7 and 4:6 using the same ultrasound horn for 90 s at varied applied power intensities of 0.27 w/mL, 0.81 W/mL and 1.35 W/mL, corresponding to specific energy values of 24.5 J/mL, 73.1 J/mL and 121.7 J/mL, respectively.

The mean droplet size measurements showed that W1/O:W2 loading did not have a major effect on the oil droplet size of the double emulsions (Figure 5.3A). Higher W1/O:W1 ratios have previously been reported to result in larger secondary double emulsion droplets at lower energy densities (~ 1.3 J/mL) (Leong, Zhou, et al., 2017) attributed to the applied ultrasound energy being diluted in relation to volume of material to be emulsified (Leong, Zhou, et al., 2017). However, at the much higher specific energy densities used in this study (>24.5 J/mL) the size of the droplets was not affected, with the applied energy being sufficient to cause similar size reductions at all loadings tested.

In contrast, the power intensity had a considerable effect on the oil droplet size of the double emulsions, with the increased shear forces leading to better disintegration of emulsion droplets into smaller sizes. A power intensity of 0.27 W/mL applied for 90 s was insufficient for this formulation, producing double emulsions with a mean oil droplet size >300 μm. Sonication at 1.35 W/mL produced double emulsion oil droplets with a mean size close to that of the native milk fat globules, however the size distribution was bimodal, with peaks around 0.1-1 μm and 1-10 μm. The presence of submicron size droplets in the double emulsion may affect the textural properties of cheese if they were to be used as a milk fat replacer. Small fat droplets are believed to act as inert weak points in the otherwise strong protein matrix, reducing curd firmness (Michalski et al., 2003). In comparison, at the intermediate power intensity of 0.81 W/mL, the majority of the oil droplets in the double emulsion were ~10 μm, with a few larger

droplets inflating the volume mean droplet size. Increasing the sonication time from 90 s to 120 s at 0.81 W/mL produced more uniformly sized droplets of ~10 μm in diameter, but a further increase in time to 150 s resulted in the formation of submicron sized droplets (data not shown).

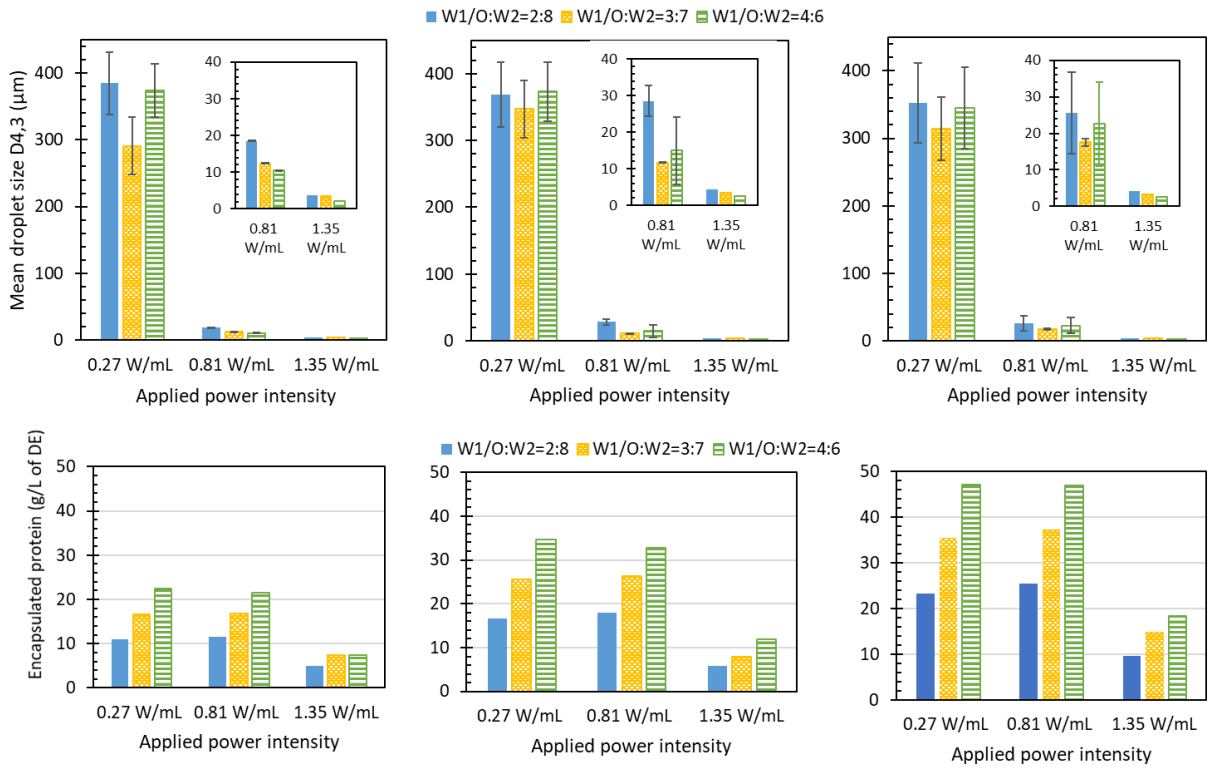


Figure 5.3: (A) Volume mean droplet size and (B) the amount of whey protein encapsulated in the inner aqueous phase of the double emulsions (in g of encapsulated protein per L of double emulsion) formed by loading the W1/O single emulsion made with 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions into the secondary aqueous phase (containing 5% w/w WPC) at W1/O:W2 ratios of 2:8, 3:7 and 4:6.

Optical microscopy images of double emulsions formed at 0.81 W/mL confirmed the presence of inner droplets encapsulated within the double emulsion oil droplets (Figure 5.4A). When the oil soluble fluorescent dye, Nile red, was dissolved in the sunflower oil, the inner droplet did not emit fluorescence, confirming that the double emulsion droplet contained an internal aqueous phase (Figure 5.4B). Contrary to previous observations of irregularly shaped internal aqueous droplets of skim milk (Leong, Zhou, et al., 2017), the internal aqueous droplets were spherical in this study. This could be due to the different emulsifiers used, with Span 80 employed in the study by Leong and co-workers, a surfactant which has been reported to interact with milk proteins such as BSA to form gel like complexes (Gaiti et al., 1994). The irregular shape of the internal aqueous phase of skim milk reported by Leong and co-workers

was believed to be a result of interactions between Span 80 molecules at the oil-water interface and whey proteins in the aqueous phase (Leong, Zhou, et al., 2017). They later reported that using PGPR (1-5% w/w in the oil phase) as a lipophilic emulsifier could mitigate such aggregation of the internal skim milk droplets (Leong et al., 2018), consistent with the use of PGPR in the current study and the observed spherical shape of the inner aqueous droplets.

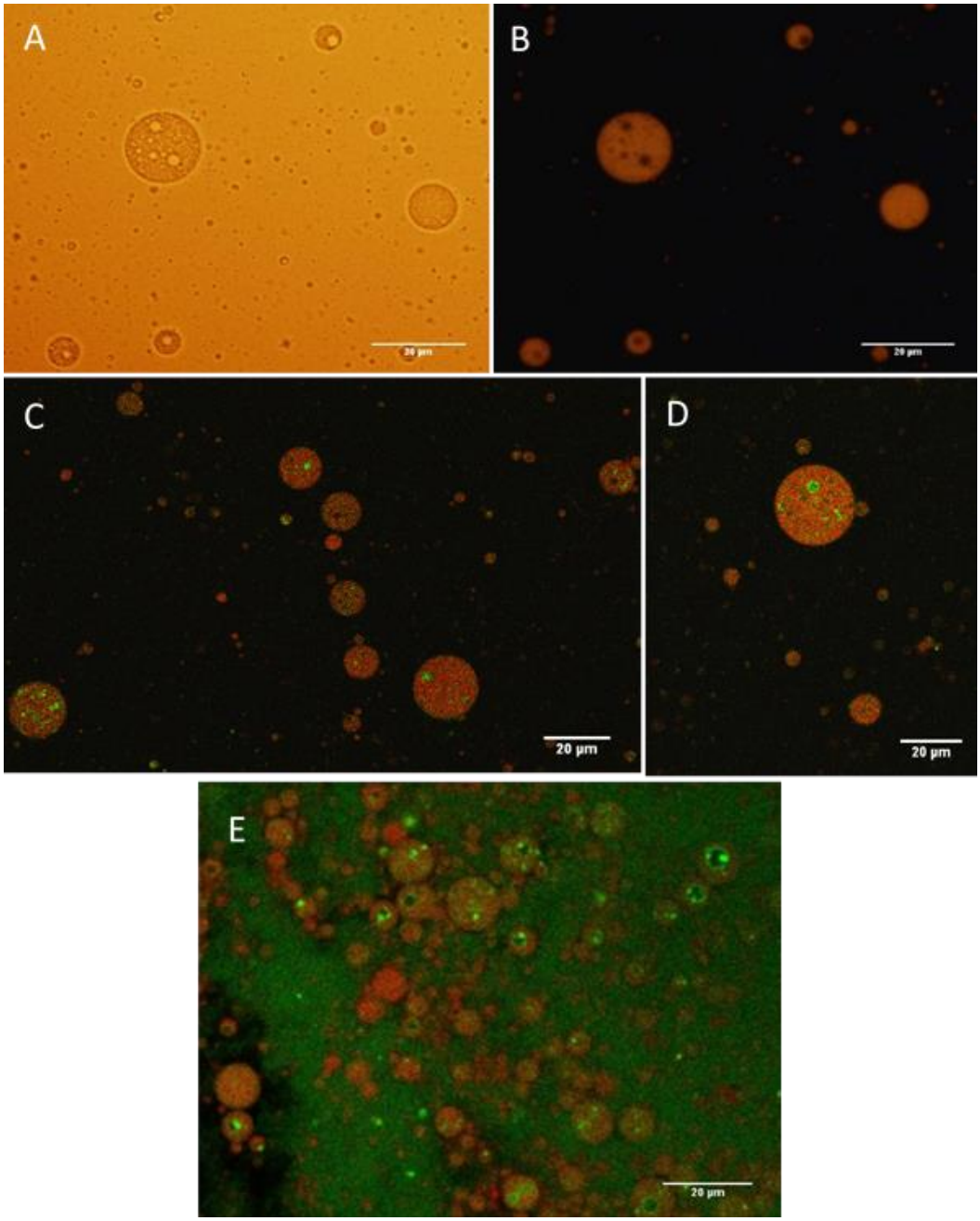


Figure 5.4 – Optical (A), fluorescent (B) and confocal laser scanning (C, D) microscopy images of W1/O/W2 double emulsions prepared with 40% w/w WPC solution, sunflower oil and 5% w/w WPC solutions as W1, O and W2 phases, respectively. E is a confocal laser scanning microscopy image of a skim milk rennet gel filled with a W1/O/W2 emulsion. The orange areas in (B) represent the oil phase containing fluorescent Nile red, while the dark areas represent the aqueous phase. In the confocal laser

scanning microscopy images proteins and fat are stained in green (Fast green) and red (Nile red), respectively. The scale bars are 20 μm.

When proteins in the internal aqueous phases were stained with Fast green, the confocal microscopy images revealed that the internal aqueous phase was fluorescent, confirming that proteins were indeed encapsulated within the double emulsion droplets (Figure 5.4 C, D). However, surface active whey proteins were concentrated near the oil-water interface and aggregates were also present (Figure 5.4 C, D, E). Ultrasonic treatment of WPC solutions was previously reported to cause protein denaturation and subsequent aggregation (Chandrapala et al., 2011), and this may be the reason for the observed aggregates in the internal aqueous phase.

Understanding how to maximise the amount of whey protein encapsulated in the double emulsions is a primary aim of this study. To quantify the amount of WPC solution encapsulated within the double emulsion droplet, the extent of sodium chloride released from the inner aqueous phase was determined by measuring the conductivity of the double emulsion. Based on this, the amount of whey protein encapsulated is the double emulsion was calculated and compared in relation to the ultrasonic emulsification parameters used. The results revealed reduction in the whey protein encapsulation yields at higher ultrasonic power intensities (Figure 5.3B). This reduction in yield can be correlated to the reduced size of the oil droplets obtained from emulsification at higher power intensities, indicating that excessive power led to the escape of the internal water droplets from the W1/O emulsion during secondary emulsification. While the amount of whey protein encapsulated varied significantly between different formulations, from 5 to almost 60 $g_{encapsulated}$ protein/mL of double emulsion, even the lowest value obtained here (4.7 g/L of double emulsion) is significantly higher than the maximum previously reported protein encapsulation rate (calculated to be ~0.2% g/L of double emulsion) achieved by Leong and co-workers (Leong, Zhou, et al., 2017). Given the formulation tested here had an aqueous outer phase containing 50 g/L protein, the highest encapsulation rate (of almost 60 g/L for the W1/O:W2 = 4:6, 40% WPC) represents more than a doubling in the total protein content due to the protein encapsulated within the double emulsion.

It should be noted that quantification of encapsulation using conductivity measurements may be subject to the relative diffusion rates of whey protein, water and sodium chloride through the oil and surfactant boundaries. As the concentrations of NaCl and whey protein are higher in the inner aqueous droplet than the external aqueous phase, there is a driving force for diffusion out of the encapsulated droplets. In general, water molecules will diffuse across

semipermeable membranes (oil and surfactant boundaries in this case) faster than Na^+ or Cl^- ions that diffuse together to maintain charge neutrality (Hancock & Cath, 2009). Although the relative rate of whey protein diffusion across oil and surfactant boundaries has not been previously reported, it is likely to diffuse much more slowly than water or NaCl due to its much higher molecular weight. Although the conductivity measurements were applied reasonably soon after emulsification (~15-30 minutes), this may have been sufficient time for preferential diffusion to lead to an underestimation of the whey protein encapsulation.

In addition, the osmotic pressure in the internal droplet is higher than that of the external aqueous phase due to the higher concentrations of whey protein and NaCl, which may lead water to diffuse into the internal aqueous phase with time. Extensive transfer of water from the external aqueous phase to the internal droplet could lead to swelling of the internal droplets, which can no longer be retained in the oil droplets resulting in the collapse of the secondary droplet over time (Wen & Papadopoulos, 2001). This would represent a loss in encapsulation over time, however it would be largely an artefact resulting from the use of NaCl as a marker, which would not be used in real formulations. Regardless, as conductivity was measured at similar times after emulsion preparation in all the systems studied, it is possible to attribute the reduction in whey protein encapsulation with sonication power in systems with similar WPC concentrations and W1/O:W2 loading to shear-induced disintegration of the internal aqueous phase.

5.3.4. Formation of whey-protein enriched cheese curds containing double emulsions

This study was aimed at enabling whey protein enrichment of cheese via double emulsion encapsulation. To investigate whether whey protein encapsulated double emulsions could be successfully incorporated into cheese making, cooked curds were produced from cheese milk containing a whey protein-stabilised and whey-protein encapsulated double emulsion in place of the milk fat. A control curd was also prepared which contained a single emulsion of sunflower oil stabilised with whey protein. While both cheese milks had similar fat and protein contents, ~ 42% w/w of the whey protein in the double emulsion cheese milk was encapsulated inside the oil droplets.

The effect of including a double emulsion in the cheese milk on rennet gelation was examined using oscillatory rheometry. The results did not reveal any a significant difference in either the rate of rennet gelation or the gel strengths between the double emulsion and single emulsion formulations (Figure 5.5).

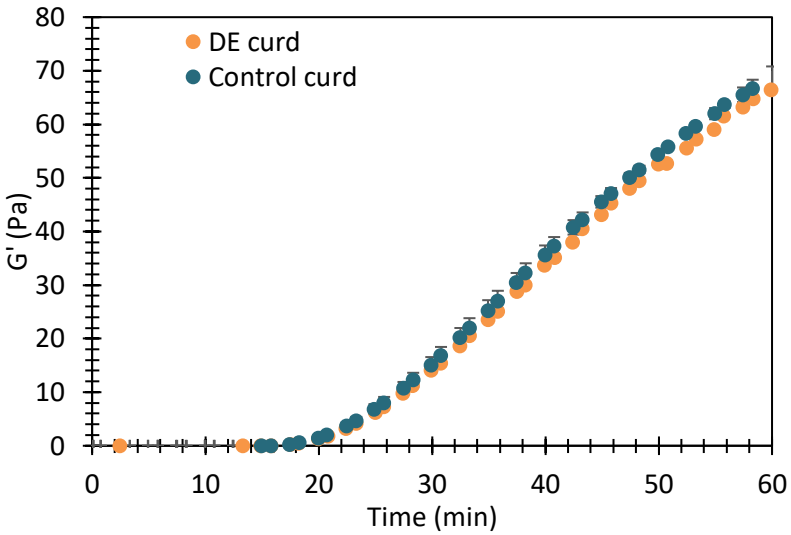


Figure 5.5: Comparison of the rate of increase in the storage modulus with time between rennetted double emulsion cheese milk ( ) and a control single emulsion cheese milk ( ).

