Impact of micellar calcium phosphate concentration on the casein micelle structure, stability and functionality

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Abstract

Caseins, the major group of milk proteins, are individually disordered proteins with a great degree of hydrophobicity and low stability in an aqueous medium, thus they combine in a very complex structure termed the casein micelle. Many properties of milk depend on the state of the casein micelles, and the presence of calcium phosphate clusters in these casein micelles has a vital impact on the structure of the protein particles as well as their technological functionality. Calcium distribution between the colloidal and soluble phases is influenced by environmental conditions like temperature, pH, or the addition of chelators, all of which can modify the amount of micellar calcium phosphate (MCP). Changes in the micellar calcium phosphate (MCP) level within the casein micelle influences the structure and properties of casein micelles, potentially expanding the application range of milk ingredients in various domains such as food, cosmetics, and medicine.

Thus, the primary aim of the study was to examine the influence of varied micellar calcium phosphate concentrations on the structure, stability, and integrity of the casein micelle. To achieve this, the micellar calcium phosphate (MCP) content of the casein micelles was modified through pH adjustment followed by dialysis. The study assessed the turbidity, zeta potential, and particle size distribution of casein micelles, along with the partitioning of calcium and milk proteins between the colloidal and soluble phases of the obtained milk. Additionally, changes in the secondary structure of proteins resulting from the adjustment of MCP content were examined using Fourier Transform Infrared (FTIR) spectroscopy. Furthermore, protein structure was characterized through ^{1}H NMR, while organic and inorganic phosphorus were investigated using ^{31}P NMR. Results showed that MCP adjustment had a notable effect on the calcium concentration, with its amount being significantly reduced by lowering the pH of initial adjustment below 6.7 or enriched by increasing the pH above 6.7. Smallest particle size was observed in the sample with the greatest reduction in MCP content. Concentration of individual caseins in the soluble phase gradually increased with decreasing MCP levels, indicating casein micelle disintegration during adjustment. At ~60% MCP removal, FTIR revealed a critical stage of structural rearrangement, and ^{31}P NMR exhibited an increase in signal intensity for calcium-free Ser-P, further intensifying with decreasing MCP concentration. This study underscored the significance of MCP in preserving micellar structure and its influence on the integrity of the casein micelle.

In the subsequent phase, the thermal stability of MCP-adjusted skim milk samples was studied, given that heat treatment is a common practice in the dairy industry and the thermal stability of milk proteins holds significant importance in these processes. Micellar calcium phosphate plays a crucial role in maintaining the stability of milk proteins. In this regard, four MCP-adjusted samples, comprising 67%, 96%, 100%, and 113% of the original MCP content, were subjected to heat treatment (90 °C for 10 min) at different pH values (6.3, 6.6, 6.9, and 7.2), with subsequent analysis of particle size, turbidity, protein distribution, and structure. The results revealed a substantial influence of MCP level and pH on heat-induced alterations in milk properties, with MCP_{67} samples demonstrating the highest thermal stability. Precisely, a 33% reduction in MCP content (MCP_{67}) exhibited a less pronounced increase in non-sedimentable κ-casein and a reduced decrease in α_{s2}-casein concentrations after heating, in contrast to other samples. The lower MCP content contributed to a moderate increase in average particle size and turbidity, accompanied by lower loading of the β-turn structural component after heating at low pH (6.3). In contrast, MCP_{113} exhibited instability during heating, evidenced by an increase in particle size, turbidity, notable changes in protein concentrations, and a slight elevation in non-sedimentable κ-casein concentration. FTIR results revealed higher loading of intermolecular β-sheet, β-turn, and random coil structures, along with lower loading of α-helix and β-sheet structures in MCP-enhanced skim milk samples. These observations imply substantial alterations in the secondary structure of milk proteins and an enhanced formation of larger aggregates.

This thermal stability is vital in diverse dairy applications, especially in procedures such as ultra-high temperature (UHT) treatment and other sterilization processes. The application of intense temperatures in sterilization (115–120°C for 5–15 min) or UHT (135–150°C for 1–10 s) induces various structural alterations and can pose challenges and concerns in the industry, including protein denaturation, the Maillard reaction, vitamin degradation, and sediment formation on surfaces. Considering the intricate relationship between heat stability and micellar calcium phosphate (MCP) content, the present study investigated the thermal stability of MCP-adjusted skim milk samples including MCP_{67} (33% MCP-depleted), MCP_{113} (13% MCP-enriched), and the control (MCP_{100}) at 120°C for 5 second and 140°C for 1 second across pH levels 6.3-7.2. MCP_{67} exhibited the least decrease in non-sedimentable individual casein levels, displaying lowest particle size and turbidity after heating, indicative of exceptional thermal stability. Conversely, MCP_{113} displayed the highest decrease in these casein levels, with the highest particle size and turbidity, implying comparatively lower thermal stability. Heating at

both 120°C and 140°C at pH6.3, MCP-enhanced and native skim milk samples demonstrated coagulation, while MCP-depleted skim milk remained stable. The results reveal the substantial influence of MCP and pH level on heat-induced alterations in UHT milk.

Moreover, the acid-induced gelation process in milk is a crucial step extensively employed in the production of yogurt and various other fermented dairy products. The amount of micellar calcium phosphate has a substantial impact on the sensory and functional properties of acid-induced gels. Therefore, this study explored how the concentration of micellar calcium phosphate (MCP) and the extent of heating at different pH levels influence the properties of acid-induced gels. Three MCP-adjusted samples, ranging from 67 to 113% of the original MCP content, heated at two temperatures (80 or 90°C) for 10 minutes at various pH values (6.3, 6.6, 6.9, or 7.2). Gelation was initiated by adding GDL at 30°C and stopped when the pH reached 4.5. The distribution of calcium and proteins between colloidal and soluble phases was examined, and small amplitude oscillatory rheology was employed to determine viscoelastic behavior. In skim milk samples depleted of micellar calcium phosphate (MCP), the concentrations of non-sedimentable caseins and whey proteins were found to be higher compared to both control and MCP-enhanced skim milk samples. The influence of MCP adjustment on gelation was contingent upon pH variations. Notably, a 33% MCP-depleted sample exhibited the greatest G' at low pH among all samples, while at other pH levels, MCP100 resulted in the maximum G' surge. Furthermore, the readjusted pH of skim milk played a significant role in gel properties after heating. At alkaline pH values (6.9, 7.2), higher temperatures led to a notable reduction in gelation time. Conversely, at lower pH values (6.3, 6.6), the heating temperature had no observable impact on either gelation time or gelation pH. In summary, this study underscores the substantial impact of MCP adjustment on acid gelation, highlighting a pronounced dependency of the MCP adjustment effect on pH variations.

Overall, this project has established that the combination of FTIR, 1H NMR, and 31P NMR spectroscopies testing holds significant potential as useful tools for achieving a comprehensive understanding of the conformational changes occurring due to micellar calcium phosphate (MCP) adjustment. These changes impact the structure, integrity, and stability of casein micelles in skim milk. Additionally, the study contributes to a better understanding of the role of MCP in various milk processing methods, including heating, ultra-high temperature treatment, and acid-induced gelation. This newfound knowledge can be particularly beneficial in the dairy industry.

Declaration

I, Elaheh Ahmadi, declare that the PhD thesis by Publication entitled "IMPACT OF MICELLAR CALCIUM PHOSPHATE CONCENTRATION ON THE CASEIN MICELLE STRUCTURE, STABILITY AND FUNCTIONALITY" is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures.

[redacted]

Elaheh Ahmadi

Date: 07/03/2024

Dedicated to my beloved husband, parents and brother

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VICTORIA UNIVERSITY

DETAILS OF INCLUDED PAPERS: THESIS WITH PUBLICATION

Please list details of each scholarly publication and/or manuscript included in the thesis submission. Copies of published scholarly publications and/or manuscripts submitted and/or final draft manuscripts should also be included in the thesis submission.

This table must be incorporated in the thesis before the Table of Contents.

Chapter No.	Publication Title	Publication Status • Published • Accepted for publication • In revised and resubmit stage • Under review • Manuscript ready for submission	Publication Details • Citation, if published • Title, Journal, Date of acceptance letter and Corresponding editor's email address • Title, Journal, Date of submission
3	Structural Properties of Casein Micelles with Adjusted Micellar Calcium Phosphate Content	Published	Foods, SJR Q1
4	Thermal stability of skim milk containing various levels of micellar calcium phosphate	Published	Molecules, SJR Q1
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Abbreviations

˚C = degree Celsius

α-La=α-Lactoalbumin

ANOVA= Analysis of variance

β-Lg=β-Lactoglobulin

Ca = Calcium

CCP = colloidal calcium phosphate

CN = casein

FTIR = fourier transform infrared spectroscopy

g = gram

GDL= Glucono delta-lactone

GLM = general linear model

G'= Storage modulus

G″= Loss modulus

h = hour

^{1}H NMR= Proton nuclear magnetic resonance

HPLC= High-performance liquid chromatography

ICP= Inductively coupled plasma atomic emission spectroscopy

kDa = kilodalton

SAS = statistical analysis software

SDS = sodium dodecyl sulphate

SH = thiol group SS = disulphide

SPSS=Statistical Package for Social Sciences

UHT = ultra-high temperature

V = volts

WPC = whey protein concentrate

α = alpha

β = beta

κ = kappa

μg = microgram

μL = microliter

Chapter 1

Chapter 1. Introduction to the thesis

This chapter indicates an introduction to the thesis, providing background information, outlining the aim and specific objectives, and presenting the overall structure of the thesis.

1.1. Background

Milk contains high levels of essential nutritional minerals, such as calcium, magnesium, iron, and zinc. These minerals exist in various forms, either as soluble compounds or as colloidal (micellar) salts (Burrow et al., 2018; Holt et al., 2013; Markoska at al., 2020). The amount of calcium significantly influences the processing and functional attributes of dairy products. In milk, these minerals, along with individual caseins, maintain a dynamic equilibrium between the micellar and serum phases. Soluble or serum calcium exists in either ionic form or forms complexes with citrate and phosphate ions. In the micellar phase, calcium appears as a colloidal or micellar calcium phosphate (MCP) (Gaucheron, 2005) bound to phosphorylated serine residues of the casein micelles (Dalgleish & Corredig, 2012; Huppertz et al., 2017). Approximately 70% of the calcium in milk is incorporated in the casein micelles and about 30% is found in the serum phase (Gaucheron, 2005; Dalgleish & Corredig, 2012).

Caseins, a major group of milk proteins, are individually disordered proteins with a great degree of hydrophobicity, which renders them highly unstable in aqueous solution. For this reason, caseins tend to achieve greater stability by forming colloidal aggregates in milk, which are termed casein micelles. Casein micelle includes four types of caseins named αs_1-, αs_2-, β-, and κ-caseins (Holt et al., 2013; Huppertz, 2013; Grewal, et al., 2018; Grewal, et al., 2020; Markoska at al., 2020). The interactions among caseins and other components in diverse dairy systems play a crucial role in milk functionality, stability and milk processing (Corredig et al., 2019). The ability of caseins micelle to bind minerals such as calcium depends on the environmental conditions including temperature, pH or addition of chelators, which influences the equilibrium between colloidal and serum phases and can alter the amount of MCP (Gaucheron, 2005).

Numerous research outcomes have been published on the interactions between minerals and casein molecules, acknowledging that these bindings hinge on various factors. These factors encompass the form of casein molecules, their phosphorylation state, charge, distinct positive charge of cations, and the nature of binding, whether electrostatic or coordinative. Additionally, the physico-chemical environment, such as pH, ionic strength, and temperature, plays a crucial role in these interactions (Dalgleish, 2011; Broyard & Gaucheron, 2015; Bijl et al., 2013). In this regard, changing pH is important modifications in the salt distribution between aqueous and micellar phases. These modifications rely on the nature of the aqueous phase, the type of added chelators, the added quantities, and the final pH (Broyard & Gaucheron, 2015).

It is still unknown how micellar calcium concentration affects behaviour of individual caseins and assists them in building such a complex structure called casein micelle. It is known that αs_1 casein is calcium sensitive (De Kruif & Holt, 2003). Another casein is αs_2 casein, which is the most calcium sensitive protein compared to others. β-casein is also a calcium sensitive protein due to its ability to bind calcium ions to phosphorylated residues that leads to its precipitation (Huppertz, 2013). The last casein is κ-casein which is recognized to be on the surface of the micelle with the main role to prevent further growth of the micelle. The distribution of calcium phosphate between the casein micelle and serum phase has a large effect on the structure and stability of casein micelles (Dalgleish & Corredig, 2012).

The dissociation of MCP from caseins in milk has been a main topic in a number of studies. The maintenance of the casein micelle structure is closely tied to the content and stability of Ca^{2+} and MCP. Attempts to remove Ca^{2+} ions from the aqueous environment have been shown to result in the disaggregation of micelles. In 1972, Lin reported the possibility of some micellar depletion of Ca^{2+} while retaining partial structural integrity of the casein micelle (Griffin and Griffin, 1988). In 1985, Griffin aimed to estimate the total refractive index of the casein micelles, considering contributions from both protein and inorganic constituents. This endeavour started with the intention of identifying these different contributions through selective removal of micellar calcium phosphate. Griffin found that significant removal of Ca^{2+} was not achievable without some micellar disaggregation. Anema (2009) manipulated the quantity of micellar calcium phosphate (MCP) while maintaining a constant level of serum calcium, and subsequently investigated the gelation properties of acid gels. The findings from this study indicated that MCP played a role in the initial phases of milk gelation during acidification at various temperatures (Anema, 2009).

Silva et al. (2013) introduced a notable approach that involved the preparation of casein suspensions with varied amounts of MCP, maintaining a constant pH and ionic environment in the serum milk phase. Their study delved into the examination of diverse physicochemical and foaming properties exhibited by casein suspensions with varying levels of mineralization, primarily MCP. The outcomes were discussed in the context of the distinct amounts of MCP utilized. The process of demineralization led to the dissociation of casein micelles and a subsequent reduction in foaming ability, attributed to the formation of smaller protein particles within the casein suspensions.

As the importance of calcium roles, consideration of the methods available for the quantification of its effect on the structure and stability of casein micelles is of interest (Corredig et al. 2019). A great deal of studies concentrated on the understanding of its effects via different methods and approaches (Dalgleish, 2011; Holt et al., 2013). Centrifugation and ultrafiltration are frequently employed techniques in the literature; however, these methods are limited and fail to capture milk mineral and protein interactions in their natural state (Burrow et al., 2018). A primary criticism of centrifugation is its tendency to allow low molecular weight peptide fractions to remain in the supernatant, leading to an underestimation of the bound mineral content. Hence, this project aimed to establish a new method for monitoring the structural changes of casein micelles resulting from MCP manipulation. This could be achieved by utilizing Nuclear Magnetic Resonance (NMR) in conjunction with Fourier Transform Infrared Spectroscopy (FTIR). Due to some limitations, NMR could be used to improve predictive modelling achieved by FTIR, which has never been attempted before.

FTIR has been previously successfully established in milk proteins analyses. FTIR could be employed for identification and quantification of different structural modifications of the secondary structure of the caseins (Boiani et al., 2018a; b; Grewal, et al., 2018; Grewal, et al., 2020; Mediwaththea,et al., 2018). NMR is another great technique which was applied as an analytical method in dairy industry for the first time, for studying milk components using spin-lattice relaxation time by Odeblad and Westin (1958). NMR was employed for first time by Williamson in 1985 to determine the globular protein structure (Maher & Rochfort, 2014). NMR method does not need separation and chemical modification and comprehensive information regarding the chemical components of mixtures can be rapidly and directly provided (Gaucheron, 2005; Markoska at al., 2020). There are a very few NMR studies on the individual caseins in the literature. In addition to studies on specific segments on caseins, NMR has also been successfully utilized to detect the position and interactions between colloidal calcium phosphate nanoclusters and phosphoserine regions in caseins using ^{31}P NMR (Belloque, 2008; Holt et al., 2013; Markoska at al., 2020). However, understanding the effect of calcium adjustment on the casein micelle and its structure has not been examined by this technique in literature yet.

In recent years, multiple studies have focused on producing micellar casein with reduced calcium content (Schäfer et al., 2019a; 2020). The initial step to diminish calcium content in milk involves solubilizing calcium, typically achieved through acidification, cooling, or the addition of calcium chelators (Gaucheron, 2005). Subsequently, microfiltration, with or

without a diafiltration (DF) modus, is employed to eliminate solubilized calcium via the permeate from the retentate. Multiple DF stages are commonly utilized to thoroughly wash out soluble calcium (Schäfer, 2019a). Schäfer et al. also noted that a reduction in the calcium content of a skim milk retentate by more than 50% was attainable.

Moreover, in the case of fresh cheese processed from microfiltration (MF) skim milk retentates with varied calcium contents, a reduction of approximately 57% in calcium content before fermentation resulted in a significant decrease of perceived bitterness by over 50% and a reduction of bitter peptide content by approximately 67%, compared to fresh cheese produced from non-calcium-reduced MF milk retentate (Schäfer et al., 2019b). In another study, Schäfer et al. (2020) investigated the processing of micellar casein concentrate (MCC) powders with protein contents of 60% (MCC60) and 85% (MCC85), including a calcium-reduced MCC85. This was achieved through microfiltration (MF) and diafiltration (DF) of skim milk, followed by spray-drying. The calcium-reduced MCC85 was obtained by adjusting the pH of the starting milk to 6.2 and the MF-retentate to 5.6 before MF and DF, resulting in a product with approximately 50% of the total calcium. Calcium reduction enhanced the solubility of MCC85 without affecting viscosity significantly, and it led to a slight increase in casein micelle size. Additionally, acid gels produced from calcium-reduced MCC85 exhibited greater strength compared to other products.

As indicated most of these studies used one of accepted approaches to alter properties of MCP and focused on the impact of these modifications on the processing parameters or a final product. Rarely these studies included a thorough elucidation of structural properties of such modified casein micelles, which is the main focus of the proposed research.

1.2. Research aims and objectives

Protein-mineral interactions play a crucial role in determining the stability of casein micelles, influencing the biological and structural functions of milk. A significant interaction occurs between micellar calcium phosphate (MCP) and individual caseins, leading to the formation of micelles. Since the colloidal behavior of caseins in this context is an actively researched area, the primary objective of this study was to evaluate the impact of adjusting micellar calcium phosphate in skim milk, while preserving the integrity of the casein micelle, on the structural characteristics, stability, and functionality of the casein micelle. This project aimed to advance this field with a focus on establishing and understanding the link between properties of MCP

adjusted by several methods and properties of the casein micelle and skim milk using traditional and modern methods of structural elucidation.

More specific aims were:

- To establish an impact of MCP-adjustment using pH modification followed by dialysis on the conformational structure and integrity of casein micelle examining by FTIR and NMR spectroscopy
- To establish an impact of MCP-adjustment on the properties of MCP, soluble and colloidal phases and structural characteristics of the resulting casein micelle
- To determine the structural changes in the casein micelle caused by mineral interactions by utilizing the Nuclear Magnetic Resonance (NMR) Spectroscopy alone or in combination with Fourier Transform Infrared Spectroscopy (FTIR)
- To elucidate how these MCP-adjustments impact the functional properties of the casein micelle including heat stability (behavior during heating)
- To determine how these MCP-adjustments impact the thermal stability of the MCP-adjusted skim milk in ultra-high temperature conditions
- To evaluate how these MCP-adjustments influence acid-gelation properties of the MCP-adjusted skim milk

1.3. Thesis outline

This thesis is organized into seven chapters. Chapter 1 serves as an introduction, outlining the project and its objectives. In Chapter 2, a comprehensive literature review is presented, summarizing the current scientific knowledge relevant to the thesis. Chapters 3 to 6 encompass complete research articles, including both published and submitted works. Finally, Chapter 7 presents an overall conclusion derived from the study, along with suggestions for future research directions.

The thesis contains following chapters:

Chapter 1-Presents the background and general information of the study including aim, objectives and the structure of the thesis.

Chapter 2-Depicts a literature review with detailed information.

Chapter 3- Focuses on conformational changes of MCP-adjusted skim milk obtained by pH modulation.

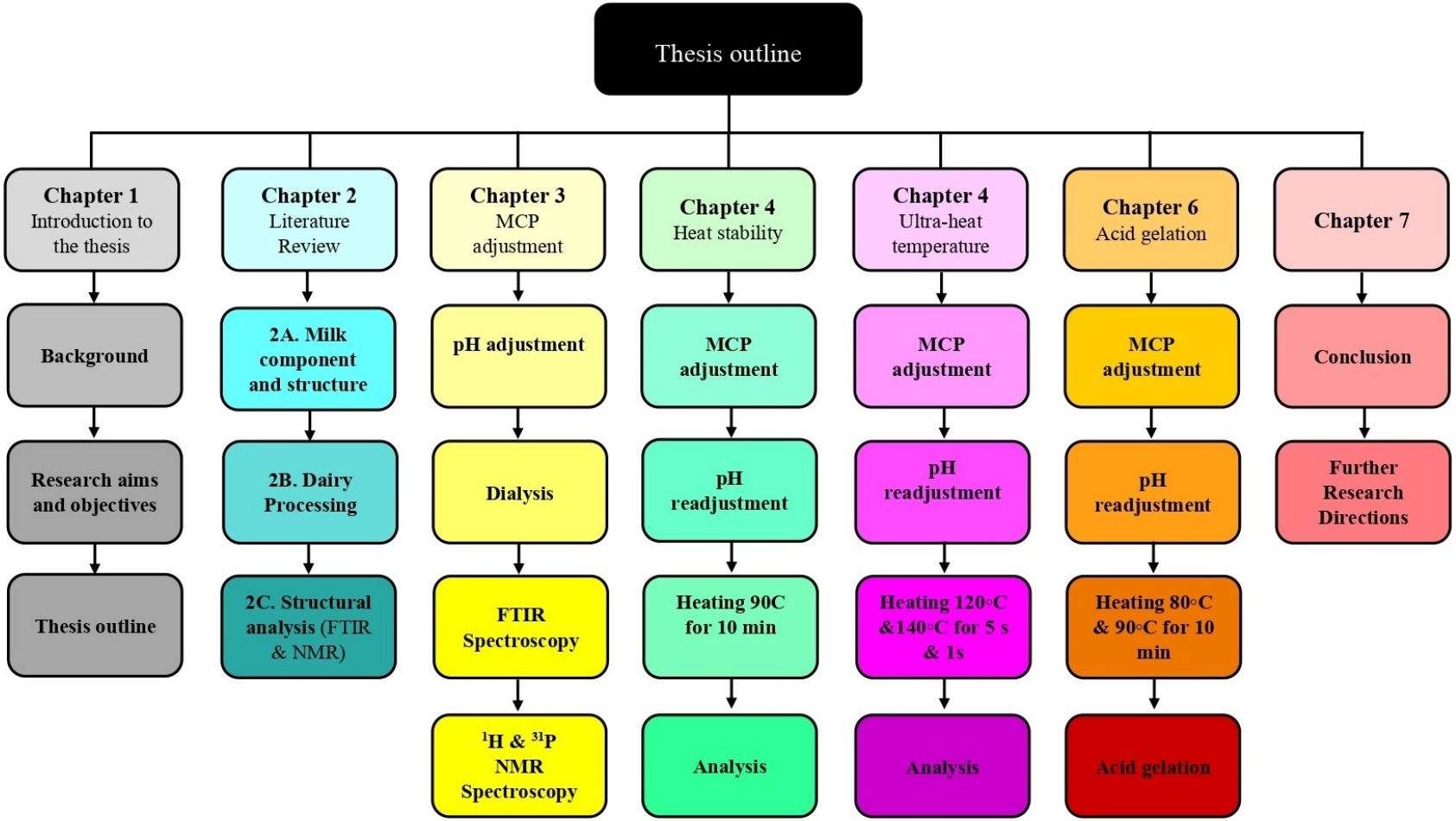
Chapter 4-Focuses on thermal stability of micellar calcium phosphate (MCP) adjusted skim milk.

Chapter 5 - Focuses on ultra-high temperature stability of the micellar calcium phosphate (MCP) adjusted skim milk.

Chapter 6 - Focuses on acid gelation of the micellar calcium phosphate adjusted skim milk

Chapter 7 – Conclusions and future directions

Figure 1.1. Outline of thesis



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Chapter 2

Chapter 2. Supplementary literature review

This chapter provides supplementary literature related to the study, elucidating fundamental concepts and presenting key research findings.

2.1 Milk

Milk is a highly nutritious food, is excreted by lactating cows with its principal function being the fulfilment of the comprehensive the nutritional needs of mammals (de la Fuente & Juarez, 2015; Fox, 2003; O'Mahony & Fox, 2014). Milk containing many macronutrients such as proteins (caseins, whey proteins), fat and carbohydrates, as well as micronutrients more specifically minerals and importantly calcium. Bovine milk comprises approximately 87% water, 3.9% fat, 3.5% protein, 4.6% lactose, 0.65% mineral substances, and various minor constituents including enzymes (such as peroxidase, catalase, phosphatase, and lipase), gases (including oxygen and nitrogen), and vitamins (A, C, D, thiamine, riboflavin) (Fox, 2003). Across the globe, over 6 billion people consume milk and dairy products, with the majority residing in developing nations. To meet the rising demand, milk production must increase by nearly 2% annually. Approximately 150 million households worldwide are involved in milk production, primarily small-scale farmers in developing countries. This activity significantly supports household livelihoods, food security, and nutrition, offering quick returns and a vital source of cash income for small-scale producers. Over the past 30 years, global milk production has surged by more than 50%, rising from 500 million tons in 1983 to 769 million tons in 2013 (Kapaj, 2018). Milk is a liquid with a white or yellowish-white colour, where its components - including proteins, lactose, fat, and ash - function as solutes, while water serves as the solvent in the milk matrix (Holt, 1997; Fox, 2003). The physical properties of milk, resembling water but altered by the presence of various solutes, undergo modification in the continuous phase due to the dispersion of emulsified and colloidal components (Fox & McSweeney, 1998).

2.1.1 Major milk constituents

2.1.1.1 Water

Among these constituents, water plays a pivotal role, serving as the primary solvent and carrier for other components. On average, cow's milk, which is the most commonly consumed type, typically contains about 87% to 90% water. The water content in milk varies widely depending on factors such as species, breed, lactation stage, and environmental conditions. Additionally, variations can occur due to processing methods and the removal or addition of certain components in the production of different types of milk products. Water is crucial in maintaining the solubility and bioavailability of essential nutrients in milk, particularly

calcium. Calcium in milk exists primarily in a soluble form, making it highly accessible for absorption by the human body. This high water content helps ensure that calcium and other nutrients are effectively distributed throughout the milk, enhancing its nutritional value (Swaisgood, 1995).

2.1.1.2 Lactose

Lactose, the predominant carbohydrate in milk, is a unique disaccharide composed of galactose and glucose linked by a β1-4 glycosidic bond. This specific arrangement distinguishes lactose as a carbohydrate exclusive to milk. From a nutritional perspective, lactose plays a vital role as a source of energy for neonates. It contributes to approximately 30% of the calories in milk and serves as an alternative to energy-dense lipids (Fox, 2003). This dual function underscores the importance of lactose as a key component in the nutritional profile of milk, particularly in supporting the energy needs of newborns (Huppertz, 2022). Lactose also facilitates calcium absorption in the small intestine. This interaction enhances the bioavailability of calcium, making it easier for the body to absorb and utilize this essential mineral. The presence of lactose in milk not only provides an energy source but also optimizes the nutritional value of milk by enhancing calcium uptake, which is crucial for bone development and overall health (Areco et al., 2015).

Table 2.1 Composition of milk (Jensen et al., 1991).

Milk Component	Average content (w/w, %)		
	Total	Colloidal	Serum phase
Water	87	8	79
Lipids	4.07	4.06	0.02
Protein	3.23	2.67	0.56
Carbohydrates (lactose)	4.60	-	4.60
Mineral substance	0.70	0.20	0.48
Organic acids (Citrate, Others)	0.19	0.03	0.16

2.1.1.3 Milk Fat

Milk fats serve as a crucial energy source for neonates, offering essential fatty acids and fat-soluble vitamins. Beyond their nutritional role, milk fats contribute significantly to the sensory attributes of milk and dairy products, imparting flavor and mouthfeel. Nearly 99% of milk

lipids exist in the form of milk fat globules, lipid spheres with diameters ranging from 0.1 to 15 µm, averaging around 4 µm (Fox & McSweeney, 1998; Heid & Keenan, 2005). In commercial milk processing, homogenization is employed to enhance stability during storage by reducing the size of fat globules. This process involves pumping milk through a small orifice at high pressure and temperatures exceeding 37°C. Homogenized milk exhibits improved characteristics, such as the absence of a cream layer upon storage and enhanced flavour and texture. This technological intervention ensures a more consistent and appealing product for consumers (Chandan, 2011).

Table 2.2 Fat composition of milk (Jensen et al., 1991).

Component	Average content (w/w, %)		
Lipids	Total	Colloidal	Serum phase
Triglycerides	4.0	-	-
Diglycerides	0.01	0.01	-
Phospholipids	0.035	0.025	0.01
Sterols	0.014	0.012	0.002
Free fatty acids	0.01	0.008	0.002
Cerebrosides	0.005	0.003	0.002

2.1.1.4 Vitamins

Milk is a good source of various vitamins, providing essential nutrients that contribute to overall health. The vitamin content in milk can be affected by factors such as processing methods, storage conditions, and the cow's diet. Additionally, some vitamins may be lost during the processing of certain dairy products (Fox et al., 2015b). Cow's milk naturally contains vitamin D, which is essential for calcium absorption and bone health. Milk is a good source of vitamin A in the form of retinol. Vitamin A is crucial for maintaining healthy skin, vision, and immune function. Riboflavin is important for energy metabolism and helps maintain healthy skin, eyes, and nerve functions. Milk is a significant source of riboflavin. Milk contains vitamin B_{12}, a vital nutrient for the formation of red blood cells and neurological function (Voronina et al., 2022). Niacin is involved in energy metabolism, and milk contributes to niacin intake in the diet. Pantothenic acid, important for energy metabolism, is found in milk. Pyridoxine is involved in the metabolism of amino acids and is present in milk. Milk contains small amount of vitamin E, an antioxidant that helps protect cells from damage (Fox et al., 2015b).

2.1.1.5 Minerals

Bovine milk is reported to contain around 8-9 g L^{-1} (0.7% w/w) of minerals (Walstra et al., 1999; Gaucheron, 2005). Beyond their nutritional significance for human health, these minerals play a pivotal role in shaping the properties of milk, ultimately influencing the quality of dairy products. Noteworthy is the strong interaction between minerals and casein, a key milk protein. Changes in the concentration or equilibrium of minerals can lead to alterations in the structure of casein, consequently impacting the overall properties of milk (Fox & McSweeney, 1998; Holt, 1997). The intricate interplay between minerals and milk components underscores the importance of mineral composition in defining the characteristics of dairy products. Calcium is a predominant mineral in milk and plays a central role in bone and teeth formation, blood clotting, and nerve transmission. The concentration of calcium can vary between milk types and species. Calcium provides stability to milk and influences its textural properties. Calcium is a principal mineral contributing to the structural integrity of milk, forming complexes with casein proteins to create the micellar structure. Phosphorus, often in conjunction with calcium, is essential for bone formation and mineralization, influencing the structural composition of milk by contributing to the development of the casein micelle. Potassium and sodium act as an essential electrolyte, contributing to the osmotic balance of milk (Gaucheron, 2005).

Table 2.3 Mineral composition of milk (Gaucheron, 2005).

Component	Average content (w/w, %)		
Minerals	Total	Colloidal	Serum phase
Calcium	0.117	0.080	0.037
phosphate	0.21	0.1	0.11
Potassium	0.143	0.011	0.132
Magnesium	0.011	0.004	0.007
Sodium	0.048	0.002	0.046
Chloride	0.11	-	0.11
Sulphate	0.01	-	0.01
Bicarbonate	0.01	-	0.01

2.1.1.6 Milk Proteins

Bovine milk comprises approximately 3.5% protein (Fox & McSweeney, 1998). The primary role of milk protein is to provide essential amino acids necessary for the growth of young

neonates. Proteins in milk play a crucial role in shaping the characteristics of the milk by influencing its physical properties and engaging in interactions with other components. There are two major categories of milk proteins that are defined by their chemical composition and physical properties: casein and whey (Damodaran, 1997). Milk protein primarily consists of caseins and a smaller portion of whey proteins. Casein accounts for about 80% of the total protein content, while whey proteins make up the remaining 20% (Huppertz et al., 2017) (Table 2.5).

Table 2.4 Protein composition of milk (Walstra et al., 2005).