The retention of the encapsulated whey protein through processing to a cooked curd was evaluated. It was also compared to the whey protein retained in the control single emulsion, in which whey protein was in the external aqueous phase. Compositional analysis of the cooked curds showed that the double emulsion curds had 14% and 15.5% increases in yield and the protein content, respectively, compared to the control (Table 5.1). The protein retention in the control cheese was 68.1 ± 3.5 % w/w, similar to the casein content in cheese milk, reflecting that the majority of free whey protein was drained out during syneresis. Note that the casein content in cheese milk was lower than the casein content in skim milk (~80%) as whey protein was added to achieve a casein:whey protein ratio of ~2:1. SDS-PAGE of the whey (Figure 5.6) confirmed there was an insignificant amount of casein lost in in whey. Protein retention was much higher in the double emulsion curds, at 85.8 ± 4.3 % w/w. This was higher than the amount of casein in the cheese milk, that whey protein encapsulated within oil the oil was retained, leading to an increase in the whey protein retention. The protein concentration in the double emulsion whey was in fact lower (1.23 ± 0.09% w/w) than in the whey of the control (1.74 ± 0.08% w/w), with a corresponding equal reduction in the intensity of all SDS-PAGE protein bands (Figure 5.6). However, a significant increase in the individual whey protein components in the curds could not be detected by the densitometry of SDS-PAGE gel images (data not shown), possibly due to the comparatively low increase in the intensity of the whey protein contents relative to the high concentration of caseins. Although there was a some variability between the analyses, RP-HPLC analysis of the curds provided direct evidence of an increase in the β-LG and α-lactalbumin contents (Figure 5.7).

Table 5.1: Compositional analysis of double emulsion and control curds.

Curd system	DE curd	Control curd
Mass of cheese milk (g)	10.06 ± 0.01[a]	10.03 ± 0.01[a]
Protein content in cheese milk (% of wet mass)	4.37 ± 0.00[a]	4.34 ± 0.00[b]
Casein content in cheese milk (% of total protein)	64.46 ± 0.01[a]	65.15 ± 0.09[b]
Whey protein content in cheese milk (% of total protein)	35.54 ± 0.01[a]	34.85 ± 0.09[b]
Mass of cooked curd (g)	3.30 ± 0.04[a]	2.88 ± 0.09[b]
Moisture content in cooked curd (% of wet mass)	71.48 ± 0.30[a]	70.14 ± 0.03[b]
Protein content in cooked curd (% of dry matter)	40.0 ± 1.9[a]	34.3 ± 2.6[b]
Mass of drained whey (g)	6.04 ± 0.04[a]	6.39 ± 0.23[a]
Total solids in drained whey (% of wet mass)	7.57 ± 0.25[a]	7.94 ± 0.31[a]
Protein content in drained whey (% of wet mass)	1.23 ± 0.09[a]	1.74 ± 0.08[b]
Curd yield (% of cheese milk)	32.8 ± 0.3[a]	28.7 ± 0.9[b]
Protein retention in curd (% of protein in cheese milk)	85.8 ± 4.3[a]	68.1 ± 3.56[b]
Protein loss in whey (% of protein in cheese milk)	16.9 ± 1.5[a]	25.5 ± 0.6[b]

*Standard deviation of replicate samples measured from duplicate systems are indicated next to the mean. **Significantly different values ($p < 0.05$) across a row are denoted using superscript letters.

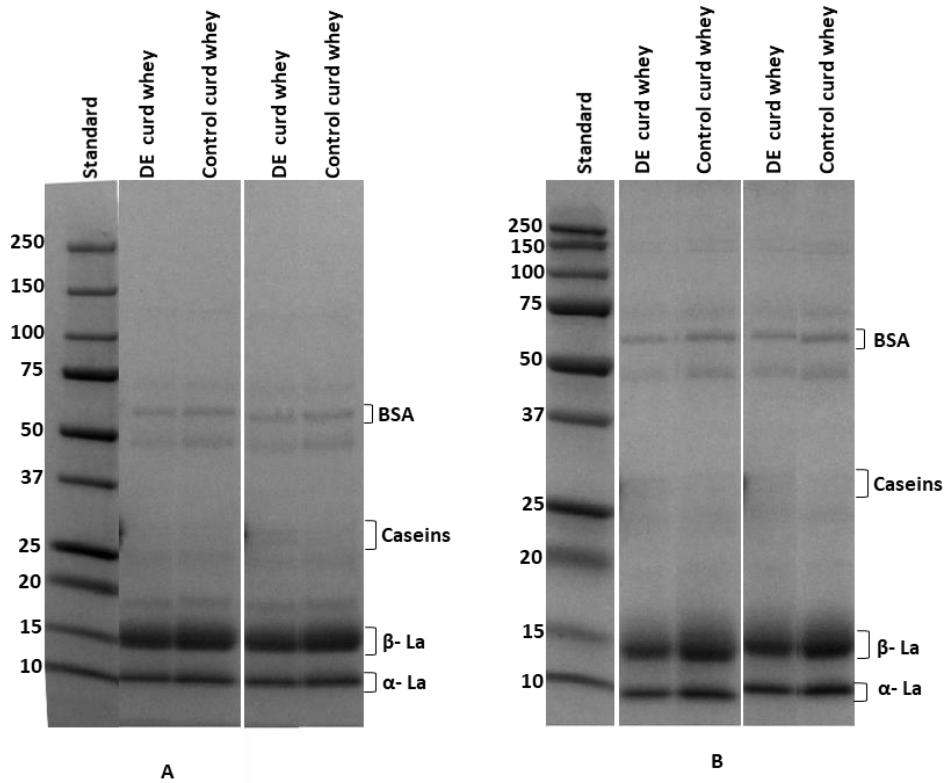


Figure 5.6: SDS-PAGE profiles of whey drained from double emulsion and single emulsion control curds. A and B are lanes from a Biorad 4–20% Criterion TGX precast protein gel and a Biorad 16.5% Criterion tris-tricine gel respectively. Please note that lanes extracted from each gel image are rearranged in the figures to facilitate visual comparison.

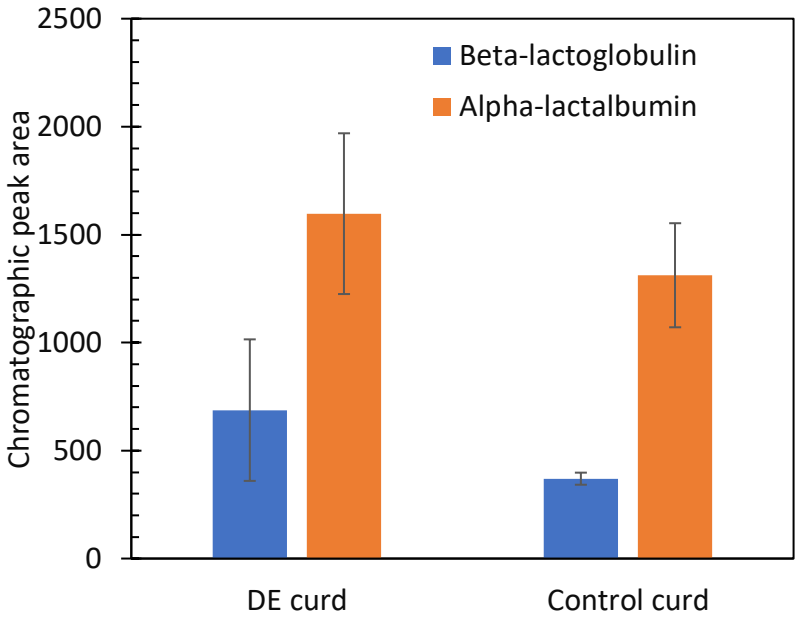


Figure 5.7: HPLC peak areas of β-lactoglobulin and α-lactalbumin in double emulsion and control single emulsion curd samples.

CLSM images (Figure 5.8) also confirmed the presence of protein encapsulated within oil droplets in the cooked curds. Oil droplets were segregated into pockets in the protein matrix, suggesting that using non-interactive whey proteins as an emulsifier instead of casein-rich skim milk could reduce previously reported active interactions between the casein network and the oil droplets (Leong et al., 2020), increasing the mobility of fat droplets within the casein matrix.

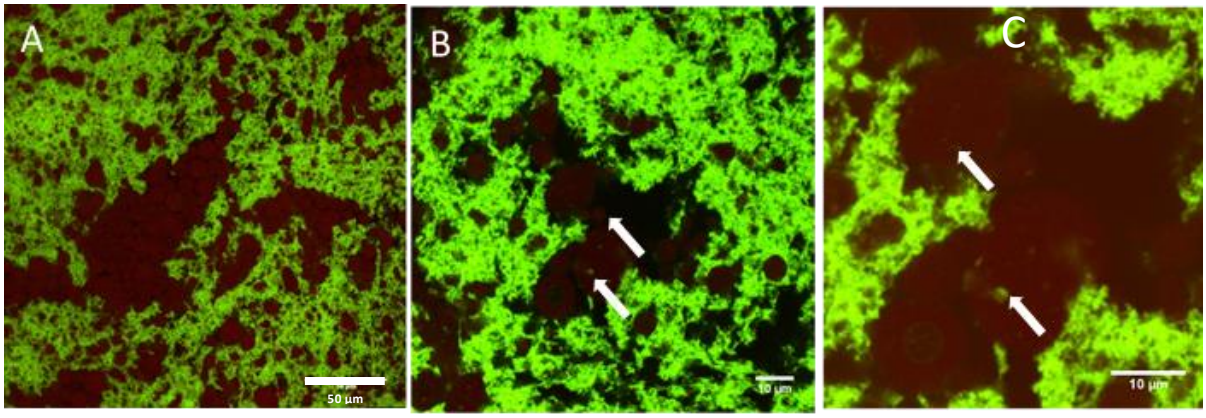


Figure 5.8: Confocal laser scanning microscopy images of cooked curds prepared using whey protein-enriched double emulsions at lower (A) and higher (B,C) magnifications. Areas of green florescence inside the red oil droplets (indicated by arrows) represent encapsulated whey protein. Note that higher laser gains were applied in order to visualise encapsulated proteins inside the oil droplets (B,C), which was low in concentration compared to the protein-rich casein matrix.

5.4 Conclusions

In this chapter, ultrasound was successfully applied in the formation of whey protein-enriched water-in-oil-in-water double emulsions using minimal amounts of food-grade emulsifiers. These emulsions had a markedly higher rate of protein encapsulation than previously reported studies. The results also demonstrate the possibility of manipulating emulsion formulation and ultrasonic emulsification parameters to form double emulsions of varying size distributions and protein contents. Whey protein-rich double emulsions were successfully incorporated into cooked curds formed by rennet gelation, without impairing gelation kinetics to increase the retention of whey proteins which are otherwise lost during syneresis. The understanding developed in this chapter provides a novel means of utilising whey protein and increasing the nutritional content of dairy products including cheese.

5.5 References

Barłowska, J., Szwajkowska, M., Litwińczuk, Z., & Król, J. (2011). Nutritional value and technological suitability of milk from various animal species used for dairy production. *Comprehensive reviews in food science and food safety, 10*(6), 291-302.

Brown, R. J., & Ernstrom, C. A. (1982). Incorporation of ultrafiltration concentrated whey solids into Cheddar cheese for increased yield. *Journal of dairy science, 65*(12), 2391-2395.

Chandrapala, J., Zisu, B., Palmer, M., Kentish, S., & Ashokkumar, M. (2011). Effects of ultrasound on the thermal and structural characteristics of proteins in reconstituted whey protein concentrate. *Ultrasonics sonochemistry, 18*(5), 951-957.

Chouinard, P., Girard, V., & Brisson, G. (1998). Fatty acid profile and physical properties of milk fat from cows fed calcium salts of fatty acids with varying unsaturation. *Journal of Dairy Science, 81*(2), 471-481.

Cipolat-Gotet, C., Cecchinato, A., Stocco, G., & Bittante, G. (2016). The 9-MilCA method as a rapid, partly automated protocol for simultaneously recording milk coagulation, curd firming, syneresis, cheese yield, and curd nutrients recovery or whey loss. *Journal of dairy science, 99*(2), 1065-1082.

Creusot, N., & Gruppen, H. (2007). Hydrolysis of whey protein isolate with Bacillus licheniformis protease: Fractionation and identification of aggregating peptides. *Journal of Agricultural and Food Chemistry, 55*(22), 9241-9250.

Crum, L. A. (1982). Nucleation and stabilization of microbubbles in liquids. *Applied Scientific Research, 38*(1), 101-115.

Desrumaux, A., & Marcand, J. (2002). Formation of sunflower oil emulsions stabilized by whey proteins with high-pressure homogenization (up to 350 MPa): effect of pressure on emulsion characteristics. *International journal of food science & technology, 37*(3), 263-269.

DiNicolantonio, J. J., & O'Keefe, J. H. (2017). Good Fats versus Bad Fats: A Comparison of Fatty Acids in the Promotion of Insulin Resistance, Inflammation, and Obesity. *Missouri medicine, 114*(4), 303.

Felfoul, I., Bornaz, S., Baccouche, A., Sahli, A., & Attia, H. (2015). Low-fat Gouda cheese made from bovine milk-olive oil emulsion: physicochemical and sensory attributes. *Journal of food science and technology, 52*(10), 6749-6755.

Gaikwad, S. G., & Pandit, A. B. (2008). Ultrasound emulsification: effect of ultrasonic and physicochemical properties on dispersed phase volume and droplet size. *Ultrasonics sonochemistry, 15*(4), 554-563.

Gaiti, N., Aserin, A., & Cohen, Y. (1994). Mechanistic considerations on the release of electrolytes from multiple emulsions stabilized by BSA and nonionic surfactants. *Journal of Controlled Release, 29*(1-2), 41-51.

Galus, S., & Kadzińska, J. (2016). Whey protein edible films modified with almond and walnut oils. *Food Hydrocolloids, 52*, 78-86.

Giroux, H. J., Constantineau, S., Fustier, P., Champagne, C. P., St-Gelais, D., Lacroix, M., & Britten, M. (2013). Cheese fortification using water-in-oil-in-water double emulsions as carrier for water soluble nutrients. *International Dairy Journal, 29*(2), 107-114.

Gülseren, İ., & Corredig, M. (2012). Interactions at the interface between hydrophobic and hydrophilic emulsifiers: Polyglycerol polyricinoleate (PGPR) and milk proteins, studied by drop shape tensiometry. *Food hydrocolloids, 29*(1), 193-198.

Hancock, N. T., & Cath, T. Y. (2009). Solute coupled diffusion in osmotically driven membrane processes. *Environmental science & technology, 43*(17), 6769-6775.

Heino, A. T., Uusi-Rauva, J. O., Rantamaki, P. R., & Tossavainen, O. (2007). Functional properties of native and cheese whey protein concentrate powders. *International Journal of Dairy Technology, 60*(4), 277-285.

Hinrichs, J. (2001). Incorporation of whey proteins in cheese. *International Dairy Journal, 11*(4), 495-503.

Kaltsa, O., Michon, C., Yanniotis, S., & Mandala, I. (2013). Ultrasonic energy input influence on the production of sub-micron o/w emulsions containing whey protein and common stabilizers. *Ultrasonics sonochemistry, 20*(3), 881-891.

Kethireddipalli, P., Hill, A. R., & Dalgleish, D. G. (2010). Protein interactions in heat-treated milk and effect on rennet coagulation. *International Dairy Journal, 20*(12), 838-843. doi:https://doi.org/10.1016/j.idairyj.2010.06.003

Leman, J., Kinsella, J., & Kilara, A. (1989). Surface activity, film formation, and emulsifying properties of milk proteins. *Critical Reviews in Food Science & Nutrition, 28*(2), 115-138.