Component	Average content (w/w, %)		
Protein	Total	Colloidal	Serum phase
Casein	2.6	2.6	-
αs_1-casein	1.02	1.02	-
αs_2-casein	0.28	0.28	-
β-casein	0.93	0.93	-
κ-casein	0.37	0.37	-
Whey protein	0.56	-	0.56
β-Lactoglobulin	0.32	-	0.32
α-Lactalbumin	0.12	-	0.12
Bovine serum albumin	0.04	-	0.04
Immunoglobulins	0.08	-	0.08

2.1.1.6.1 Whey Proteins

Whey proteins, derived from the liquid portion of milk after cheese production, are known for their diverse and intricate characteristics. Comprising a mixture of individual proteins, whey exhibits a rich protein profile, each component contributing distinct properties to its overall functionality. The primary categories of whey proteins include α-Lactalbumin (α-LA), β-Lactoglobulin (β-LG), Immunoglobulins (Ig), and Lactoferrin. These proteins contribute to various physiological and functional properties of whey, making it a valuable ingredient in food and beverage formulations.

2.1.1.6.1.1 α-Lactalbumin (α-LA)

α-Lactalbumin (α-LA) is a significant component comprising approximately 20-25% of whey proteins. It is synthesized and secreted by mammary epithelial cells and plays a crucial role in lactose synthesis and milk production. α-Lactalbumin is a globular protein with a relatively

compact structure stabilized by disulfide bonds. It consists of a single polypeptide chain folded into a well-defined three-dimensional shape, with a molecular weight typically around 14 kDa. α-La is composed of 123 amino acid residues. Structurally characterized by its alpha-helix-rich composition, α-lactalbumin plays a pivotal role in diverse biological processes. One of its primary functions is involvement in lactose synthesis, contributing to the carbohydrate composition of milk. Additionally, α-lactalbumin has been implicated in immune regulation, showcasing its multifaceted roles in supporting the nutritional and immune aspects of milk. The intricate structure and functional versatility of α-lactalbumin underscore its importance in the complex matrix of whey proteins within milk. α-Lactalbumin is relatively heat-stable compared to other whey proteins, retaining its structural integrity and functional properties under moderate heat treatment. However, excessive heat can denature α-Lactalbumin, leading to changes in its structure and functionality (Fox et al., 2015 a) (Figure 2.1).

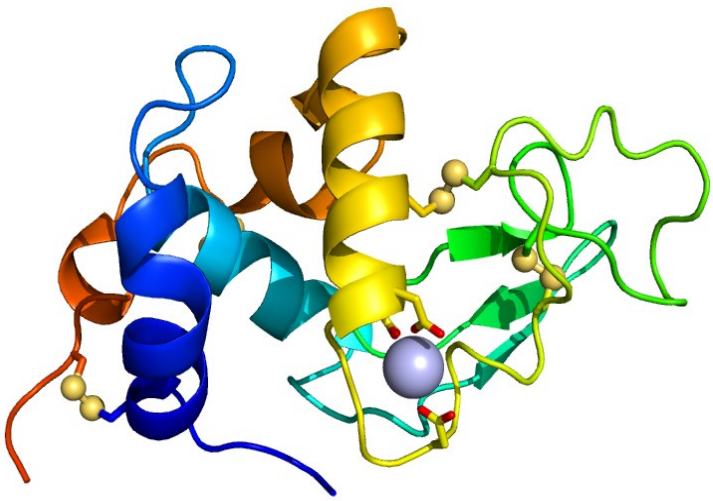

Figure 2.1 Structure of α-lactalbumin (α-LA) (Jøhnke, & Petersen, 2012)

2.1.1.6.1.2 β-Lactoglobulin (β-LG)

β-Lactoglobulin stands out as a beta-sheet-rich protein, holding the title of the most abundant whey protein, making up approximately 50-60% of the whey protein content in milk. It is a protein with 162 amino acid residues and a molecular weight of approximately18 kDa. β-Lactoglobulin contains all the essential amino acids required by humans, making it a high-quality protein source with excellent nutritional value. It is particularly rich in hydrophobic amino acids, such as leucine, isoleucine, and valine. β-Lactoglobulin is a globular protein with a well-defined tertiary structure. It consists of a single polypeptide chain folded into a compact,

globular shape, stabilized by intramolecular disulfide bonds (Edwards & Jameson, 2014; Wijayanti et al., 2014). Renowned for its role in binding hydrophobic molecules, beta-lactoglobulin significantly contributes to the nutritional value of milk. Its beta-sheet-rich structure highlights its functional versatility, enabling interactions with various molecular entities and, consequently, playing a pivotal part in enhancing the overall nutritional profile of milk (Fox et al., 2015a) (Figure 2.2).

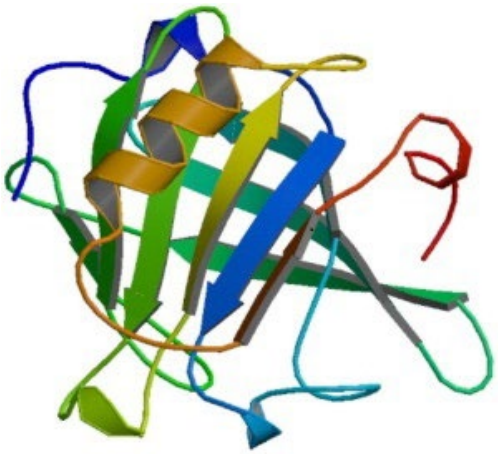

Figure 2.2. Structure of β-lactoglobulin (β-LG) (Cheison, Leeb, Toro-Sierra, & Kulozik, 2011).

2.1.1.6.1.3 Immunoglobulins (Ig)

Immunoglobulins, also known as antibodies, exhibit diverse structures contributing to their vital role in the immune system. Although their percentage in milk is relatively low, their significance cannot be understated. Immunoglobulins play a crucial role in immune defense by binding to pathogens, actively participating in the body's immune response (Wijayanti et al., 2014). Despite their lower abundance in milk, the presence of immunoglobulins underscores the importance of milk as a source of immune-supporting elements, providing additional value beyond its nutritional components (Wijayanti et al., 2014; Fox et al., 2015 a).

2.1.1.6.1.4 Lactoferrin

As a globular protein with the unique ability to bind iron, lactoferrin plays a vital role in milk, albeit in relatively small amounts. Its function extends beyond simple iron absorption, as lactoferrin exhibits potent antimicrobial properties. This multifaceted protein contributes to the

overall biological activity of milk, showcasing the complexity of its components beyond their nutritional aspects. The presence of lactoferrin underscores the intricate balance of bioactive elements within milk, offering additional health-related benefits to consumers (Pereira et al., 2007; Fox et al., 2015 a).

2.1.1.6.2 Caseins

Caseins, the major group of milk proteins, are individually disordered proteins with a great degree of hydrophobicity and low stability in an aqueous medium (Tuinier and Kruif, 2002). These caseins interact with each other and together with calcium phosphate resulting in complex structures named casein micelles (Huppertz, 2013). Casein is specifically defined as the protein that precipitates at pH 4.6. Additionally, caseins coagulate in response to the action of rennet, which involves chymosin and other proteinases. These phosphorylated proteins, with an average phosphorous content of 0.85%, possess the ability to bind significant amounts of calcium (Fox et al, 2015a). The molecular weight of caseins ranges from 20 to 25 kDa. Caseins exhibit relative heat stability, although heating above 120°C induces chemical changes that render them insoluble (Walstra et al., 2005). This heat stability is attributed to their less ordered structural organization, including secondary and tertiary structures, largely influenced by a high proline content. However, the complete elucidation of the secondary and tertiary structure of caseins remains incomplete due to challenges in their crystallization. The casein micelle consists of four main types of individual caseins including αs_1-casein, αs_2-casein, β-casein, and κ-casein each contributing to the unique properties and functionality of the micelle (Swaisgood, 2003; Pritchard & Kailasapathy, 2011; Huppertz, 2013).

Table 2.5 Key characteristics of four caseins (Huppertz, 2013).

	αs_1-casein	αs_2-casein	β-casein	κ-casein
Molecular weight (Da)	23599	25206	23973	19052
Number of amino acid residues	199	207	209	169
Serine	16	17	16	13
Proline	17	10	35	20
Cysteine	0	2	0	2
Lysine	14	24	11	9
Positively charged residues	25	33	20	17
Negatively charged residues	40	39	28	28
Aromatic residues	20	20	14	14

2.1.1.6.2.1 αs_1-casein

αs_1-casein has a molecular weight in the range of approximately 24 to 25 kDa. Like other casein proteins, αs_1 Casein undergoes phosphorylation. Phosphate groups are added to the protein, contributing to the unique functional properties of casein micelles, including their ability to bind calcium and stabilize the colloidal structure (Swaisgood, 2003; Huppertz, 2013).

The conformational structure of αs_1-casein, like other casein proteins, is complex and involves both ordered and disordered regions. Caseins are amphiphilic proteins, meaning they have both hydrophilic and hydrophobic regions. αs_1-casein has a unique amphiphilic structure, where hydrophilic (water-attracting) and hydrophobic (water-repelling) regions coexist. This amphiphilic nature allows caseins to form micelles in milk. Caseins, including αs_1-caseins are known for having highly disordered or unstructured regions. These disordered regions make the casein micelles flexible and dynamic (Huppertz, et al., 2018).

αs_1-casein undergoes phosphorylation, where phosphate groups are added to specific amino acid residues. Phosphorylation occurs at multiple sites within the protein. This phosphorylation is crucial for the stabilization of casein micelles and their ability to bind calcium. The phosphorylated regions of αs_1-casein play a significant role in binding calcium ions. This interaction is fundamental to the colloidal stability of casein micelles and the supersaturation of calcium in milk. The disordered regions in the conformation of αs_1-casein contribute to the structural flexibility of casein micelles. This flexibility allows the micelles to adapt to changes in environmental conditions and processing (Holt, 1992).

2.1.1.6.2.2 αs_2-casein

αs_2-casein is another major type of casein protein found in bovine milk. αs_2-casein has a molecular weight similar to other casein proteins, typically ranging from 25 to 27 kDa (Farrell et al., 2004). Similar to other caseins, αs_2-casein undergoes phosphorylation, where phosphate groups are added to specific amino acid residues. This phosphorylation is essential for the functional properties of casein micelles. αs_2-casein, as part of the casein micelles, contributes to the colloidal stability of milk. Casein micelles are crucial for dispersing and stabilizing fat

globules and other components in milk, preventing their aggregation. αs_2-casein, like other caseins, has an amphiphilic structure, meaning it has both hydrophilic and hydrophobic regions. The structural flexibility of caseins allows them to form micelles with dynamic properties (Pritchard & Kailasapathy, 2011).

2.1.1.6.2.3 β-casein

β-casein, like other caseins, serves as a crucial source of essential amino acids, providing the necessary building blocks for growth, especially in neonates. It consists of 209 amino acids and possessing a molecular weight of approximately 24 kDa, β-casein stands out as the most hydrophobic among all caseins (Swaisgood, 2003). Structurally, it is a phosphoprotein, containing phosphate groups. β-casein exhibits a complex structure, and its characteristics can vary due to genetic polymorphisms, resulting in different variants such as A_1 and A_2 β-casein. In cow's milk, β-casein constitutes a significant portion of the total protein content. β-casein contributes to the structure of casein micelles. During processing, β-casein can interact with other milk components, affecting the physical properties of milk and dairy products (Pritchard & Kailasapathy, 2011).

2.1.1.6.2.4 κ-casein

κ-casein is one of the four main types of casein proteins found in bovine milk and has a molecular weight of approximately 19 kDa. It consists of 169 amino acids (Pritchard & Kailasapathy, 2011; Holt, 1992). Similar to other caseins, κ-casein undergoes phosphorylation, where phosphate groups are added to specific amino acid residues. Phosphorylation is critical for the functional properties of casein micelles, including their interaction with calcium. κ-casein plays a crucial role in the stabilization of casein micelles in milk. It is responsible for preventing the aggregation and coagulation of casein micelles, contributing to the colloidal stability of milk. κ-casein, along with other caseins, interacts with calcium ions, contributing to the unique properties of casein micelles. This interaction is essential for the stability and functionality of milk. κ-casein, like other caseins, has an amphiphilic structure with both hydrophilic and hydrophobic regions. This structural feature allows caseins to form micelles in milk (McMahon & Oommen, 2008; Huppertz, 2013).

2.1.1.6.2.5 Casein micelles

Casein micelles are characterized as heterogeneous, dynamic, polydisperse spherical structures with an average diameter of 200 nm (Dalgleish & Corredig, 2012; Horne, 2009). The manner in which caseins interact among themselves and the other components in various dairy systems affects milk functionality including stability, nutritional properties, and technological/processing characteristics (Burrow et al., 2018; Corredig et al., 2019). They are composed of four types of caseins named αs1-, αs2-, β-, and κ-caseins (CNs), connected via various interactions and, more importantly, linked through calcium phosphate deposits described as micellar calcium phosphate (MCP) nanoclusters via phosphoserines located on these proteins. This colloidal casein-calcium phosphate complex consists of 94% protein and 6% inorganic constituents (Holt 1992; Horne, 2003) (Figure 2.3). Casein micelles are complex structures found in bovine milk and are primarily composed of various types of casein proteins. These micelles play a crucial role in the unique properties of milk, particularly its ability to maintain high concentrations of calcium and phosphate. These proteins are amphiphilic, meaning they have both hydrophilic and hydrophobic regions. Electron microscopy studies have revealed that casein micelles are generally spherical, with diameters ranging from 40 to 500 nm. The molecular weight of casein micelles is in the range 106 - 109 Da (Walstra, Wouters & Geurts, 2006; De Kruif & Holt, 2003). Casein proteins within the micelles are phosphorylated, meaning they contain phosphate groups. This phosphorylation contributes to the micelles' ability to bind calcium and helps maintain their stability. Casein micelles contribute to the colloidal nature of milk, preventing the aggregation of proteins and keeping them dispersed in the liquid. This dispersion contributes to the white appearance of milk and its overall stability (Fox & McSweeney, 1998; Holt et al., 2013; Malacarne et al., 2014). Phosphates within the casein micelle contribute to this unique structure, existing as either Micellar calcium phosphate (inorganic phosphate) or covalently bound to caseins as phosphate groups (organic phosphate) (Fox & McSweeney, 1998). This intricate arrangement allows casein micelles to effectively sequester and stabilize calcium within the milk matrix, influencing the overall composition and properties of milk. The ability of casein micelle to maintain its structural integrity under the influence of adverse environmental conditions is referred to as intramicellar stability (Liu & Guo, 2008). The unique structure of casein micelles allows milk to be supersaturated with respect to calcium. Modifying the MCP level of casein micelles in milk can impact their structure and properties, potentially expanding the application

range of milk ingredients in various domains such as food, cosmetics, and medicine. (Liu and Guo, 2008; Holt et al., 2013).

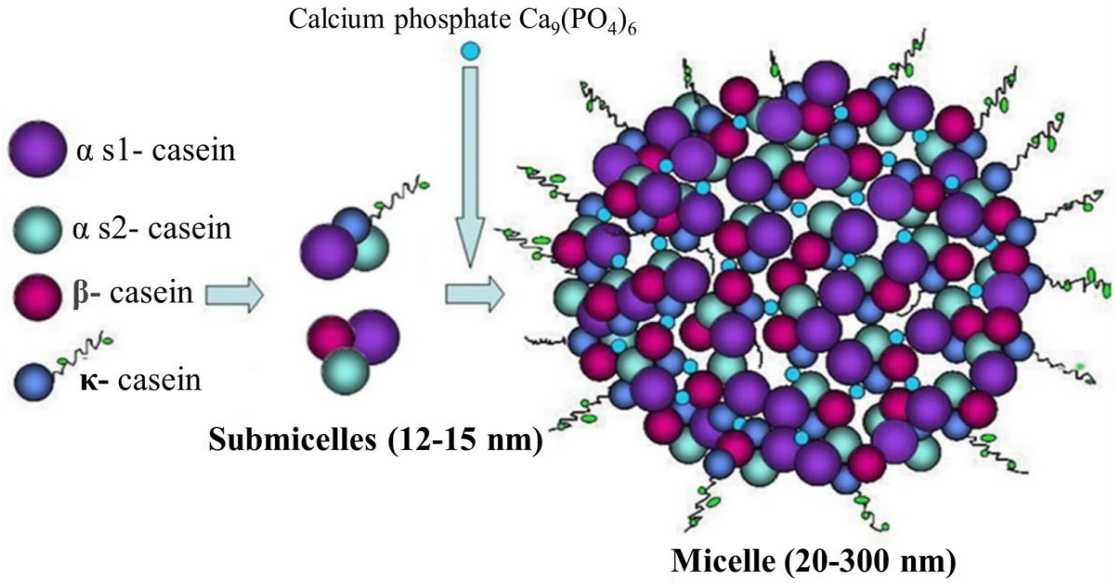

Figure 2.3 Casein micelle structure (Petrova et al., 2022).

2.1.1.6.2.6 Casein micelle structure

The casein micelle in milk is a complex and dynamic structure with a unique organization of proteins and minerals (De Kruif, 1999). While the exact structure is challenging to characterize due to its dynamic nature, several models have been proposed to provide insights into the organization of the casein micelle. Several models and their refinements have described the casein micelle structure over the years but all of them agree that the k-caseins are found on the surface of the micelles to form a hairy layer, providing stability to the micelle (De Kruif et al., 2012; Huppertz et al., 2017).

The submicelle model, introduced by Schmidt in 1982, suggests that casein micelles consist of smaller protein subunits linked via colloidal calcium phosphate (CCP). Within the casein micelle, submicelles lacking κ-casein are situated in the micelle's interior, whereas submicelles with elevated levels of κ-casein are positioned on the micelle's surface. The glycosylated hydrophilic segments of κ-casein extend from the micelle surface like 'hairs,' contributing to stability through steric and electrostatic repulsion (Schmidt, 1982). Walstra later proposed that CCP is inside the submicelle, linked through hydrophobic interactions (Walstra, 1999).

Following the submicelle model, the dual-binding model proposes that individual caseins are cross-linked through hydrophobic regions, and clusters of calcium phosphate act as bridges facilitating the assembly of the casein supramolecule. Electrostatic repulsion hinders the growth of hydrophobically bonded proteins. κ-casein is integrated into the casein supramolecule through hydrophobic bonding of its N-terminal region (Horne, 1998, 2002, 2006, 2008, 2014).

In the internal structure model, κ-casein has an uneven distribution on the micelle surface, creating clefts exposing other caseins to serum. The micelle's interior contains subassemblies of αs, some β-casein, and CCP. β-casein acts as a crucial surfactant for stabilizing water within the micelles (Dalgleish, 2011).

In the nanocluster model, the casein micelle is viewed as a homogeneous matrix with dispersed colloidal calcium phosphate (CCP) nanoclusters resembling 'cherry stones'. Phosphorylated amino acid residues of caseins bind to the surface of the nanocluster. The tails of caseins create a protein matrix with density fluctuations at the 2nm scale through weak interactions. κ- casein limits self-association, contributing to micelle stabilization (Holt, 1992; Holt & Horne, 1996). The process of casein binding to calcium phosphate nanoclusters serves as the initiation step for the formation of the casein supramolecule. This idea has been supported by various studies (De Kruif & Holt, 2003; de Kruif, et al, 2012; Holt, 1992; Holt & Horne, 1996; Holt, 1998). One commonly accepted representation describes the casein micelle as a network of primary casein particles linked by micellar calcium phosphate nanoclusters (Huppertz et al., 2017).

2.1.1.6.2.7 Micellar calcium phosphate

Micellar calcium phosphate (MCP) refers to the fraction of inorganic constituents within the casein micelle, predominantly composed of calcium and phosphate ions. MCP exists primarily in the form of nanoclusters, characterized by an amorphous calcium phosphate core with a diameter of several nanometers. These nanoclusters are surrounded by a stabilizing shell consisting of αs_1-, αs_2-, and β-caseins (Huppertz & de Kruif, 2007). In milk, calcium phosphates exist in various forms with different Ca/P ratios and structural arrangements, encompassing both amorphous and crystallized states (Gaucheron, 2005). Table 2.6 outlines potential forms of calcium in milk. Determining the composition of micellar calcium phosphate (MCP) in the colloidal phase proves challenging experimentally due to the inseparability of calcium bound to organic or inorganic phosphates.

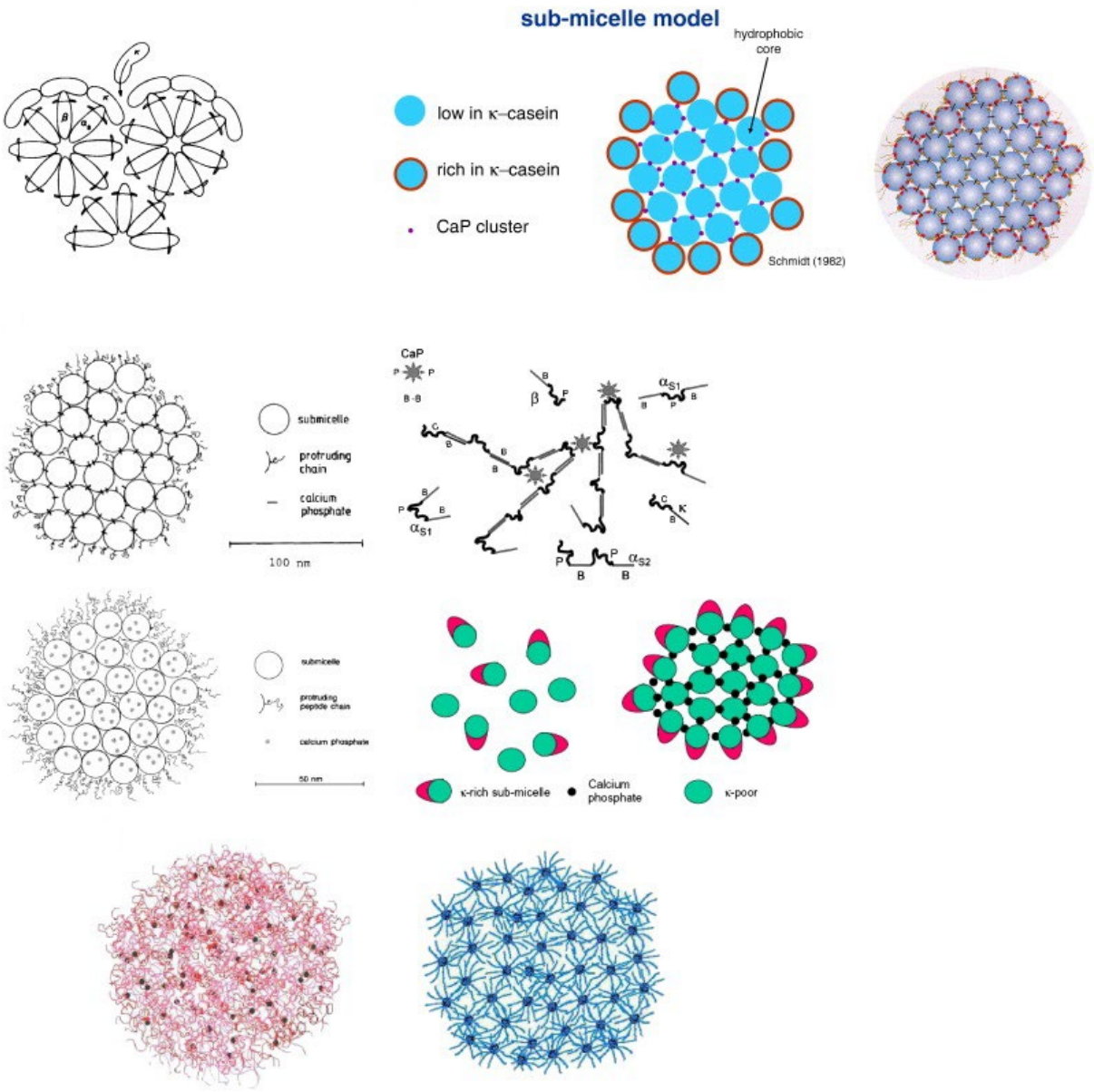

Figure 2.4 Casein micelle structure models (De Kruif et al., 2012).

It is the ability of the caseins to sequester calcium (Ca) and thus deliver it in a concentration far above its solubility. This is possible since the majority of Ca in milk is in the form of micellar calcium phosphate (MCP) complex within the structure of the casein micelle. A casein micelle contains 10^4 polypeptide chains of casein molecules associated with about 3×10^3 MCP nanoclusters. Approximately 2/3 casein is directly bound to the MCP via negative charges of the phosphoserine residues and reducing the electrostatic repulsion in the casein micelles (Gaucheron, 2005; Dalgleish & Corredig, 2012; Huppertz et al., 2017). Approximately two-thirds of the total calcium content exists in a colloidal form associated with the micelles. This

association occurs in two primary forms: Micellar calcium phosphate (MCP) and calcium ions bound to the phosphoserine residue (Deeth & Lewis, 2015; Flynn & Cashman, 1997).

Table 2.6 The Ca/P ratios of different calcium salts in milk (Gaucheron, 2005)

Compound	Ca/P
Dicalcium phosphate	1
Dicalcium phosphate dihydrate	1
Micellar calcium phosphate	1.1
β-tricalcium phosphate	1.5
Octacalcium phosphate	1.33
Hydroxyapatite	1.67
Tricalcium citrate dihydrate	N.A
Amorphous calcium phosphate	1.45

This complex has a vital impact on the structure of the protein particles as well as their technological functionality (Tuinier and Kruif, 2002). Out of total calcium content, approximately 70% is incorporated in the casein micelles (protein-bound calcium, colloidal) and about 30% is found in the serum phase (diffusible calcium). A part of the soluble calcium is present in an ionic form or as complexes with citrate and phosphate ions. Calcium distribution between these two phases is in equilibrium and influenced by environmental conditions such as temperature, pH, or addition of chelators, all of which can alter the amount of MCP (Gaucheron, 2005; Dalgleish, 2011; Huppertz, Fox, & Kelly, 2018; Schäfer, et al., 2020). The distinctive structure of casein micelles imparts the ability for milk to be supersaturated with respect to calcium. Alteration of the MCP level of the casein micelle in milk can affect the structure and property of casein micelles, which may broaden the application range of milk ingredients in food, cosmetic, and medicine domains (Liu and Guo, 2008).

2.1.1.6.2.7.1 Micellar calcium phosphate adjustment

The amount of calcium is a significant factor influencing the sensory and functional characteristics of fermented dairy products as well as dairy powders (Lucey & Fox, 1993; Mistry & Maubois, 2017). In addition, the amount of soluble calcium affects rennetability, thermal stability, and rheological properties of various dairy products. The high calcium content can reduce the melting ability of cheddar cheese (Mistry & Maubois, 2004) and

increase the perceived bitterness level (Schäfer et al., 2019b). In powder processing, the reconstitution properties of milk protein concentrate (MPC) powders can be affected negatively by $CaCl_2$ (Schokker et al., 2011), thus, calcium reduction was proposed as a way to improve the functionality of the powder. In recent years, researchers have worked to modify the content of MCP (Schäfer et al., 2019; 2021) and milk (Pyne & McGann, 1960; Eshpari et al., 2015; Huppertz & Lambers, 2020).

In the context of adjusting MCP content, McGann and Pyne (1960) introduced a technique involving pH modification, using NaOH for an increase and GDL for a decrease. Dialysis against bulk milk after this process restores soluble calcium content, with MCP content remaining either low or high, leading to an overall decrease or increase in calcium content (McGann and Pyne, 1960). This method has been applied in various studies, including investigations into the influence of MCP concentration on milk heat stability, rennet and acid gelation properties (Anema, 2009; Ozcan, Horne, & Lucey, 2011).

Early investigations suggested that alterations in micellar calcium phosphate (MCP) levels within the casein micelle could improve the thermal stability of skim milk (Fox & Hoynes, 1975). These studies underscored the importance of understanding the interplay between MCP levels and the thermal characteristics of dairy systems, providing valuable insights into potential strategies for optimizing the heat stability of milk (Fox & Hoynes, 1975; Singh, & Fox, 1987). Fox and Hoynes (1975) demonstrated that with a reduction in MCP content, there was an extension in the heat coagulation time (HCT) of milk. Singh and Fox (1987) noted that modifying MCP had a negligible effect on non-sedimentable nitrogen levels but slightly elevated the concentration of non-sedimentable N-acetylneuraminic acid (NANA), which appeared to persist at higher levels upon heating. Anema and Li (2000) demonstrated the impacts of different MCP levels and selected pH on the heat-induced alterations in reconstituted skim milk. Their findings indicated that heightened MCP levels had minimal effects on the dissociation of casein micelles, whereas a decrease in MCP content led to more significant micellar dissociation. These studies underscored the vital role of MCP in preserving micellar structure and influencing micelle stability during heating.

Modification in micellar calcium phosphate (MCP) levels within the casein micelle have the potential to impact milk gelation (Famelart et al., 2009; Ozcan et al., 2007; Peng, Horne, & Lucey, 2009). In a prior investigation, the modification of MCP by introducing varying trisodium citrate (TSC) levels to fermented reconstituted skim milk, inoculated with a yogurt

starter culture, resulted in heightened gel stiffness and reduced whey separation with low TSC levels. The authors postulated that the minimal removal of CCP at low levels facilitated an enhanced rearrangement and molecular mobility of the micelle structure, potentially contributing to increased crosslink formation between strands in yogurt gel networks (Ozcan et al., 2007).

Alterations in micellar calcium phosphate (MCP) levels within the casein micelle could impact milk gelation (Famelart et al., 2009; Ozcan et al., 2007; Peng et al., 2009). Famelart et al. (2009) conducted a study on the acid gelation of calcium-depleted skim milk through dialysis against milk permeate containing a cation-exchange resin, followed by heating and acidification with a yogurt culture. Their findings indicated that a 30% calcium depletion elevated the gelation pH, whereas more extensive depletion led to a decrease in gelation pH (Famelart et al., 2009). In a related exploration, Peng, Horne, & Lucey investigated the effects of pre-acidifying the pH before heating and fermentation by a yogurt starter culture. They observed that lower pH levels resulted in weaker gels (Peng et al., 2009). Anema (2009) observed that gradually removing MCP from milk before heat treatment and acidification minimally affected gelation pH but significantly reduced G'. These results imply that G' is influenced by MCP levels during acidification, with higher MCP contributing to the formation of a more elastic gel. In another study, Ozcan, Horne, & Lucey increased the MCP content of milk using NaOH and examined the impact of MCP-enriched skim milk on acid gels produced by yogurt culture (Ozcan et al., 2011).

2.1.2 Secondary structure of milk proteins

Over the last three decades, insightful revelations about the secondary structure of individual caseins and their micellar states have emerged through spectral investigations and predictive modelling (Huppertz, 2013). This progress has led to a redefinition of their classification, transitioning from 'random coil proteins' to 'rheomorphic proteins' with attributes such as an open, flexible, and mobile conformation (Holt & Sawyer, 1993). The secondary structures of various caseins, both in their individual forms and within the micellar state, have been ascertained through diverse methods.

Within the realm of caseins, κ-casein (κ-CN) stands out as having the highest amount of secondary structure. The proposed structure of the C-terminal half of α_{s2}-casein includes a

globular conformation with both α-helix and β-sheet components, while the N-terminal region assumes a randomly structured hydrophilic tail. αs1-casein (α_{s1}-CN) displays a limited α-helix content but a notable presence of β-sheets and β-turns (Fox et al., 2015). In comparison, β-lactoglobulin (β-Lg) exhibits a lower proportion of α-helix (~12%) compared to α-lactalbumin (α-La) with 36% α-helix. However, β-Lg surpasses α-La in β-sheet content, registering at 50%. Structurally, β-Lg adheres to the typical lipocalin architecture, a protein family present in gram-negative bacteria, vertebrate cells, invertebrate cells, and plants, involved in transporting small hydrophobic molecules such as steroids, bilins, retinoids, and lipids. The β-Lg structure features a β-barrel composed of eight antiparallel β-strands labelled A–H, accompanied by a three-turn α-helix parallel to three of the β strands. Strands A–D constitute one surface of the barrel, while strands E–H form the other (Kontopidis et al., 2004).

2.2 Milk processing

The journey of milk from the farm to the dairy factory encompasses several crucial stages. Reception and Testing is the first step when raw milk is received from dairy farms. Samples are tested for quality, including fat content, bacterial load, and other parameters Milk may be pasteurized at this stage to eliminate harmful bacteria. Dairy processing in the industry involves various techniques to transform raw milk into a wide range of products (Burke et al., 2018). There are different types of milk vary in fat content. The main types of milk you'll find in stores include whole milk, 2% milk, 1% milk, and skim milk. Full fat milk (Whole milk) typically contains around 3.5% fat. This is the highest fat content among the commonly available types of milk. Reduced-Fat milk contains approximately 2% fat. For this purpose, milk separated into cream and skim milk using centrifugal separators. It has less fat than whole milk but more than 1% and skim milk. Low-Fat Milk (Light) is another type, which milk has around 1% fat. The last one is skim Milk (Fat-Free or Non-Fat Milk). Skim milk has had most or all of the fat removed, typically containing less than 0.5% fat. It is the lowest-fat option among the common types of milk. The choice between these milk types often depends on personal preferences, dietary needs, and health considerations. Individuals looking to reduce their fat intake may opt for skim or low-fat milk, while those who enjoy the creamier taste of whole milk may choose that option (Burke et al., 2018).

2.2.1 Heating

Heating is a fundamental process in dairy processing, serving multiple critical roles to ensure the safety, quality, and functionality of various dairy products. Heating plays a vital role in various stages of dairy processing (Deeth & Lewis, 2017a).

2.2.1.1 Thermisation

Thermisation is a heat treatment process used in the dairy industry to pasteurise raw milk partially without compromising its sensory and nutritional qualities. The purpose of thermisation is to reduce the microbial load in the milk, including pathogens and spoilage bacteria, while maintaining the desired characteristics of the final dairy product.

The thermisation process involves heating raw milk to a temperature below that used in traditional pasteurization. Typically, the temperature ranges between 60°C to 69°C. The duration of thermisation is relatively short, usually ranging from 15 to 30 seconds. This temperature is sufficient to inactivate certain bacteria, particularly psychrotrophs, yeasts, and molds. The key conditions in thermisation include carefully controlling the temperature and duration to strike a balance between achieving microbial reduction and preserving the sensory and nutritional qualities of the milk. Importantly, these treatments bring about negligible changes in the overall composition of the milk (Walstra et al., 2005; Deeth & Lewis, 2017a).

2.2.1.2 Pasteurisation

The standard temperature and time conditions for pasteurization are generally governed by regulations and guidelines, but they may vary depending on the specific dairy product and local requirements. The most common method is High-Temperature Short-Time (HTST) pasteurization, which involves heating the milk to a temperature of about 72°C to 74°C for a duration of around 15 to 20 seconds. Another pasteurization method is Low-Temperature Long-Time (LTLT) pasteurization, where the milk is heated to a lower temperature, typically around 63°C, and held at that temperature for a more extended period, often 30 minutes or more. The process effectively eliminates or reduces pathogenic bacteria, yeasts, and molds, improving the safety of dairy products for consumers. Pasteurization has played a crucial role

in reducing the risk of foodborne illnesses associated with raw milk consumption, ensuring that dairy products are safer for widespread distribution and consumption (Walstra et al., 2005; Deeth & Lewis, 2016).

High pasteurisation is another heat treatment method, involving an intensity of 20 seconds at 85°C, specifically designed to eliminate the activity of the enzyme lactoperoxidase. This treatment is highly effective in eradicating virtually all vegetative microorganisms, although it does not affect spores. It also leads to the inactivation of the majority of enzymes, with the exception of plasmin and certain bacterial proteinases and lipases. While high pasteurisation imparts a cooked flavour to the milk, it also results in partial denaturation of whey proteins and the destruction of the bacteriostatic properties of the milk (Mulvihill & Grufferty, 1995; Deeth & Lewis, 2016).

2.2.1.3 Ultra High Temperature (UHT) Treatment

Ultra High Temperature (UHT) treatment, also known as ultra-high-temperature processing, is a heat treatment method extensively used in the dairy industry. The primary purpose of UHT treatment is to sterilize milk, by rapidly heating them to a very high temperature to achieve microbial inactivation and extend shelf life. UHT treatment involves exposing the milk to temperatures above 135°C. Typically, temperatures range from 135°C to 150°C (Deeth & Lewis, 2016, 2017b). The high temperature is applied for a very short duration, typically ranging from 2 to 5 seconds. The key objective of UHT treatment is to attain commercial sterility, eradicating harmful bacteria and spores present in milk. This rapid heating and cooling process significantly extends the shelf life of the product, allowing it to be stored for an extended period without the need for refrigeration (Deeth & Lewis, 2016).

Products treated with UHT can be stored in aseptic packaging, which further ensures the prevention of contamination and extends the shelf life of the dairy items. Despite its efficacy in inactivating all milk enzymes except bacterial lipases and proteinases, it is associated with drawbacks such as browning due to extensive Maillard reactions, a characteristic sterilized milk flavour, the loss of some available lysine, denaturation of whey proteins, alterations in caseins, vitamin loss, and a decrease in milk pH by 0.2 units. On the other hand, UHT treatment induces fewer chemical changes, a mild cooked flavour, and does not result in the inactivation of plasmin, proteinases, and bacterial lipases (Deeth & Lewis, 2016, 2017b).

2.2.1.3.1 Physicochemical changes of milk during UHT treatment

During UHT (Ultra High Temperature) treatment of milk, several chemical changes occur, including alterations in pH and mineral balance. Heating is known for its capacity to lower the pH of milk, primarily due to the production of formic acid resulting from lactose breakdown (Berg & van Boekel, 1994). The pH of milk decreases with the increase in temperature, reaching values as low as 5.6 at 140 °C (Walstra et al., 1984). It's important to note that while a decrease in pH is expected to increase ionic calcium concentration. The exact relationship between pH and Ca^{2+} concentration at 140°C is suggested to influence the heat stability of milk at this temperature (Deeth & Lewis, 2017a). This occurrence can be attributed to the reduced solubility of calcium phosphate as the temperature increases (Fox & McSweeney, 1998). This decline in the solubility of calcium phosphate during UHT processing may lead to the formation of deposits on heat exchanger walls or their association with casein micelles, termed as fouling (Deeth & Lewis, 2017a).

Fouling is a common issue in the context of UHT (Ultra High Temperature) treatment in milk processing. Milk contains proteins, primarily caseins, which are prone to denaturation and can adhere to the surfaces of heat exchangers during UHT treatment. This protein fouling forms a layer that reduces heat transfer efficiency and requires frequent cleaning and maintenance. These deposits can make it challenging to achieve the desired product outlet temperature without raising the temperature of the heating medium to an undesirable level. Minerals present in milk, such as calcium and magnesium, can contribute to fouling. When subjected to high temperatures during UHT, these minerals can form deposits on equipment surfaces, diminishing heat transfer effectiveness and potentially leading to equipment damage. (Ramsey & Swartzel, 1984; Deeth & Lewis, 2016).

One of the significant alterations in milk proteins resulting from UHT (Ultra High Temperature) treatment or any heat processing is the denaturation of whey proteins. The level of denaturation for whey proteins is lower in UHT milk when compared to the complete denaturation observed in sterilization. The extent of denaturation is influenced by the specific processing method and the temperature-time profile during the heating process. Among whey proteins, immunoglobulins (Ig) and bovine serum albumin (BSA) are completely denatured by

sterilisation processes, followed by β-lactoglobulin (β-Lg) and alpha-lactalbumin (α-La) (Deeth & Lewis, 2016).

The denaturation process involves the unfolding of whey proteins, exposing reactive sulfhydryl groups and hydrophobic regions. This results in sulfhydryl-disulphide and hydrophobic interactions between proteins. The primary interaction of significance involves crosslinking between β-Lactoglobulin (β-Lg) and κ-casein. The denaturation of β-Lg involves the unfolding of its globular monomer, revealing hydrophobic amino acids and a free sulfhydryl group. This reactive non-native monomer then reacts with κ-casein on the surface of the casein micelle, resulting in the formation of complexes in the serum phase (Deeth & Lewis, 2016). The distribution of denatured whey proteins is affected by the pH of heating, with more proteins attaching to the casein micelle at lower pH values. The micelle with an increased amount of α-La attached becomes more hydrophobic, affecting water-holding capacity, an important consideration products (Deeth & Lewis, 2016).

2.2.2 Milk Gelation

The process of gelation in milk entails the formation of a continuous, three-dimensional network comprising interconnected molecules or particles within a liquid phase (Walstra, Van Vliet & Bremar, 1991). This process is driven by the coordinated interplay of protein–protein and protein–solvent interactions, resulting in the immobilization of a substantial amount of water by a relatively small proportion of proteins. The initial stage in the development of milk gels includes the destabilization of milk proteins, facilitating their interaction and aggregation (De Kruif et al., 1995; Schmidt, 1981). The formation of a gel occurs when the extent of protein aggregation surpasses a critical threshold, enabling the creation of a self-supporting network capable of entrapping the solvent (Lucey, 2009).

The development of a gel network relies on achieving a delicate equilibrium between the attractive and repulsive forces among protein molecules, influenced by factors such as pH, ionic strength, and temperature Excessive attractive forces typically lead to the creation of a random network, termed a coagulum, incapable of retaining water. Conversely, an absence of a network occurs when repulsive forces prevail. Hence, a critical balance between these opposing attractive and repulsive forces is essential for the successful formation and stabilization of a network (Kinsella, Rector & Phillips, 1994; Lucey, 2009).

2.2.2.1 Rennet-Induced Gelation

Rennet-induced gelation is a critical process in the dairy industry, particularly in cheese production. The primary purpose of rennet-induced gelation in the dairy industry is to coagulate the milk proteins, mainly casein, to form curd. This curd formation is a crucial step in cheese production, as it separates the liquid whey from the solid curds. This process involves the use of rennet, an enzyme complex derived from the stomach lining of young ruminants (chymosin), to coagulate milk proteins and form a gel-like structure. These enzymes hydrolyze the C-terminal part of the κ-casein, leading to a reduction in electrostatic and steric stabilization of the casein micelles and promoting their aggregation (Hyslop, 2003).

The temperature at which rennet-induced gelation occurs can vary based on the type of cheese being produced. However, it generally ranges from 30°C to 40°C. The temperature affects the rate of enzymatic activity and the texture of the resulting curd. The primary function of rennet is to eliminate the "hairy layer" of the casein micelle, resulting in a decrease in the net negative charge and steric repulsion of the micelle. The casein micelle comprises αs- and β-casein, bound within the micelle through hydrophobic interactions and colloidal calcium phosphate (CCP). In contrast, κ-casein is located on the micelle surface, playing a crucial role in stabilizing the micelle through electrostatic and steric repulsion via the hydrophilic "hairy layer" (Hyslop, 2003; Lucey, 2009).

2.2.2.2 Acid-Induced Gelation

Acid gelation is a process in the dairy industry, particularly in the production of certain dairy products like yogurt and acid-set cheeses. Milk can undergo acidification through various methods, including the fermentation of lactose to lactic acid by bacterial cultures or direct addition of mineral acids like hydrochloric acid (HCl), and the use of the acidulant glucono-δ-lactone (GDL), which hydrolyses to form gluconic acid (Lucey and Singh, 1997; Lucey, 2016). Acid gelation often involves the addition of starter cultures containing lactic acid bacteria, such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* or through the addition of chemical acids like HCl or glucono delta-lactone (GDL) (Lucey & Singh, 1997). These bacteria contribute to the fermentation process, converting lactose into lactic acid. The temperature at

which acid gelation occurs can vary based on the specific dairy product being produced. For example, in yogurt production, temperatures are typically maintained around 43°C to 45°C.

GDL (glucono-delta-lactone) serves as an acid precursor, gradually undergoing hydrolysis in aqueous solutions to generate gluconic acid. Consequently, introducing GDL to milk leads to a gradual decrease in the milk's pH over time. The acidification rate is influenced by both the temperature of acidification and the concentration of GDL. Specifically, for a given type of milk, the acidification rate rises with an increase in acidification temperature at a constant GDL concentration, and it also increases with a higher GDL concentration at a consistent acidification temperature (de Kruif, 1997; Anema, 2009). As the pH of the milk decreases through acidification, colloidal calcium phosphate solubilizes from casein micelles, leading to micellar disintegration. Subsequently, the caseins associate to form a gel network (Pyne, 1962; Heertje et al., 1985).

Gels formed in milk through the aggregation of casein upon acidification to its isoelectric point are known as acid-induced milk gels (Lucey & Singh, 1997). At the isoelectric point, the casein micelles lack charge, leading to increased hydrophobic interactions and subsequent aggregation of casein particles (Lucey, 2009). In unheated milk, gelation occurs at approximately pH 4.6, corresponding to the isoelectric point (pI) of casein. The formation of acid-induced milk gels in casein gels is fundamentally rooted in the impact of pH. Lucey (2009) categorizes the alterations in casein micelles with decreasing pH into three regions.

In the pH range from 6.7 to 6.0, the reduction in pH leads to a decrease in the net negative charge on casein micelles, resulting in diminished electrostatic repulsion. Since only a small amount of colloidal calcium phosphate (CCP) is dissolved above pH 6.0, the structural characteristics of the micelle remain relatively unaffected (Kinsella et al., 1994; Lucey, 2009). Within the pH range from 6.0 to 5.0, the charged κ-casein "hairs" on the micelle surface may shrink or collapse due to their charged nature. This change contributes to a reduction in both electrostatic repulsion and steric stabilization. Complete dissolution of CCP in the micelle occurs by approximately pH 5.0. As the pH drops below 5.0, approaching the isoelectric point (pH 4.6), the net negative charge of the casein micelles decreases, leading to the aggregation of casein particles through charge neutralization (Lucey and Singh, 1997; Lucey, 2009).

Acidification elevates the ionic calcium concentration and the dissolved calcium in the soluble phase of milk (Deeth & Lewis, 2015; Lucey et al., 1996). Reducing the pH augments the

concentration of H^+ ions in the soluble phase of milk, leading to alterations in the calcium equilibrium. This increase in H^+ ions may cause a shift in equilibrium towards the protonation of $HPO4^{2-}$ to $H2PO4^-$ (Gaucheron, 2005). Previous studies have thoroughly explored the impact of compositional and processing parameters on the textural properties of acid milk gels (Pyne, 1962; Lucey and Singh, 1997; 2003).

2.2.2.2.1 Factors influencing acid gelation

2.2.2.2.1.1 Preheat treatment

The preheat treatment is a common practice in the preparation of acid-induced milk gels, aimed at enhancing gelation properties. This step also helps ensure the desired texture, flavor, and consistency in the final product (Vasbinder, van de Velde & De Kruif, 2004). In unheated acid-induced milk gels, gelation is exclusively associated with casein, whereas preheated acid milk gels incorporate both casein and whey proteins, facilitated by the denaturation of whey proteins induced by heat. The surface properties of casein micelles play a pivotal role in shaping the interactions between denatured whey proteins and casein micelles during the heating process (Dalgleish & Corredig, 2012). Consequently, conditions influencing the surface properties of casein micelles, such as pH and temperature, significantly impact the effects of preheat treatment on the gelation of milk proteins (Dalgleish & Law, 1988).

Preheating milk at its natural pH raises the gelation pH of acid milk gels (Anema, 2009; Lucey, Tamehana, Singh & Munro, 2000). In unheated milk, whey proteins remain in their stable native forms and play a minimal role in gelation, which predominantly occurs as the pH approaches the isoelectric point (pI) of casein around 4.6 (Anema et al., 2004). Conversely, preheating milk at its natural pH results in the denaturation of whey proteins, leading to their complexation with the micellar κ-casein through hydrophobic interactions and intermolecular disulphide bonds (Anema, 2009; Schorsch, Wilkins, Jones & Norton, 2001). This interaction causes whey proteins to coat the casein micelle, altering the isoelectric pH at the surface of the casein micelle from 4.6 to approximately 5.2, corresponding to the pI of β-lactoglobulin (Anema et al., 2004).

The interaction between whey proteins and casein micelles, resulting in the formation of a complex, has been recognized as a substantial factor in the cross-linking of casein particles

during acidification, consequently causing an elevation in G′ (Dalgleish & Corredig, 2012; Lucey, 2002). As acidification progresses, electrostatic repulsion diminishes, and the whey protein-κ-casein complexes serve as pivotal attachment points for the casein micelles by forming bridges between protein particles. This bridging effect is attributed to the higher surface hydrophobicity of whey proteins (Dalgleish & Corredig, 2012).

2.2.2.2.1.2 pH

The pH of milk plays a crucial role in acid gelation, influencing the process through several mechanisms. Casein micelles, the primary protein structures in milk responsible for gel formation, carry a net negative charge at neutral pH. As the pH decreases (becomes more acidic), the negative charge on the casein micelles increases. pH of milk influences acid gelation by affecting the charge and conformation of proteins, the availability of calcium ions, and the solubility of whey proteins. Higher preheating pH can cause the lower levels of associated denatured whey proteins can be attributed to the increased dissociation of κ-casein (Anema et al., 2004). The higher pH during heating resulted in reduced attachment of β-lactoglobulin to the casein micelles. Consequently, denatured β-lactoglobulin aggregated in the serum phase, leading to an increased level of soluble aggregated β-lactoglobulin (Lakemond & van Vliet, 2008). Elevating the pH during the heating process, resulting in the aggregation of denatured whey proteins in the serum phase, has been reported to correlate with higher gelation pH and G' of acid milk gels (Dalgleish & Corredig, 2012; Lakemond & van Vliet, 2008). This increase in gelation pH is attributed to the enhanced dissociation of κ-casein, leading to a reduction in the "hairy" layer of the casein micelle, thereby promoting hydrophobic interactions. As a result, this initiates the gelation of the aggregated whey proteins at their isoelectric point (pH 5 to 5.5) in the serum phase (Anema et al., 2004; Vasbinder et al., 2004).

2.2.2.2.1.3 Temperature

Different temperatures can have varying effects on the acid gelation of milk. At lower temperatures, the rate of acid gelation is typically slower while at higher temperatures, the rate of acid gelation may be accelerated. Increasing the temperature during gelation has been demonstrated to improve gel strengths in both whey protein and casein gels. In general, higher

temperatures lead to a more rapid movement of molecules, resulting in a higher frequency of collisions. Additionally, higher temperatures may promote the denaturation of whey proteins, allowing them to interact more readily with casein micelles and enhance gel formation (Dalgleish & Corredig, 2012).

2.2.2.2.1.4 Minerals

The influence of minerals on the gelation of milk proteins is substantial, as the presence of ions impacts the balance between attractive and repulsive forces among the proteins. At the natural pH of milk, both whey proteins and casein micelles bear a negative charge. Therefore, the introduction of positively charged ions such as Ca^{2+} and Na^{+} serves to screen the protein charges, reducing electrostatic repulsion and promoting hydrophobic interactions and aggregation. The addition of salts not only changes the ionic strength of the solution but may also cause shifts in the calcium equilibrium within milk, potentially affecting the stability of the proteins (Lucey, 2009).

2.2.2.3 Rheological measurements

Rheological measurements help assess and control the physical properties of dairy products during manufacturing processes, ensuring consistency and meeting quality standards. Rheology is a crucial aspect in understanding and optimizing the texture, consistency, and overall quality of various dairy products. Gels, characterized by their viscoelastic nature, display a combination of solid-like and liquid-like behavior. The viscoelastic behavior observed in macromolecular gels is intricately linked to the nature and speed of configurational rearrangements of the macromolecules, along with the type and quantity of intermolecular bonds formed. Consequently, examining the rheological properties provides valuable insights into the features of a gel, offering information on its molecular structure (Auty et al., 2005).

Dynamic oscillatory measurements help in understanding how materials respond to alternating forces and deformations, providing insights into their viscoelastic properties. Dynamic oscillatory measurements are valuable for studying changes during gelation as the deformations induced are typically minimal, having a negligible impact on the structure. These measurements yield two distinct parameters: the storage modulus (G′), indicating the amount

of energy stored elastically in the structure, and the loss modulus (G″), representing the energy loss or viscous response per cycle of deformation (Lucey & Singh, 1998).

The gel point refers to a critical stage during the gelation process when a liquid or sol transforms into a gel, acquiring a more solid-like structure. This transition is marked by the formation of a three-dimensional network within the material, resulting in increased viscosity and elasticity. This shift involves the transformation from a disconnected state to a networked structure. Various methods have been proposed to determine the gelation point, with some suggesting that the crossover of G′ and G″ at a specified frequency serves as an indicator of gelation (Stading & Hermansson, 1990). The accurate method for detecting the gel point involves observing the increase in G′ to a level surpassing the experimental noise, typically exceeding 1 Pa (Meletharayil, Patel, Metzger, & Huppertz., 2016). Another criterion is identifying the point of rapid ascent in G′ values (Hsieh, Regenstein & Anandha Rao, 1993).

2.3 Conformational and structural analysis

Conformational and structural analysis of milk proteins is a critical aspect of understanding their functionality and behavior in various applications. Milk component, such as proteins, and minerals, undergo complex interactions and structural arrangements that influence the properties of dairy products (Wang, Sun, Pu, & Wei, 2017). Analyzing their structures involves a combination of both traditional and advanced techniques, requiring sophisticated methods for studying their structure. Fourier Transform Infrared Spectroscopy (FTIR) is a powerful analytical technique used in the conformational and structural analysis of various substances, including milk proteins (Nicolaou, & Goodacre, 2010). Nuclear Magnetic Resonance (NMR) spectroscopy is another technique that can offer detailed information about the three-dimensional arrangement of atoms within the protein (Belloque, & Ramos, 1999).

2.3.1 Fourier-Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a powerful analytical technique used in the conformational and structural analysis of various substances, including milk (Grewal, Huppertz, & Vasiljevic, 2018). FTIR measures the absorption of infrared radiation by a sample, providing information about the functional groups present in the molecules and their chemical

environment. This absorption is dependent on the vibrational frequencies of the bonds within the molecule, which, in turn, are determined by factors such as the mass of the constituent atoms, bond strength, geometric arrangement, and vibrational coupling periods (Karoui, Downey, & Blecker, 2010). This sensitivity renders FTIR spectroscopy a potent technique for examining alterations in the structure and interactions of food components, especially proteins, lipids, and lactose, across diverse physicochemical conditions during processing and subsequent storage. FTIR is also used to monitor structural changes induced by factors like pH, temperature, or the presence of other substances. Such investigations are pivotal for the dairy industry, contributing to the comprehension of the quality and shelf life of dairy products (Kher et al., 2007; Nicolaou, & Goodacre, 2010).

FTIR can be applied to determine the interactions between different milk protein components or with other molecules. By examining shifts in specific bands or the emergence of new bands in the FTIR spectra, researchers can deduce changes in molecular interactions. FTIR also can help determine protein concentration and assess the quality of dairy products based on the spectral characteristics of their protein content. FTIR is versatile and can be applied to study proteins in various states, including solutions, gels, powders, and films (Van De Voort, Sedman, Emo, & Ismail, 1992).

FTIR spectroscopy, commonly utilized in the Mid-Infrared region, offers valuable insights into the structure and intra- and intermolecular bonding of organic molecules. The positions of bands in FTIR spectra exhibit sensitivity to the molecular environment, providing additional information about structure and bonding (Karoui et al., 2010). FTIR reveals distinctive features associated with the major components of milk through its mid-infrared spectra. In the mid-infrared spectra of milk, specific spectral regions are attributed to different milk components after successful water subtraction. These regions include 3000-2800 cm^{-1} (associated with milk lipids), 1800-1700 cm^{-1} (related to lipids), 1700-1500 cm^{-1} (corresponding to milk proteins' amide I and II bands), 1500-1200 cm^{-1} (involving contributions from milk proteins, carbohydrates, and interactions between different milk components), 1200-900 cm^{-1} (including contributions from milk minerals, milk fat, lactose, and the fingerprint region) (Karoui et al., 2010).

In the context of milk proteins, FTIR is employed to study their secondary structure, composition, and interactions. It can identify characteristic absorption bands associated with different protein secondary structures, such as alpha-helices, beta-sheets, turns, and random

coils. FTIR can detect changes in the amide I and amide II bands of proteins, which are sensitive to alterations in protein composition and conformation (Grewal et al., 2017a). FTIR detects changes in the amide I and amide II bands of proteins through the absorption of infrared radiation by specific functional groups in the protein backbone. These bands are associated with vibrational modes of the amide bonds (C=O and N-H) in the peptide backbone. The amide I (1700-1600 cm^{-1}) band primarily corresponds to C=O stretching vibrations, while the amide II (1600-1500 cm^{-1}) band involves a combination of N-H bending and C-N stretching vibrations. The amide I band is particularly informative about the secondary structure of proteins (Karoui, Mazerolles, & Dufour, 2003; Grewal et al., 2017b; 2018). Different secondary structures, such as alpha-helices, beta-sheets, turns, and random coils, exhibit characteristic peaks within the amide I region. Changes in the amide I band can occur due to alterations in the protein's secondary structure. For example, shifts in peak positions or changes in peak intensity may indicate variations in the types and amounts of secondary structure elements. The amide II band is sensitive to changes in the protein's overall conformation. It involves a combination of N-H bending and C-N stretching vibrations, providing information about the arrangement of amino acid residues in the protein. Like the amide I band, changes in the amide II band can signify modifications in the protein's conformation or interactions with other molecules. Shifts or alterations in peak characteristics may reflect changes in the protein's dynamic state (Iñón, Garrigues, & de la Guardia, 2004). Deconvolution techniques can be applied to the FTIR spectra to quantitatively determine the proportion of each secondary structure element in a protein. This helps in understanding the folding patterns and structural changes in proteins under various conditions (Iñón, Garrigues, & de la Guardia, 2004).

In the fingerprint region, absorptions arise from bending and skeletal vibrations, which are sensitive to large wavenumber shifts. Peaks in this region include one around 1159 cm^{-1} associated with C-O vibrations of milk fat and areas between 1250 and 800 cm^{-1} with characteristic peaks of various C–O vibrations in carbohydrates, mainly lactose. Bands around 995 and 987 cm^{-1} suggest changes in stretching vibrations of the $-PO_3^{2-}$ moiety of the serine-phosphate residue in milk. In summary, Fourier Transform Infrared Spectroscopy is a valuable tool in the analysis of milk proteins. Its ability to provide information on secondary structure, composition, and molecular interactions makes it a versatile technique for researchers studying the conformational and structural aspects of milk proteins and their behavior in different conditions (Grewal et al., 2017b).

2.3.2 Nuclear Magnetic Resonance spectroscopy (NMR)

Nuclear Magnetic Resonance spectroscopy (NMR) is a powerful analytical technique used in chemistry, physics, and biochemistry to study the magnetic properties of atomic nuclei. NMR (Nuclear Magnetic Resonance) is a nonselective, invasive technique that uses the magnetic properties of atomic nuclei to obtain detailed information about the structure, composition, and dynamics of molecules. The term "nonselective" implies that NMR can provide insights into a broad range of molecular species within a sample, without the need for specific targeting. NMR spectroscopy detects signals from specific atomic nuclei, allowing researchers to elucidate the three-dimensional structures of molecules and gain insights into the composition and behavior of complex biological systems, including milk (Pavia et al., 2001; Markoska, Vasiljevic, & Huppertz, 2020).

2.3.2.1 High-field proton (^{1}H) Nuclear Magnetic Resonance spectroscopy

High-field proton (^{1}H) NMR spectroscopy in milk analysis is a technique that utilizes the magnetic properties of hydrogen nuclei to investigate the composition and structure of milk components. Nuclei with either an odd mass or odd atomic number exhibit both spin and a magnetic moment (Pavia et al., 2001). Nuclear magnetic resonance occurs when these nuclei align in a magnetic field, and a radiofrequency is applied to excite them. After excitation, the nuclei gradually release their excitation energy and return to their original spin state through a process known as relaxation (Pavia et al., 2001). The protons within a molecule experience shielding from surrounding electrons. Consequently, the resonance frequency of each proton in a molecule varies depending on the local environment, resulting in a phenomenon known as chemical shift. This chemical shift (δ) is quantified as the deviation in parts per million (ppm) from Sodium trimethylsilyl-[2,2,3,3- 2H4]-1-propionate (TSP), where protons are defined to have a chemical shift of 0.00 ppm (Pavia et al., 2001). Proton (^{1}H) NMR spectroscopy is an attractive method in metabolomics analyses due to its non-selective nature, minimal sample preparation requirements, and its capability to detect all mobile hydrogen-containing molecules. This technique has been applied in various areas of food science, including the examination of fruit juices (Eads and Bryant, 1986; Belton et al., 1996; Koda et al., 2012), milk (Eads and Bryant, 1986; Hu et al., 2004; Hu et al., 2007), honey (Schievano et al., 2009), and wine (Son et al., 2009; Lopez-Rituerto et al., 2012). Proton NMR is particularly useful for studying lactose, the primary carbohydrate in milk (Eads and Bryant, 1986; Hu et al., 2004).

This includes assessing lactose concentration and potential variations in lactose conformation. Changes induced by processing methods, such as pasteurization or homogenization, can be monitored using proton NMR. This aids in assessing the impact of processing on the molecular structure and stability of milk components. The mobility and distribution of water molecules in milk can be assessed through proton NMR. This information is crucial for understanding the physical properties of milk, including its hydration state and potential interactions with other components. In the context of milk protein analysis, NMR can be employed to investigate the structural and conformational aspects of proteins, providing information about their interactions and dynamics. By detecting signals from hydrogen nuclei in amino acid side chains, researchers can analyze protein folding, interactions, and modifications (Lubke et al., 2002). NMR spectroscopy has demonstrated its value as an analytical tool for characterizing structural changes in caseins (Humphrey and Jolley, 1982; Leslie et al., 1969), casein micelles (Kakalis et al., 1990; Griffin and Roberts, 1985; Rollema and Brinkhuis, 1989), and whey proteins (Belloque and Smith, 1998; Kuwata et al., 1998; Lubke et al., 2002; Markoska et al., 2020). ^{1}H NMR is particularly useful for studying the fat composition in milk. It can provide detailed information about the types of fatty acids present in triglycerides, allowing for the characterization of the lipid profile in milk fat (Kalo et al., 1996). Proton NMR can be used for metabolite profiling, allowing the identification and quantification of various small molecules present in milk. This is valuable for understanding the nutritional and bioactive composition of milk (Klein et al., 2010; Klein et al., 2012).

2.3.2.2 ^{31}P Nuclear Magnetic Resonance spectroscopy (NMR)

^{31}P NMR spectroscopy is a technique that utilizes the magnetic properties of phosphorus-31 nuclei to study the structure and composition of molecules containing phosphorus. In the context of milk analysis, ^{31}P NMR is particularly valuable for investigating phosphorus-containing compounds, such as phospholipids and other phosphorylated molecules present in milk (Belloque & Smith, 1998; Belloque et al., 2000; Belton, Humphrey & Jolley, 1982; Griffin & Roberts, 1985; Kakalis et al., 1990; Kuwata et al., 1998; Leslie et al., 1969; Lyster, & Richards, 1985; Lubke et al., 2002; Rollema & Brinkhuis, 1989). Phospholipids are a major component of milk fat globule membranes. ^{31}P NMR spectroscopy allows researchers to analyze the specific types and amounts of phospholipids present in milk, providing insights into the lipid composition and structural characteristics of these molecules (Belton et al., 1985). Milk proteins, including caseins and whey proteins, contain phosphorylated residues. ^{31}P NMR

spectroscopy can be employed to study the phosphorylation state of proteins, offering information about the extent and distribution of phosphate groups within the protein structure. ^{31}P NMR spectroscopy can be used to monitor the levels of inorganic phosphate in milk. Changes in the concentration of inorganic phosphate may reflect alterations in the milk's mineral content or other environmental factors (Belton et al., 1985).

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Chapter 3

Chapter 3. Micellar calcium adjustment

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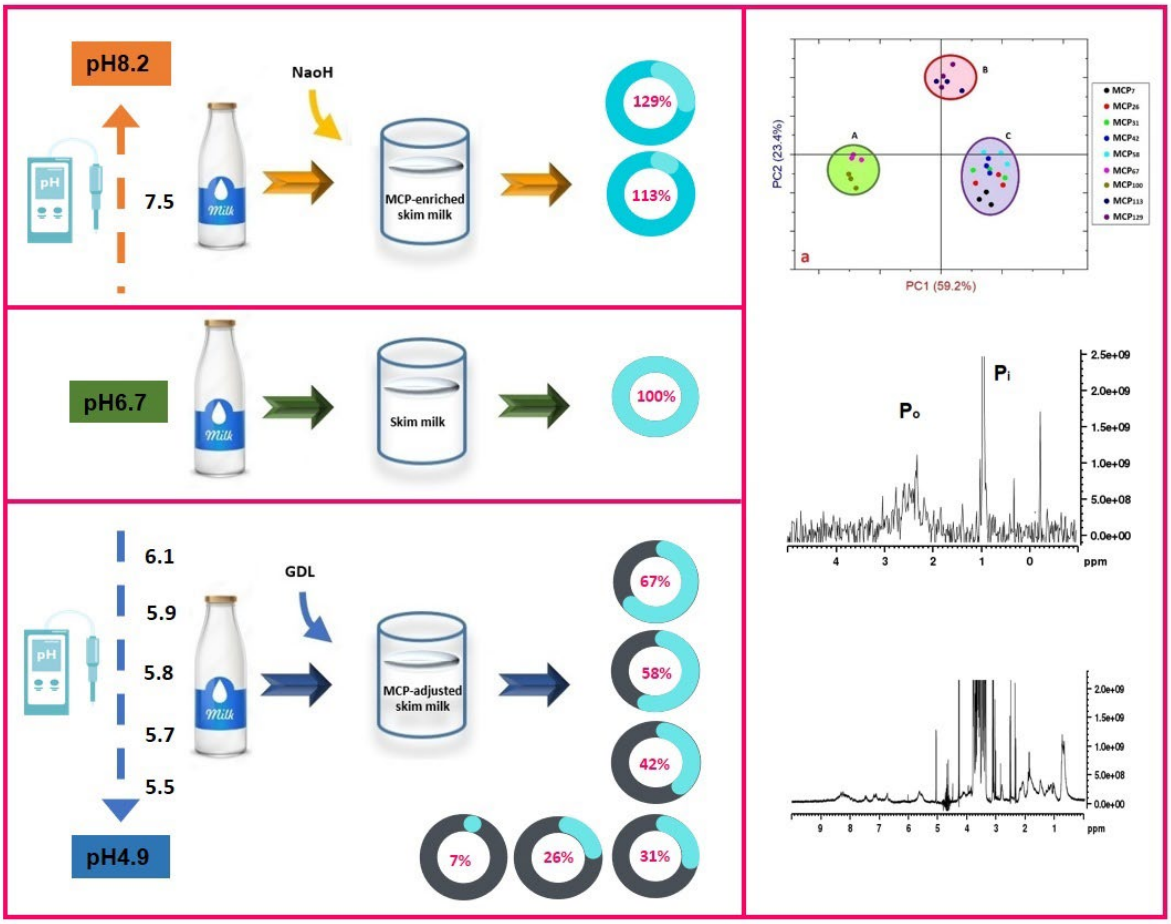

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Structural Properties of Casein Micelles with Adjusted Micellar Calcium Phosphate Content

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Abstract: Micellar calcium phosphate (MCP) content of skim milk was modified by pH adjustment followed by dialysis. Turbidity, casein micelle size and partitioning of Ca and caseins between the colloidal and soluble phases of milk were determined. Protein structure was characterised by Fourier transform infrared (FTIR) spectroscopy and proton nuclear magnetic resonance (^{1}H NMR), whereas organic and inorganic phosphorus were studied by phosphorus-31 nuclear magnetic resonance (^{31}P NMR). The sample with the lowest MCP content (MCP7) exhibited the smallest particle size and turbidity, measuring 83 ± 8 nm and 0.08 ± 0.01 cm^{-1}, respectively. Concentrations of soluble caseins increased with decreasing MCP levels. At ~60% MCP removal, FTIR analysis indicated a critical stage of structural rearrangement and ^{31}P NMR analysis showed an increase in signal intensity for Ca-free Ser-P, which further increased as MCP concentration was further reduced. In conclusion, this study highlighted the importance of MCP in maintaining micellar structure and its impact on the integrity of casein micelle.