Leong, T. S. H., Juliano, P., & Knoerzer, K. (2017). Advances in ultrasonic and megasonic processing of foods. *Food Engineering Reviews, 9*(3), 237-256.

Leong, T. S. H., Ong, L., Gamlath, C. J., Gras, S. L., Ashokkumar, M., & Martin, G. J. O. (2020). Formation of cheddar cheese analogues using canola oil and ultrasonication – a comparison between single and double emulsion systems *International Dairy Journal*, 104683.

Leong, T. S. H., Zhou, M., Kukan, N., Ashokkumar, M., & Martin, G. J. (2017). Preparation of water-in-oil-in-water emulsions by low frequency ultrasound using skim milk and sunflower oil. *Food Hydrocolloids, 63*, 685-695.

Leong, T. S. H., Zhou, M., Zhou, D., Ashokkumar, M., & Martin, G. J. (2018). The formation of double emulsions in skim milk using minimal food-grade emulsifiers–A comparison between ultrasonic and high pressure homogenisation efficiencies. *Journal of food engineering, 219*, 81-92.

Li, M., & Fogler, H. (1978). Acoustic emulsification. Part 2. Breakup of the large primary oil droplets in a water medium. *Journal of Fluid Mechanics, 88*(3), 513-528.

Lobato-Calleros, C., Reyes-Hernández, J., Beristain, C., Hornelas-Uribe, Y., Sanchez-Garcia, J., & Vernon-Carter, E. (2007). Microstructure and texture of white fresh cheese made with canola oil and whey protein concentrate in partial or total replacement of milk fat. *Food Research International, 40*(4), 529-537. doi:https://doi.org/10.1016/j.foodres.2006.10.011

Lobato-Calleros, C., Rodriguez, E., Sandoval-Castilla, O., Vernon-Carter, E., & Alvarez-Ramirez, J. (2006). Reduced-fat white fresh cheese-like products obtained from W1/O/W2 multiple emulsions: Viscoelastic and high-resolution image analyses. *Food Research International, 39*(6), 678-685.

Lobato-Calleros, C., Sosa-Pérez, A., Rodríguez-Tafoya, J., Sandoval-Castilla, O., Pérez-Alonso, C., & Vernon-Carter, E. (2008). Structural and textural characteristics of reduced-fat cheese-like products made from W1/O/W2 emulsions and skim milk. *LWT-Food Science and Technology, 41*(10), 1847-1856.

Mahdi Jafari, S., He, Y., & Bhandari, B. (2006). Nano-emulsion production by sonication and microfluidization—a comparison. *International journal of food properties, 9*(3), 475-485.

Martin, G. J. O., Williams, R., & Dunstan, D. (2007). Comparison of casein micelles in raw and reconstituted skim milk. *Journal of dairy science, 90*(10), 4543-4551.

Matsumoto, S., Kita, Y., & Yonezawa, D. (1976). An attempt at preparing water-in-oil-in-water multiple-phase emulsions. *Journal of colloid and interface science, 57*(2), 353-361.

Michalski, M.-C., Gassi, J.-Y., Famelart, M.-H., Leconte, N., Camier, B., Michel, F., & Briard, V. (2003). The size of native milk fat globules affects physico-chemical and sensory properties of Camembert cheese. *Le Lait, 83*(2), 131-143.

Puhan, Z., & Gallmann, P. (1981). Ultrafiltration in the manufacture of quarg. *Northern European Dairy Journal, 1*, 4-5.

Punidadas, P., Feirtag, J., & Tung, M. (1999). Incorporating whey proteins into mozzarella cheese. *International journal of dairy technology, 52*(2), 51-55. doi:https://doi.org/10.1111/j.1471-0307.1999.tb02070.x

Ramos, M. J., Fernández, C. M., Casas, A., Rodríguez, L., & Pérez, Á. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource technology, 100*(1), 261-268.

Schkoda, P., & Kessler, H. (1997). Revolutionary new process for the manufacture of fresh cheese. *European Dairy Magazine, 2*(8), 26-28.

Shanmugam, A., & Ashokkumar, M. (2014). Ultrasonic preparation of stable flax seed oil emulsions in dairy systems–physicochemical characterization. *Food Hydrocolloids, 39*, 151-162.

Singh, H., & Waungana, A. (2001). Influence of heat treatment of milk on cheesemaking properties. *International Dairy Journal, 11*(4-7), 543-551. doi:https://doi.org/10.1016/s0958-6946(01)00085-1

Siso, M. G. (1996). The biotechnological utilization of cheese whey: a review. *Bioresource technology, 57*(1), 1-11.

Stang, M., Schuchmann, H., & Schubert, H. (2001). Emulsification in high-pressure homogenizers. *Engineering in Life Sciences, 1*(4), 151-157.

Thompson, L., & Doraiswamy, L. (1999). Sonochemistry: science and engineering. *Industrial & Engineering Chemistry Research, 38*(4), 1215-1249.

Vasbinder, A. J., Rollema, H., & De Kruif, C. (2003). Impaired rennetability of heated milk; study of enzymatic hydrolysis and gelation kinetics. *Journal of dairy science, 86*(5), 1548-1555.

Wen, L., & Papadopoulos, K. D. (2001). Effects of osmotic pressure on water transport in W1/O/W2 emulsions. *Journal of colloid and interface science, 235*(2), 398-404.

6 Conclusions and recommendations for future work

In this chapter, insights gained from the experimental chapters of the thesis are presented together to develop a collective understanding of whey protein-casein interactions during rennet gelation of bovine milk. This thesis developed new understanding of the effects of adding high concentrations of whey (>20% w/w) to cheese milk in either a native or denatured form. Whey proteins are small, compact proteins that can interact with other colloids via hydrophobic interactions and adsorb to oil-water interfaces. Although whey proteins are lost during cheese syneresis, there is increasing interest in utilising them by incorporating into food products including cheese, due to their unique nutritional properties.

It was revealed that when high concentrations of native whey proteins are included in cheese milk, they impair the rate of rennet gelation and prolong gelation time (Chapter 3). However, when whey protein is heat-denatured into aggregates, the impact on rennet gelation is diminished, in relation to the size of the aggregates. In addition, the whey aggregates can be better incorporated into cheese, both improving the yield and resulting in irregularities in the otherwise firm non-fat cheese microstructure, giving the possibility of improving cheese texture (Chapter 4). An alternative and novel approach, is to stably encapsulate whey protein inside water-in-oil-in-water double emulsions, for inclusion in cheese matrices with markedly high protein contents without impacting on the rennet gelation kinetics (Chapter 5). The results from this thesis will be useful for developing approaches to effectively utilise whey proteins, improve cheese yields and nutritional properties, and increase production efficiency during cheese manufacturing.

6.1 Key conclusions

The key conclusions of the thesis are summarised as follows:

Native whey proteins inhibit κ-CN hydrolysis by chymosin and impairs the rate of aggregation of para-casein micelles during rennet gelation

Chapter 3 demonstrated that native whey proteins impair enzymatic hydrolysis by rennet and significantly delay subsequent aggregation of casein micelles. These observations were independent of any changes in the ionic balance or viscosity of the modified cheese milks. As binding between whey protein and casein micelles, or whey protein and rennet were not observed, it was proposed that whey proteins passively occupy the gaps in the casein micelle surface arising from enzyme hydrolysis. The whey proteins provide a steric hindrance to rennet

reaching the casein micelle surface and a barrier to intimate contact between destabilised casein micelles leading to slower gelation. While the higher concentrations of whey protein examined in the study may not be relevant in a commercial setting (whey protein:casein of 4:1), they enabled clear trends to be observed that helped reveal the underlying mechanisms of the inhibitory roles of whey protein during rennet gelation of milk. Also, the results obtained using milk with reduced whey protein content (whey protein:casein of 0.03:1) are of practical relevance to the use of microfiltration retentates in cheese making. The results demonstrated that the rate of gelation can be increased when native whey proteins are not present, which would translate to more efficient and productive cheese vats. Alternatively, as the gelation rate is improved in casein-rich streams, conventional rennetting times in cheese production could be achieved using a reduced amount of rennet. This reduces the requirement for clotting enzymes that are not recoverable in the process. Further, the upstream separation of whey proteins from cheese milks enables the formation of minimally processed whey protein streams that maintain their native protein confirmation and functionality. This reduces the amount of acid whey produced as a waste stream during syneresis, whose processing is problematic due to the acidic pH and the high organic load.

Forming whey proteins into sufficiently large whey protein aggregates can avoid the negative effect on the rate of rennet gelation.

Chapter 4 demonstrated that if whey proteins are formed into sufficiently large aggregates, for instance using heat or heat plus ultrasound treatment, the negative effects on the rate of rennet gelation in whey protein-rich cheese milks can be minimised. Further, these treatments on whey protein-rich cheese milks can improve whey protein retention in cheese. Irrespective of changes in the soluble calcium in the systems, larger aggregates were found to reduce the steric barrier created by native whey proteins and smaller whey protein aggregates. Further, more hydrophobic aggregates can interact with the casein matrix, which reduces the expulsion of whey protein during syneresis. It also facilitates a more open cheese microstructure, which helps to mitigate the increased hardness that is observed in low-fat cheeses. In summary, this chapter demonstrated the potential of using heat and ultrasound treatment to increase the retention of whey protein and improve the texture of low-fat cheese.

Whey protein enrichment of cheese by incorporation of whey encapsulated water-in-oil-in-water double emulsions

In Chapter 5, ultrasound was successfully used to form whey protein-enriched water-in-oil-in-water double emulsions using minimal amounts of food-grade emulsifiers. These emulsions

had a markedly higher protein encapsulations than previously reported studies. The results also demonstrated the possibility of manipulating emulsion formulation and ultrasonic emulsification parameters to form double emulsions of varying size distributions and protein contents. Whey protein-rich double emulsions were successfully incorporated into cooked curds formed by rennet gelation, without impairing gelation kinetics to increase the retention of whey proteins which are otherwise lost during syneresis. The understanding developed in this chapter provided a novel means of utilising whey protein and increasing the nutritional content of dairy products including cheese.

6.2 Recommendations for future work

Based on the knowledge developed from this research study, the following recommendations for future investigation are suggested.

Effect of forming cheddar cheese from whey protein-depleted microfiltered milk streams on cheese properties.

Chapter 3 demonstrated the possibility of using whey protein-depleted microfiltered milk streams to reduce clotting times during cheese making. However, the scope of the study was limited to the rennet gelation phase of cheese making. Although it has previously been reported that the yield of cheese produced from microfiltered cheese milk is similar to that of regular cheese (Neocleous et al., 2002), the effect on the textural and sensory properties during ripening has not yet been investigated. These aspects are of practical importance for the use of microfiltration during commercial cheese production.

A comparison of different technologies that can be used to alter the particle size of concentrated denatured whey protein slurries.

Chapter 5 investigated the potential of using power ultrasound to alter the functional properties of whey protein aggregates. An alternative technology, which is already widely used in the dairy industry, that can be used in this regard is high-pressure homogenisation. A comprehensive comparison between these two technologies in terms of the extents of size reduction, conformational changes of milk proteins and power consumption may help selection of one technology over the other.

The impact of the mode of whey incorporation on post-gelation processing and the ripening of cheeses

This thesis presents three strategies to incorporate whey proteins into cheese matrices as a means of adding value to cheese whey and improving the nutritional attributes of cheese.

However, significant changes in the curd strength, moisture content and the microstructure of cheese matrices were observed during the incorporation of differently processed whey protein matrices. Textural attributes in cheese curd play an important role during the post gelation processing stages of cheeses such as shredding and melting where the physical properties of cheeses are altered by applying energy. Further, the changes in moisture content affect the growth of cheese cultures during ripening and could lead to changes in cheese flavour and texture. These aspects need further investigation.

Bioavailability of whey proteins in the cheese matrix as affected by process-induced structural changes

Although whey proteins are successfully incorporated into cheese matrices in our study, the nutritional value is determined by the bioavailability of whey proteins during digestion. This aspect was beyond the scope of the current research study. As whey proteins undergo conformational changes during heat denaturation and ultrasound treatment, enzyme-protein interactions during digestion are likely to be altered resulting in the formation of peptides that are different from those formed by native whey protein digestion. Therefore, the behaviour of whey proteins in cheese matrices during digestion needs to be further investigated in order to more fully evaluate the benefits of whey protein included cheeses.

The time dependent stability of whey protein-enriched double emulsions in cheese matrices

In Chapter 5, whey protein encapsulated double emulsions were incorporated into cheese matrices to form whey protein-enriched cheese. An extension of this study would be investigating the impact of storage conditions and storage time on the stability of these emulsions during ripening. It is important to understand the rate of release of encapsulated whey protein and the subsequent effects of this on proteolysis, peptide development, and flavour development. This would help determine the optimum ripening conditions to obtain acceptable sensory and textural attributes.

In conclusion, this thesis demonstrates that whey proteins can be successfully incorporated in cheese matrices using controlled particle formation and double emulsion encapsulation. However, the properties of the included whey protein stream influence the milk microenvironment and impact the hydrolysis and aggregation of casein micelles during rennet gelation and alter the composition and microstructural properties of cheeses. This thesis developed knowledge of the interactions between caseins and whey proteins during rennet

gelation as affected by the state of whey protein aggregation and the method of whey protein inclusion. These findings in particular, highlight the impact of whey proteins in the kinetics of rennet gelation, which has largely been overlooked in literature to date. Further, an effective strategy to utilise the amphiphilic nature of whey proteins to ultrasonically generate whey protein-encapsulated, whey protein-stabilised double emulsions and whey-protein enriched cheese curd matrices was established.

References

Neocleous, M., Barbano, D., & Rudan, M. (2002). Impact 9 of Low Concentration Factor Microfiltration on Milk Component Recovery and Cheddar Cheese Yield. *Journal of Dairy Science, 85*(10), 2415-2424.

7 Appendix 1

Supporting experiments were performed to optimise experimental conditions and better understand interactions between casein and whey proteins during rennet gelation. While the results of these experiments were beyond the key research questions addressed in the thesis, for completeness, they have been appended to the thesis. These experiments are reported as independent sections addressing short research questions. Each section contains a brief introduction, details of the experimental procedure used and a description of the key research findings.

7.1 Identifying individual whey proteins responsible for impaired casein hydrolysis and aggregation.

Chapter 3 demonstrated that native whey proteins inhibited the enzymatic action of chymosin, and delayed the subsequent rate of casein aggregation. In order to identify which whey proteins (α-lactalbumin, β-lactoglobulin or bovine serum albumin) contribute to the inhibition of enzyme hydrolysis and aggregation, milk systems were prepared with casein and individual whey proteins, to match the casein and individual whey proteins concentrations found in a milk system of casein:whey protein ratio (CN:WP) 4:1 (the system demonstrating the most delayed gelation in the previous studies) reported in section 3.2.3. The systems are hereby designated as CN + α-la, CN + β-lg and CN + BSA.

Oscillatory rheometry results from rennet gelation of these systems showed that β-lactoglobulin (β-lg) caused a significant delay in the onset of gelation and an impairment of the subsequent aggregation, compared to the other whey protein isolates considered (Figure. 7.1 a). Kinetic studies of enzyme hydrolysis (measured by the rate of casein macropeptide release) performed on CN + β-lg and CN + BSA systems (with protein concentrations similar to the samples used for rheology) agreed with the rheology results, as systems with β-lg resulted in a slower rate of enzyme hydrolysis (Figure 7.1 b).