Keywords: micellar Ca phosphate; casein micelle; secondary structure of proteins; FTIR; NMR

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1. Introduction

Milk is a highly nutritious food containing both macronutrients, such as proteins (caseins and whey proteins) and fats, as well as micronutrients, including minerals such as Ca. The capacity of caseins to bind calcium in milk enables them to transport calcium and phosphate at concentrations significantly exceeding the solubility of calcium phosphate. This is possible because the majority of Ca and inorganic phosphate (P_i) in milk is in the form of micellar calcium phosphate (MCP) within the casein micelles. Casein micelles are heterogeneous, dynamic, polydisperse spherical structures with an average diameter of 200 nm [1]. They are composed of the four caseins, α_{s1}-, α_{s2}-, β-, and κ-casein, which are connected via various intermolecular interactions, as well as via MCP nanoclusters that connect to caseins via phosphoserines located on the former three caseins. The dry matter of the casein micelle consists of ~94% protein and ~6% inorganic material [2].

Of the total Ca content in bovine milk, ~70% is found in the casein micelles (protein-bound Ca, micellar) and ~30% is found in the serum phase (diffusible Ca) [3]. Serum Ca is partially present in ionic form, but also as complexes with citrate and phosphate ions. Conditions such as temperature, pH, as well as the addition of Ca-sequestering salts (CSS) can alter the amount of MCP, but also affect the Ca species in the serum phase. Alteration of the MCP level of the casein micelles can affect the structure and properties of casein micelles, which may further broaden the application range for milk protein ingredients in the food, cosmetic, and medicine domains [3].

The amount of soluble calcium affects rennetability, thermal stability, and rheological properties of various dairy products thus, calcium reduction was proposed as a way to improve the functionality of various dairy products. Due to the requirement for specific techno-functional properties of caseins as well as their nutritional benefits, in recent years, various studies have been conducted and different process options for calcium reduction in skim milk were compared quantitatively and qualitatively to produce casein micelles with an altered MCP content, e.g., in skim milk retentates [4] and in milk [5–11]. While functional properties of MCP-adjusted milks, such as thermal stability [12–14] and digestion [10] have been studied in detail, detailed studies on MCP-adjusted casein micelles have been more limited and the secondary structure of the proteins in MCP-adjusted skim milk was not investigated. This study aimed to investigate how modifying the MCP content in skim milk influences the structural characteristics of casein micelles, specifically focusing on its impact on micellar integrity.

2. Materials and Methods

2.1. Materials

Freshly pasteurised skim milk was sourced from Warrnambool Cheese and Butter—Saputo in Warrnambool, Victoria, Australia. Sodium azide (0.02%, *w*/*w*), glucono-delta-lactone (GDL), sodium hydroxide (NaOH), deuterium oxide (D_2O) and Pronase (protease mixture) were procured from Sigma-Aldrich in St. Louis, MO, USA. Additionally, high retention seamless cellulose dialysis tubing (14 kDa MWCO) was obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Sample Preparation

Samples were prepared from freshly pasteurised skim milk obtained from Warrnambool Cheese and Butter—Saputo (Warrnambool, Victoria, Australia) on three separate occasions. To prevent bacterial growth, sodium azide (0.02%, *w*/*w*) was added to the milk on receipt. In order to prepare samples varying in MCP content, the skim milk was subjected to a protocol described previously [5], whereby pH of skim milk was first either lowered to 4.9, 5.5, 5.7, 5.8, 5.9 or 6.1 by the addition of predetermined amounts of glucono-delta-lactone (GDL) or increased to 7.5 or 8.2 by adding 1.0 M NaOH at 5 °C. After the pH was stabilised, the pH-adjusted samples, as well as a control sample maintained at pH 6.7, were dialysed using a high retention seamless cellulose dialysis tubing (14 kDa MWCO, Sigma-Aldrich, St. Louis, MO, USA), against 2 × 20 volumes of original pasteurised skim milk for 72 h at 5 °C [5,10]. After the dialysis, the samples were removed from dialysis tubing and analysed. The study design of the present study and associated sample coding is shown in Figure 1. The coding was based on the estimate of micellar Ca relative to that of the control. Micellar Ca was defined as the amount of Ca that sedimented on ultracentrifugation (Section 2.3).

2.3. Sample Fractionation

The sedimentable and non-sedimentable phase of milk samples were separated by ultracentrifugation at 100,000× *g* for 1 h at 20 °C using a Beckman Ultra L-70 centrifuge (Beckman Coulter, Australia Pty. Ltd., Gladesville, Australia). The clear supernatant was then collected carefully with a syringe from each tube. To prepare 10 kDa-permeable fractions, part of the ultracentrifugal supernatant was subsequently filtered through a centrifugal 10 kDa filter at 4000× *g* for 4 h (Corning Spin-X UF concentrators, Merck, Darmstadt, Germany) using an Eppendorf Model 5810 centrifuge (Hamburg, Germany). A 10 kDa-permeable fraction was also prepared, similar to outlined above, from milk samples which had been incubated with 0.4 mg mL^{-1} of the protease mixture Pronase from *Streptomyces griseus* type XIV (Sigma-Aldrich, ST. Louis, MO, USA) for 24 h at 20 °C.

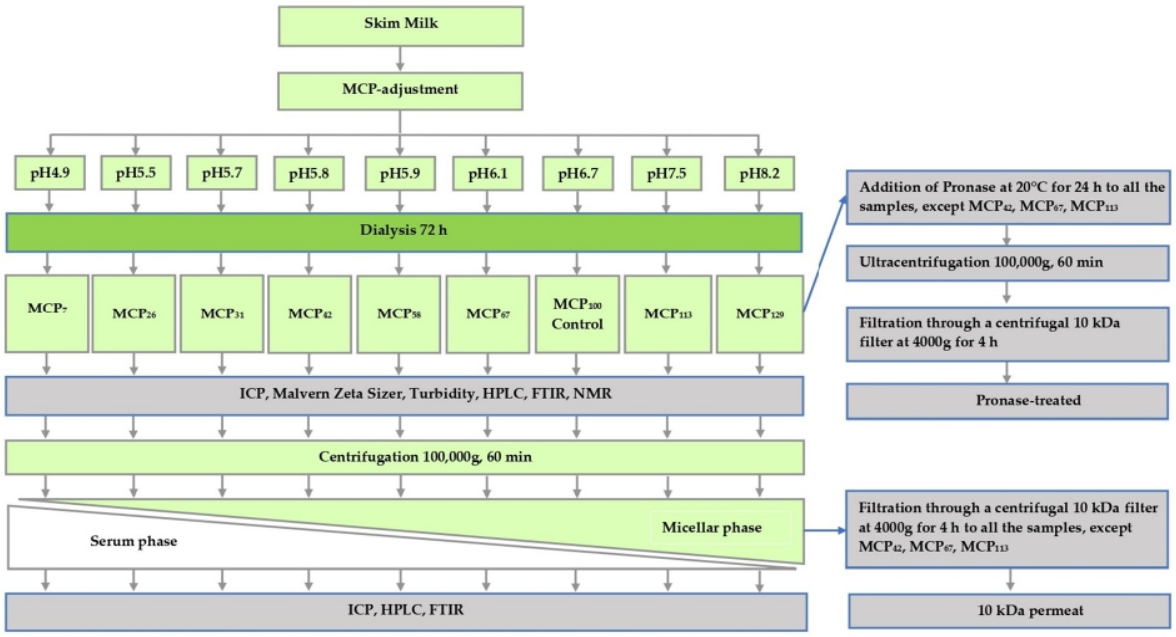

Figure 1. Experimental design of this study.

2.4. Sample Analysis

2.4.1. Ca Determination

The concentration of Ca in the whole samples, ultracentrifugal supernatant, and the 10 kDa permeates of milk with and without prior pre-treatment with Pronase was determined using an inductively coupled plasma atomic emission spectrometer (ICP-AES, ICPE-9000 system, Shimadzu Corporation, Kyoto, Japan) [15]. Micellar Ca was defined as the amount of sedimentable Ca (i.e., total Ca–Ca in the ultracentrifugal supernatant). The amount of nanocluster-associated Ca was determined as the difference between total Ca and the amount of Ca in the 10 kDa permeate of Pronase-treated milk.

2.4.2. Particle Size Distribution and Turbidity

Particle size (Z-average diameter) was determined by dynamic light scattering (Zetasizer-Nano, Malvern instruments Ltd., Malvern, UK) at a scattering angle of 90° at 25° C. Samples were diluted in simulated milk ultrafiltrate (SMUF) [16] in a ratio of 1:100. Refractive indexes for casein micelle and SMUF of 1.57 and 1.342, respectively, were used [17]. Turbidity of the bulk samples was measured at 860 nm using 1 mm path length quartz cuvettes using a UV–Visible spectrophotometer (Biochrom Ltd., Cambridge, UK) following the previously described method [15]. Turbidity of the milk serum after centrifugation was also measured.

2.4.3. High-Performance Liquid Chromatography

Caseins in whole samples and ultracentrifugal supernatants were analysed using a reversed-phase high-performance liquid chromatography (RP-HPLC) at room temperature by a Shimadzu HPLC system (Model Prominence-i, LC- 2030 C, Shimadzu Corporation, Kyoto, Japan) with a Varian 9012 system controller (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with a RI detector (Varian, 9050) and a C_4 column (Aeris Widepore, 150 mm × 4.6 mm, 3.6 µm particle size, 300 Å porosity, Phenomenex, Torrance, CA, USA). Samples were prepared and analysed according to the method described previously [15].

2.4.4. Fourier Transform Infrared (FTIR) Spectroscopy

For FTIR spectroscopy measurements, all milk samples were analysed using an FTIR spectrometer (Frontier 1, PerkinElmer, Boston, MA, USA) in the range of 4000–600 cm^{-1}.

At the start of measurement, the background spectrum was scanned with a blank (SMUF) to resolve changes in milk proteins [18]. In addition, corresponding ultracentrifugal supernatant samples were analysed under the same instrumental conditions as for the milk sample spectra acquisition [18]. The spectrum of ultracentrifugal supernatants was subtracted from corresponding spectrum of milk samples to reveal structural features of the micellar casein fraction. In the Amide I band region of the FTIR spectra between 1700 and 1600 cm^{-1}, six features corresponding to main protein secondary structures were assigned: side chains (1608–1611 cm^{-1}), β-sheet (1620–1631 cm^{-1}), random coils (1640–1649 cm^{-1}), α-helix (1658–1666 cm^{-1}), β-turns (1668–1681 cm^{-1}), and aggregated β-sheet (1689–1694 cm^{-1}) [19]. By subtracting the supernatants from the corresponding bulk samples, the aim was to visualise the changes associated with the micellar casein phase.

2.4.5. NMR Spectroscopy

NMR analysis was carried out using a 600 MHz Bruker Avance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). For the ^{31}P NMR analysis, the samples were prepared by mixing 0.1 mL of deuterium oxide and 0.9 mL of milk sample, whereas for the ^{1}H NMR analysis the samples were mixed by 0.9 mL of deuterium oxide 10% and 0.1 mL of milk. The ^{1}H NMR spectra were recorded using 32 scans and spectral width of 9615 Hz. The water signal was suppressed using excitation sculpting with gradients allowing for presaturation during relaxation delay in cases of radiation damping [19]. The ^{31}P NMR spectra were acquired at frequency of 242 MHz with power-gated proton decoupling, acquisition time of 0.3 s and 36 number of scans. The spectra were analysed using TopSpin 4.1.1 software (Bruker BioSpin). The FID was corrected by 0.3 Hz line-broadening parameter and phase correction by 0th and 1st order correction for pk. For both analyses, ^{1}H and ^{31}P NMR, each sample was analysed in triplicate.

2.5. Statistical Analysis

All experiments assessed the impact of MCP content on the selected parameters. SPSS software v. 26 (IBM Inc. Chicago, IL, USA) was used for one-way analysis of variance (ANOVA) to establish differences among means followed by Tukey's multicomparison of the means. The level of significance was pre-set at $p < 0.05$. The data were replicated three times on three different occasions. In addition, the FTIR data, processed as described previously, was analysed by Principal Component Analysis (PCA) with Origin Pro 2021, v. 95E software (OriginLab Corporation, Northampton, MA, USA), as described previously [18].

3. Results

3.1. Ca Distribution

Table 1 shows the concentrations of Ca in the different fractions of MCP-adjusted skim milk samples. The total Ca content of the control skim milk (MCP_{100}) was ~32.2 mmol L^{-1}, out of which 21.5 mmol L^{-1} was colloidal, 10.7 mmol L^{-1} was found in the ultracentrifugal supernatant and 9.7 mmol L^{-1} was 10 kDa permeable. Concentrations of Ca in the ultracentrifugal supernatants were slightly higher than the corresponding levels in the 10 kDa-permeable fractions, due to fact that whey proteins and caseins in the ultracentrifugal supernatant bind some Ca. In agreement with previous studies [5,10], MCP levels decreased in samples that were dialysed after acidification, whereas it increased in samples that were alkalinised prior to dialysis (Table 1). In addition to micellar Ca, we also estimated nanocluster-associated Ca, as previous studies indicated that not all micellar Ca is in the MCP nanoclusters [20]. To do this, the level of Ca in the 10 kDa-permeable fraction of milk that treated with Pronase was determined.

Table 1. Ca concentration in milk, its supernatant (100,000× *g* for 60 min), 10 kDa permeate and the 10 kDa permeate of milk after treatment with Pronase for pasteurised skim milk samples with their MCP adjusted to 7% (MCP_7) to 129% (MCP_{129}) by either acidification or alkalisation followed by exhaustive dialysis against bulk milk. MCP content was relative to the control based on the nanocluster associated Ca.

Sample Code [1]	pH of Milk Prior to Dialysis	Total Ca (mmol L^{-1})	Supernatant Ca (mmol L^{-1})	10 kDa Permeable Ca (mmol L^{-1})	10 kDa-Permeable Ca of Pronase-Treated Milk (mmol L^{-1})	Nanocluster-Associated Ca (mmol L^{-1})
MCP_7	4.9	14.74 ± 1.01^{E}	13.24 ± 0.50^{A}	12.49 ± 0.26^{A}	12.65± 1.56	2.09 ± 0.06^{F}
MCP_{26}	5.5	18.24 ± 3.19^{DE}	12.53 ± 2.19^{AB}	11.48 ± 0.94^{AB}	13.54 ± 1.38	4.70 ± 0.20^{E}
MCP_{31}	5.7	19.23 ± 5.06^{DE}	12.51 ± 2.30^{AB}	11.64 ± 0.53^{AB}	12.84 ± 1.33	6.39 ± 0.09^{D}
MCP_{42}	5.8	21.38 ± 3.42^{CD}	12.32 ± 2.07^{AB}	N/A	N/A	N/A
MCP_{58}	5.9	23.68 ± 3.38^{CD}	11.08 ± 0.72^{AB}	11.49 ± 2.38^{AB}	13.21± 1.14	10.47 ± 0.06^{C}
MCP_{67}	6.1	25.42 ± 1.55^{C}	10.92 ± 0.87^{AB}	N/A	N/A	N/A
MCP_{100}	6.7	32.23 ± 1.79^{B}	10.72 ± 0.67^{AB}	9.78 ± 0.09^{B}	11.72± 2.33	20.51 ± 0.02^{B}
MCP_{113}	7.5	35.43 ± 2.83^{AB}	11.04 ± 0.48^{AB}	N/A	N/A	N/A
MCP_{129}	8.2	38.17 ± 3.19^{A}	10.47 ± 0.28^{B}	11.63 ± 1.58^{AB}	13.92± 1.57	24.25 ± 0.10^{A}

[1] The subscript numbers indicate proportion of retained MCP relative to that of the control; The superscript capital letters indicate significant differences within the rows across treatment by Tukey's honestly significant difference procedure ($p < 0.05$); results are expressed as the means ± standard deviation; N/A—not assessed.

Treatment of milk with this enzyme mixture is known to hydrolyse milk proteins to free amino acids and small peptides, which can permeate through a 10 kDa membrane, whereas the Ca phosphate nanoclusters remain intact [21,22]. Hence, Ca associated with other parts of the caseins would permeate through the 10 kDa membrane after hydrolysis with Pronase. Using this approach, the concentration of nanocluster-associated Ca was estimated to be 20.5 mmol L^{-1} in the control milk. With decreasing MCP content, the concentration of nanocluster-associated Ca decreased significantly, down to 2 mmol L^{-1} of the nanocluster associated Ca for sample MCP_7 (Table 1).

3.2. Physicochemical Properties of Skim Milk with the Altered MCP Content

Particle size and turbidity of all samples are presented in the Table 2, whereas the particle size distribution of the milk samples is shown in Figure 2. The average particle size in control skim milk was 163 nm (Table 2), in line with previous reports [10]. With decreasing MCP content, the turbidity of skim milk samples decreased progressively, with the turbidity of the sample MCP_7 close to that of the milk serum (Table 2), indicating a very high degree of disintegration of the casein micelle. On the other hand, increasing the MCP content did not alter the turbidity (Table 2).

Table 2. Particle diameter and turbidity for pasteurised skim milk samples with their MCP adjusted from 7% (MCP_7) to 129% (MCP_{129}) by either acidification or alkalisation followed by exhaustive dialysis against bulk milk For sample details, see Figure 1.

Sample	Particle Diameter (nm)	Turbidity (cm^{-1})
MCP_7	83 ± 8^{D}	0.08 ± 0.01^{I}
MCP_{26}	115 ± 7^{C}	0.14 ± 0.01^{H}
MCP_{31}	140 ± 17^{B}	0.23 ± 0.00^{G}
MCP_{42}	158 ± 14^{A}	0.26 ± 0.00^{F}
MCP_{58}	163 ± 8^{A}	0.29 ± 0.00^{E}
MCP_{67}	162 ± 5^{A}	0.32 ± 0.00^{D}
MCP_{100} (control)	163 ± 4^{A}	0.41 ± 0.00^{A}
MCP_{113}	169 ± 11^{A}	0.40 ± 0.00^{B}
MCP_{129}	165 ± 5^{A}	0.39 ± 0.00^{C}

The capital letters indicate significant differences within the rows across treatment by Tukey's honestly significant difference procedure ($p < 0.05$); results are expressed as the means ± standard deviation.

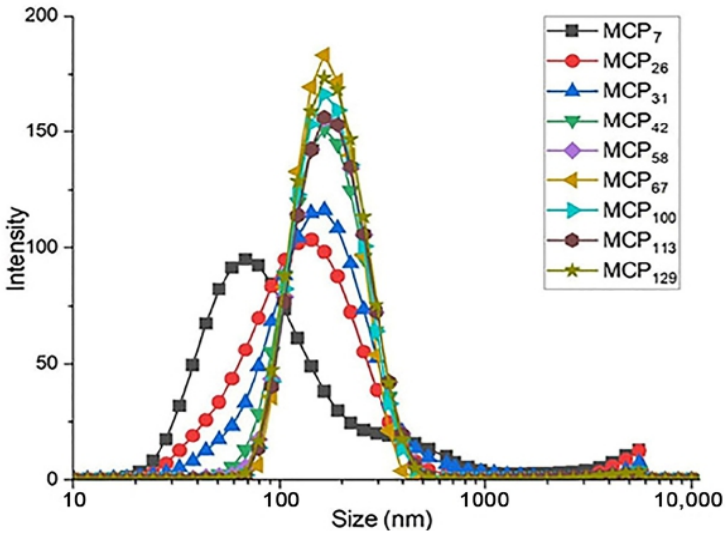

Figure 2. Particle size distribution of MCP modified skim milk samples. MCP content was adjusted from 7% (MCP_7) to 129% (MCP_{129}) relative to the control by either acidification or alkalisation followed by exhaustive dialysis against bulk milk. Graph is representative of two replicate samples.

3.3. Distribution of Individual Caseins in the MCP-Adjusted Skim Milk

Results for the protein composition of the ultracentrifugal supernatants of the MCP-adjusted skim milk samples as determined by HPLC are shown in Figure 3. The percentage of non-sedimentable α_{S1}- α_{S2}-, κ-, and β-casein increased with decreasing MCP content (Figure 3), particularly from sample MCP_{67}. The highest concentrations of non-sedimentable caseins were found in sample MCP_7. Increasing the MCP content caused slight decrease in the concentrations of individual soluble caseins in comparison to the control (MCP_{100}) (Figure 3). Comparable trends were observed for all caseins.

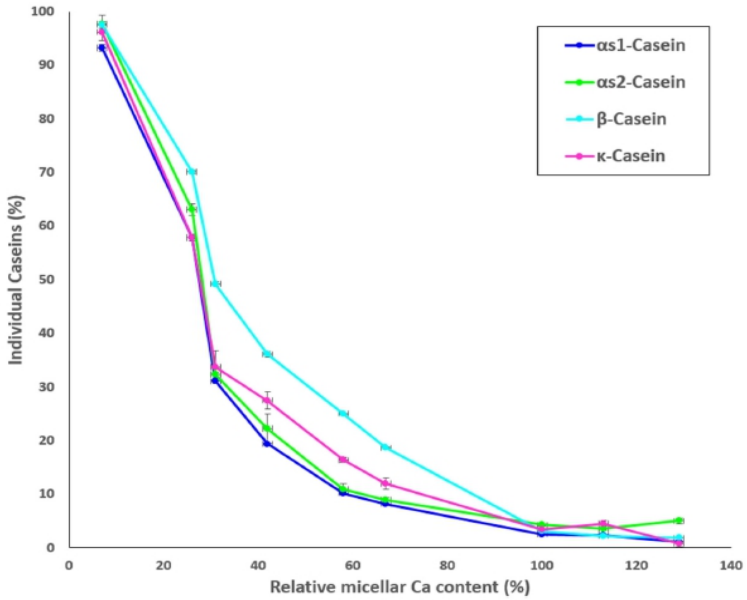

Figure 3. Proportion (%) of individual caseins present in the supernatant to skim milk as a function of the relative micellar Ca content, calculated as a ratio of micellar Ca content of a sample after pH adjustment and dialysis to micellar Ca content of the control.

3.4. Structural Characterisation of MCP Adjusted Skim Milk Samples by FTIR

The structural changes in the Amide I region of milk proteins and the micellar casein fraction in the samples are shown in Table S1 and Table 3, respectively. FTIR spectra in the region between 4000 and 650 cm^{-1} are presented in Figure S1. The most substantial difference as a result of adjusting the MCP content of skim milk was observed in the side chains of the amino acids (1608–1611 cm^{-1}), with the greatest intensity observed for samples MCP_{113} and MCP_{129} (Table S1; Table 3). The intensity of side chains in the micellar casein fraction decreased significantly at two key points when MCP content was reduced (Table 3). The first notable decrease was observed between samples MCP_{100} and MCP_{67}, and the second significant change was observed between samples MCP_{58} and MCP_{42} (Table 3). It is important to note that the side chains of amino acids can contribute to the structural integrity of proteins through weak interactions, such as ionic or hydrogen bonds [23].

Table 3. Changes in structural features of individual caseins in Amide I region of MCP-adjusted skim milk samples as determined by Fourier transform Infrared Spectroscopy. MCP-adjusted skim milk samples containing from 7% (MCP_7) to 129% (MCP_{129}) of MCP relative to that of the control were obtained by either acidification or alkalisation followed by exhaustive dialysis against bulk milk. The spectra were obtained after the background adjustment using the corresponding supernatants obtained by ultracentrifugation.

Band Assessment	Side Chain	β-Sheet	Random Coil	α-Helix	β-Turn	Aggregated β-Sheet
Band Frequency (cm^{-1})	1608–1611	1620–1631	1640–1649	1658–1666	1668–1681	1689–1694
MCP_7	1.1 ± 0.02^{g}	11.8 ± 0.20^{ab}	16.9 ± 1.34^{a}	24.2 ± 1.88^{abc}	44.8 ± 0.98^{ab}	1.2 ± 0.21^{ef}
MCP_{26}	1.9 ± 0.23^{fg}	6.6 ± 0.35^{d}	15.0 ± 2.28^{ab}	21.6 ± 0.49^{bc}	52.3 ± 2.20^{a}	2.4 ± 0.13^{def}
MCP_{31}	3.8 ± 0.11^{e}	6.7 ± 0.03^{d}	11.4 ± 0.59^{ab}	22.5 ± 0.03^{bc}	52.0 ± 0.02^{a}	3.5 ± 0.43^{de}
MCP_{42}	2.3 ± 0.09^{f}	1.9 ± 0.33^{e}	13.9 ± 1.27^{ab}	33.3 ± 1.72^{a}	48.4 ± 2.76^{ab}	0.1 ± 0.02^{f}
MCP_{58}	9.5 ± 0.45^{d}	3.2 ± 0.33^{e}	9.9 ± 0.42^{b}	23.5 ± 1.81^{bc}	50.6 ± 2.75^{a}	2.9 ± 0.42^{de}
MCP_{67}	10.3 ± 0.03^{d}	9.8 ± 0.67^{bc}	11.9 ± 3.61^{ab}	30.5 ± 1.09^{ab}	34.0 ± 5.57^{cd}	4.8 ± 0.03^{d}
MCP_{100}	15.2 ± 0.28^{c}	6.9 ± 2.09^{cd}	11.1 ± 1.57^{ab}	15.1 ± 6.27^{c}	40.8 ± 0.54^{bc}	10.8 ± 1.80^{c}
MCP_{113}	32.6 ± 0.18^{a}	13.2 ± 0.18^{a}	10.1 ± 0.22^{b}	2.6 ± 0.10^{d}	21.9 ± 0.05^{e}	19.5 ± 0.37^{b}
MCP_{129}	24.1 ± 0.42^{b}	6.5 ± 0.02^{d}	17.2 ± 0.13^{a}	2.8 ± 0.00^{d}	26.7 ± 0.25^{de}	22.7 ± 0.29^{a}

The subscripts indicate proportion of retained MCP relative to that of the control; The small letters show significant differences within the columns ($p < 0.05$); the results are expressed as the means ± standard deviation.

The greatest structural changes could be assigned to the micellar casein fraction. The proportion of side chains, which contribute to the structural integrity of proteins through weak interactions, such as ionic or hydrogen bonds [23], was greatest at the MCP level above that of the control, it however consistently declined concomitant with the reduction in the MCP content. On the contrary, the proportion of β-turns followed an inversed pattern to that of the side chains with the lowest values obtained at high MCP content. Random coil and β-sheet structures, with some exceptions, remained fairly consistent across the whole MCP range (Table 3). α-Helical structures significantly increased when lowering MCP content down to 42% of the original level, after which it declined by almost 30% (Table 3). At the same time, contributions of the β-sheet and aggregated β-sheet structures were the lowest for MCP_{42}.

From these observations, sample MCP_{42} appeared to be a key point at which the structural components of the proteins underwent substantial changes as a result of changes in the MCP content. PCA analysis also confirmed differences in structural components in Amide I region based on MCP-adjustment, classifying the samples into three groups (Figure 4). The PC1 differentiated the MCP adjusted skim milk samples in three groups including the MCP-enriched samples (MCP_{113} and MCP_{129}) from the low-MCP samples (MCP_7—MCP_{58}), and samples MCP_{67}—MCP_{100} (Figure 4).

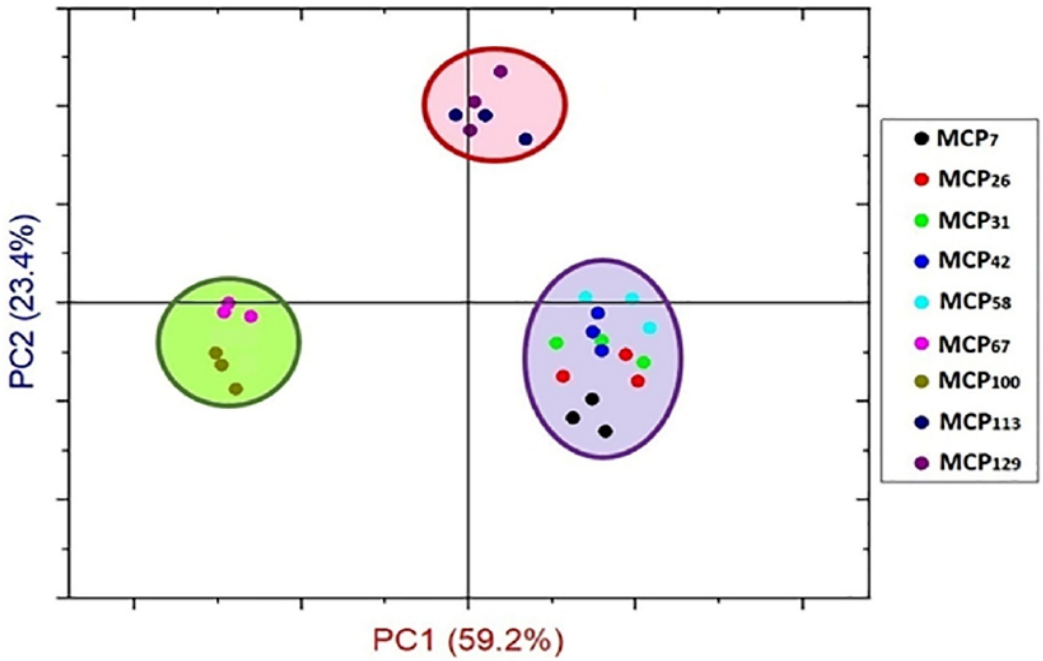

Figure 4. Principal component scores for Amide I region (1700–1600 cm^{-1}) of MCP-adjusted skim milk samples containing from 7% (MCP_7) to 129% (MCP_{129}) of MCP relative to that of the control achieved by either acidification or alkalisation followed by exhaustive dialysis against bulk milk.

H and ^{31}P NMR

The MCP-adjusted milk samples were analysed by ^{31}P and ^{1}H NMR spectroscopy. The ^{31}P NMR spectra provide information on the changes in the state of phosphate in milk and the spectra presented in Figure 5 are characterised by two distinct peaks, one narrow peak at δ = 1–1.5 ppm assigned to P_i and a broader peak at around 3 ppm usually assigned to P_o. In the control samples, the P_o signal at 2.0–3.5 ppm was very broad and started to become more prominent at MCP_{42} (Figure 5D) with the greatest intensity detected at MCP_7 (Figure 5A). The signal shape and intensity of this peak depends on the different chemical environments and mobility of SerP [24], with the signal disappearing concomitant with the phosphorus immobilisation. The Po signal appeared to be noticeable different for the samples containing the least amount of MCP (Figure 5A–C), whereas the Pi peak appeared consistently across these ranges of MCP. The width of the Pi peak is impacted by the MCP concentration; in samples with higher MCP content (Figure 5H,I), a broader width is observed.

The ^{1}H NMR spectra showed an intense signal for lactose at 3.2–4.0 ppm (Figure 6) [25]. Signal intensity in the aliphatic region (0.5–2.0 ppm) decreased as the MCP concentration was increased. The methyl signal that appears at ~0.5 ppm was most intense in MCP_7 and the least in MCP_{129} (Figure 6). This resonance arises from the methyl protons of alanine, leucine, isoleucine and threonine and their intensity could be related to their greater presence in the soluble phase [26]. Similarly, the aromatic region of the spectrum (6–9 ppm) [27], depicting ring protons of phenylalanine, tyrosine, tryptophan and histidine, resonated greatly at lower MCP content (Figure 6A–E), which again was attributed to a greater proportion of soluble proteins in the past [28]. The Hα and Hβ regions of the ^{1}H NMR spectra also showed variation in the signal intensity when the MCP concentration was altered (Figure 6). Changes were observed in the doublet at 2.5 ppm, doublet at 3.0 ppm, singlet at 4.2 ppm, singlet at 5.2 ppm and multiple signals at 4.7 ppm. The chemical shifts in the MCP_{31} sample at 2.5, 3.0, 4.2 and 5.2 ppm show lower signal intensity compared to the rest of the samples. Thus, the signal in this region could be due to involvement in hydrogen

interactions guided by the Hα and Hβ of the backbone. The signals at 4.7 ppm could not be interpreted accurately as the water suppression interfered with the signal intensity.

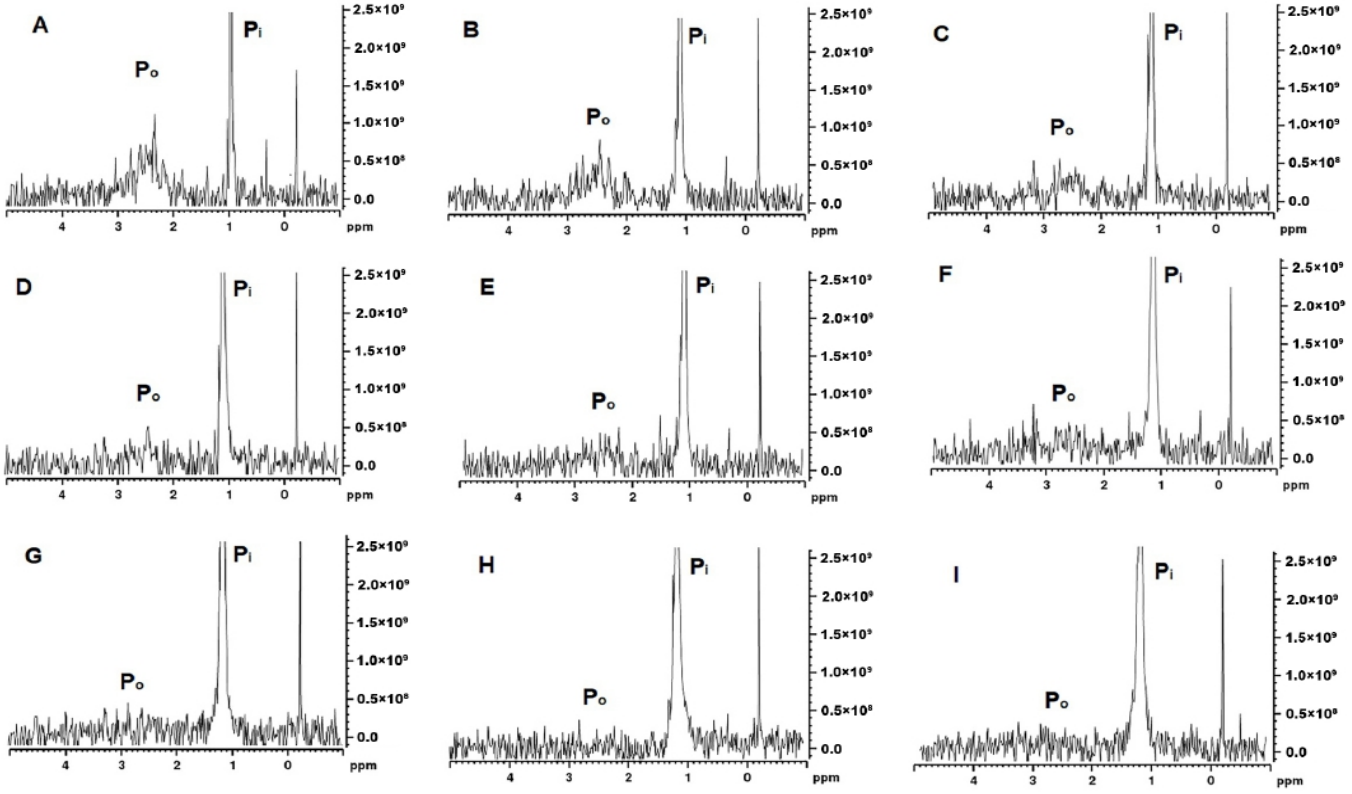

Figure 5. ^{31}P NMR spectra for MCP_7 (**A**), MCP_{26} (**B**), MCP_{31} (**C**), MCP_{42} (**D**), MCP_{58} (**E**), MCP_{67} (**F**), MCP_{100} (**G**), MCP_{113} (**H**), and MCP_{129} (**I**).

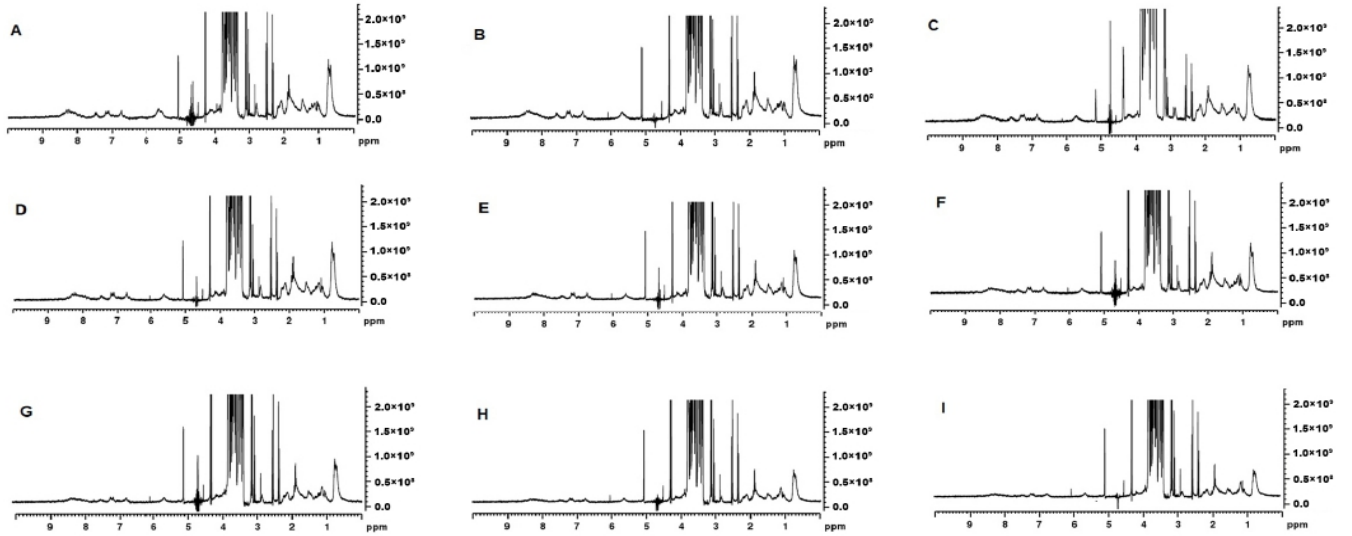

Figure 6. ^{1}H NMR spectra for MCP_7 (**A**), MCP_{26} (**B**), MCP_{31} (**C**), MCP_{42} (**D**), MCP_{58} (**E**), MCP_{67} (**F**), MCP_{100} (**G**), MCP_{113} (**H**), and MCP_{129} (**I**).

4. Discussion

4.1. Impact of Micellar Calcium Phosphate Levels on the Casein Micelle Structure

Casein micelles are in a dynamic equilibrium with the surrounding serum; thus, they can exchange proteins and minerals under various saturation conditions, and rearrange their structure in different chemical environments [29]. De Kruif et al. [30] showed that the previously proposed nanocluster model [31] captures the main features of the casein micelle. In this model, the MCP is dispersed by competent phosphopeptides to form equilibrium core-shell nanoclusters [20]. Based on this model, phosphorylated caseins bind to the growing nanoclusters [30]. The proteins associated with the nanoclusters protrude out and interact with other proteins via weak interactions, including hydrophobic interactions, hydrogen bonding, ion bonding and weak electrostatic interactions, which results in formation of a more or less homogeneous protein matrix [30]. Previously, Holt et al. [32] stated that micellar Ca appears to exist in two forms—as a Ca phosphate salt and as Ca^{2+} bound to the protein. Similarly, originally it was believed that phosphorus appeared in 2 chemical forms in casein micelle: as the MCP and phosphorylated serine (phosphoserine) in the caseins [33,34]. However, more recently Hindmarsh and Watkinson showed using ^{1}H ^{31}P cross-polarisation magic angle spinning (CP-MAS) NMR that in addition to these two forms, other immobile phosphorus bodies exist within the casein micelle that have not yet been classified [35].

4.2. Micellar Calcium Phosphate Adjustment: Insights from FTIR Analysis

The state of MCP has a notable effect on the properties of the casein micelle. The results of the current study confirmed previous findings [8–10,15] that the MCP adjustment changes Ca equilibrium (Table 1), resulting in a substantial decline of the average particle size below MCP_{42}. Interestingly, from the data in Table 2 it seems that turbidity declined almost linearly with decreasing MCP content. The caseins and MCP are responsible for the light-scattering properties of the casein micelle [36] thus decreasing MCP concentration reduces the refractive index of the casein micelles, and thus turbidity of the milk. However, a decrease in turbidity was also associated with a release of individual caseins [2], which indicated changes in the concentration of non-sedimentable caseins in the serum phase (Figure 3). β-Casein appeared to be the most affected micellar protein initially, with almost 40% of its initial micellar concentration released into the serum (Figure 3). This could be related to its positioning in the casein micelle, as some studies indicated that β-casein may either be present or be close to the surface [37,38]. The role of MCP in maintaining β-casein association with the micelle was also linked to its greater solubility at low temperature [39].

Structurally, the main changes observed were associated with the micellar and the non-sedimentable caseins. Only limited changes in structural elements took place when all milk proteins were assessed involving side chains, β-sheets and random coils while α-helix, β-turns and aggregated β-sheets remained fairly consistent (Table S1). Changes were likely influenced by dissociation of individual caseins from the micelle, as whey proteins likely remain unaffected by the whole process of MCP adjustment. On the other hand, the casein micelle underwent substantial structural changes (Table 3), with changes becoming very prominent around residual MCP content of 42 to 58% of the original. The Amide I signal of the casein micelle was previously reported to contain ~40% of β-turns at the natural pH of milk [40], similar to what was observed in the current study (Table 3). The substantial increase of β-turns upon the MCP reduction could in part be attributed to dissolution of β-casein from the micelle since it contains approximately 20% of β-turns [40,41]. In addition, β-casein contains a large proportion of β-sheets [41] thus the decline in this structural feature of the casein micelle could have been caused also by the β-casein solubilisation. Considering other physical properties, it is likely that the structure of the casein micelle remains fairly stable up to a certain point, around MCP_{42}, although the protein and mineral composition may have changed. This could be related to the proposed structure of MCP and how it interacts with the phosphoserine residues of the individual caseins.

4.3. Micellar Calcium Phosphate Adjustment: Insights from NMR Analysis

As established by ^{31}P NMR, milk contains about 23 mM of inorganic phosphate (P_i) and approximately 10 mM-phosphate esters, mainly as SerP residues of the caseins [42]. These phosphate assignments are clearly depicted as $\delta \sim +1.9$ originating from P_i with, in general, a broad peak at approximately $\delta = +3.2$ arising from SerP of the casein (Figure 5) [42]. Depending on the type of ^{31}P NMR analysis, four types of phosphorus have been identified in literature including organic phosphorus from phosphoserine residues, organic phosphorus from serine to the MCP, inorganic phosphorus in the MCP and free inorganic phosphorus in the serum [43]. The MCP adjustment in our study appears to initially affect the width of the P_i peak (Figure 5) as it was wider at a higher MCP concentrations and then became narrower as the concentration of the MCP declined. This could be related to the types of P_i present in the MCP. Van Dijk [44] suggested that the MCP consisted of two parts—the stable arm, which crosslinks with SerP, and the unstable arm, which is in equilibrium with soluble salts in milk serum. Kolar et al. [45] extended on this suggestion and proposed that P_i appeared as three types including free inorganic phosphate in the serum, inorganic phosphate belonging to the unstable arm of the MCP and in the equilibrium with the serum P_i, and inorganic phosphate as a part of the stable arm of the MCP. Such a model has been termed C_2-SerP_3 ion cluster and depicts 2 'free' unstable and 2 stable arms firmly connecting 2 peptide chains [45]. It is likely that partial dissolution of the MCP would lead to solubilisation of the unstable MCP arms, which would not affect the integrity of the micelle greatly. However, once the stable arm is dissolved and SerP is exposed the micellar integrity appears to be compromised, which likely started to occur at MCP_{42} (Figure 5D). At this point, P_o started to appear, indicated a greater mobility of SerP (Figure 5D). The peak at +3.2 ppm became the most prominent at the lowest MCP content. This is also confirmed by ^{1}H-NMR (Figure 6) as the peaks in 2 identified regions (0.5–3.5 ppm and 5.5–9.0 ppm) started to change. The resonance around 1 ppm, which arises from the methyl protons of Ala, Leu, Ile and Thr residues, appeared to increase concomitant with the MCP decrease. At the same time, the peaks in the 5.5–9.0 ppm region, derived from amide main and side chain and aromatic side chain protons from caseins, started to become more distinct at $MCP_{42–58}$ (Figure 6D,E). The spectrum in the 7.5 and 8.5 ppm region has been assigned to the casein micelle backbone previously [46] and the peaks in this region were very broad but started to appear at MCP below 67% (Figure 6F). All of this indicates that either parts of the casein micelle became more mobile (resonated more) or the micelle lost its integrity, which resulted in greater resonance.

Increasing the MCP content by 13 or 29% had a rather minimal impact on the observed physical properties. However, it appears that the individual caseins are drawn from the soluble phase into the micellar structure. This resulted in the further immobilisation of organic phosphorus and widening of the P_i peak (Figure 5H,I), decline in the resonance associated with free amino acids depicted by ^{1}H-NMR (Figure 6H,I) and decline in the concentration of non-sedimentable caseins (Figure 3). While it may be plausible that additional P_i could be incorporated into the unstable arm of the MCP, it is not clear how the individual caseins would be further incorporated into the casein micelle. This is especially in reference that the particle size did not change significantly thus one of the assumptions could be that they would be positioned somewhere in the interior. It is however evident that even increasing the MCP content above its native level would cause structural changes in the casein micelle including rise in side chains and aggregated β-sheets and decline in the α-helical and β-turn structures (Table 3). How these structural changes may impact the properties and behaviour of the casein micelle under various processing conditions remains to be determined.

5. Conclusions

Several important physicochemical changes in milk proteins take place when the content of MCP in the casein micelle is altered. The casein micelle integrity did not appear substantially affected by the decline in MCP concentration following its adjustment down

to 42% of its original level, although the level of individual caseins increased in the serum phase of milk. The MCP content of 42% of the initial appears to be a minimum level to maintain the integrity of the casein micelle. While physical changes were not considerably noticeable above this level, the conformational changes in the casein micelle were substantially impacted by the adjustment. The MCP levels were also increased by adjustment at elevated pH although there appeared to be a limit to which additional Ca can be incorporated into the nanoclusters. This study provided some insights into the conformational and physicochemical changes in pasteurised milk with adjusted MCP.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods13020322/s1, Table S1: Changes in structural features of proteins in the Amide I region in MCP-adjusted skim milk samples as determined by Fourier transform Infrared Spectroscopy. MCP-adjusted skim milk samples containing from 7% (MCP_7) to 129% (MCP_{129}) of MCP relative to that of the control were obtained by either acidification or alkalisation followed by exhaustive dialysis against bulk milk. The spectra were obtained after the background adjustment. Figure S1. The original FTIR spectra (4000–650 cm^{-1}) of MCP adjusted skim milk varied containing from 7% (MCP_7) to 129% (MCP_{129}) of MCP relative to that of the control achieved by either acidification or alkalisation followed by exhaustive dialysis against bulk milk.

Author Contributions: E.A. prepared this study, the research questions and the methodology and performed the experiments and formal analysis, visualisation, writing original draft. T.M. assisted with data analysis, review and editing, T.V. and T.H. supervised this study and provided critical feedback, analysis, review and editing and funding acquisition and resources. All authors have read and agreed to the published version of the manuscript.

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Supplementary data

Table S1. Changes of structural features of proteins in Amide I region in MCP-adjusted skim milk samples as determined by Fourier transform Infrared Spectroscopy. MCP-adjusted skim milk samples containing from 7% (MCP_7) to 129% (MCP_{129}) of MCP relative to that of the control were obtained by either acidification or alkalisation followed by exhaustive dialysis against bulk milk. The spectra were obtained after the background adjustment.

Band Assessment	Band Frequency (cm^{-1})	MCP_7	MCP_{26}	MCP_{31}	MCP_{42}	MCP_{58}	MCP_{67}	MCP_{100}	MCP_{113}	MCP_{129}
Side chain	1608-1611	12.9±0.86^{d}	16.3±0.10^{c}	12.7±0.49^{d}	12.2±1.24^{de}	13.2±0.60^{c}	9.9±0.56^{de}	9.5±0.15^{e}	23.1±1.76^{b}	27.3±0.35^{a}
β-sheet	1620-1631	34.6±5.03^{a}^{bc}	35.3±0.18^{ab}	38.7±3.70^{a}^{b}	41.7±3.03^{a}	32.2±3.32^{a}^{bc}	32.3±1.63^{a}^{bc}	30.1±2.5^{bc}^{d}	19.2±1.10^{d}	23.7±0.35^{cd}
Random coil	1640-1649	13.2±0.84^{a}^{b}	16.4±0.13^{ab}	13.7±0.95^{a}^{b}	17.4±6.74^{ab}	21.5±5.55^{a}^{b}	22.2±3.54^{a}^{b}	23.5±6.32^{a}	16.2±3.72^{ab}	7.5±0.15^{b}
α-helix	1658-1666	5.5±0.60^{a}	2.8±0.12^{ab}	3.7±0.81^{ab}	1.9±0.01^{b}	2.7±1.66^{ab}	2.7±0.86^{ab}	2.7±1.29^{ab}	3.5±0.97^{ab}	5.1±0.24^{a}
β-turn	1668-1681	23.8±1.89^{a}^{bc}	21.6±0.34^{bc}	23.7±2.31^{a}^{bc}	17.8±1.61^{c}	22.4±2.70^{a}^{bc}	24.9±0.57^{a}^{bc}	25.3±0.53^{a}^{bc}	29.9±4.08^{a}	29.4±0.51^{ab}
Aggregated β-sheet	1689-1694	10.0±0.81^{a}	7.6±0.17^{a}	7.6±1.03^{a}	8.8±0.85^{a}	7.7±0.44^{a}	8.0±1.10^{a}	8.7±2.13^{a}	8.1±0.06^{a}	7.1±0.10^{a}

The subscripts indicate proportion of retained MCP relative to that of the control; [2]The small letters show significant differences within the columns ($p < 0.05$); the results are expressed as means ± standard deviation.

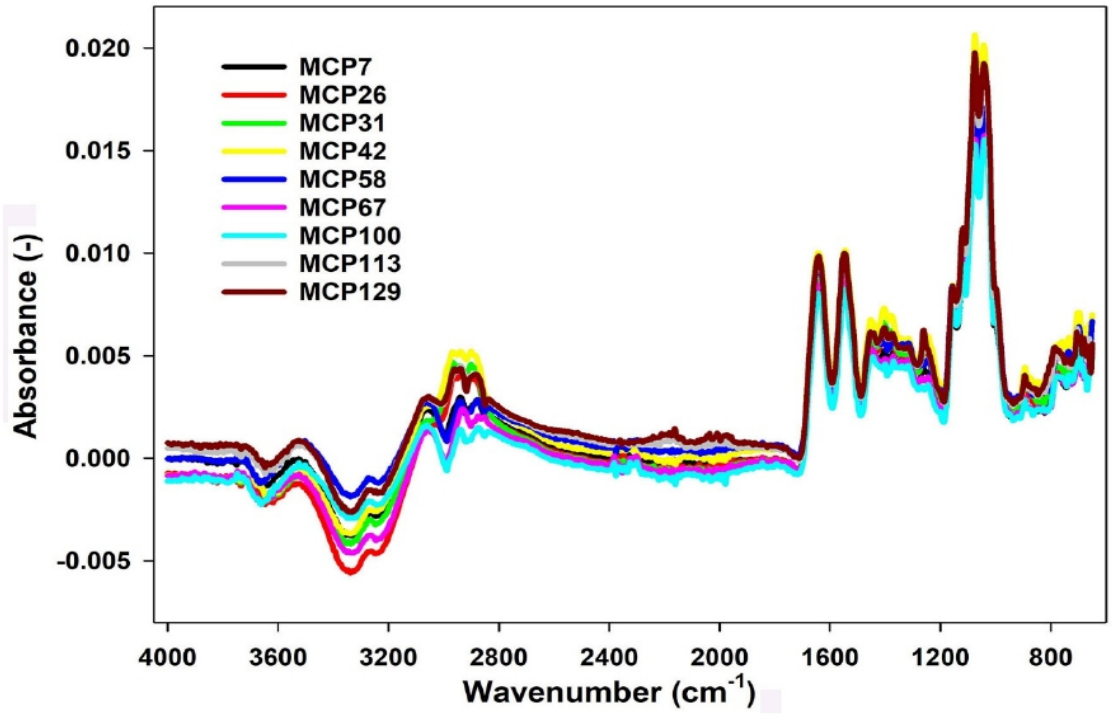


Figure S1. The original FTIR spectra (4000-650 cm^{-1}) of MCP adjusted skim milk varied containing from 7% (MCP_7) to 129% (MCP_{129}) of MCP relative to that of the control achieved by either acidification or alkalisation followed by exhaustive dialysis against bulk milk.

Figure S2. SDS-PAGE electropherograms of the MCP modified skim milk (M) and supernatants (L). Figs A and C are the non-reducing and Figs B and D are reducing SDS PAGE of MCP modified skim milks varied by pH adjustment (4.9-8.2) after dialysis against original skim milk. Lanes M1(Milk pH 4.9); L1(Supernatant pH 4.9); M2(Milk pH 5.5); L2(Supernatant pH 5.5); M3(Milk pH 5.7); L3(Supernatant pH 5.7); M4(Milk pH 5.8); L4(Supernatant pH 5.8); M5(Milk pH 5.9); L5(Supernatant pH 5.9), M6(Milk pH 6.1); L6(Supernatant pH 6.1); M7(Milk pH 6.4); L7(Supernatant pH 6.4); M8(Milk pH 6.7); L8(Supernatant pH 6.7); M9(Milk pH 7.5); L9(Supernatant pH 7.5); M10(Milk pH 8.2); L10(Supernatant pH 8.2). The MCP content was adjusted at these pH levels and equated to 7% at pH 4.9, 26% at pH 5.5, 31% at pH 5.7, 42% at pH 5.8, 58% at pH 5.9, 67% at pH 6.1, 96% at pH 6.4, 100% (control) at pH 6.7), 113% at pH 7.5, 129% at pH 8.2

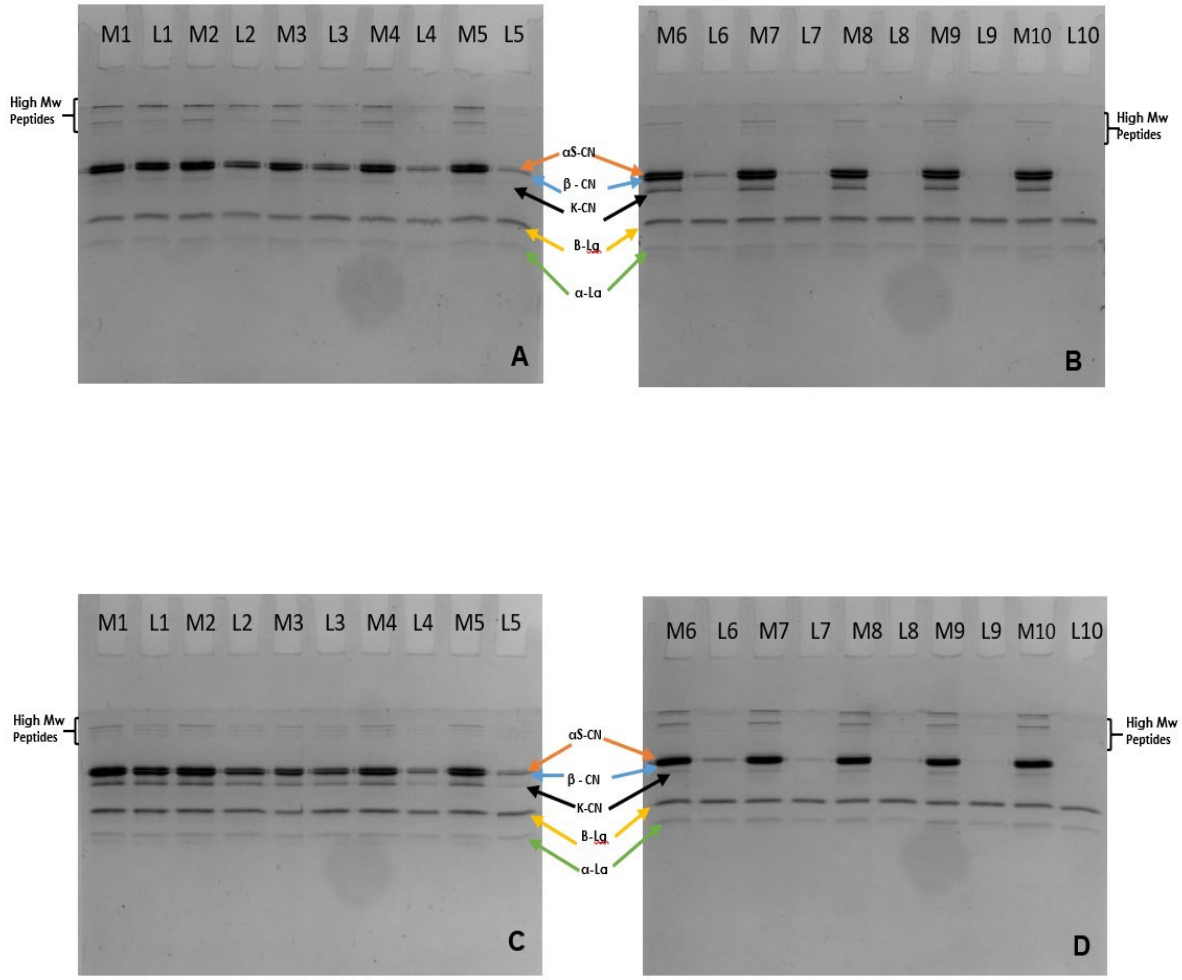


Figure S3. Comparison of the High-performance liquid chromatograms of different MCP modified skim milk samples varied by pH adjustment (4.9-8.2) after dialysis against original skim milk (A) and their supernatants (B). The MCP content was adjusted at these pH levels and equated to 7% at pH 4.9, 26% at pH 5.5, 31% at pH 5.7, 42% at pH 5.8, 58% at pH 5.9, 67% at pH 6.1, 96% at pH 6.4, 100% (control) at pH 6.7), 113% at pH 7.5, 129% at pH 8.2

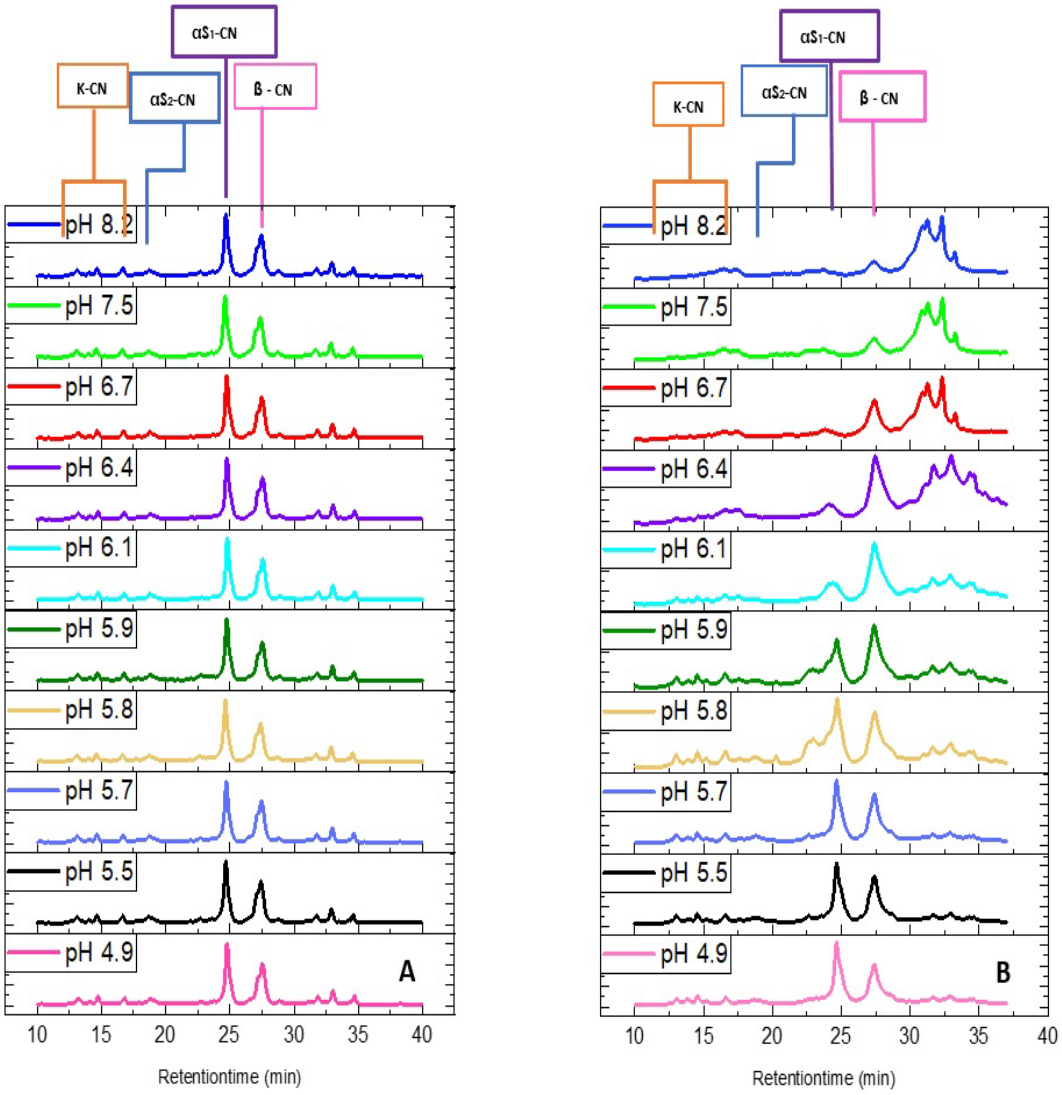


Figure S4. Second derivative of the FTIR spectra (Amide Ⅰ region 1700-1600nm) (A) and the spectra region (1200-900 nm) (B) of MCP modified skim milk varied by acidified pH adjustment (4.9-5.9). Principal component scores for Amide Ⅰ region (C) and Principal component score for the region (1200-900nm) (D). Loading plot for the region (Amide Ⅰ region 1700-1600nm) and (1200-900nm) for MCP modified skim milk by different pH (4.9-5.9) E and F, respectively. The MCP content was adjusted at these pH levels and equated to 7% at pH 4.9, 26% at pH 5.5, 31% at pH 5.7, 42% at pH 5.8, 58% at pH 5.9, 100% (control) at pH 6.7).