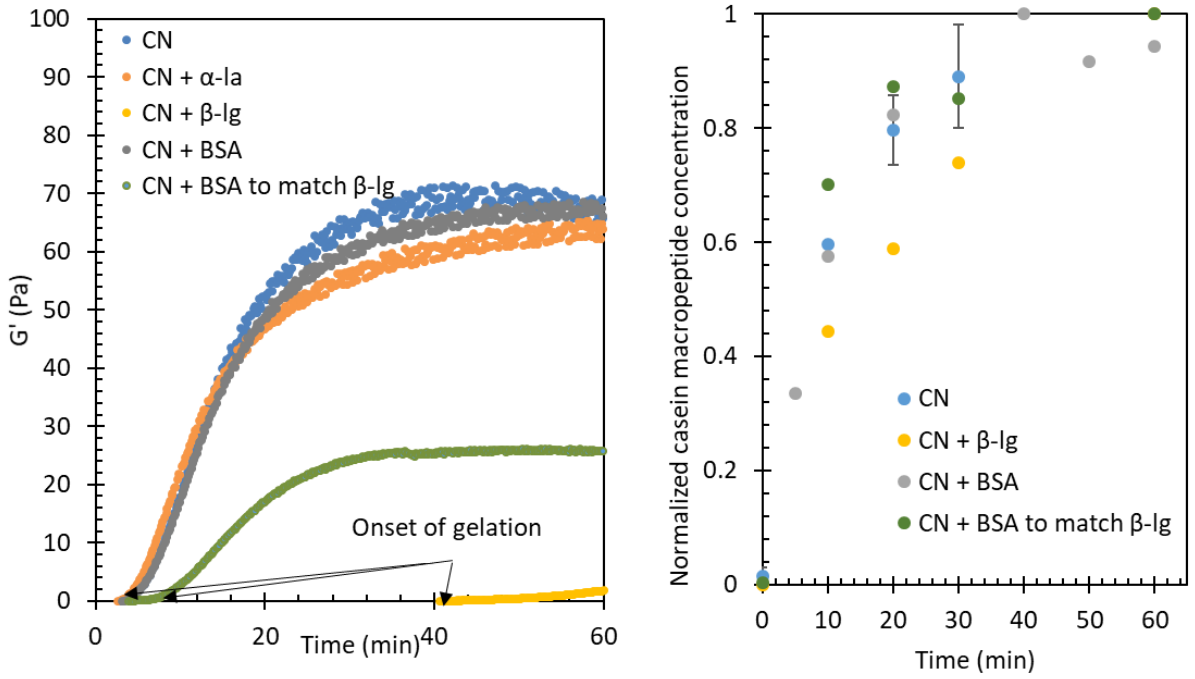


Figure 7.1: (a) The rate of overall gelation and (b) kinetics of enzyme hydrolysis of systems with CN or CN with individual whey proteins.

However, the concentrations of α-la and BSA in milk is lower than that of β-lg (which represents more than 50% of the total whey protein content of milk). Therefore, the impaired gelation caused by β-lactoglobulin could be due to a higher number of protein molecules occupying the bulk solution, obstructing the chymosin molecules from diffusing to the casein micelle surface for enzyme hydrolysis.

To establish that the degree of inhibition is dependent on type of whey protein independent of differences in their native concentration, a system with casein and BSA was prepared to match the concentrations of CN + β-lg. The new system was labelled 'CN+BSA to match β-lg'. Interestingly the onset of gelation the 'CN+BSA to match β-lg' was close to that of a pure casein (CN) system, although the final gel strength was lower (Figure 7.1 a). The low final gel strength could be due to the slower aggregation and the changed gel structure resulting from the differences in the bulk concentration of the two systems. These results differed largely from the CN + β-lg system which had a significant delay in the onset of gelation as well as a very low final gel strength after I hr. The kinetic studies agreed with the rheology results as the 'CN + BSA to match β-lg' system did not demonstrate a significant low rate of enzyme hydrolysis compared to the CN + β-lg system. (Figure 7.1 b).

The delayed onset of gelation and the slower rate of the hydrolysis when β-lg is present, could be due to two main reasons; (a) possible binding between the active site of the enzyme (chymosin and whey proteins hindering the enzyme activity or (b) whey proteins occupying the spaces created by the cleavage of the κ-casein layer, obstructing the enzymes from approaching the remaining κ-casein hairs for hydrolysis. However, a native polyacrylamide gel electrophoresis done on mixtures of rennet and individual whey proteins did not reveal any chymosin-whey protein complexes (Figure 7.2). This confirmed the previous speculation that whey proteins tend to occupy the spaces created by the cleavage of the κ-casein layer, obstructing the enzymes from approaching the remaining κ-casein hairs. Further, this effect was seen to be more prominent with β-lg compared to α-la or BSA.

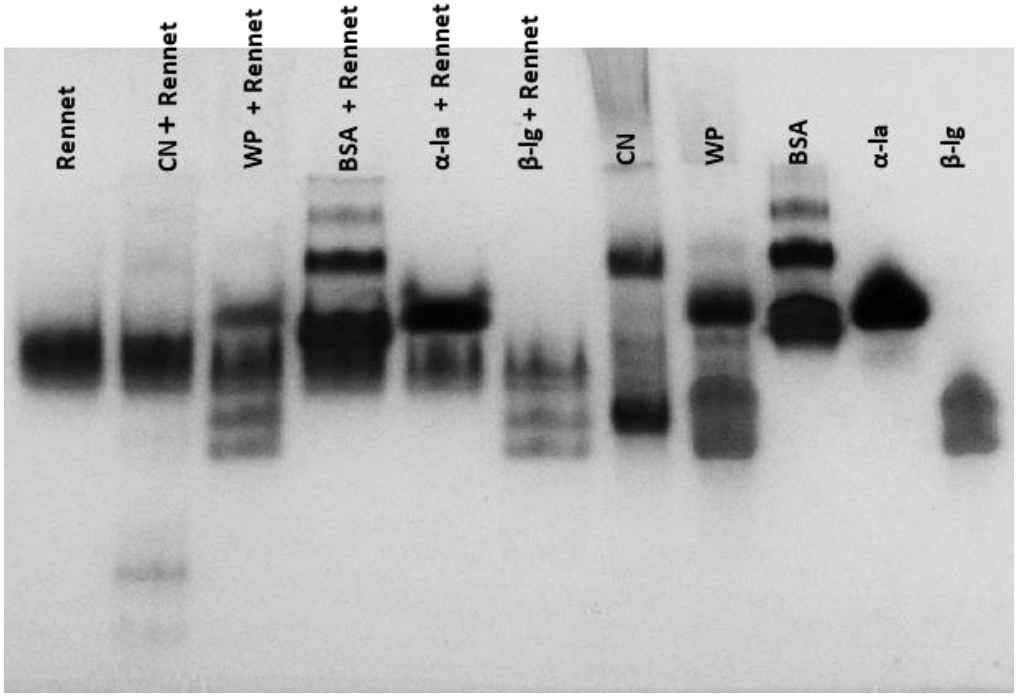


Figure 7.2. Native polyacrylamide gel electrophoresis images of mixtures of rennet individual whey proteins.

Highlights

- β-lg causes a more significant delay in the onset of gelation and an impairment of the subsequent casein aggregation, compared to α-la or BSA.
- β-lg impairs casein hydrolysis by chymosin.
- There is no evidence of molecular binding between casein and individual whey protein isolates.

7.2 Effect of sample size and composition on the temperature profile of whey protein solutions during heat denaturation

Chapter 4 investigated the effect denatured whey protein aggregates had on the kinetics of rennet gelation and properties of model cheddar cheese analogues. During this work, two types of whey protein sources; membrane filtered whey protein (MFWP) and whey protein concentrate (WPC) were denatured. During preliminary experiments they were denatured by placing 25 mL of (20% w/w) whey protein solutions in an 85 ºC water bath for 15 mins. Although this treatment produced large (~ 10-900 µm) aggregates in MFWP solutions, WPC solution turned into a solid clog due to the presence of a greater amount of denatured aggregates in the original solution. Therefore a 10% w/w WPC solution was used for denaturation using the same thermal treatment. The reduction in WPC concentration (from 20% w/w to 10% w/w) did not change the temperature profile of the sample (Figure 7.3)

Further, cheese milk samples initially prepared for gelation experiments (~20 mL) were scaled up (~100 g or 10 kg) to conduct cheese trials. Consequently, heat treatment of whey protein concentrate (WPC) solutions were scaled up from 25 mL to 50 mL. In order to achieve the same temperature profile in 25 WPC samples, a higher water bath temperature (95 ºC) was used (Figure 7.3).

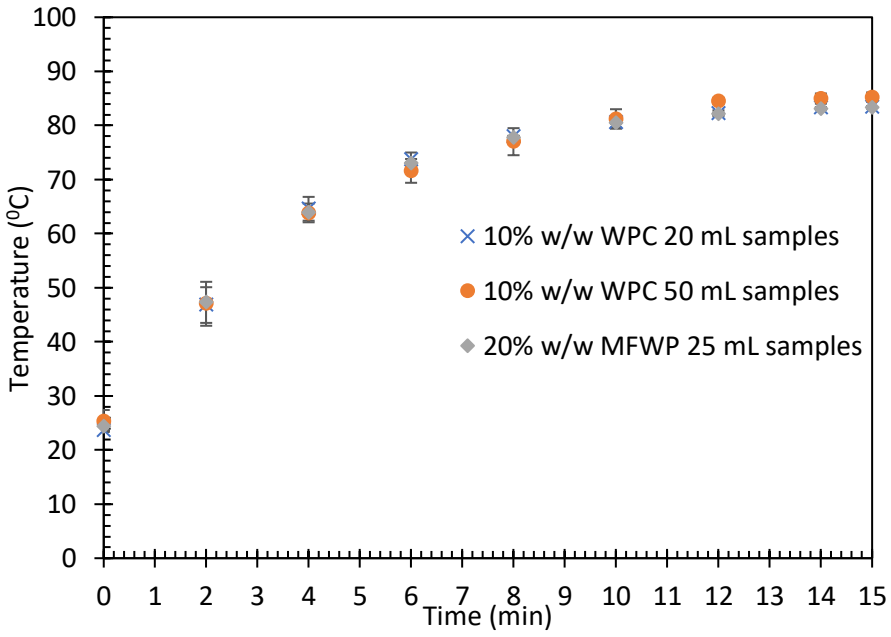


Figure 7.3: Temperature vs. time profiles of 25 mL 10% w/w WPC (■), 50 mL 10% w/w WPC (●), and 25 mL 20% MFWP (◆) samples subjected to heating.

Highlights

- Reduction in WPC concentration form 20% w/w to 10% w/w did not change the temperature profile of the sample.
- Similar temperature profiles were achieved by heating 25 mL and 50 mL WPC samples at 85 ºC and 95 ºC respectively.

7.3 Effect of water:oil ratio on water-in-oil single emulsions

As discussed in section 5.3.1, changes in the single emulsion droplet size caused by different water:oil ratios and whey protein concentrations in the aqueous phase was investigated. Figure 7.4 provides representative images used for image analysis and the determination of droplet size distribution.

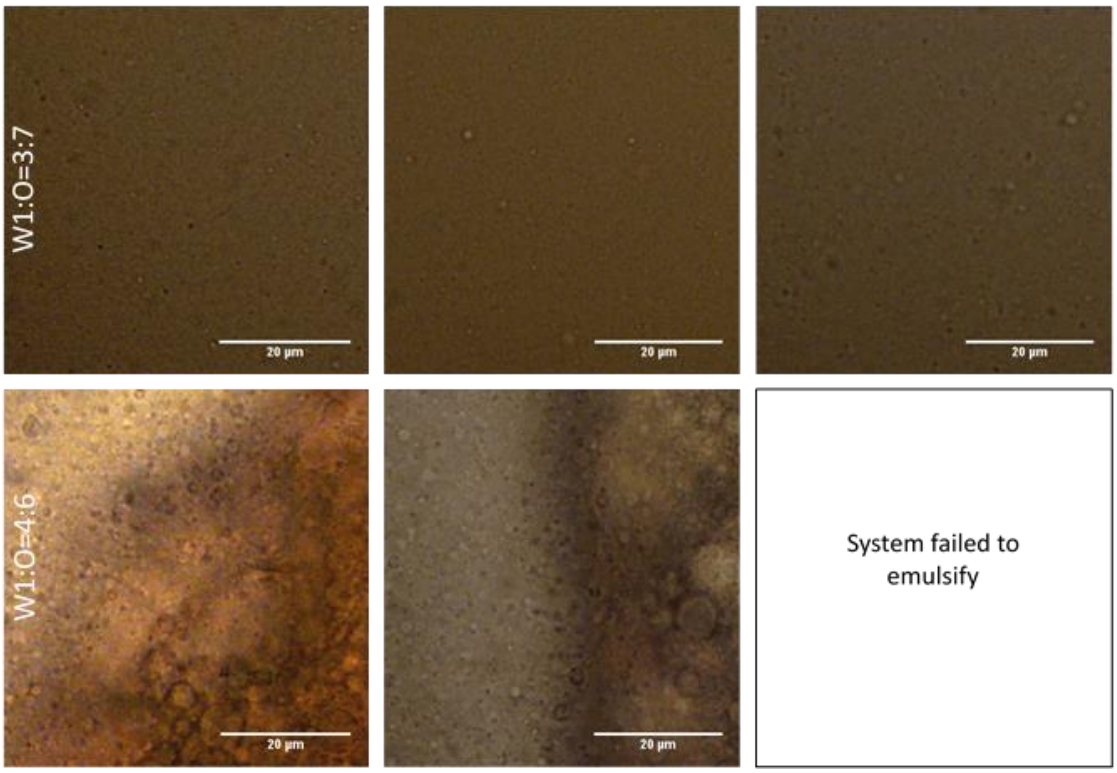


Figure 7.4: Optical microscopy images representing of the W1/O emulsions formed using 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions and sunflower oil at W1:O ratios of 3:7 (top) and 4:6 (bottom). In the microscopy images, emulsion droplets can be identified as white spots in the dark background. Data collected from multiple images (at least 3) were pooled together to determine the size distributions.

7.4 Effect of lipophilic emulsifier concentration on water-in-oil single emulsions

As discussed in section 5.3.2, changes in the single emulsion droplet size caused by different PGPR and whey protein concentrations in aqueous phase was investigated. Figure 7.5 provides

representative images used for image analysis and the determination of droplet size distribution.

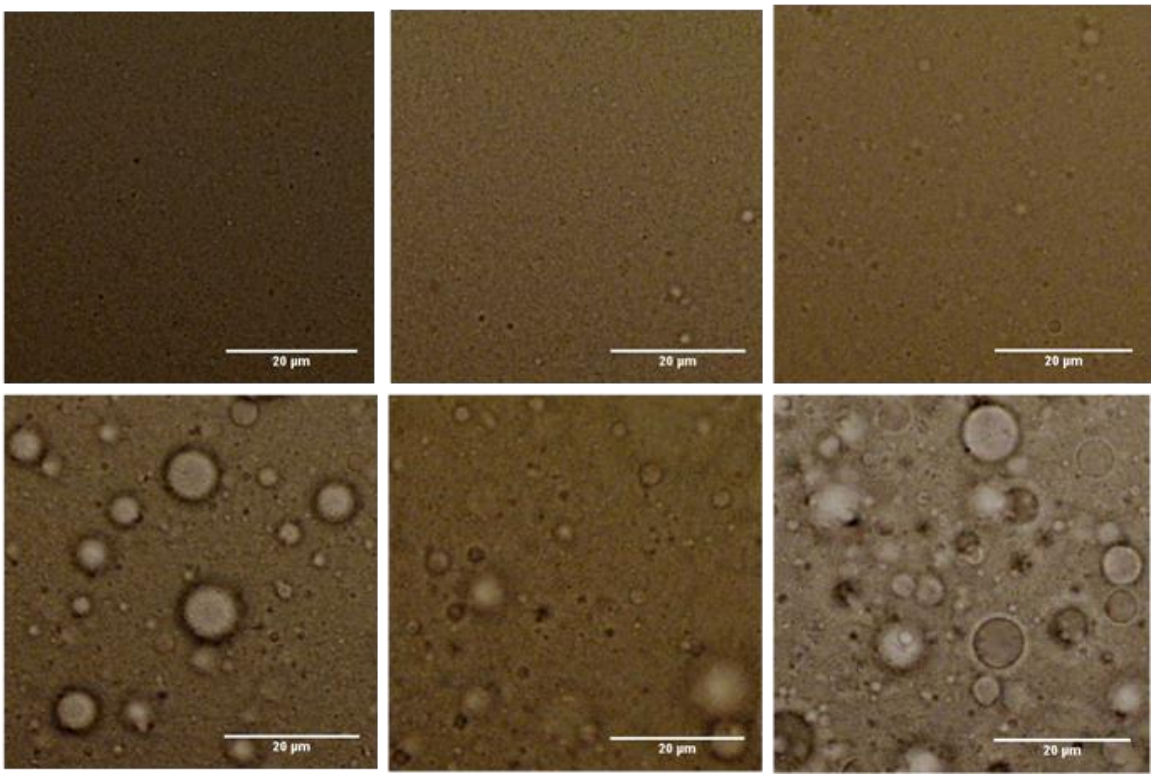


Figure 7.5: Optical microscopic images representing the W1/O emulsions formed using 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions and sunflower oil at W1:O ratios of 3:7, using 3% w/w (top) and 2% w/w PGPR (bottom) in the oil phase while maintaining a lecithin concentration of 1%. In the microscopy images, emulsion droplets can be identified as white spots in the dark background. Data collected from multiple images were pooled together to determine the size distributions.