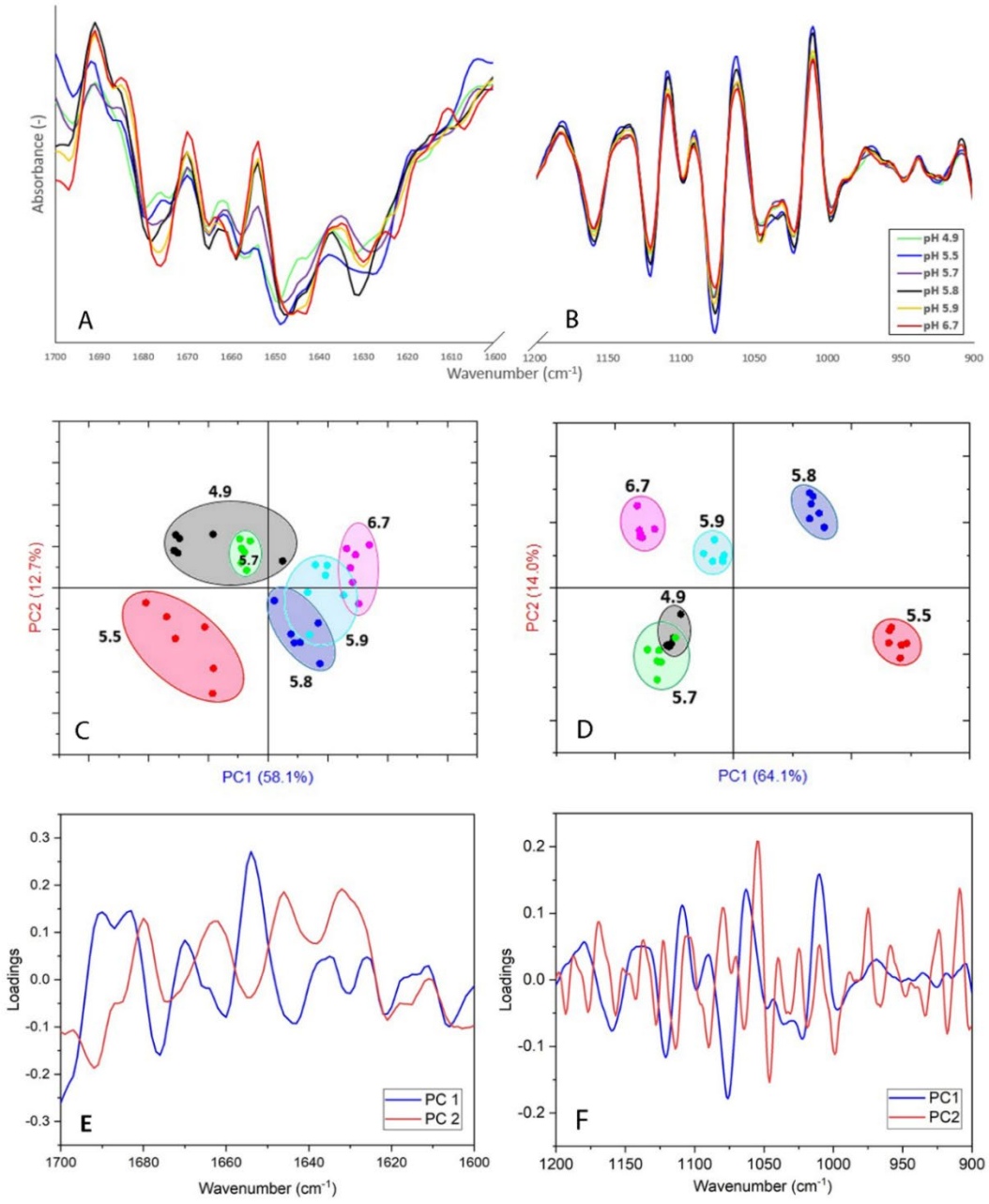


Figure S5. Second derivative of the FTIR spectra (Amide Ⅰ region 1700-1600nm) (A) and the spectra region (1200-900 nm) (B) of MCP modified skim milk varied by pH adjustment (6.1-6.7). Principal component scores for Amide Ⅰ region (C) and Principal component score for the region (1200-900nm) (D). Loading plot for the region (Amide Ⅰ region 1700-1600nm) and (1200-900nm) for MCP modified skim milk by different pH (6.1-6.7) E and F, respectively. The MCP content adjusted at these pH levels equalled to 67% at pH 6.1, 96% at pH 6.4, 100% (control) at pH 6.7

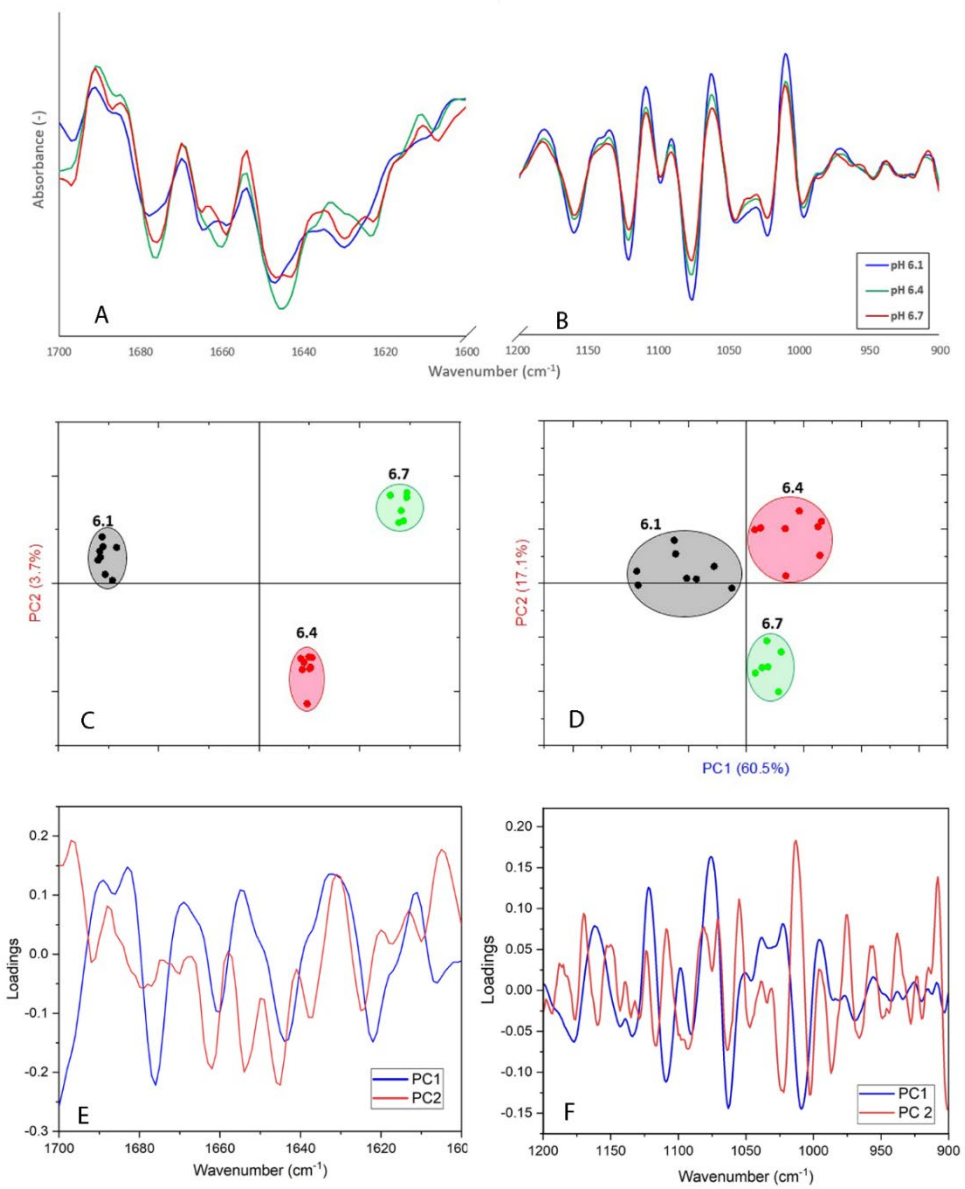
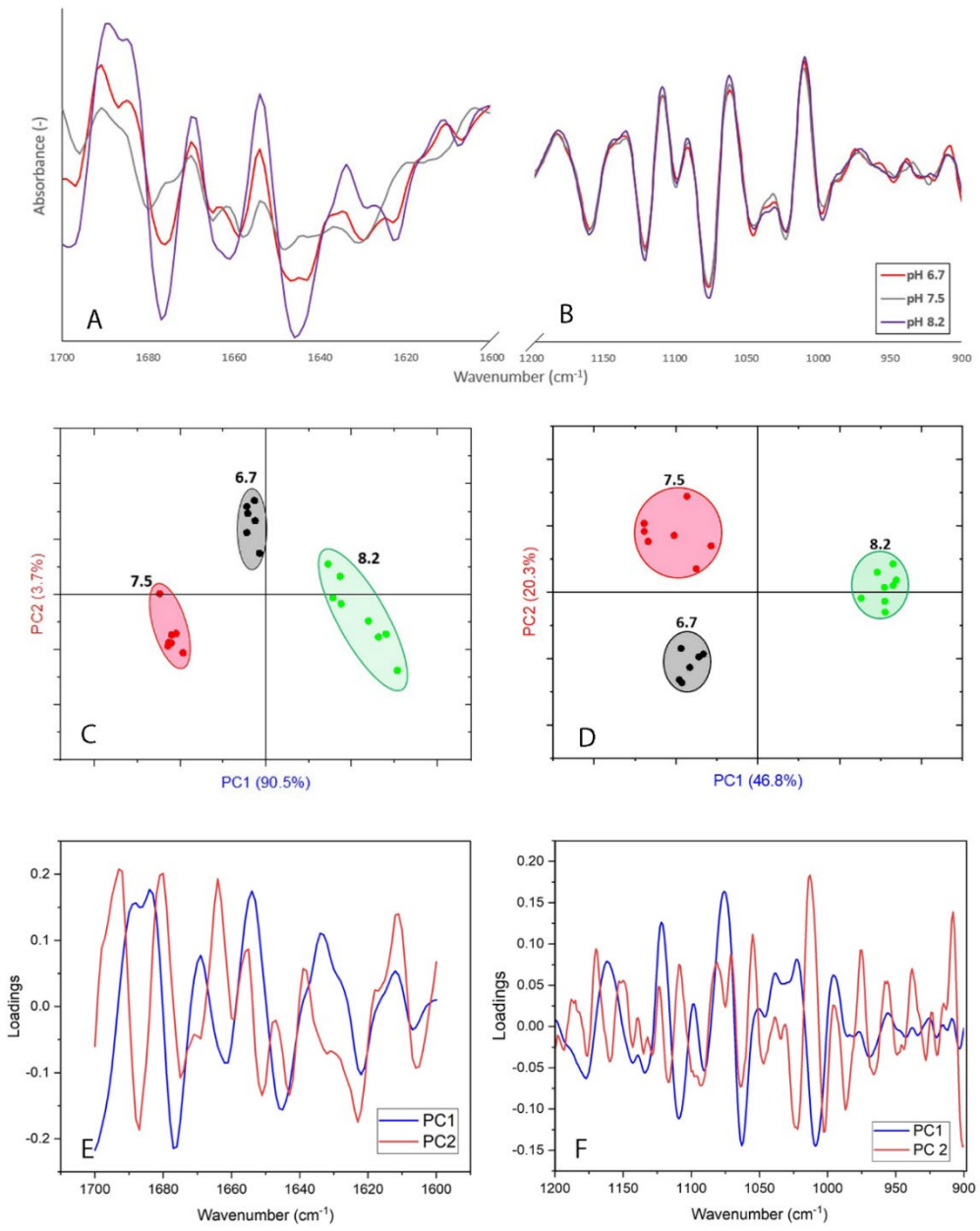


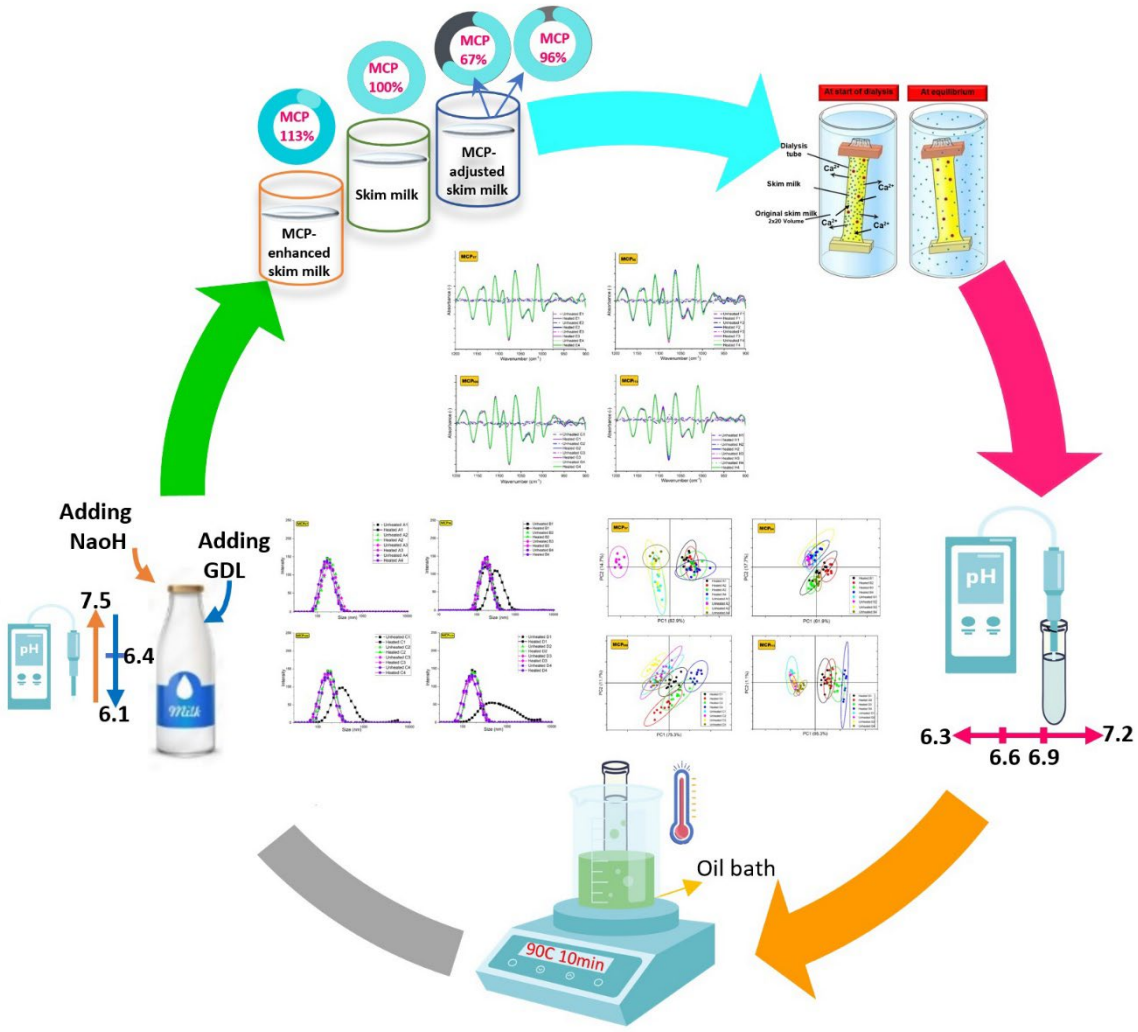
Figure S6. Second derivative of the FTIR spectra (Amide Ⅰ region 1700-1600nm) (A) and the spectra region (1200-900 nm) (B) of MCP modified skim milk varied by alkaline pH adjustment (6.7-8.2). Principal component scores for Amide Ⅰ region (C) and Principal component score for the region (1200-900nm) (D). Loading plot for the region (Amide Ⅰ region 1700-1600nm) and (1200-900nm) for MCP modified skim milk by different pH (6.7-8.2) E and F, respectively. The MCP content adjusted at these pH levels was equal to 100% (control) at pH 6.7, 113% at pH 7.5, 129% at pH 8.2



Chapter 4

Chapter 4. Heat Stability of MCP-adjusted skim milk

This chapter has been published as a research paper entitled “Influence of pH on Heat-Induced Changes in Skim Milk Containing Various Levels of Micellar Calcium Phosphate” by Elaheh Ahmadi, Todor Vasiljevic, and Thom Huppertz in the peer-reviewed Molecules, 2023, 28(19), 6847. https://doi.org/10.3390/molecules28196847



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Todor Vasiljevic	10	Concept development, experimental design, manuscript editing, revision and supervision		07/03/2024
Thom Huppertz	10	Concept development, experimental design, manuscript editing, revision, submission and supervision		07/03/2024

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Influence of pH on Heat-Induced Changes in Skim Milk Containing Various Levels of Micellar Calcium Phosphate

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Abstract: The present study investigated the effect of micellar calcium phosphate (MCP) content and pH of skim milk on heat-induced changes in skim milk. Four MCP-adjusted samples, ranging from 67 to 113% of the original MCP content, were heated (90 °C for 10 min) at different pH values (6.3, 6.6, 6.9, and 7.2), followed by determining changes in particle size, turbidity, protein distribution, and structure. The results demonstrate a strong effect of MCP level and pH on heat-induced changes in milk, with the MCP_{67} samples revealing the greatest thermal stability. Specifically, decreasing MCP content by 33% (MCP_{67}) led to a smaller increase in non-sedimentable κ-casein and a lower decrease in αs_2-casein concentrations after heating compared to other samples. Lower MCP content resulted in a moderate rise in the average particle size and turbidity, along with lower loading of β-turn structural component after heating at low pH (pH 6.3). Notably, MCP_{113} exhibited instability upon heating, with increased particle size, turbidity, and a significant decrease in non-sedimentable αs_2-casein concentration, along with a slight increase in non-sedimentable κ-casein concentration. The FTIR results also revealed higher loading of intermolecular β-sheet, β-turn, and random coil structures, as well as lower loading of α-helix and β-sheet structures in MCP-enhanced skim milk samples. This suggests significant changes in the secondary structure of milk protein and greater formation of larger aggregates.

Keywords: thermal stability; micellar calcium phosphate; adjustment; skim milk; FTIR

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1. Introduction

Heat treatment is one of the most widely used unit operations in the dairy sector and includes different heat intensities, such as thermisation (~62–65 °C for 10–20 s), pasteurisation (~72–80 °C for 15–30 s), ultra-high temperature (UHT) treatment (~135–150 °C for 1–10 s), and sterilisation (~110–120 °C for 10–30 min) [1,2]. Heat treatment is used to eliminate pathogenic microorganisms, increase the shelf life of milk, and/or to impart some desirable functional properties during further industrial processing of dairy products [3]. During thermal processing, milk can be exposed to conditions which alter milk protein structure, solubility, and functionality. Whey proteins play a key role in the heat-induced destabilisation of skim milk, via interactions with the casein micelles and aggregation, but dissociation of individual caseins from the casein micelles are also similarly important. In addition to heating time and temperature, factors such as the pH of the milk and the concentration of the soluble calcium also influence thermal stability of milk proteins [4].

Milk salts play a significant role in heat-induced changes in milk, particularly in relation to the heat-induced interactions between denatured whey proteins and casein micelles [5]. Early investigations revealed that modifications in micellar calcium phosphate (MCP) levels within the casein micelle could enhance the thermal stability of skim milk [6]. These studies emphasized the importance of understanding the interplay between MCP

levels and the thermal characteristics of dairy systems, offering insights into potential strategies for optimizing heat stability of milk [6,7]. For example, Fox and Hoynes [6] showed that heat coagulation time (HCT) of milk was extended upon reduction in the MCP content, while it was reduced upon enhancing the MCP content in comparison to the original skim milk. In addition, the HCT clearly showed a pH dependence, with the greatest stability moving slightly above the natural pH of milk. Singh and Fox [7] reported that MCP adjustment had a minor impact on the levels of non-sedimentable N but somewhat increased the concentration of non-sedimentable N-acetylneuraminic acid (NANA), which appeared to remain elevated upon heating. Anema and Li [8] also explored the impact of varying MCP levels and selected pH on heat-induced changes in reconstituted skim milk, finding that increased MCP levels had minimal impact on the casein micelle dissociation, whereas reduced MCP content resulted in greater micellar dissociation. At pH 6.5, they observed that levels of non-sedimentable k-CN were lower than those at pH 7.1 after heating at 90 °C for 10 min. Other caseins experienced the opposite behaviour—greater concentration at pH 6.5 than those at pH 7.1 [8]. These studies highlighted the importance of MCP in maintaining micellar structure and its impact on the stability of the micelle upon heating [6–8].

As has been stated previously [9], the heat stability of milk is influenced by various factors, including the structural integrity of the casein micelle, particularly the dissociation of κ-casein (k-CN), pH fluctuations that occur during heating [10], and the involvement of ionic calcium. However, so much has been left to be explored when it comes to these impacts, especially the combined effect of MCP adjustment and pH; thus, the investigation is still ongoing. In order to provide further understanding of the behaviour and conformational changes of skim milk during heating, we have designed our investigation to include varying levels of the MCP (67 to 113% of the original) using pH adjustment followed by dialysis and influence of subsequently varying pH levels, ranging from low (pH 6.3) to high (pH 7.2) [11]. This comprehensive approach would contribute to a more holistic understanding of the interplay between MCP content, pH, and heat-induced changes in skim milk, shedding light on its potential applications in the dairy industry.

2. Results

2.1. Calcium Content of MCP-Adjusted Skim Milk

The effect of MCP adjustment on total calcium in unheated skim milk samples is shown in Table 1. The total calcium content of skim milk in the control milk (MCP_{100}) was ~30–31 mmol L^{-1} and adjustment of MCP content clearly influenced the amount of total calcium, as expected. The lowest calcium level was observed in the sample acidified to pH 6.1 prior to dialysis, i.e., sample MCP_{67}, and the highest concentration of Ca was found in sample MCP_{113}, which had been adjusted to pH 7.5 prior to dialysis. Adjustment of pH to 6.3, 6.6, 6.9, or 7.2 after dialysis had no major effect on the total calcium content (Table 1).

2.2. Physiochemical Changes in MCP-Adjusted Skim Milk Samples after Heat Treatment

The Z-average particle size of the samples is shown in Table 1, and particle size distributions of the samples are shown in Figure 1. The average particle size of sample MCP_{100} before heat treatment was ~163 nm (Table 1) and the particle size distribution of all dispersions had a main peak appearing between ~50 and ~400 nm, relating to that of native casein micelles in the MCP-adjusted skim milk samples (Figure 1). Indicatively from Table 1, turbidity was not affected; thus, the appearance of skim milk, such as its colour, did not change. The pH adjustment did not affect the average particle size and turbidity of unheated samples (Table 1).

After heating the control samples (MCP_{100}) at pH 6.6, 6.9, or 7.2 at 90 °C for 10 min, particle size decreased by 12–22 nm (Table 1) and the particle size distribution shifted towards somewhat smaller particles (Figure 1). This heat-induced reduction in the average particle size of control milk is in agreement with the results previously reported [12]. Subjecting samples MCP_{67}, MCP_{96}, and MCP_{113} to heat treatment had no effect on the

particle size at pH 6.6, whereas heating at pH 6.9 and 7.2 also resulted in a reduction in the particle size (Table 1). Heat treatment at pH 6.3 increased average particle size substantially, by 47–321 nm, with the extent of the heat-induced increase in particle size at this pH increasing with enhanced MCP content (Table 1; Figure 1). Along with these heat-induced increases in particle size, increases in turbidity were also observed (Table 1). The largest particle size (484 nm) and greatest turbidity (40.22) were observed for the milk sample MCP_{113} heated at pH 6.3 (Table 1). Although it is commonly observed that whey proteins undergo denaturation upon heating and interact with caseins [13], the substantial increase in particle size and turbidity observed after heat treatment of MCP_{113} at pH 6.3 exceeds the anticipated effects of whey protein aggregation alone in milk and rather suggests heat-induced aggregation of casein micelles.

Table 1. Calcium concentration in milk before heat treatment, particle size of MCP-adjusted milk before and after heat treatment for pasteurised skim milk samples with their MCP adjusted from 67% (MCP_{67}) to 113% (MCP_{113}) by either acidification or alkalisation followed by exhaustive dialysis against bulk milk [1,2]. For sample details, see Section 4.1 [1].

MCP-Adjusted Samples	pH	Total Ca (mmoL L^{-1})	Particle Size Unheated (nm)	Particle Size Heated (nm)	Turbidity Unheated (cm^{-1})	Turbidity Heated (cm^{-1})
MCP_{67}	6.3	21.76 ± 0.05^{E}	160 ± 1^{Ab}	207 ± 1^{Da}	0.29 ± 0.01^{Ab}	0.53 ± 0.03^{Ca}
	6.6	21.76 ± 0.11^{E}	161 ± 2^{Aa}	157 ± 1^{Ea}	0.34 ± 0.01^{Aa}	0.36 ± 0.02^{Da}
	6.9	22.01 ± 0.05^{E}	162 ± 1^{Aa}	152 ± 1^{Eb}	0.27 ± 0.02^{Ab}	0.32 ± 0.01^{Da}
	7.2	21.81 ± 0.20^{E}	160 ± 1^{Aa}	150 ± 3^{Eb}	0.26 ± 0.03^{Aa}	0.29 ± 0.02^{Da}
MCP_{96}	6.3	25.70 ± 0.05^{D}	161 ± 1^{Ab}	296 ± 5^{Ca}	0.29 ± 0.00^{Ab}	0.90 ± 0.07^{Ba}
	6.6	26.10 ± 0.00^{CD}	161 ± 0^{Aa}	156 ± 1^{Ea}	0.32 ± 0.02^{Aa}	0.35 ± 0.01^{Db}
	6.9	25.90 ± 0.13^{D}	161 ± 1^{Aa}	154 ± 1^{Eb}	0.31 ± 0.01^{Aa}	0.32 ± 0.02^{Db}
	7.2	24.53 ± 0.07^{D}	162 ± 1^{Aa}	149 ± 2^{Eb}	0.26 ± 0.03^{Aa}	0.33 ± 0.01^{Db}
MCP_{100}(Control)	6.3	27.85 ± 0.08^{C}	162 ± 0^{Ab}	325 ± 6^{Ba}	0.36 ± 0.03^{Ab}	0.94 ± 0.02^{Ba}
	6.6	27.46 ± 0.05^{C}	163 ± 1^{Aa}	151 ± 1^{Eb}	0.35 ± 0.04^{Aa}	0.37 ± 0.02^{Da}
	6.9	27.65 ± 0.05^{C}	165 ± 1^{Aa}	145 ± 1^{Eb}	0.32 ± 0.02^{Aa}	0.34 ± 0.01^{Da}
	7.2	26.83 ± 0.55^{C}	164 ± 1^{Aa}	142 ± 2^{Eb}	0.32 ± 0.02^{Aa}	0.31 ± 0.00^{Da}
MCP_{113}	6.3	31.25 ± 0.18^{A}	163 ± 1^{Ab}	484 ± 3^{Aa}	0.34 ± 0.02^{Ab}	1.60 ± 0.04^{Aa}
	6.6	32.31 ± 0.12^{A}	165 ± 1^{Aa}	160 ± 1^{Ea}	0.35 ± 0.00^{Ab}	0.42 ± 0.01^{Da}
	6.9	30.46 ± 0.02^{B}	165 ± 1^{Aa}	147 ± 1^{Eb}	0.34 ± 0.01^{Aa}	0.38 ± 0.03^{Da}
	7.2	32.76 ± 0.12^{A}	165 ± 1^{Aa}	143 ± 1^{Eb}	0.34 ± 0.02^{Aa}	0.33 ± 0.03^{Da}

[1] The subscripts indicate proportion of retained MCP relative to that of the control; [2] the capital letters indicate significant differences across entire rows. The small letters show significant differences between two correlated samples across the columns ($p < 0.05$).

2.3. Heat-Induced Changes in the Protein Distribution of MCP-Adjusted Skim Milk

The proportion of individual caseins and whey proteins in the supernatant of milk samples is shown in Table 2. Before heat treatment, ~4, 13, 18, 7, 88, and 72% of αs_1-, αs_2-, β-, and κ-CN, α-lactalbumin, and β-lactoglobulin, respectively, were found in the supernatant of sample MCP_{100} at pH 6.6. A reduction in MCP content resulted in an increase in the proportion of αs_1-, αs_2-, β-, and κ-CNs in the supernatant of unheated skim milk samples (Table 2), whereas non-sedimentable α-lactalbumin and β-lactoglobulin remained constant. MCP enrichment before heating did not significantly change the level of non-sedimentable αs_1-CN at pH 6.6, whereas the levels of αs_2-, β-, and κ-CNs in the supernatant decreased significantly compared to sample MCP_{100} (Table 2). Our results are in alignment with a previous study [7]. Adjusting pH to 6.3, 6.6, 6.9, or 7.2 of skim milk samples before heating only caused noticeable changes in levels of non-sedimentable αs_2- and κ-CN. The level of non-sedimentable αs_2-CN increased with decreasing pH (Table 2). In MCP-reduced unheated skim milk samples, non-sedimentable κ-CN increased when pH was reduced to 6.3, while its concentration was reduced in MCP-enhanced skim milk samples when pH was adjusted to 6.3. Adjusting pH above the natural pH of milk resulted in a reduction

in non-sedimentable κ-CN independent of the MCP content (Table 2). Heat treatment of sample MCP_{113} at pH 6.3 and 6.6 resulted in a notable increase in non-sedimentable αs_1-CN. A similar increase was noticed in MCP_{100} at pH 6.6. On the other hand, in all other skim milk samples, heat treatment decreased the levels of non-sedimentable αs_1-CN (Table 2; Figure 2). Heat treatment also reduced levels of non-sedimentable α_{s2}-CN and β-CN in all samples at all pH values, except for sample MCP_{113} at pH 6.3, in which the heat treatment led to an increase in the level of non-sedimentable α_{s2}-CN (Table 2; Figure 2). In all heated samples, levels of non-sedimentable α_{s2}-CN and β-CN appear to decrease with rise in pH, while non-sedimentable α_{s1}-CN varied only slightly (Table 2), which is in agreement with the previously reported findings [8]. That study reported on the impact of heating temperature on properties of reconstituted skim milk with adjusted MPC content at pH 6.5 and 7.1 [8].

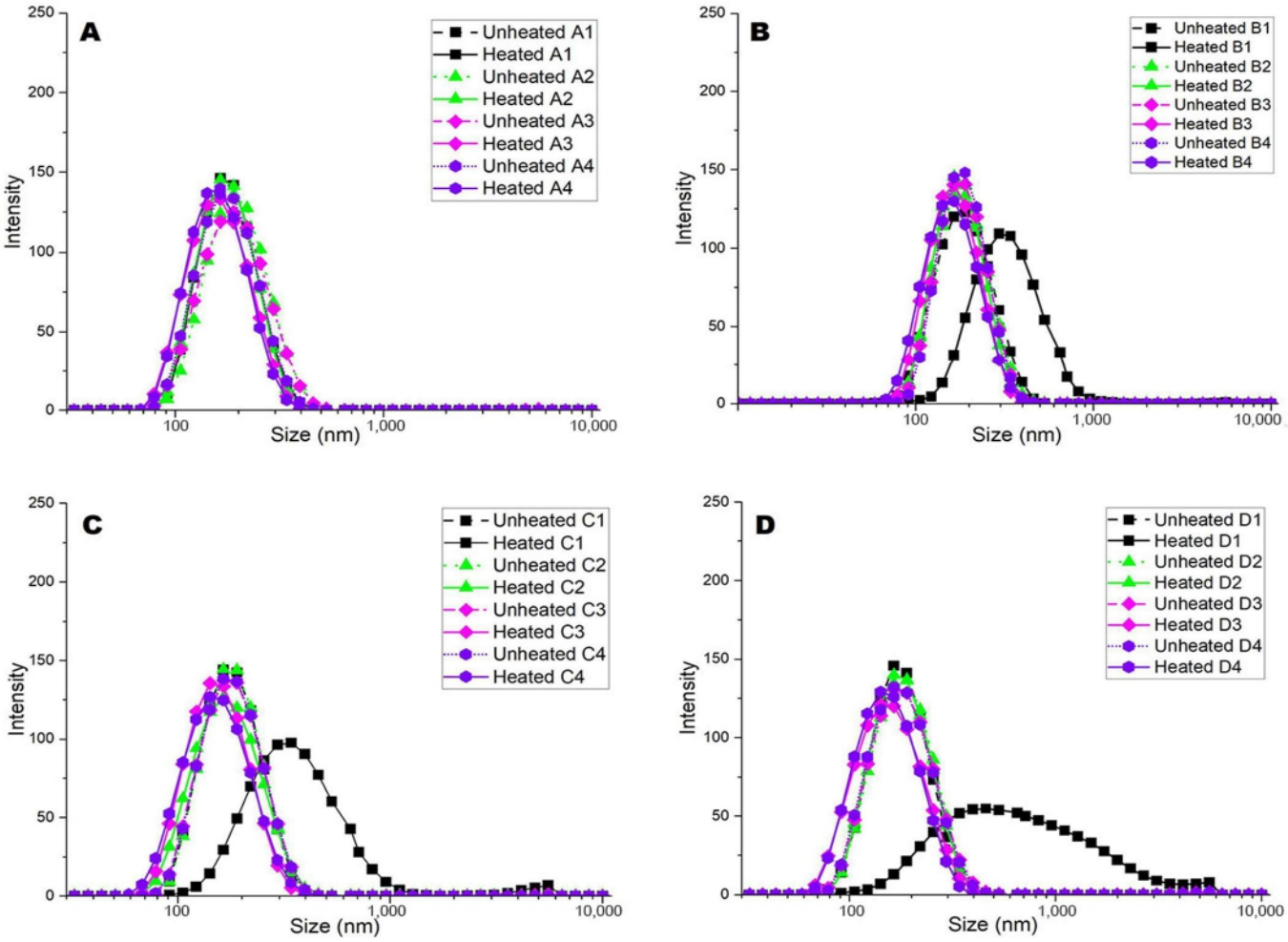

Figure 1. Particle size distribution of MCP-adjusted skim milk samples. MCP content was adjusted, varied by (**A**) 67% (MCP_{67}), (**B**) 96% (MCP_{96}), (**C**) 100% (MCP_{100} or Control), and (**D**) 113% (MCP_{113}) relative to the control by either acidification or alkalisation followed by dialysis against original skim milk. Numbers 1 to 4 represent different readjusted pH, including 1 (pH 6.3), 2 (pH 6.6), 3 (pH 6.9), and 4 (pH 7.2). Graph is representative of two replicate samples.