7.5. Quantification of the casein fraction in the pH 4.5 precipitable milk proteins

In section 4.2.2, proteins were precipitated at pH 4.6 in order to quantify the casein content in milk protein streams. In skim milk and MFWP systems where denatured whey proteins are not present, only caseins precipitate at pH 4.6. However, in WPC systems where denatured whey proteins are precent, both caseins and denatured whey protein aggregates precipitate at pH 4.6. In order to accurately quantify the fraction of caseins in the pH 4.6 precipitated of WPC, an SDS PAGE was performed on the precipitate (Figure 7.6). The percentage of proteins were calculated in the mean percentage intensity of casein bands. The results indicated that only 25.1% of the proteins in the precipitate were caseins. This value was used in the calculation of total casein and whey protein contents of WPC.

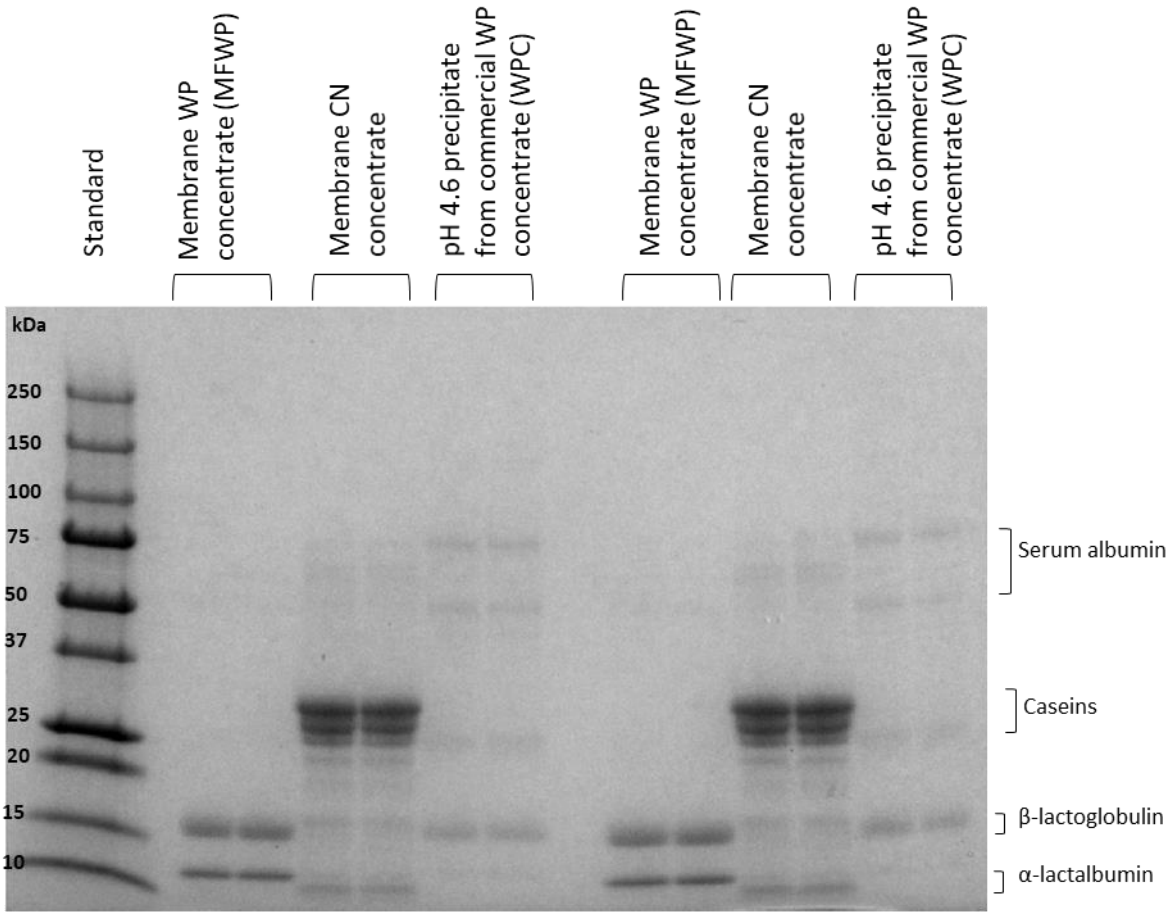


Figure 7.6: SDS-PAGE profiles of pH 4.5 precipitate of WPC. The samples were compared against MFWP and membrane filtered casein concentrate to identify the protein bands.

Table 7.1: Calculation of the percentage intensity of casein bands in the pH 4.5 precipitate of WPC

Replicate number	Intensity of casein bands as a percentage of all protein bands
1	23.1
2	19.2
3	32.8
4	25.4
Mean	25.1
Standard deviation	5.7

8 Appendix 2

In addition to the 3 main experimental chapters presented in the thesis, two other preliminary investigations were conducted:

- To investigate the interactions between caseins and whey proteins when milk protein-stabilised fat droplets were included in rennet gels and model cheddar cheese systems.
- To investigate the impact of rennet hydrolysis on the stability of skim milk-stabilised oil-in-water emulsions.

These two preliminary studies are presented here as two appendix sections.

8.1 Effects of incorporating milk protein-stabilised fat droplets in rennet gels and model cheddar cheese systems.

8.1.1 Introduction

The entrapped fat in cheese contributes to the structural and mechanical properties of the matrix and the distinctive soft texture of cheese, which is not obtained if rennet gels are made with protein alone. During proteolysis and lipolysis when cheese is aged, the native milk fat membrane is deformed and fat moves through the protein structure, aligning and coalescing. Although native milk fat is highly nutritious, recent attention has been drawn to replacing milk fat by other nutritious oils such as sunflower and olive oils to produce healthier cheeses. However, incorporating such oils cheese requires an additional emulsification step. Casein and whey proteins being natural all-dairy emulsifiers, are a good choice to be used for the purpose of emulsifying fat droplets prior to incorporating them in cheeses. However, as the fat membrane layer directly interacts with the casein matrix during rennet gelation and cheese aging, the type and nature of the fat membrane layer could affect key aspects in cheese making such as the kinetics of rennet gelation, fat and protein retention, texture and the microstructure. Therefore, this study was aimed at:

- Producing stable, emulsified milk fat droplets of similar size to native milk fat globules using low-frequency high-power ultrasound.
- Investigating how fat droplets with different membrane layers interact with the casein micelles during rennet gelation.

- Comparing fat retention and its mobility in cheeses made with milk fat emulsified with casein and whey proteins.

8.1.2 Materials and methods

Emulsion preparation

Membrane filtered native whey protein and casein concentrate powders were reconstituted (5% w/w) in filtered and deionised water. The solutions were stirred for two hours in at 40 ºC temperature for complete solubilisation. Anhydrous milk fat (obtained from an Australian supplier) at 40 ºC was mixed with the whey protein solution so that the final volume fraction of fat was 20%. For all experiments excluding cheese trials, a coarse emulsion was made by homogenising a 25 mL sample using an Ultraturrox for 30 s. The sample was then directly transferred to a sonication cell with a 30 ºC water circulation and sonicated at 50% amplitude (33.8W power) for different time intervals.

Measurement of emulsion droplet size

The volume mean diameter of emulsion droplets was measured according to section 5.2.6

Confocal laser scanning microscopy (CLSM)

When CLSM was used to investigate the morphology of the fat droplet membrane material, 20 µL of the emulsion was diluted in in 980 µL of simulated milk ultrafiltrate (SMUF). 10 µL of Nile Red (0.5 mg/mL in Methanol) and 12 µL of Fast Green (1mg/mL in water) was added to the solution and kept for 15 minutes. 10 µL of sample was transferred to a cavity slide, covered with a coverslip and secured with nail polish. CLSM images were taken using a Leica SP5 microscope (Leica Microsystems, Heidelberg, Germany) according to section 5.2.9.

To track casein fat interactions during rennet gelation, emulsified fat was added to skim milk to achieve a final fat content of 4% w/w. The solutions were stained similar to the previous section. Milk systems were diluted two folds using SMUF and equilibrated to 31 ºC in a water bath. They were renneted with 0.5 v/v 3.5 IMCU/mL rennet and 10 µL of sample was immediately transferred to a cavity slide, covered with a coverslip and secured with nail polish. The slide was placed at the microscopic chamber maintained at 31 ºC and images were taken at the same spot on the sample over a period of 1 hour.

Model cheddar cheese systems

Three cheeses were made with whole milk, skim milk containing whey protein-stabilised fat and skim milk containing casein-stabilised fat. Cheese milks were formulated using whole milk or skim milk with protein-stabilised emulsions in such a way that all systems had 3.3% w/w fat and 3.5% w/w protein (2.6% w/w casein and 1% whey protein). Cheeses were prepared from 10 L batches of these milks using a cheddar cheese model adapted from Ong et al. . (Ong et al., 2012, 2013) as explained in section 4.2.9.

Fat content in cheese

Cheeses were shredded into fine particles and tempered to room temperature for 15 minutes. 9 g of cheese was measured into a 50 mL falcon tube and 10 mL of distilled water at 60 °C was added into it. 17.5 mL of 97% sulphuric acid was added in increments and mixed after each addition until all the contents dissolved resulting in a chocolate brown solution. The samples were centrifuged at 839 g for 5 minutes. 5 mL of distilled water at 60 °C water was added and centrifuged for another 2 minutes. 5 mL of distilled water at 60 °C was added again and centrifuged again for 1 minute. The tubes were tempered in a water bathe at 50 °C for 5 minutes. 10 mL from the top layer was transferred to a 15 mL falcon tube and centrifuged again at 839 g for 5 minutes. The height of the fat layer and the diameter of the tube was measured. The volume and then the mass of fat in the cheese sample was calculated later. The fat retention was calculated as a percentage of the fat content in the cheese milk

Protein content in cheese

Protein analysis of the cheese was performed based on the Dumas combustion method using a LECO Trumac CNS analyser (LECO Corporation, Michigan, USA) according to section 4.2.2

8.1.3 Results and discussion

At a constant applied power, increasing the sonication time produced smaller and therefore more stable emulsion droplets (Figure 8.1). After investigating a range of sonication times at the same applied power, 1 min sonication was selected as the optimum treatment time to produce emulsified fat droplets which have a similar size to the native milk fat droplet (Figure 8.2). In order to evaluate whether the emulsified fat droplets flocculate over time as a result of colloidal interactions, the size distributions of emulsions were measured after 3 hours of preparation, however a significant change in the size was not observed (data not shown).

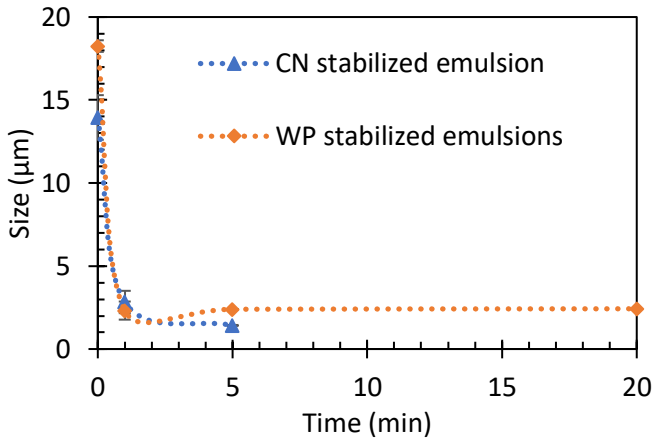


Figure 8.1: Volume mean average size of CN- and WP-stabilised emulsion droplets as a function of sonication time. 0 minutes indicates the samples that were homogenised only. Error bars provide the standard deviation of at least duplicate samples.

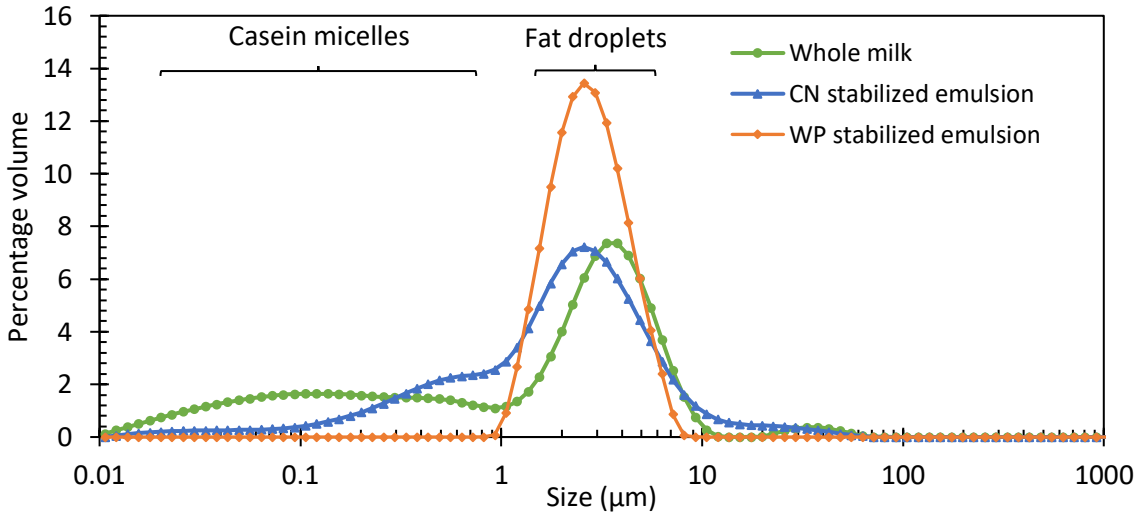
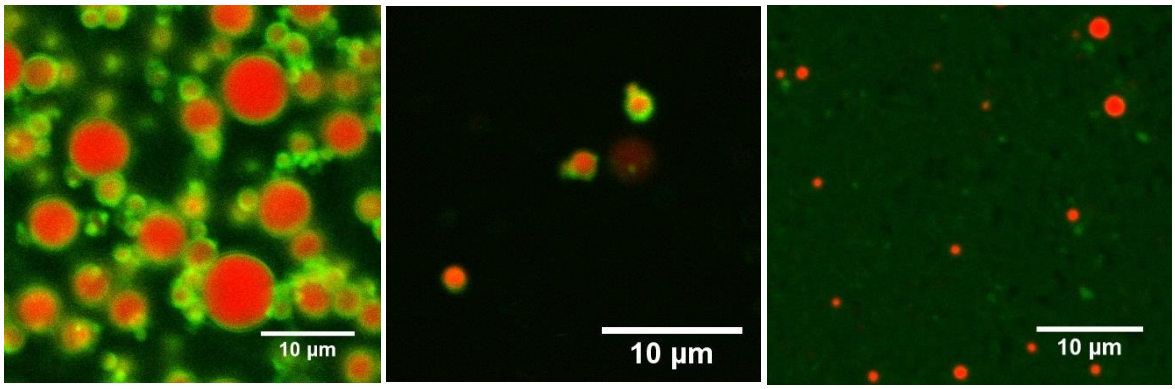


Figure 8.2: Size distribution of whole milk (●) and emulsions prepared using casein (CN) (▲) and whey proteins (WP) (♦) as emulsifiers. The distributions are representative of the measurements of least duplicate samples.