Table 2. Influence of heat treatment (90 °C for 10 min) on levels of non-sedimentable caseins and whey proteins in MPC-adjusted skim milk adjusted to pH 6.3, 6.6., 6.9, or 7.2 prior to heating [1,2].

MCP-Adjusted Samples	pH	Milk Protein Concentration (%)											
		αs_1-Casein		αs_2-Casein		β-Casein		κ-Casein		α-Lactalbumin		β-Lactoglobulin	
		Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
MCP_{67}	6.3	14.2 ± 0.1^{Ba}	5.7 ± 0.2^{Ab}	27.5 ± 0.3^{Aa}	13.6 ± 0.5^{Ab}	36.9 ± 0.1^{Aa}	20.8 ± 0.2^{Ab}	21.4 ± 0.1^{Ab}	26.9 ± 0.1^{Ga}	84.0 ± 0.3^{Ba}	20.2 ± 0.02^{Gb}	69.4 ± 0.4^{Da}	44.6 ± 0.2^{Bb}
	6.6	14.0 ± 0.1^{Bb}	5.8 ± 0.4^{Ab}	20.3 ± 0.1^{Ba}	11.1 ± 0.1^{Bb}	36.3 ± 0.0^{Aa}	18.6 ± 0.1^{Bb}	15.1 ± 0.0^{Cb}	24.9 ± 0.1^{Ha}	79.1 ± 0.9^{CDa}	24.2 ± 1.0^{Fb}	70.0 ± 0.2^{Da}	54.2 ± 0.3^{Ab}
	6.9	14.5 ± 0.1^{Ba}	5.8 ± 0.1^{Ab}	16.1 ± 0.0^{Ca}	1.3 ± 0.0^{Db}	36.5 ± 0.1^{Aa}	10.7 ± 0.1^{Fb}	16.5 ± 0.1^{Bb}	44.4 ± 0.1^{Aa}	82.8 ± 0.2^{Ba}	79.2 ± 0.4^{Ab}	72.0 ± 0.2^{Ca}	56.1 ± 0.8^{Ab}
	7.2	16.1 ± 0.0^{Aa}	5.8 ± 0.2^{Ab}	13.1 ± 0.1^{Da}	2.0 ± 0.1^{Db}	36.3 ± 0.1^{Aa}	11.6 ± 0.1^{Fb}	15.8 ± 0.0^{BCb}	43.7 ± 0.1^{Ba}	80.4 ± 0.2^{Ca}	60.4 ± 0.1^{Eb}	70.8 ± 0.3^{Da}	56.3 ± 0.1^{Ab}
MCP_{96}	6.3	5.2 ± 0.1^{Ca}	3.8 ± 0.9^{Bb}	16.1 ± 0.0^{Ca}	8.6 ± 0.1^{Cb}	17.4 ± 0.0^{BCa}	16.8 ± 0.3^{Cb}	7.6 ± 0.1^{Db}	23.1 ± 0.1^{Ja}	81.8 ± 0.1^{Ca}	9.5 ± 0.1^{Hb}	76.4 ± 0.1^{BCa}	32.7 ± 0.3^{Eb}
	6.6	5.9 ± 0.1^{Ca}	3.7 ± 0.3^{Bb}	16.3 ± 0.1^{Ca}	9.2 ± 0.1^{Cb}	18.5 ± 0.1^{Ba}	16.8 ± 0.1^{Cb}	7.3 ± 0.1^{Db}	23.2 ± 0.0^{Ja}	81.7 ± 0.1^{Ca}	24.2 ± 0.2^{Fb}	77.8 ± 0.1^{Ba}	32.4 ± 0.1^{Eb}
	6.9	5.1 ± 0.1^{Ca}	2.2 ± 0.1^{Cb}	17.0 ± 0.2^{Ca}	1.3 ± 0.0^{Db}	19.0 ± 0.1^{Ba}	7.3 ± 0.1^{Hb}	5.9 ± 0.0^{DEb}	39.6 ± 0.1^{Da}	83.7 ± 1.6^{Ba}	70.1 ± 0.3^{Bb}	69.2 ± 0.2^{Da}	44.8 ± 0.4^{Bb}
	7.2	5.5 ± 0.4^{Ca}	4.2 ± 0.1^{Bb}	6.7 ± 0.1^{Ea}	1.9 ± 0.1^{Db}	17.6 ± 0.2^{BCa}	9.4 ± 0.1^{Gb}	5.6 ± 0.1^{DEb}	37.1 ± 0.1^{Ea}	81.5 ± 0.1^{Ca}	63.5 ± 0.5^{Db}	67.8 ± 0.1^{Ja}	31.5 ± 0.2^{Eb}
MCP_{100}(Control)	6.3	3.8 ± 0.1^{Da}	3.7 ± 0.1^{Ba}	11.1 ± 0.1^{DEa}	8.3 ± 0.1^{Cb}	18.0 ± 0.0^{BCa}	12.8 ± 0.3^{Eb}	4.2 ± 0.0^{Eb}	19.7 ± 0.1^{Ka}	83.0 ± 0.4^{Ba}	10.5 ± 0.1^{Hb}	76.3 ± 0.0^{BCa}	39.8 ± 0.1^{Cb}
	6.6	3.6 ± 0.1^{Db}	4.0 ± 0.5^{Ba}	12.9 ± 0.0^{Da}	8.9 ± 0.0^{Cb}	17.9 ± 0.1^{BCa}	13.2 ± 0.1^{Eb}	6.7 ± 0.1^{Db}	19.4 ± 0.1^{Ka}	87.6 ± 0.2^{Aa}	23.1 ± 0.1^{Fb}	72.2 ± 0.0^{Ca}	37.0 ± 0.2^{Db}
	6.9	3.5 ± 0.0^{Da}	2.4 ± 0.3^{Cb}	15.6 ± 0.1^{Ca}	1.2 ± 0.0^{Db}	19.8 ± 0.0^{Ba}	7.5 ± 0.0^{Hb}	6.3 ± 0.1^{Db}	35.8 ± 0.0^{Fa}	86.5 ± 0.1^{Aa}	67.0 ± 1.4^{Cb}	73.8 ± 0.1^{Ca}	43.9 ± 0.2^{Bb}
	7.2	3.9 ± 0.1^{Da}	3.7 ± 0.1^{Bb}	8.3 ± 0.0^{Ea}	1.5 ± 0.0^{Db}	12.1 ± 0.0^{CDa}	8.5 ± 0.1^{GHb}	5.3 ± 0.0^{DEb}	35.8 ± 0.1^{Fa}	79.9 ± 1.3^{Ca}	63.4 ± 0.1^{Db}	72.5 ± 0.3^{Ca}	32.7 ± 0.1^{Eb}
MCP_{113}	6.3	3.0 ± 0.1^{Db}	4.1 ± 0.1^{Ba}	5.5 ± 0.2^{EFb}	7.5 ± 0.1^{Ca}	13.3 ± 0.1^{Cb}	15.1 ± 0.1^{Da}	4.2 ± 0.1^{Eb}	23.9 ± 0.1^{Ia}	88.8 ± 0.2^{Aa}	10.2 ± 0.0^{Hb}	83.9 ± 0.2^{Aa}	37.1 ± 0.1^{Db}
	6.6	3.4 ± 0.1^{Db}	4.0 ± 0.1^{Ba}	7.6 ± 0.1^{Ea}	8.1 ± 0.1^{Ca}	14.8 ± 0.1^{Ca}	13.4 ± 0.1^{Eb}	5.7 ± 0.1^{DEb}	23.8 ± 0.1^{Ia}	87.1 ± 0.9^{Aa}	19.9 ± 0.2^{Gb}	77.2 ± 0.2^{BCa}	22.6 ± 0.1^{Fb}
	6.9	3.6 ± 0.3^{Da}	1.3 ± 0.1^{Cb}	6.7 ± 0.1^{Ea}	0.6 ± 0.0^{DEb}	10.2 ± 0.1^{Da}	6.3 ± 0.0^{HIb}	3.5 ± 0.0^{Eb}	40.8 ± 0.1^{Ca}	88.7 ± 0.3^{Aa}	63.6 ± 0.2^{Db}	76.1 ± 0.1^{BCa}	38.8 ± 0.0^{Cb}
	7.2	3.0 ± 0.1^{Da}	1.4 ± 0.1^{Cb}	6.7 ± 0.0^{Ea}	0.0 ± 0.0^{Eb}	10.3 ± 0.1^{Da}	7.3 ± 0.0^{Hb}	3.8 ± 0.1^{Eb}	37.0 ± 0.1^{Ea}	86.1 ± 0.3^{Aa}	60.3 ± 0.3^{Eb}	78.8 ± 0.0^{Ba}	24.6 ± 0.1^{Fb}

[1] The subscripts indicate proportion of retained MCP relative to that of the control; [2] the capital letters indicate significant differences across entire rows. The small letters show significant differences between two correlated samples across the columns ($p < 0.05$).

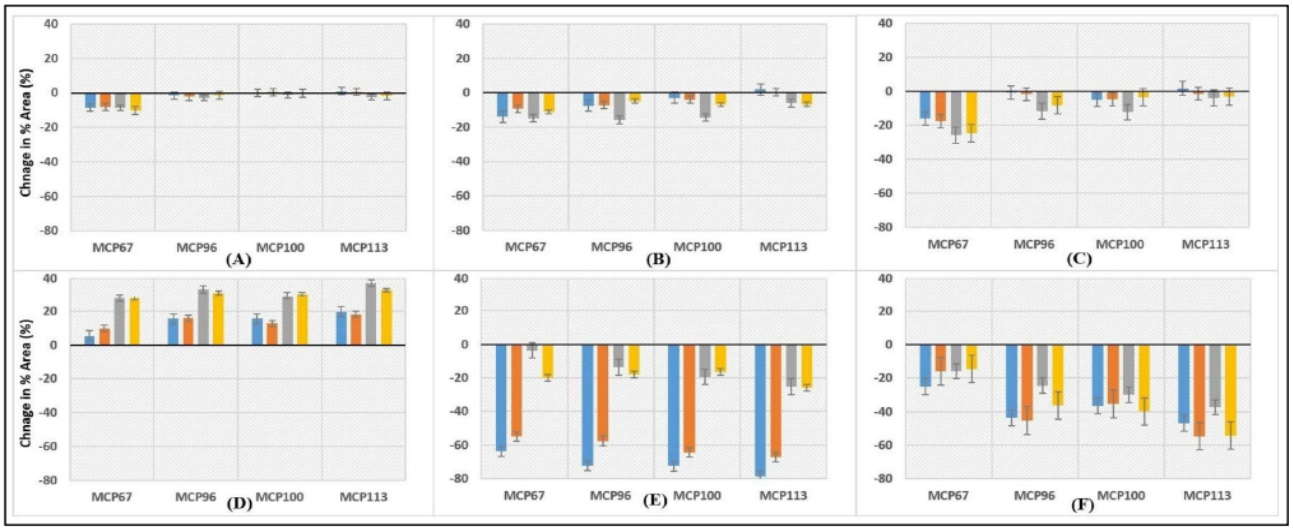

Figure 2. The total percentage areas of various proteins: (**A**) αs_1-CN, (**B**) αs_2-CN, (**C**) β-CN, (**D**) κ-CNs, (**E**) α-La, and (**F**) β-Lg in the supernatant of MCP-adjusted skim milk in relation to the entire milk were calculated by differentiating between heated and unheated skim milk samples. The MCP content of skim milk samples was adjusted, varied by 67% (MCP_{67}), 96% (MCP_{96}), 100% (MCP_{100} or Control), and 113% (MCP_{113}) relative to the control by either acidification or alkalisation followed by dialysis against original skim milk. Supernatant obtained from unheated and heated milk at 90 °C for 10 min at pH 6.3 ■; pH 6.6 ■; pH 6.9 ■; and pH 7.2 ■.

Contrary to the other caseins, the concentration of non-sedimentable κ-CN substantially increased after heat treatment, which was observed across all the samples (Table 2, Figure 2). Levels of non-sedimentable κ-CN in samples heated at pH 6.9 and 7.2 were considerably higher than those in the samples heated at pH 6.3 and 6.6. Only small differences at a given pH were observed between heated samples of different MCP contents (Table 2; Figure 2). These observations appear in agreement with a previous study, which showed a greater dissociation of κ-CN at pH 7.1 in comparison to that at pH 6.5 and governed by MCP content [8].

Levels of non-sedimentable α-La and β-Lg decreased after heat treatment, which is expected due to heat-induced denaturation and aggregation of whey proteins. MCP-adjustment had a notable impact on the distribution of the whey proteins compared to the control sample (MCP_{100}) and levels of non-sedimentable whey proteins in heated samples decreased with increasing MCP content (Figure 2). In addition, it was observed that the final pH adjustment had a more pronounced impact on reducing the concentration of whey proteins at lower pH levels (pH 6.3), whereas, at higher pH levels (pH 6.9 and 7.2), the decrease in non-sedimentable whey protein was lower.

2.4. FTIR Fingerprinting

2.4.1. Region I: Amide I (1700–1600 cm^{-1})

FTIR was used to fingerprint the changes in the secondary structure of milk proteins as a result of the heat treatment (Figure 3). The FTIR spectra in the region between 1700 and 1600 cm^{-1} arising from the absorption associated with the amide I region (1700 and 1600 cm^{-1}) has six features: intermolecular/aggregated β-sheets (1700–1681 cm^{-1}), β-turns (1680–1660 cm^{-1}), α-helix (1652–1641 cm^{-1}), random coils (1640–1631 cm^{-1}), intramolecular β-sheet (1630–1620 cm^{-1}), and side chains (1618–1608 cm^{-1}) [14]. Figure 3 shows the dominant effect of heat treatment on the secondary structure in skim milk samples, particularly in the area of intermolecular β-sheets (1700–1681 cm^{-1}), presumably due to heat-induced aggregate formation. PCA analysis confirmed the distinction between heated and unheated samples by effectively separating them (Figure 4). Heated MCP-enhanced

skim milk samples exhibited greater loading for intramolecular β-sheet structures, whereas, in the MCP-depleted samples, the changes were not substantial compared to unheated original skim milk samples (Figure 3). Furthermore, the intramolecular β-sheet structures also displayed pH dependence; at pH 6.3, there was a noticeable shift in loading with increased peak intensity, indicating a higher presence of intermolecular β-sheet structures (Figure 3).

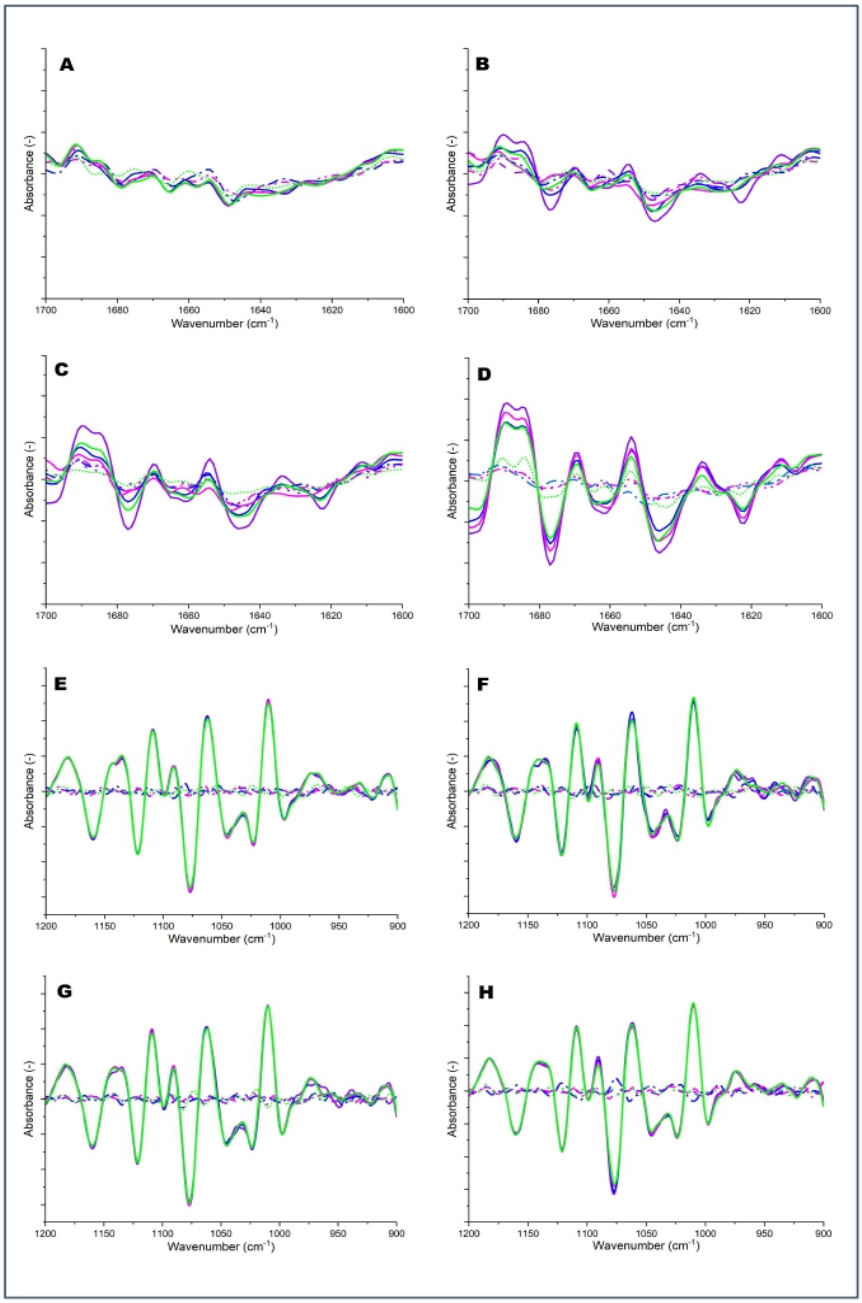

Figure 3. Second derivative of the FTIR spectra (Amide I region 1700–1600 cm^{-1}) (**A**) MCP_{67}, (**B**) MCP_{96}, (**C**) MCP_{100}, (**D**) MCP_{113} and the spectra region (1200–900 nm) (**E**) MCP_{67}, (**F**) MCP_{96}, (**G**) MCP_{100}, and (**H**) MCP_{113}. – – Unheated 1; —— Heated 1; – · – Unheated 2; —— Heated 2; – · – Unheated 3; —— Heated 3; ····· Unheated 4; —— Heated 4. Numbers 1 to 4 represent 1: pH 6.3, 2: pH 6.6, 3: pH 6.9, and 4: pH 7.2.

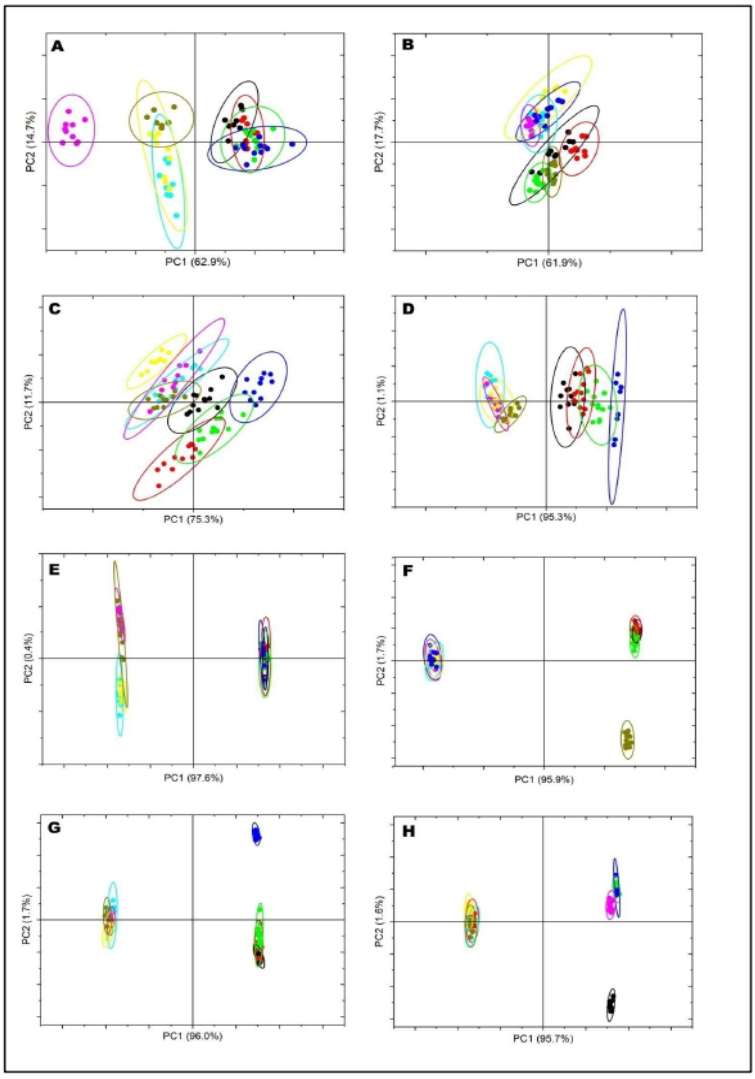

Figure 4. Principal component scores for Amide I region (1700–1600 cm^{-1}) (**A**) MCP_{67}, (**B**) MCP_{96}, (**C**) MCP_{100}, (**D**) MCP_{113} and the spectra region (1200–900 nm) (**E**) MCP_{67}, (**F**) MCP_{96}, (**G**) MCP_{100}, and (**H**) MCP_{113}. ● Unheated 1; ● Heated 1; ● Unheated 2; ● Heated 2; ● Unheated 3; ● Heated 3; ● Unheated 4; ● Heated 4. Numbers 1 to 4 represent 1: pH 6.3, 2: pH 6.6, 3: pH 6.9, and 4: pH 7.2.

Heat-induced changes in milk caused an increase in the intensity of β-turn peaks (1660–1680 cm^{-1}), as observed in FTIR analysis. The presence of β-turns in milk proteins is considered a consequence of protein unfolding from higher-order structures [15]. Adjustment of MCP content influenced the intensity of peaks in the β-turn region. In heated MCP_{113} samples, an increase in β-turn was observed (Figure 3). However, in the heated MCP-depleted skim milk samples (MCP_{67}), there were no significant changes compared to unheated milk samples. The changes in β-turns were also influenced by the pH adjustment. The intensity of the β-turn peak showed a negative correlation with pH, indicating that, at lower pH levels (pH 6.3), there was a higher presence of β-turns (Figure 3). In addition, there was a substantial decrease in the intensity of the peak in the α-helix region (1652–1641 cm^{-1}) in heated MCP-enhanced samples (MCP_{113}), whereas the random coils (1640–1631 cm^{-1}) exhibited a remarkable increase after heating (Figure 3). Notably, the absorbance at the random coil area was significantly lower in skim milk samples with a reduced MCP content, which corresponded to the higher heat stability of these samples. The most prominent change in the α-helix and random coil was observed when the sample

pH was adjusted to 6.3 (Figure 3). Moreover, decreases in ordered secondary structure, mainly β-sheet (1630–1620 cm^{-1}), upon heating were expected. Interestingly, the intensity of the peak in highly MCP-reduced samples (MCP_{67}) was close to the original unheated milk, while β-sheet rose in the control and MCP-enriched samples, which has been related to substantial aggregation [15]. In particular, the lowest loading of intramolecular β-sheet (1630–1620 cm^{-1}) was at lower pH (6.3) in heated skim milk samples, which exhibited substantial denaturation and aggregation of proteins. The rise in the loadings around 1618–1608 cm^{-1} has also been attributed to disruption of intramolecular hydrogen bonds within a secondary structure [16], leading to formation of new stronger intermolecular hydrogen bonds (Figure 3).

2.4.2. Region II (1200–900 cm^{-1})

Region II (1200–900 cm^{-1}) of analysis was able to detect changes in both milk carbohydrates and minerals, as shown in Figure 4. The intensity of peaks around 1200 and 1100 cm^{-1} depicts various carbohydrate vibrations, with lactose being the primary component [17], which did not change substantially as a result of MCP adjustment, pH adjustment, or heat treatment. Decreasing the pH caused a slight decrease in the loading at 1089–1058 cm^{-1}, which indicates the dissociation of phosphate from casein micelle. Conversely, higher pH demonstrated increased absorption (Figure 3). The absorption values of this peak (1089–1058 cm^{-1}) were found to be lower for heated milk when compared to unheated milk (Figure 3). PCA also confirmed the difference in this region by classifying the samples into two groups. Principal Component 1 (PC1) separated the heated skim milk samples from unheated samples (Figure 4).

Changes in the intensity of the peaks around 995 and 987 cm^{-1} in Figure 3 depict changes in stretching vibrations of the $–PO3^{2-}$ moiety of phosphoserine [17] and are related to the dissociation of MCP. As expected, MCP-reduced skim milk samples (MCP_{67}) exhibited a lower intensity of peaks at 995–987 cm^{-1}, while MCP-enhanced samples (MCP^{113}) showed a higher intensity of peak around this area (995 and 987 cm^{-1}). The pH adjustment to a low value (6.3) led to a decrease in the intensity of the peak (995 and 987 cm^{-1}), which likely depicted the dissociation of MCP from the phosphoserine residues. Heat treatment also caused lower loading at 995 and 987 cm^{-1}, which is indicative of the impact of heating on calcium solubility, as illustrated in Figure 3. The PCA analysis also supported the disparity in the mineral regions by dividing the samples into two distinct groups. PC1 was able to differentiate between heated skim milk samples and unheated samples (Figure 4).

3. Discussion

In addition to time and temperature, the thermal stability of milk is strongly influenced by pH and minerals [2]. The present study examined the thermal behaviour of the MCP-adjusted skim milk samples, with the MCP content varying from 67 to 113% of that found in the control milk at pH adjusted to 6.3, 6.6, 6.9, or 7.2. Based on the previous research, pH adjustment followed by dialysis is a recognized method for modulating micellar calcium content of milk [11]. Our preliminary study revealed that MCP-reduced skim milk samples appeared to retain casein micelle integrity when MCP content was reduced by up to 33% (MCP_{67}). Such an adjustment of the MCP content within the casein micelle has been reported to affect the thermal stability of skim milk expressed as HCT [6]. The relationship was inverse—lowering or enhancing MCP content prolonged or shortened HCT, respectively [6]. Singh and Fox [7] further emphasized the inverse relationship between levels of micellar calcium phosphate (MCP) and the thermal stability of skim milk at alkaline pH. They also stated that the behaviour of k-CN may not be fully driven by MCP content and suggested that some of this protein may be attached to the micelle through some other electrostatic attractions [7]. Anema and Li [8] investigated how changing MCP levels and pH affected heat stability of reconstituted skim milk. They observed that lowering of MCP levels led to substantial micellar dissociation, while MCP levels

greater than the original MCP content had very little effect on the micelle integrity [8]. When considering the impact of heating on skim milk, it is important to note that the reduction in calcium phosphate solubility occurs as the temperature increases [5,18]. κ-CN can interact with whey proteins on the surface of casein micelles [19] and in the serum phase of milk in the form of soluble complexes [20]. In the present study, κ-CN displayed more pronounced changes compared to the other caseins upon MCP adjustment at different pH levels (Figure 2). The concentration of non-sedimentable κ-CN significantly increased by reducing the MCP content, similar to results previously reported [8]; at the same time, no significant change in particle size and turbidity was observed at pH above 6.6; however, both parameters increased significantly when the samples were heated at low pH (Table 1). These observations indicate that the MCP appears to be an important factor in maintaining the micellar integrity, while some other factors are involved when it comes to pH-dependant behaviour. However, it seems obvious that milk proteins behave differently below the natural pH of milk that could also be indirectly governed by the MCP content.

It is well known that during heating of milk at 90 °C, two main phenomena take place—k-CN dissociates from the micelle, which is illustrated by its concentration rise in the serum phase (Table 2) and denaturation of whey proteins, which consequently create sedimentable and non-sedimentable aggregates with k-CN and/or themselves (Table 2). The presence of aggregated proteins is evidenced by the level of loading at 1700–1681 cm^{-1}, suggesting the formation of intramolecular β-sheet aggregates (Figure 3). The lower loading at these wavelengths appears to be related to the reduced MCP content.

Furthermore, lowering of the MCP content leads to greater micellar dissociation and greater content of individual caseins in the soluble phase, which may also start participating directly or indirectly in these complex reactions (Table 2). For example, the concentration of non-sedimentable αs_2-CN decreased after heating at pH 6.9 and 7.1. While its concentration also decreased in comparison to the original levels, the levels of non-sedimentable αs_2-CN remained high after heating at pH 6.6 and 6.3 (Table 2, Figure 2). This suggests that sedimentable aggregates created at elevated pH contained more αs_2-CN linked via its sulfhydryl groups to other particles, including either complexes with k-CN and whey proteins or reattachment to the casein micelle. The particle size (and turbidity) remained fairly consistent across most of the MCP/pH range, which changed once pH before heating was adjusted to 6.3. A notable trend was observed that the particle size and turbidity were MCP-dependent at this pH—being larger at higher MCP content (Table 1). Reduction in all heat-reactive proteins, including αs_2-CN, k-CN, β-lactoglobulin, and even α-lactalbumin, in the soluble phase after heating is indicative of excessive aggregation and precipitation after centrifugation. A threefold increase in the particle size after heating of MCP_{113} at pH 6.3 is suggestive of casein–casein interactions via covalent or calcium-induced bridging due to higher Ca content in this sample (Table 1). Calcium forms complexes with the phosphate groups found on αs_2-CN [21] and the binding of Ca to αs-CNs is stronger than binding to other caseins [22], which could address the decline in non-sedimentable portion of this protein in this sample compared to the MCP_{67} sample heated at the same pH. The FTIR analysis also illustrated a notable decrease in the intensity of the peak in the α-helix region (1652–1641 cm^{-1}), accompanied by a remarkable increase in the presence of random coils (1640–1631 cm^{-1}) after increasing the MCP content in skim milk followed by heating (Figure 3). It can be related to substantial unfolding of the secondary structure of the proteins, leading to denaturation of whey proteins or greater contribution of caseins, which are mainly characterised by a large proportion of random structures [23].

Interactions among milk proteins at low MCP level heated at pH 6.3 appear to be different. While the increase in particle size was expected due to minimisation of the surface charge, the rise was not as great as the one observed with the enhanced MCP sample. The fundamental difference is in the greater presence of non-sedimentable caseins resulting from reduced MCP content that may have governed aggregation behaviour of whey proteins. For example, less β-lactoglobulin and α-lactalbumin was incorporated into sedimentable aggregates when the sample with reduced MCP content was heated at pH 6.3

(Table 2). It is known that β- and αs_1-CNs possess a high chaperone-like activity [24]. This activity is not a true chaperon activity, as it only governs the aggregation step of the reaction, leading to the formation of smaller non-sedimentable particles (Table 2, Figure 2) [25]. The decrease observed in β-turns (1660–1680 cm^{-1}) upon reducing the MCP content and heating (Figure 3) may be thus attributed to reduced interactions between κ-casein and β-lactoglobulin (predominantly). These interactions are known to contribute to the formation of loops, triple helices, and turns [23].

Previous studies indicated that the heat stability of milk was substantially reduced at lower pH levels [6,7]. As shown in our study, initial pH adjustment causes substantial structural modifications of proteins, which likely heightens their reactivity during heat treatment. In addition, heating also leads to a slight drop in the pH of the milk due to the release of hydrogen ions [10]. Therefore, it was expected that lowering the pH would intensify a greater presence of κ-CNs in the serum before heating, along with enhanced dissociation of casein micelles during heating at this pH, leading to considerable aggregation of casein micelles. Previous studies have also reported an increased amount of whey proteins complexed with the micelles at lower pH [19,26]. However, this general trend was not really observed in the samples with lower MCP content heated at low pH, as the absence of larger particles indicated different aggregation patterns, resulting in smaller particles. If the standard test for measuring heat stability of milk was used, in this case, measurement of HCT, that would likely indicate greater stability due to the absence of visible aggregation.

4. Materials and Methods

4.1. Sample Preparation

The design of the experimental work is schematically presented in Figure 5. Freshly pasteurised skim milk was obtained from a commercial dairy (Warrnambool Cheese and Butter—Saputo, Warrnambool, Australia). To prevent bacterial growth, sodium azide (0.02%, *w*/*w*) was added to the milk. According to a protocol described previously [11], a predetermined amount of glucono delta-lactone (GDL) or 1.0 M NaOH was added to lower or increase the pH of skim milk to 6.1, 6.4, 6.7, or 7.5. After pH was stabilised, the samples were dialysed using a high retention seamless cellulose dialysis tubing (14 kDa MWCO, Sigma-Aldrich, St. Louis, MO, USA) against 2 × 20 volumes of original pasteurised skim milk for 72 h at 5 °C [11,27]. After dialysis, the samples were removed from the dialysis tubing. The sample coding was based on the estimate of micellar Ca relative to that of the control as described previously [27]. Following this procedure, the MCP_{67} sample thus had its MCP content reduced from the initial 100% to 67%. Conversely, in the MCP_{113} sample, the MCP content increased by 13% compared to the initial MCP amount of 100%. Then, the pH of the samples was adjusted to 6.3, 6.6, 6.9, or 7.2. Once the pH was stable, the samples were heated in an oil bath set at 90 °C. The time to reach 90 °C was ~3 min, after which samples were held for a further 10 min at this temperature and then cooled to 20 °C by immersion in an ice bath.