Confocal microscopic images of the emulsion droplets confirmed that the fat droplets (stained in red) were uniformly emulsified with the whey protein (stained in green) (Figure 8.3a). However, casein coated fat droplets (Figure 8.3b) had an irregular protein membrane (stained in green) around it while the native milk fat globule (Figure 8.3c) did not have a noticeable protein membrane.



(a) (b) (c)

Figure 8.3: Confocal microscopic images of milk fat droplets emulsified with (a) whey protein, (b) casein compared to (c) native milk fat globule in whole milk. Protein is stained in green and fat in red.

Kinetics of rennet gelation of systems containing WP- and CN-stabilised emulsions were then observed using oscillatory rheology (Figure 8.4). Emulsion containing milk systems with 3.3% fat (S+CNEM-3.3 & S+WPEM-3.3) showed similar kinetics of gelation to the system with a similar protein composition with no fat (S). However, with an increase in fat content the rate of the increase in G' improved in systems containing both casein- and whey protein-stabilised fat. Interestingly, at higher volume fractions of fat, gelation rate was faster when fat was stabilised with whey proteins (S+WPEM-7) compared to when fat was stabilised with casein (S+CNEM-7). The presence of fat globules in milk at higher volume fractions has been observed to improve the rate of rennet gelation previously (Gaygadzhiev et al., 2009; Titapiccolo et al., 2010). However, the impact of the emulsifier on the improved rate of gelation is not clear.

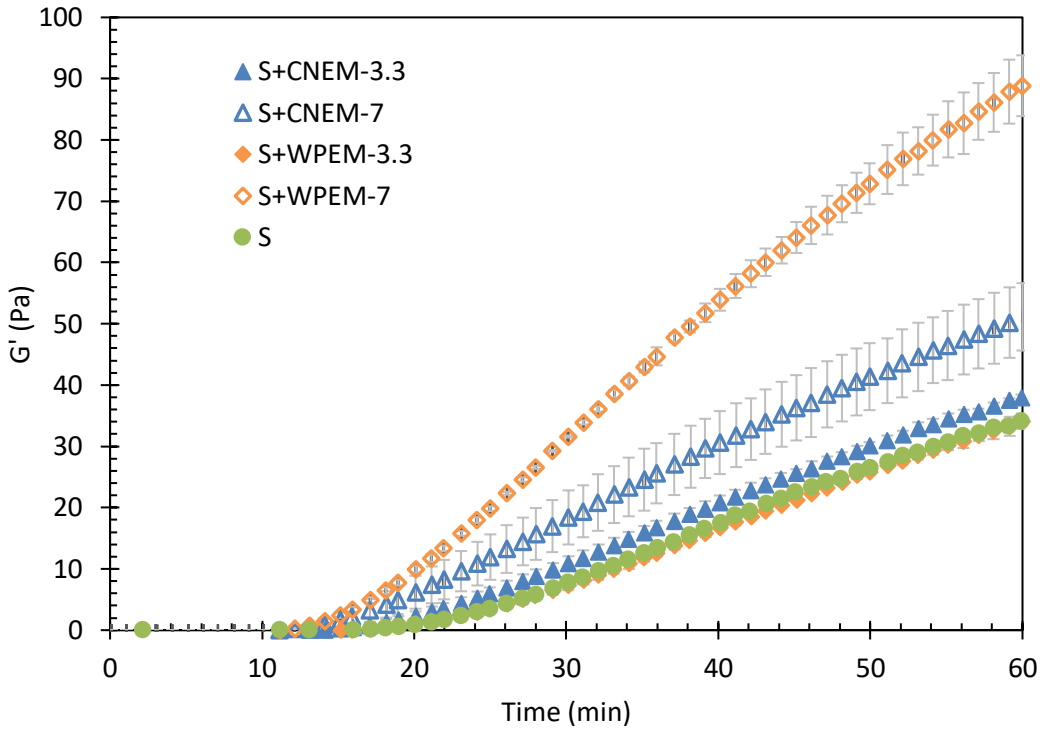
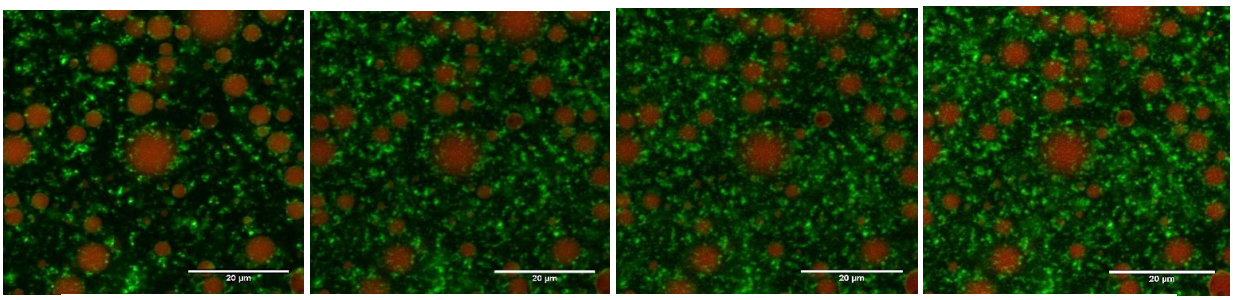


Figure 8.4: Development of G' with time in renneted S (●), S+CNEM-3.3 (▲), S+CNEM-7 (△), S+WPEM-3.3 (◆)and S+WPEM-7 (◇) systems. The error bars indicate the standard deviation of duplicate samples.

In order to understand how fat droplets coated with casein and whey protein interact with caseins during the rennet induced aggregation and gel formation, the aggregation patterns after the addition of rennet into skim milk containing each emulsion, was tracked using the confocal microscope (Figure 8.5). In milk systems containing whey protein coated fat droplets (Figure 8.4a), a large number of smaller aggregates were formed after rennetting, while no interaction was observed between the fat droplets and the casein matrix. However, in the systems with casein coated fat droplets (Figure 8.5b) the aggregate size was larger, and they seemed to grow encapsulating the fat droplets within the casein matrix. It is apparent that when the casein micelles coating the fat droplets are hydrolysed by rennet, the surface of the relatively large fat globules act as a large nucleus onto which a large number of individual cleaved micelles can migrate, thus forming larger casein aggregates around the fat droplet. When the fat droplets are coated by whey protein which are not affected by rennet, the individual micelles migrate to similar individual micelles forming smaller, yet more numerous aggregates.



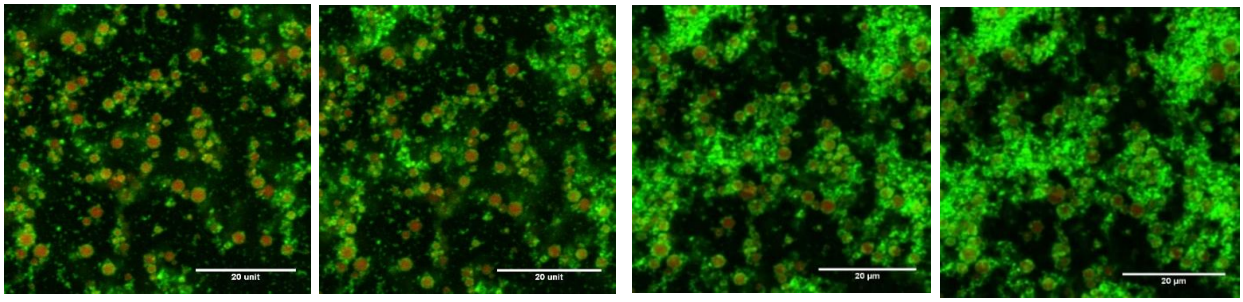


Figure 8.5: Formation of the casein matrix around fat droplets emulsified with (a) whey protein and (b) casein as observed using the confocal microscope at different time intervals after the addition of rennet.

As the whey protein emulsified fat droplets did not directly participate in the casein rennet gel formation when whey protein is used as an emulsifier, they might not be retained within the cheddar cheese matrix once incorporated into cheeses. In order to investigate whether the fat retention in the cheddar curds was affected by altering the fat droplet stabilising protein, model cooked curds were prepared incorporating the emulsified fat with a wide droplet size range into skim milk.

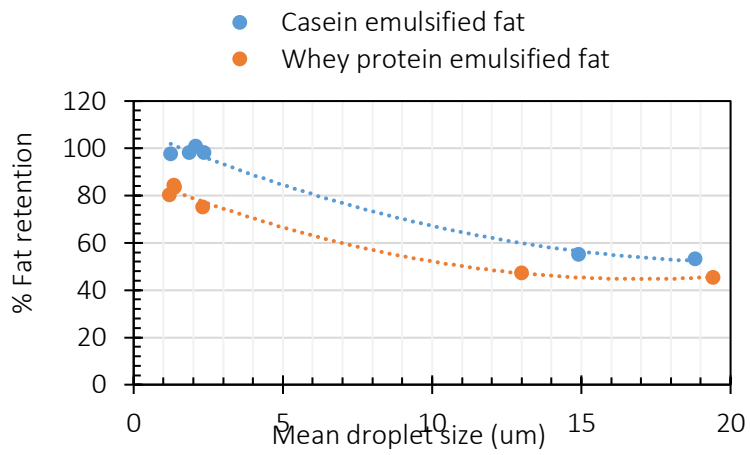


Figure 8.6: Fat retention vs emulsion droplet size in cooked curds containing casein- and whey protein-stabilised milk fat droplets.

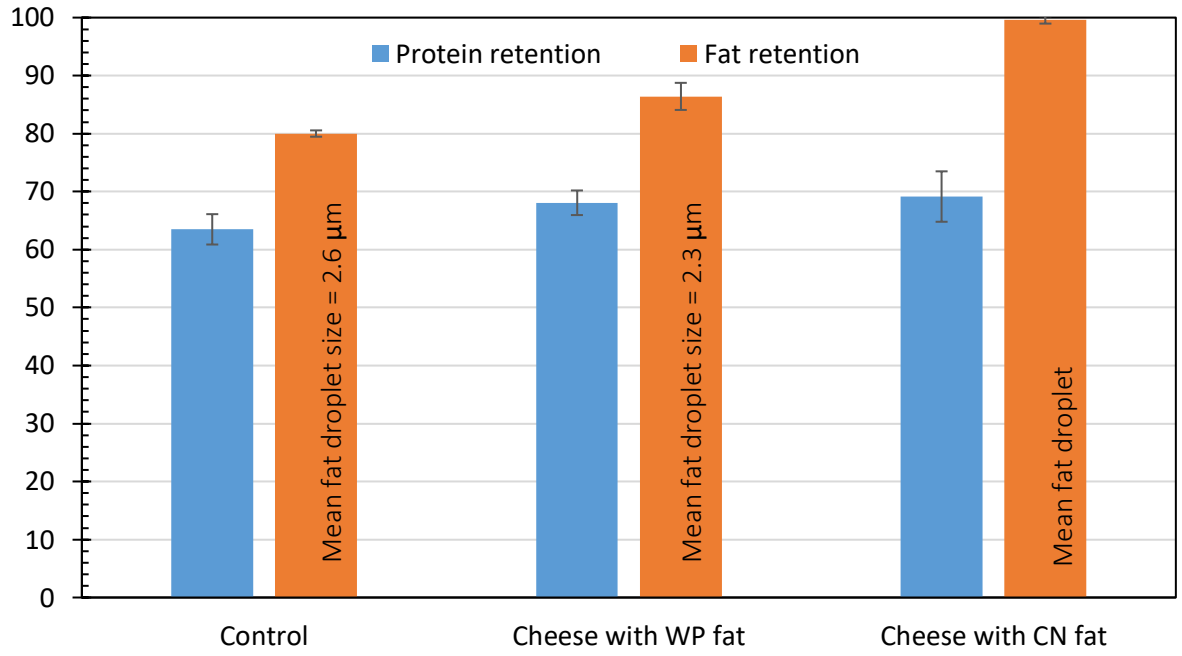


Figure 8.7: Fat and protein retentions in pressed cheeses made with unhomogenised full fat milk (control) and skim milk containing either casein or whey protein stabilised milk fat.

Interestingly, cooked curds made incorporating casein-stabilised milk fat had higher fat retention compared to WP-stabilised fat droplets, in all droplet sizes, reaching approximately 100% when droplet sizes were ~ 1-2 µm (Figure 8.6). Comparable fat retentions were reported in pressed cheeses made by incorporating whey protein- and casein-stabilised fat milk fat emulsions into skim milk. While the fat retention in whey protein-stabilised fat-filled cheeses were comparable to that in the control (made with unhomogenised whole milk), retention in casein-stabilised fat-filled cheeses reached ~100% (Figure 8.7).

Although the fat retention was improved, the distinct softness in cheese is achieved by the fat alignment in the cheese matrix post proteolysis and lipolysis. As casein-coated fat droplets are bound strongly to the casein matrix, they might not move within the casein matrix during ripening as much as a non-interacting whey protein emulsified fat droplet or a native milk fat globule would do. In order to investigate the fat alignment within the cheese matrix, confocal images of fresh pressed cheeses and those ripened 3 months were analysed (Figure 8.8).

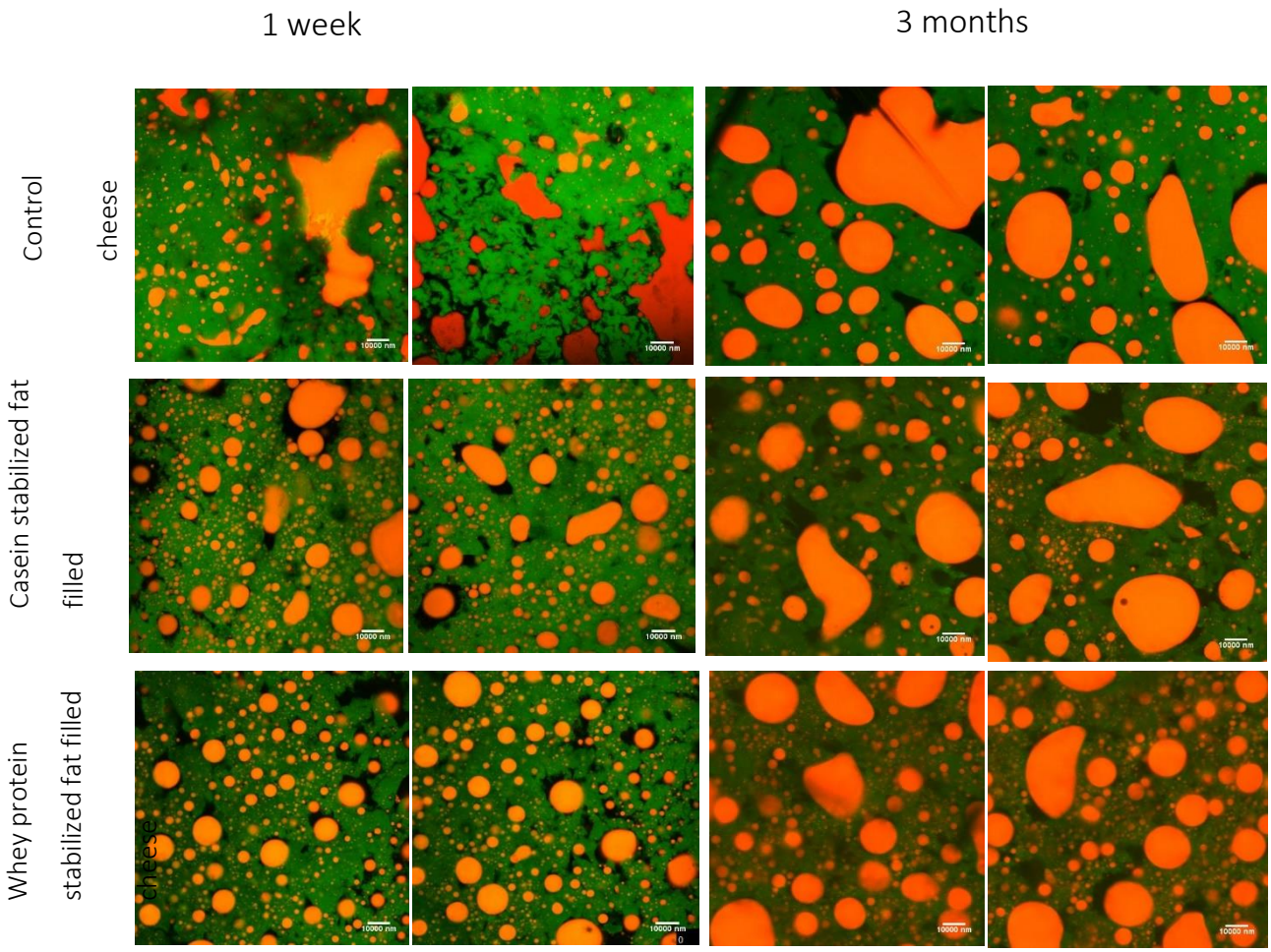


Figure 8.8: Microstructural images of whole milk and emulsion filled cheeses. Fat and protein are stained in red and green respectively. The scale bar represents 10 µm.

Microstructural images of fresh cheeses revealed that native fat droplets (in whole milk cheese) were irregular in shape in some areas. Similar irregular shaped fat-clumps are previously reported in cheddar type cheeses made with whole milk (Guinee et al., 2000). Such deformations occur as a result of shearing of the native milk fat globule membrane during cheese manufacture and shrinkage of the casein matrix during pressing (Guinee et al., 2000). However, protein-stabilised fat droplets, retained their globular shape during cheese production

and the first week of ripening (Figure 8.8). The ability of ultrasound to cause conformational changes in proteins during emulsification to provide a superior emulsion stability (Shanmugam & Ashokkumar, 2014) could be the reason for the resilience of emulsified fat droplets during cheese production. However, after 3 months of ripening the majority of fat droplets in all three cheeses had flocculated and collapsed to form larger fat-filled pockets in the cheese matrix (Figure 8.8). A quantification of the extent of fat droplet coalescence by image analysis would provide further evidence to confirm the presence or absence of subtle changes in the fat coalescence in all three cheese systems studied.

8.1.4. Highlights:

- Fat droplets emulsified with casein interact with the casein matrix during rennet gelation.
- Fat droplets emulsified with whey proteins have minimal interactions with the casein matrix during rennet gelation.
- Membrane filtered casein streams can be utilised as an effective emulsifier to increase the fat retention in cheeses, without significantly affecting fat mobility during cheese ripening.
- Casein could be a good interactive emulsifier to effectively encapsulate oil-soluble compounds in cheeses and other milk products made with casein aggregation (e.g. yoghurt).

8.1.5. References

Gaygadzhiev, Z., Corredig, M., & Alexander, M. (2009). The impact of the concentration of casein micelles and whey protein-stabilized fat globules on the rennet-induced gelation of milk. *Colloids and Surfaces B: Biointerfaces, 68*(2), 154-162.

Guinee, T. P., Auty, M., Mullins, C., Corcoran, M., & Mulholland, E. (2000). Preliminary observations on effects of fat content and degree of fat emulsification on the structure-functional relationship of Cheddar-type cheese. *Journal of texture studies, 31*(6), 645-663.

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*(1), 119-130.

Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. (2013). The effect of calcium chloride addition on the microstructure and composition of Cheddar cheese. *International Dairy Journal, 33*(2), 135-141. doi:https://doi.org/10.1016/j.idairyj.2013.03.002

Shanmugam, A., & Ashokkumar, M. (2014). Ultrasonic preparation of stable flax seed oil emulsions in dairy systems–physicochemical characterization. *Food Hydrocolloids, 39*, 151-162.

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 & technology, 90*(6), 623-639.

8.2 Formation of oil filled protein microstructures using enzyme hydrolysis of skim milk-sunflower oil emulsions

8.2.1. Introduction

Skim milk contains caseins and whey proteins that are naturally amphiphilic and are good emulsifiers for food-based water-in-oil emulsions. However, skim milk emulsions quickly become unstable during processing under extreme conditions such as high temperature. Although crosslinked microspheres can withstand extreme conditions, they are presently prepared from thermally or chemically denatured purified proteins, making their preparation expensive and less suitable for encapsulating sensitive nutraceuticals in food. Instead, proteins in milk can be crosslinked via hydrophobic interactions or covalent bonds using enzymes such as chymosin. Similar enzyme mediated protein crosslinking is commonly used in dairy industry to produce milk protein gels for cheese and yoghurt production.

The aim of this project was to form stable oil-filled protein microstructures by rennet-induced aggregation of casein in skim milk-sunflower oil emulsions. The mechanism of the formation of these microstructures, their composition and their time-dependent interactions were further investigated.

During the project, oil droplets coated with skim milk were produced by ultrasonic emulsification followed by enzyme-mediated crosslinking. Their size and stability were investigated using optical and confocal microscopy, and with Mastersizer and Lumifuge measurements. The resulting microstructured emulsions were then incorporated into dairy systems such as cheese milk to investigate their resilience and retention across different stages of processing.

8.2.2. Materials and methods

Emulsification procedure

Ultrasonic emulsification was carried out by placing the horn tip of a 20 kHz, 11 mm ultrasonic horn at the oil and water interface. The calorimetric power of the horn at the applied amplitude (30%) was 20.3 W. Unless otherwise mentioned, sonication was conducted for 30 seconds.

Enzyme-induced gelation

Unless otherwise mentioned, emulsion samples were diluted using SMUF. Each 10 mL milk system was rennetted with 100 µL of 3.5 IMCU/mL rennet solution (prepared from 200 IMCU/mL Chymax Plus FPC, Cheeselinks, Australia). When the working volume of the milk

solutions changed, the same % v/v of rennet was maintained. Samples were incubated overnight at 31 ºC.

Elemental analysis

Elemental analysis was performed based on the Dumas Combustion method using a LECO Trumac NCS analyser (LECO Corporation, Michigan, USA) according to section 4.2.2.

Oil extraction

Emulsions were prepared as previously described using undiluted skim milk and sunflower oil with an oil-to-aqueous phase loading ratio of 2:8. The emulsions were diluted twice with SMUF and divided into two samples of 10 mL each. One sample was incubated with rennet overnight while the other was held at the incubating temperature without the addition of rennet. The cream layer or the top aggregate layer of the emulsions and rennetted emulsions were separated after gravity settling overnight. SMUF was added to the cream layer in order to make a final mass of 10 g. 3 g from the diluted sample was mixed with 3 mL of methanol and 3 mL of chloroform and shaken for 1 hour in an orbital shaker. The samples were then vortexed at 1400 g for 5 min. The chloroform layer was then separated and dried in a concentrator overnight at 62 ºC. The percentage oil extracted was calculated as: final oil mass after concentrating*100/oil content in the emulsion.

Confocal laser scanning microscopy

Oil and aqueous phases were stained separately by adding 1 µL/mL of Nile red (0.5 mg/mL in ethanol) and 1 µL/mL of fast green (1 mg/mL in water). After rennet induced aggregation, 100 µL from the aggregated layer on the top of the sampling tube was diluted 10 times with SMUF. The diluted sample were mounted on a glass slide and covered with a cover slip. A confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems, Heidelberg, Germany) was used to for visualisation using the protocol reported in section 5.2.9.

8.2.3 Results and discussion

Emulsions and rennetted emulsion in different concentrations

Preliminary experiments were carried out to investigate the aggregation behaviour of emulsions formed using diluted skim milk and sunflower oil at an oil to aqueous phase loading of 2:8. In these trials, the aqueous phase contained skim milk diluted by factors of 1, 2, 4 and 10 times using SMUF to achieve final protein concentrations of 4.2 % w/w, 1.2% w/w, 1.05% w/w and

0.42% w/w in aqueous phase, respectively. The emulsions were not further diluted using SMUF prior to rennet addition.

As the concentration of protein decreased, the emulsion droplet size continuously increased (Figure 8.9). At sufficiently high protein concentrations, protein can fully cover the oil-water interface and form stable emulsions. However, as the protein concentration decreases, there is insufficient emulsifier present in the system to completely cover all the oil-water interface created by smaller droplets. This results in gaps in the interfacial membrane surrounding the droplets (McClements, 2004). There is also a possibility of protein molecules being shared by the interfaces of multiple droplets. Both the above phenomena lead to faster coalescence and increase in the emulsion droplet size.

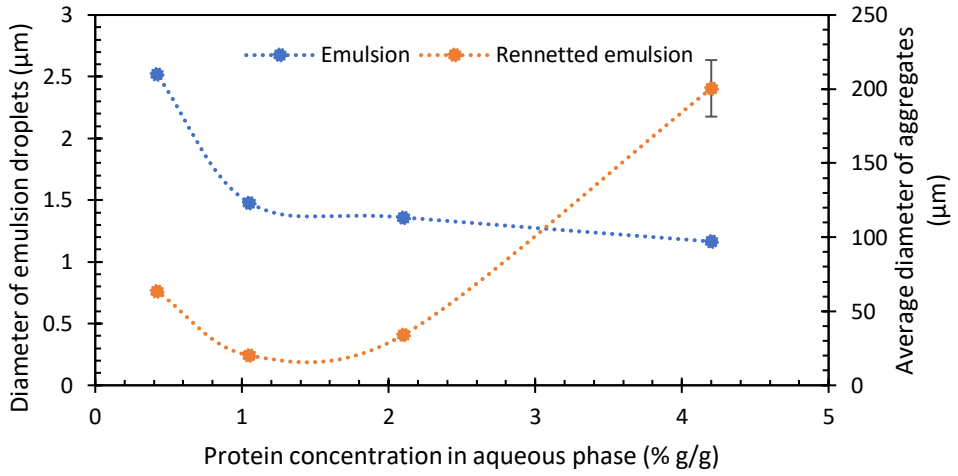


Figure 8.9: Sizes of emulsion droplets prior to rennet additions and protein-droplet aggregates after rennet addition.

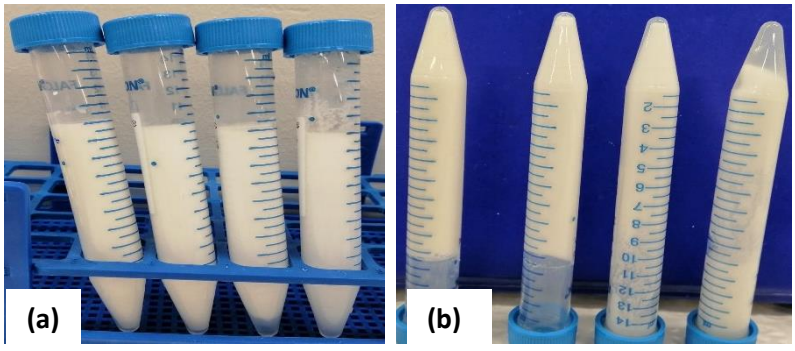


Figure 8.10: (a) Emulsions and (b) rennetted emulsions. Protein concentrations in the aqueous phases in the tubes from left to right are 4.2% w/w, 2.2% w/w, 1.05% w/w and 0.42 % w/w respectively.

When the emulsions were incubated with rennet overnight, the formation of a solid gel-like structure could be observed, particularly in systems with protein concentrations >= 2.1% w/w

(Figure 8.10). As described in this thesis, rennet-induced aggregation of milk proteins is widely studied and its mechanisms well-established. Chymosin in rennet cleaves the κ-casein hairy layer that provides electrostatic and steric stability to the casein micelle. The destabilised micelles interact and subsequently aggregate form a gel (Lucey, 2014). The strength of the gel depends on the concentration of caseins, as the presence of more casein micelles provides more protein-protein interactions and crosslinks. The gel-like structures formed in this study could thus be described as an oil filled protein gel, that is formed by the interaction and the crosslinking of cleaved casein micelles around the emulsion droplets. Although aggregates were formed at protein concentrations < 2.1 % w/w, the lower concentration may have resulted in fewer crosslinks, which were not sufficient to form a gel structure. A separate set of control experiments carried out by incubating diluted skim milk samples (without oil) overnight also confirmed that although protein concentrations < 2.1 w/w % w/w were adequate to form casein aggregates that sedimented; a strong gel structure was not formed (Figure 8.11).

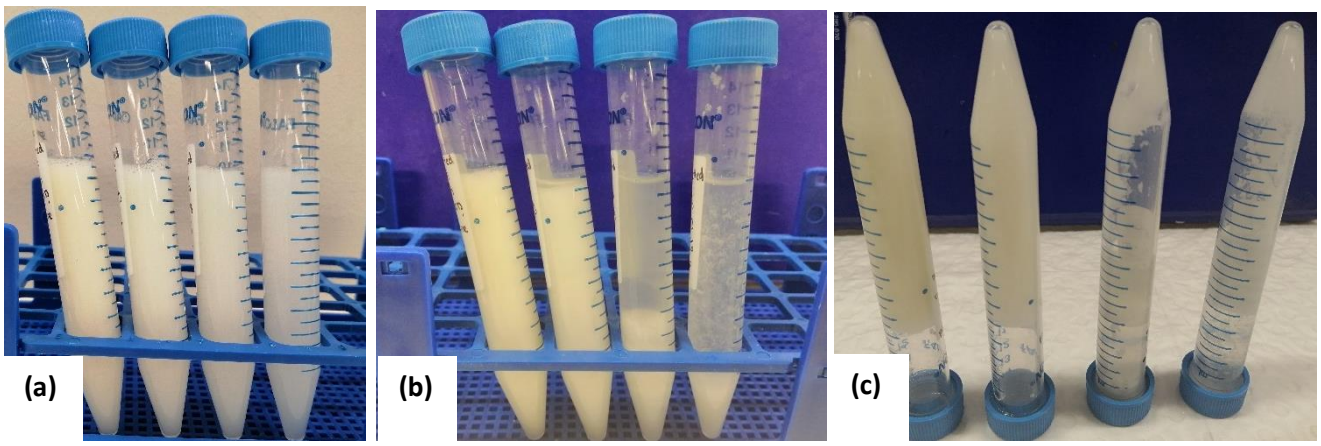


Figure 8.11: (a) Diluted skim milk and (b), (c) diluted skim rennetted overnight. Protein concentrations in the aqueous phases in the tubes from left to right are 4.2% w/w, 2.2% w/w, 1.05% w/w and 0.42 % w/w, respectively.

Protein aggregation resulted in an increase in the average particle size of emulsions after the addition of rennet (Figure 8.9). Although the emulsion droplet size decreased with the increase in protein concentration, the opposite was observed in rennetted emulsions. At sufficiently high protein concentrations (>=1.05% w/w), the particle size in rennetted emulsions increased with the increase in protein concentration (Figure 8.9). This observation agrees with the established knowledge that increase in protein concentrations leads to more casein-casein interactions and the formation of larger protein aggregates. However, at the 0.42% w/w protein concentration, there was an increase in the aggregate size compared to that at 1.05% w/w. This may have

resulted from the flocculation and the coalescence of weakly emulsified droplets during the incubation period, resulting in larger oil droplets around which the protein network was formed.

Subsequent experiments were performed to compare the heat stability and the centrifugal separation of the emulsions and rennetted emulsions. Neither the emulsions nor the rennetted emulsion showed a quantifiable separation of a distinguishable oil phase after 7 hours of heating at 70 ºC. Similarly, no distinguishable oil layer could be observed in either emulsions or rennetted emulsions after centrifugation at 7000 rpm for 20 minutes. These results indicated that both sets of samples were stable at the microscopic scale under the studied conditions.

The absence of a separate oil layer under centrifugation or heat treatment would mean that oil droplets in both rennetted and un-rennetted emulsions are strongly protected/stabilised by the milk proteins surrounding them. In order to further evaluate the stability of these emulsion droplets, a liquid-liquid extraction step was performed as a stronger de-emulsification technique. Interestingly, the extraction efficiency of oil in the non rennetted emulsions was higher than that of rennetted emulsions (data not shown), revealing that the emulsifier layer may be compromised by the incubation with rennet.

In order to investigate the changes in the proteins adsorbed to the oil-water interface after the addition of rennet, a kinetic study was performed to track the changes in the rennetted emulsions over time using confocal laser scanning microscopy and particle size analysis. During this study, homogenisation using an ultra-turrax (a rotor stator disperser at 13.5 rpm for 2 min, 64.1±0.6 W electric power measured at 1 min of operation) was also employed as an alternative emulsification technique. Emulsions were prepared using a system of undiluted skim milk and sunflower oil with an oil:aqueous phase ratio of 2:8. They were diluted twice with SMUF before enzyme addition. The rennetted emulsions were also diluted similarly. Compared to emulsions made with ultrasound, those made with the ultra-turrax had larger droplets at day one (Figure 8.12). This could be explained by the differences in mechanism of emulsification used in the two instruments.

In general, the change in the droplet size in both the ultrasound and ultra-turrax emulsions showed an increasing trend, possibly due to the flocculation and coalescence of emulsion droplets over time. Similarly, the aggregate size of the rennetted emulsions also increased with time; presumably because oil-filled protein clusters formed by the aggregation of destabilised caseins interacted over time to form larger aggregates.

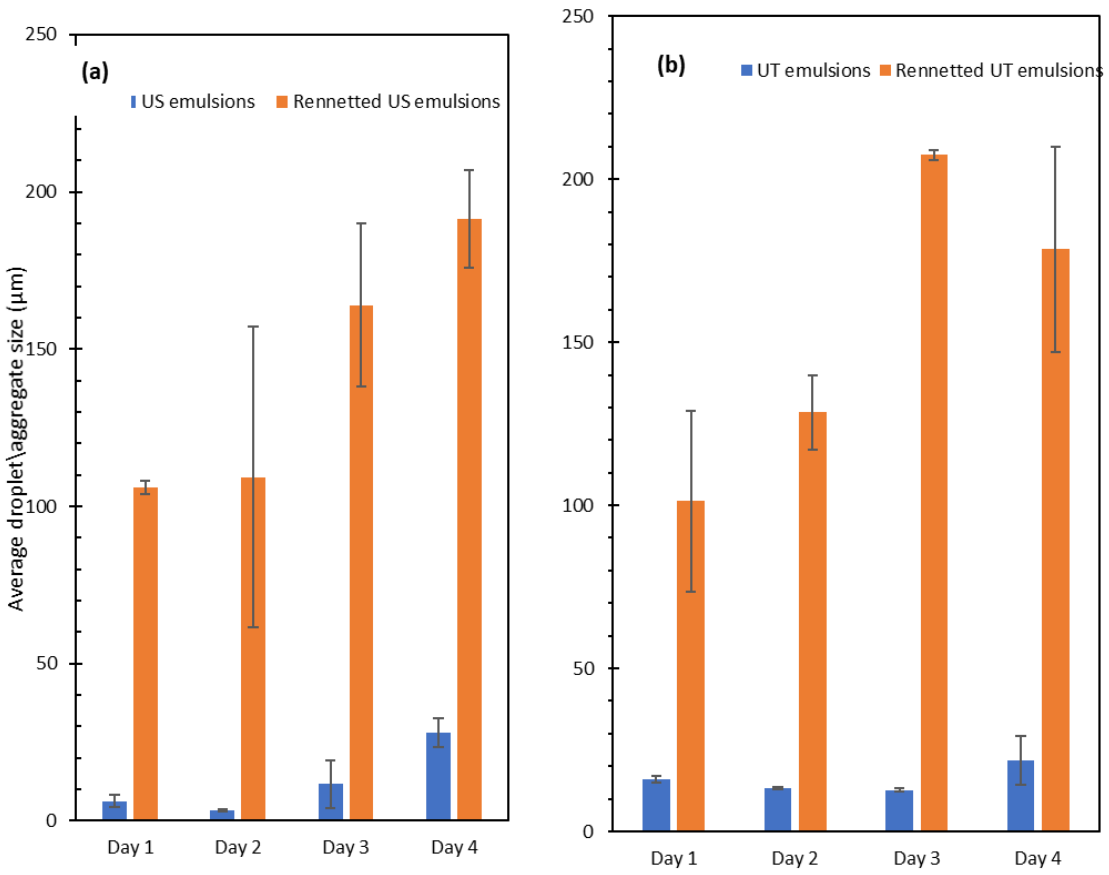


Figure 8.12: Change in the particle size of emulsions and rennetted emulsions made with (a) ultrasound and (b) ultra-turrax over time.

CLSM images also showed flocculation and coalescence of both ultrasound and ultra-turrax emulsions over time (Figure 8.13 a-c, g-i). The rennetted emulsions had an interesting microstructure where proteins (green) had aggregated surrounding oil (red) droplets. The protein clusters formed after rennetting emulsions prepared by ultrasound had more finer oil droplets embedded inside the protein aggregate (Figure 8.13 d) compared to comparable systems made by the ultraturrax (Figure 8.13 j). Oil droplets in the border of the protein aggregates were larger in size suggesting faster coalescence compared to droplets trapped inside the aggregate. With time (2nd day) the droplets in the periphery of the protein aggregates seemed to have coalesced with similar oil droplets from another protein aggregate to form larger threads connected by a continuous oil phase (Figure 8.13 e, k). In the rennetted emulsions made with the ultra-turrax, a continuous oil layer could be observed around oil-filled protein aggregates. With prolonged storage (4th day), the oil the aggregates seemed to have become larger in size with the trapped oil droplets becoming a continuous oil phase trapped inside the protein aggregate (Figure 8.13 f, l). The amount of protein (indicated by green fluorescence)

inside a cluster seemed to be higher in rennetted emulsions prepared by ultrasound (Figure 8.13 f) than in those prepared by the ultra-turrax (Figure 8.13 l).

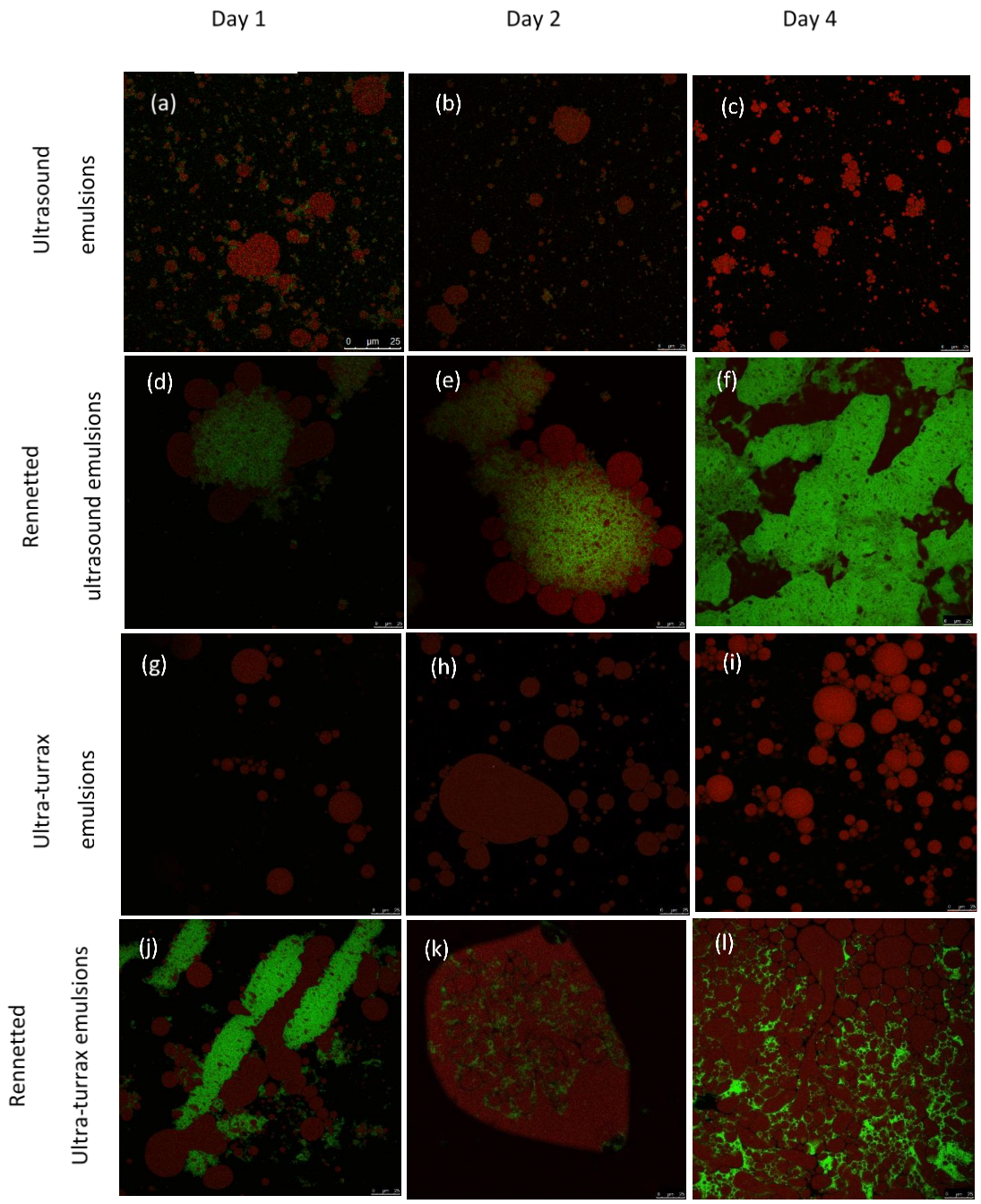


Figure 8.13 : Change in the particle size of emulsions and rennetted emulsions made with (a) ultrasound and (b) ultra-turrax over time.

The microscopic images indicated that the destabilisation and the aggregation of proteins after rennet addition destabilised the emulsion droplets over time, to form a continuous oil phase that spread inside to join the protein aggregates. This could possibly be due to the cleavage of

the hydrophilic κ-casein hairy layer of the casein micelle, that may have resulted in a change in the hydrophilic lipophilic balance and thereby the surface activity of the caseins. In a separate experiment the effect of enzyme hydrolysis on the interfacial activity of proteins in skim milk was investigated (Figure 8.14). The interfacial activity of twice diluted skim milk (two-fold) was compared against that of a similar milk system rennetted for 3 hours (until small microscopic aggregates appeared). The results showed that the interfacial activity of milk proteins (caseins being the predominant protein) was reduced by the enzyme hydrolysis of casein by rennet (Figure 8.14)

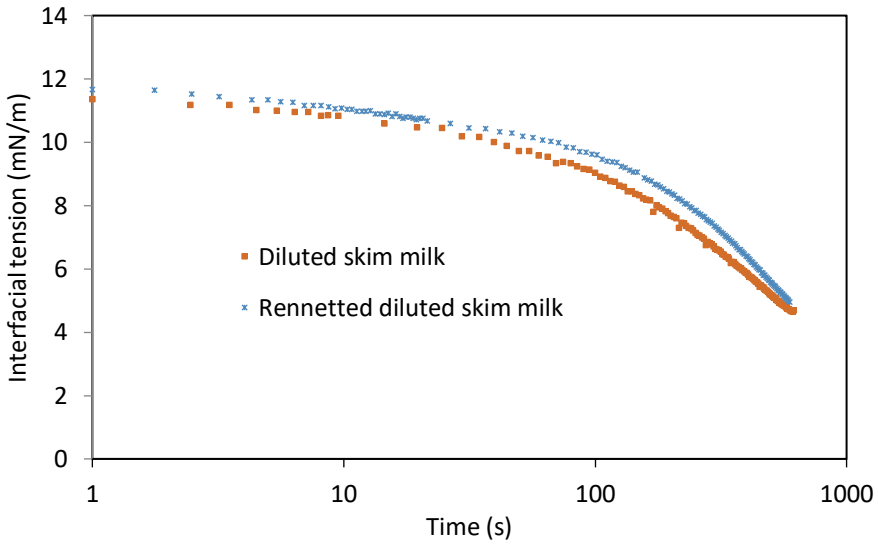


Figure 8.14: Change in the interfacial tension of skim milk systems with time.

Nonetheless, as indicated by the centrifugal and heat stability tests, the oil rich areas remained trapped inside the protein matrix and did not separate for ~6 months when stored in ambient conditions.

When centrifuged, the rennetted emulsions separated into two phases; a white top phase made of aggregates and a bottom clear phase. When compared with a similarly centrifuged unrennetted emulsion, the bottom phase of the rennetted emulsions were significantly less turbid indicating that almost all the proteins were attached to the oil droplets/clusters that migrated to the top of the sample due to density difference (Figure 8.15). This would mean that the rennet addition resulted in the formation of an oil-rich cluster that is also rich in proteins compared to a simple emulsion. These may be useful as a food additive to improve the protein content.

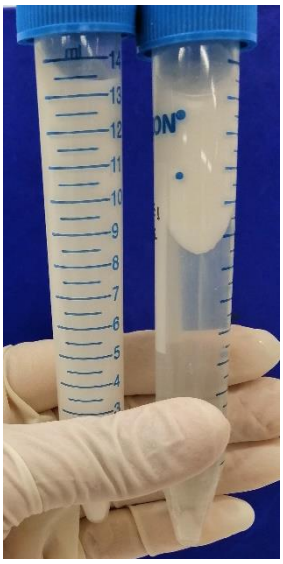


Figure 8.15: Centrifuged samples (7000 rpm 10 minutes) of emulsions (left) and rennetted emulsions (right) prepared by ultrasound.

In order to quantify the protein-to-fat ratio in the cream/aggregate phases, an elemental analysis was done to measure their nitrogen-to-carbon ratio. Sunflower oil is made primarily from triglycerides of palmitic acid, stearic acid, oleic acid and linoleic acid that are long chains of carbon, oxygen and hydrogen. Caseins in contrast are long peptide chains that are rich in nitrogen, carbon, oxygen and hydrogen. An increase in the N:C ratio in the cream/aggregate layer would therefore provide a comparison between the amount of protein trapped inside the cream/aggregate layers in the systems under study.

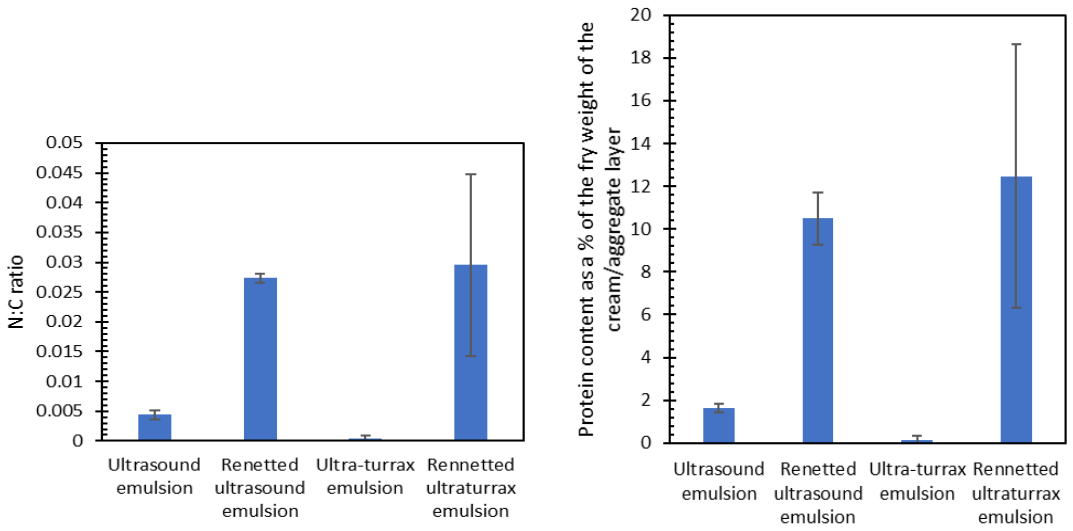


Figure 8.16: Left: N:C ratio in cream/aggregate top layer. Right: Protein content in the cream/aggregate layer as a percentage of dry weight.

As expected, the N:C ratio of the top layer from the rennetted emulsions was significantly high compared to the rennetted ones (Figure 8.16). Concomitantly, the oil-rich protein flocs produced by rennet induced aggregation had ~10% protein of its overall dry mass, confirming the possibility of their use as replacers of oil to produce high-protein low-fat products.

8.2.4 Highlights

- Stable, oil-filled protein microstructures were formed by enzyme hydrolysis of skim-milk sunflower oil emulsions.
- The globular structure of the emulsion droplets was progressively lost following rennet addition, however phase separation did not occur for up to 6 months.
- These microstructures can be separated from the bulk water phase by simple centrifugation and have a markedly higher protein content compared to creamed non-rennetted emulsions.

8.2.5 References

Lucey, J. A. (2014). Milk protein gels. In *Milk proteins* (pp. 493-523): Elsevier.

McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 9*(5), 305-313.