4.2. Sample Fractionation

Unheated and heated milk samples were fractionated by ultracentrifugation at 100,000× *g* for 1 h at 20 °C in a Beckman Ultra L-70 centrifuge (Beckman Coulter, Australia Pty. Ltd., Gladesville, Australia). After the ultracentrifugation, the clear supernatant was carefully collected from each tube using a syringe.

4.3. Sample Analysis

4.3.1. Calcium Content

The total calcium (Ca) of the unheated MCP-adjusted pH-adjusted skim milk samples was determined using an inductively coupled plasma atomic emission spectrometer (ICP-AES, ICPE-9000 system, Shimadzu Corporation, Kyoto, Japan), following the method of Bijl et al. [2].

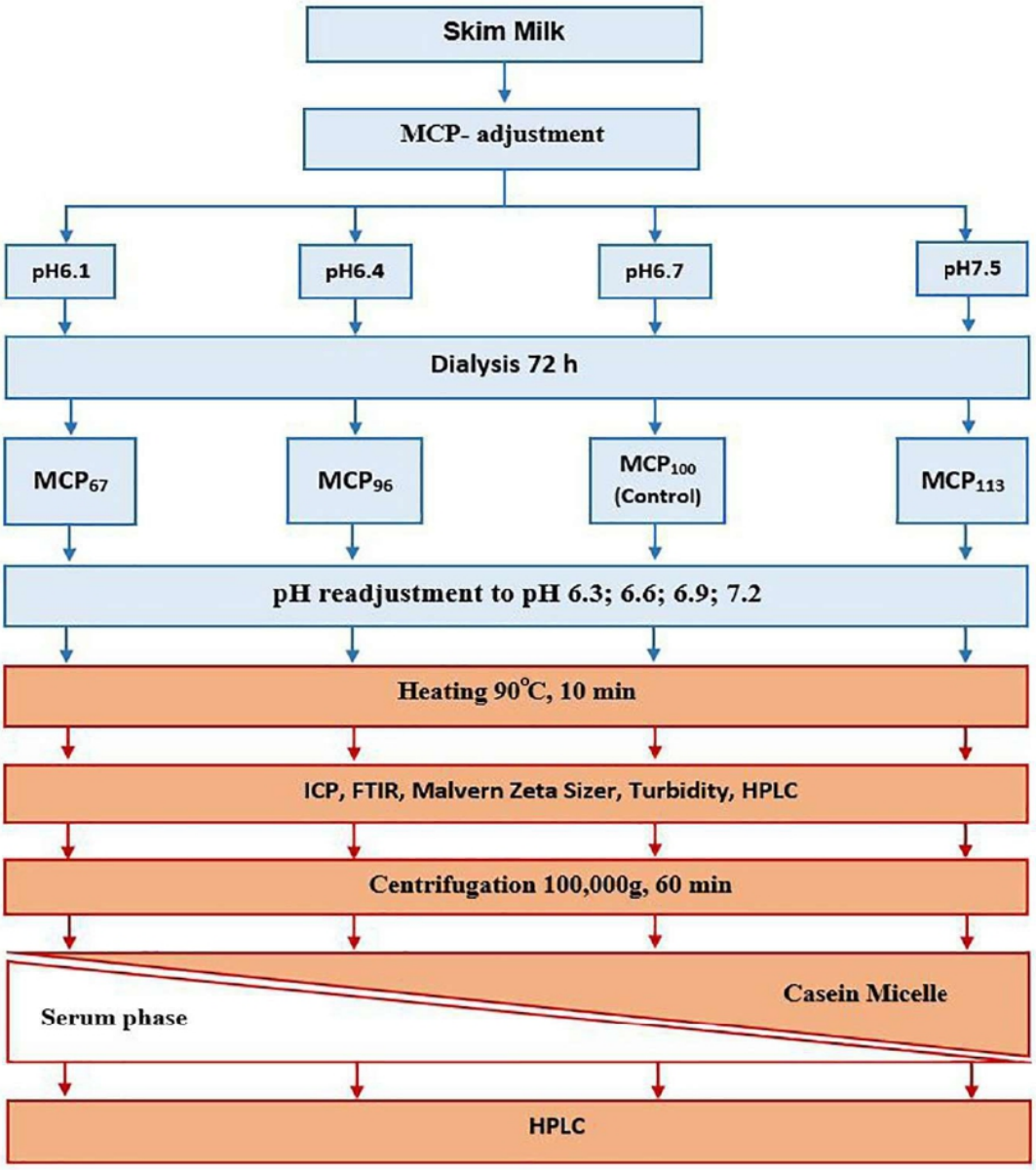

Figure 5. Experimental design of the study.

4.3.2. Turbidity

Turbidity of the samples was measured at 860 nm using a 1 mm pathlength quartz cuvette using a UV-Visible spectrophotometer (Biochrom Ltd., Cambridge, UK).

4.3.3. Particle Size Distribution

Particle size analysis of samples was performed by dynamic light scattering (Zetasizer-Nano, Malvern instruments Ltd., Malvern, UK) at a scattering angle of 90° and temperature was maintained at 25 °C. Samples were diluted in simulated milk ultrafiltrate (SMUF) [28] in a ratio of 1:100 [2].

4.3.4. High-Performance Liquid Chromatography (HPLC)

Individual caseins in whole samples and ultracentrifugal supernatants were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) at room

temperature using a Shimadzu HPLC system (Model Prominence-i, LC-2030 C, Shimadzu Corporation, Kyoto, Japan) with a Varian 9012 system controller (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with an RI detector (Varian, Palo Alto, CA, USA, 9050) and a C4 column (Aeris WIDEPORE, 150 mm × 4.6 mm, 3.6 µm particle size, 300 Å porosity, Phenomenex, Torrance, CA, USA) using pretreatment and elution conditions as described previously by Aprianita et al. [29]

4.3.5. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR measurements were conducted using an FTIR spectrometer (PerkinElmer, Boston, MA, USA) in the range of 4000–600 cm^{-1}. At the start of measurement, the background spectrum was scanned with a blank (SMUF) and corresponding ultracentrifugal supernatant using the same instrumental conditions as for the sample spectra acquisition [17]. FTIR experiments for each sample were replicated twice (on two sets of samples). Principal component analysis (PCA) was employed to better understand the changes in the conformation of caseins induced by different environments. FTIR data processed as described previously was reported with the Origin software (Origin Pro 2021, v. 95E, OriginLab Corporation, Northampton, MA, USA) [17].

4.4. Statistical Analysis

All experiments assessed the impact of heat treatment on the selected parameters in the MCP-adjusted skim milk. SPSS software (v. 26, IBM Inc. Chicago, IL, USA) was used to conduct a two-way analysis of variance (ANOVA) to establish differences among means, followed by Tukey's multi-comparison of the means. The level of significance was set at $p < 0.05$. The design was replicated three times on three different occasions.

5. Conclusions

The result of this study shows a strong influence of MCP adjustment on the thermal behaviour of milk proteins and, consequently, on the heat stability of milk. The key finding achieved by this study was that the sample containing the lowest MCP content appeared to have a high level of intact casein micelles and likely the greatest thermal stability among all samples. The pH level of the milk was also observed to have a direct relationship with its heat stability, with lowered pH levels resulting in reduced heat stability. MCP adjustment leads to differing behaviour of individual caseins, which dissociate to a certain extent at lower MCP content, while remaining at high levels at an MPC level above the initial. This leads to different interactions among proteins in the soluble and colloidal phases and different outcomes. Smaller particle size is observed at low pH and low MCP content; thus, greater heat stability may be expected. Notably, this work provided for the first time an insight into the effect of heat treatment on the conformational changes of MCP-adjusted and enriched skim milk heated at different pH. This can assist in greater understanding of the functional properties of MCP-adjusted skim milk in industrial-scale dairy processing to achieve skim milk with improved thermal and structural stability.

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Supplementary data

Table S1. Influence of heat treatment (90 °C for 10 min) on levels of caseins and whey proteins in whole milk of MPC-adjusted skim milk adjusted to pH 6.3, 6.6., 6.9, or 7.2 prior to heating [1].

MCP Adjusted sample	pH	αs_1-Casein (mg.ml^{-1})		αs_2-Casein (mg.ml^{-1})		β-Casein (mg.ml^{-1})		κ-Casein (mg.ml^{-1})		α-lactalbumin (mg.ml^{-1})		β-Lactoglobulin(mg.ml^{-1})	
		Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
MCP_{67}	6.3	13.83±0.02^{Aa}	11.88±0.07^{Bb}	2.58±0.13^{Aa}	2.38±0.09^{ABb}	13.52±0.14^{Aa}	11.87±0.07^{Ab}	2.52±0.02^{Aa}	2.07±0.28^{Ab}	1.64±0.12^{Aa}	1.47±0.30^{Ab}	3.50±0.13^{Aa}	1.24±0.06^{BCDEb}
	6.6	13.74±0.02^{Aa}	10.32±0.18^{Db}	2.61±0.10^{Aa}	2.22±0.05^{Bb}	13.54±0.02^{Aa}	10.95±0.10^{CDb}	2.51±0.14^{Aa}	1.79±0.08^{ABCb}	1.56±0.07^{Aa}	1.03±0.17^{ABCb}	3.64±0.03^{Aa}	1.72±0.19^{Ab}
	6.9	13.78±0.10^{Aa}	10.60±0.04^{Db}	2.55±0.09^{Aa}	2.42±0.05^{ABb}	13.58±0.18^{Aa}	11.19±0.08^{CDb}	2.55±0.09^{Aa}	1.74±0.10^{ABCb}	1.58±0.06^{Aa}	1.43±0.23^{Ab}	3.63±0.07^{Aa}	1.71±0.00^{Ab}
	7.2	13.77±0.07^{Aa}	12.26±0.28^{ABb}	2.60±0.14^{Aa}	2.63±0.07^{Aa}	13.54±0.09^{Aa}	11.90±0.10^{Ab}	2.52±0.04 ^{Aa}	1.81±0.06^{ABb}	1.59±0.11^{Aa}	1.52±0.15^{Ab}	3.65±0.06^{Aa}	1.58±0.13^{ABCb}
MCP_{96}	6.3	13.85±0.05^{Aa}	12.48±0.04^{Ab}	2.56±0.04^{Aa}	2.40±0.12^{ABb}	13.64±0.10 ^{Aa}	11.79±0.05^{ABb}	2.50±0.02^{Aa}	1.93±0.07^{ABb}	1.65±0.09^{Aa}	1.21±0.08^{ABb}	3.45±0.06^{Aa}	1.13±0.08^{DEb}
	6.6	13.86±0.04^{Aa}	12.37±0.09^{ABb}	2.59±0.08^{Aa}	2.54±0.14^{ABa}	13.76±0.18^{Aa}	11.92±0.08^{Ab}	2.59±0.08^{Aa}	1.79±0.08^{ABCb}	1.64±0.02^{Aa}	1.08±0.18^{ABCb}	3.55±0.05^{Aa}	1.60±0.07^{ABb}
	6.9	13.87±0.10^{Aa}	11.21±0.08^{Cb}	2.51±0.14^{Aa}	2.53±0.07^{ABa}	13.70±0.12^{Aa}	11.36±0.17^{BCb}	2.55±0.19^{Aa}	1.81±0.30^{ABb}	1.66±0.21^{Aa}	1.14±0.07^{ABCb}	3.63±0.16^{Aa}	1.59±0.11^{ABCb}
	7.2	13.78±0.10^{Aa}	10.31±0.14^{Db}	2.53±0.21^{Aa}	2.37±0.03^{ABb}	13.68±0.03^{Aa}	11.17±0.09^{CDb}	2.51±0.04^{Aa}	1.84±0.12^{ABb}	1.68±0.01^{Aa}	1.09±0.04^{ABCb}	3.63±0.04^{Aa}	1.37±0.06^{ABCDb}
MCP_{100} (Control)	6.3	13.78±0.02^{Aa}	10.26±0.22^{Db}	2.63±0.12^{Aa}	1.35±0.05^{Cb}	13.83±0.05^{Aa}	8.45±0.29^{Hb}	2.64±0.05^{Aa}	1.57±0.07^{ABCb}	1.43±0.10^{Aa}	1.04±0.15^{ABCb}	3.71±0.05^{Aa}	0.96±0.04^{Eb}
	6.6	13.80±0.03^{Aa}	10.23±0.08^{DEb}	2.67±0.13^{Aa}	1.05±0.06^{CDb}	13.85±0.03^{Aa}	10.34±0.06^{EFb}	2.67±0.13^{Aa}	1.49±0.05^{BCb}	1.45±0.17^{Aa}	0.76±0.16^{BCDb}	3.65±0.04^{Aa}	1.18±0.12^{DEb}
	6.9	13.85±0.04^{Aa}	10.61±0.04^{Db}	2.65±0.09 ^{Aa}	1.14±0.14^{CDb}	13.89±0.07^{Aa}	10.81±0.09^{Db}	2.69±0.09^{Aa}	1.45±0.14^{BCb}	1.41±0.08^{Aa}	0.83±0.02^{BCDb}	3.71±0.14^{Aa}	1.12±0.04^{DEb}
	7.2	13.82±0.03^{Aa}	9.73±0.01^{EFb}	2.61±0.08^{Aa}	1.09±0.05^{CDb}	13.87±0.04^{Aa}	10.07±0.08^{FGb}	2.62±0.03^{Aa}	1.52±0.09^{ABCb}	1.46±0.02^{Aa}	0.62±0.07^{CDb}	3.73±0.15^{Aa}	1.05±0.06^{DEb}
MCP_{113}	6.3	13.84±0.05^{Aa}	7.38±14^{Gb}	2.57±0.07^{Aa}	0.93±0.08^{Db}	13.89±0.12^{Aa}	7.85±0.10^{Ib}	2.62±0.01^{Aa}	1.23±0.15^{Cb}	1.52±0.04^{Aa}	0.47±0.03^{Db}	3.36±0.06^{Aa}	1.01±0.14^{DEb}
	6.6	13.78±0.03^{Aa}	10.63±04^{Db}	2.63±0.56^{Aa}	1.05±0.02^{CDb}	13.90±0.08^{Aa}	10.75±0.05^{DEb}	2.63±0.05^{Aa}	1.49±0.13^{BCb}	1.55±0.13^{Aa}	0.70±0.02B^{CDb}	3.38±0.14^{Aa}	1.26±0.06^{BCDEb}
	6.9	13.84±0.04^{Aa}	10.62±06^{Db}	2.59±0.08^{Aa}	1.13±0.04^{CDb}	13.81±0.04^{Aa}	10.82±0.06^{Db}	2.58±0.11^{Aa}	1.48±0.08^{BCb}	1.50±0.09^{Aa}	0.65±0.06^{CDb}	3.34±0.06^{Aa}	1.21±0.08^{CDEb}
	7.2	13.84±0.08^{Aa}	9.32±0.12^{Fb}	2.55±0.03^{Aa}	1.16±0.05^{CDb}	13.84±0.08^{Aa}	9.85±0.06^{Gb}	2.60±0.06^{Aa}	1.42±0.07^{BCb}	1.58±0.16^{Aa}	0.65±0.04^{CDb}	3.38±0.18^{Aa}	1.12±0.08^{DEb}

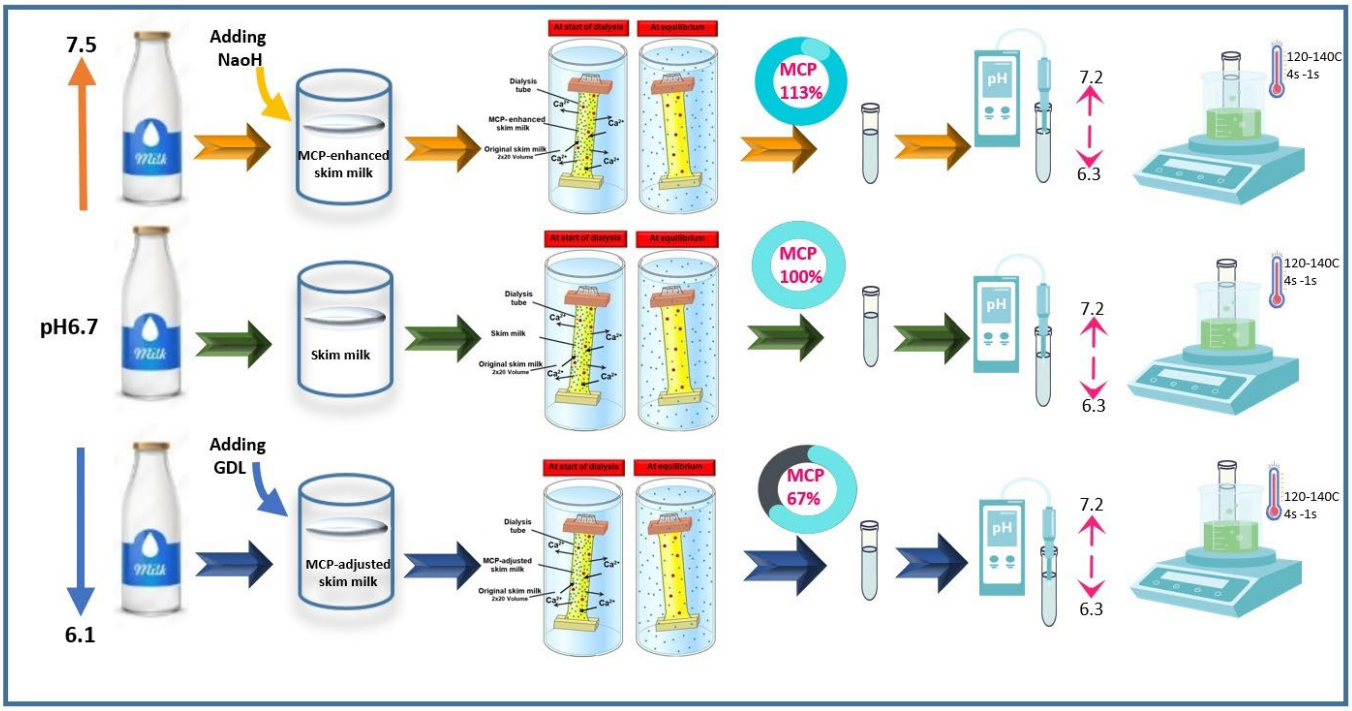
[1] The subscripts indicate proportion of retained MCP relative to that of the control; [2] the capital letters indicate significant differences across entire rows. The small letters show significant differences between two correlated samples across the columns ($p < 0.05$).

Chapter 5

Chapter 5. Ultra-high temperature (UHT) stability of MCP-adjusted Skim milk

This chapter has been published as a research paper entitled “Heat stability of skim milk containing various levels of micellar calcium phosphate” by Elaheh Ahmadi, Thom Huppertz and Todor Vasiljevic in the peer-reviewed. International Dairy Journal, 2024. 105900. https://doi.org/10.1016/j.idairyj.2024.105900

Correction note: Coagulated milk appeared thicker and showed signs of separation, with solids (curds) separating from the liquid (whey). The appearance would change from a homogeneous liquid to a lumpy or chunky texture. It was recognizable through visual inspection.



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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
Elaheh Ahmadi	80	Designed and wrote the original draft, conceptualised, reviewed, edited, revised the manuscript, designed and conducted, the experiments, and data analysis	[redacted]	07/03/2024
Thom Huppertz	10	Concept development, experimental design, manuscript editing, revision and supervision		07/03/2024
Todor Vasiljevic	10	Concept development, experimental design, manuscript editing, revision, submission and supervision		07/03/2024

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Heat stability of skim milk containing various levels of micellar calcium phosphate

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ABSTRACT

This study assessed thermal stability in micellar calcium phosphate (MCP)-adjusted skim milk samples containing 67 (MCP_{67}) or 113 (MCP_{113}) % of the MCP content of control milk (MCP_{100}) at 120 °C for 5 s or 140 °C for 1 s at pH 6.3, 6.6, 6.9 or 7.2. Sample MCP_{67} exhibited the smallest heat-induced reduction in non-sedimentable individual caseins and whey proteins, and only limited heat-induced increases in particle size and turbidity. MCP_{67} samples exhibited the highest levels of non-sedimentable k-casein post-heating, a key factor in heat coagulation. Sample MCP_{113} displayed the strongest heat-induced decrease in non-sedimentable casein, coupled with the highest heat-induced increases in particle size and turbidity, suggesting comparatively lower thermal stability. Moreover, elevated MCP levels in MCP_{113} samples might contribute to micelle instability. Milk pH at heating exhibited a linear correlation with heat stability. Overall, the findings emphasize the substantial influence of MCP and pH on heat-induced alterations in sterilized milk.

1. Introduction

The heat stability of milk relates to its ability to retain its physical and chemical characteristics when it undergoes heat treatment. This characteristic is crucial in various dairy applications, particularly in processes like ultra-high temperature (UHT) treatment and other sterilization treatments (Dumpler, Huppertz, & Kulozik, 2020). Sterilization of milk is used for preservation through either in-container sterilization (115–120 °C for 5–15 min) or by continuous UHT treatment (135–150 °C for 1–10 s) (Dumpler et al., 2020; Lewis, Grandison, Lin, & Tsioulpas, 2011). While sterilization of milk offers benefits in terms of food safety and shelf-life, there are also some challenges and issues that can arise in the industry, which is including protein denaturation, Maillard reaction, vitamin degradation, and sediment formation on the surfaces (Dumpler et al., 2020).

During sterilization of milk, several structural changes occur due to the high temperature and short duration of heating (Dumpler et al., 2020). These changes collectively contribute to the stability of sterilized milk by changing in protein structure. Sterilization causes denaturation of the whey proteins present in milk. The denatured proteins can form aggregates, resulting in changes in the viscosity, texture, and stability of the milk (Qian et al., 2017). The Maillard reaction, which is a non-enzymatic browning reaction, can also occur during UHT treatment. It involves the reaction between lactose and amino acids, resulting in the formation of brown pigments and flavour compounds. It is important to note that these changes are influenced by various factors, including minerals especially calcium, temperature, pH, lactose and amino acid concentrations, reaction time, and the presence of catalysts or inhibitors (Lewis et al., 2011; Qian et al., 2017).

In addition, thermal treatment can also cause instability of milk as a result of the heat-induced aggregation of casein micelles, which can lead to either flocculation or complete gelation of products during or shortly after heat treatment. Over the years, many investigations have focused on the pivotal elements influencing the heat stability of skim milk, such as pH, minerals, κ-casein, and whey proteins (Tessier & Rose, 1964; Fox & Hoynes, 1975; Singh & Fox, 1987; Corredig & Dalgleish, 1996; Anema & Li, 2000, 2003; Wang & Ma, 2020; Ahmadi, Vasiljevic, & Huppertz, 2023). Several studies highlighted the importance of micellar calcium phosphate (MCP, also referred to as colloidal calcium phosphate, CCP) in maintaining micellar structure and its influence on micelle stability during heating (Anema & Li, 2000; Fox & Hoynes,

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1975; Singh & Fox, 1987). Regarding the heat stability of skim milk, Fox and Hoynes (1975) demonstrated an inverse relationship between MCP content and heat coagulation time (HCT) in skim milk at 140 °C. Furthermore, the heat coagulation time (HCT) notably exhibited a dependency on pH, indicating optimal stability just above the natural pH of milk (Fox & Hoynes, 1975).

Our previous investigation (Ahmadi et al., 2023) explored into the impact of heat treatment on MCP-adjusted skim milk at temperatures <100 °C across different pH levels. Notably, we observed a strong effect of MCP content on heat-induced changes at temperatures below 100 °C. Such findings could be applicable for some thermal treatments in the dairy sector, e.g., pasteurization of milk or heating of milk for yoghurt manufacture. However, for relevance for e.g., in-container sterilization and UHT treatment, temperatures >100 °C are required. Hence, expanding on this previous study, our objective was to investigate the influence of MCP content on thermal stability and heat-induced changes in skim milk at temperatures >100 °C. Hence, the present study examined the thermal stability of the MCP-adjusted skim milk samples by varying MCP content via a 33% reduction and a 13% increase compared to the initial MCP level in control milk, to observe how it affects the thermal stability of milk. As heat stability of milk is also pH dependent, MCP-adjusted skim milk samples were also heated at different pH levels, 6.3, 6.6, 6.9, and 7.2.

2. Materials and methods

2.1. Sample preparation and fractionation

The experimental design for sample preparation and analysis is illustrated schematically in Fig. 1. Freshly pasteurized skim milk was sourced from Warrnambool Cheese and Butter – Saputo (Warrnambool, Australia) and 0.02% sodium azide was added to prevent bacterial growth. The MCP adjustment in skim milk involved adjusting the pH of the skim milk samples to 6.1 and 7.5 using predetermined quantities of GDL and 1.0 M NaOH, respectively, as described previously (Ahmadi et al., 2023). Subsequently, dialysis procedures were carried out as described previously (Huppertz & Lambers, 2020; Pyne & McGann, 1960). After dialysis, milk pH was adjusted to 6.3, 6.6, 6.9, or 7.2, then, samples were heated at 120 °C for 5 s or 140 °C for 1 s, followed by swift cooling to 20 °C. pH 6.6 was considered as the control pH.

To separate the sedimentable and non-sedimentable phases before and after heat treatment, ultracentrifugation at 100,000 × *g* for 60 min was performed as described previously (Ahmadi et al., 2023).

2.2. Sample analysis

Calcium content of whole samples and ultracentrifugal supernatants was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) with a Shimadzu ICPE-9000 system (Shimadzu Corporation, Kyoto, Japan), based on the methodology described by Bijl, van Valenberg, Huppertz, and van Hooijdonk (2013).

Particle size analysis was analysed using a Zetasizer-Nano series instrument (Malvern Instruments Ltd., Malvern, UK) as previously described (Ahmadi et al., 2023).

The turbidity was determined at 860 nm with a UV–Visible spectrophotometer (Biochrom Ltd, Cambridge, UK), using a 1 mm pathlength quartz cuvette (Ahmadi et al., 2023).

The protein distribution in the serum was assessed using Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) analysis of the whole samples and ultracentrifugal supernatants was performed at room temperature using a Shimadzu HPLC system (Model Prominence-i, LC-2030 C, Shimadzu Corporation, Kyoto, Japan), controlled by a Varian 9012 system controller (Agilent Technologies Inc., Santa Clara, CA). This system integrated an RI detector (Varian, 9050) and a C4 column (Aeris WIDEPORE, 150 mm × 4.6 mm, 3.6 μm particle size, 300 Å porosity, Phenomenex, Torrance, USA). Sample preparation followed the method outlined by Aprianita, Vasiljevic, Bannikova, and Kasapis (2014).

2.3. Statistical analysis

Statistical analysis was conducted using a split plot blocked design analysed as a General Linear Model with the MCP level as the main plot and pH adjustment and temperature/time as a

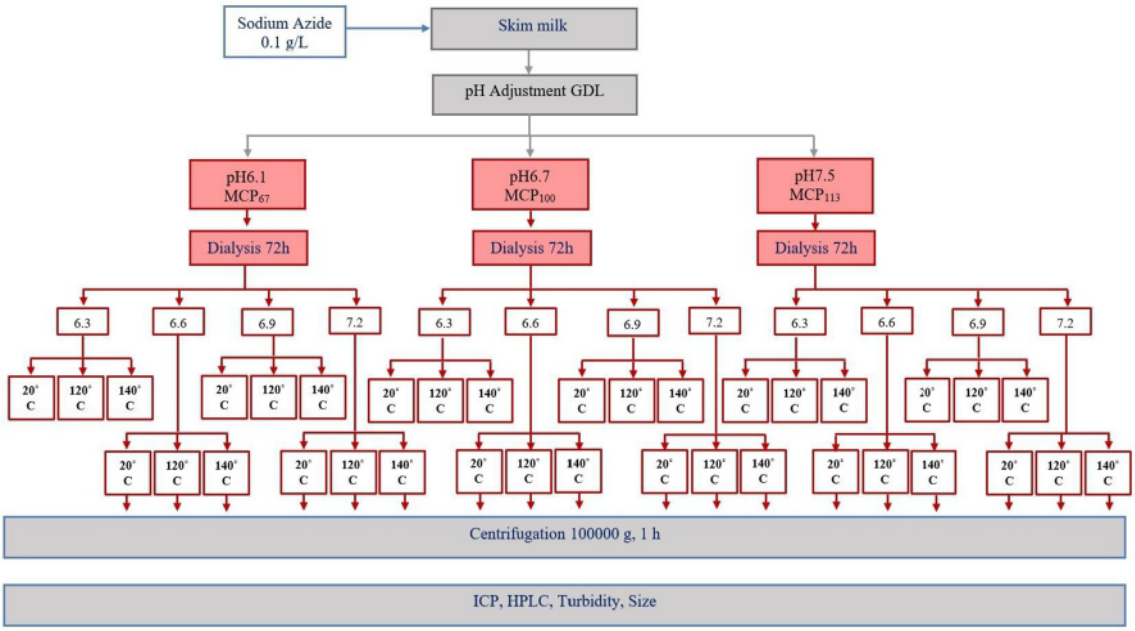

Fig. 1. Experimental design of the study.

subplot. The replications served as a block. The data was analysed using a SAS statistical software (v. 9.1 SAS Institute, Cary, NC, USA). The level of significance was established at $P < 0.05$. The experimental setup was replicated three times.

3. Results

3.1. Heat-induced changes in physicochemical properties of MCP-adjusted milk

The initial average particle size of the unheated control milk (MCP_{100}, pH 6.6) was 161 nm (Table 1) and the particle size distribution of all samples exhibited a primary peak between 50 and 400 nm (Fig. 2). MCP reduction led to a small decrease in particle size and turbidity, whereas the adjustment of milk pH after dialysis did not affect particle size and turbidity in unheated samples (Table 1).

After heat treatment at 120 °C for 5 s and 140 °C for 1 s, differences in particle size and turbidity were observed, with notable effects of pH and MCP content (Table 1). As expected, thermal stability was lowest at pH 6.3, with visible coagulation observed in samples MCP_{100} and MPC_{113} heated at this pH and significant ($P < 0.05$) increases in particle size and turbidity in sample MPC_{67} heated at pH 6.3 (Table 1). Significant increases in particle size and

Table 1
Influence of heat treatment at 120 °C for 5 s or 140 °C for 1 s at pH 6.3, 6.6, 6.9 or 7.2 on the particle size and turbidity of pasteurized skim milk samples with their micellar calcium phosphate (MCP) content adjusted to 67% (MCP_{67}) to 113% (MCP_{113}) of control milk (MCP_{100}). For sample details, see Fig. 1.

MCP content	Adjusted pH	Size (nm)			Turbidity (cm^{-1})		
		unheated	120 °C	140 °C	unheated	120 °C	140 °C
MCP_{67}	6.3	161.7^{Cab}	616.1^{Ba}	862.7^{Aa}	0.28^{Ccd}	1.71^{Ba}	1.89^{Aa}
	6.6	161.9^{Cab}	200.3^{Bbc}	241.3^{Ae}	0.30^{Cc}	0.48^{Bd}	0.70^{Ae}
	6.9	158.9^{Bab}	158.4^{Bf}	305.5^{Ac}	0.33^{Cbc}	0.38^{Be}	1.44^{Ab}
	7.2	156.8^{Bb}	151.1^{Bf}	219.1^{Af}	0.31^{Bc}	0.30^{Bf}	0.57^{Af}
MCP_{100} (control)	6.3	160.9^{Aab}	Coagulated	Coagulated	0.32^{Abc}	Coagulated	Coagulated
	6.6	161.3^{Cab}	210.4^{Bb}	244.4^{Ae}	0.34^{Cbc}	0.54^{Bc}	0.85^{Ad}
	6.9	166.8^{Ca}	179.5^{Bd}	265.5^{Ad}	0.35^{Cb}	0.40^{Be}	1.17^{Ac}
	7.2	168.6^{Ba}	168.3^{Bde}	199.6^{Af}	0.36^{Bab}	0.36^{Be}	0.48^{Ag}
MCP_{113}	6.3	164.2^{Aa}	Coagulated	Coagulated	0.37^{Aab}	Coagulated	Coagulated
	6.6	162.5^{Cab}	193.6^{Bcd}	310.9^{Ac}	0.39^{Ca}	0.70^{Bb}	1.39^{Ab}
	6.9	166.6^{Cab}	180.9^{Bd}	557.9^{Ab}	0.35^{Cb}	0.54^{Bc}	1.32^{Ab}
	7.2	162.2^{Bab}	171.1^{Bde}	209.9^{Af}	0.41^{Ca}	0.48^{Bd}	0.54^{Af}

Lower- and upper-case superscript letters indicate significant difference ($P < 0.05$) within a row and a column, respectively.

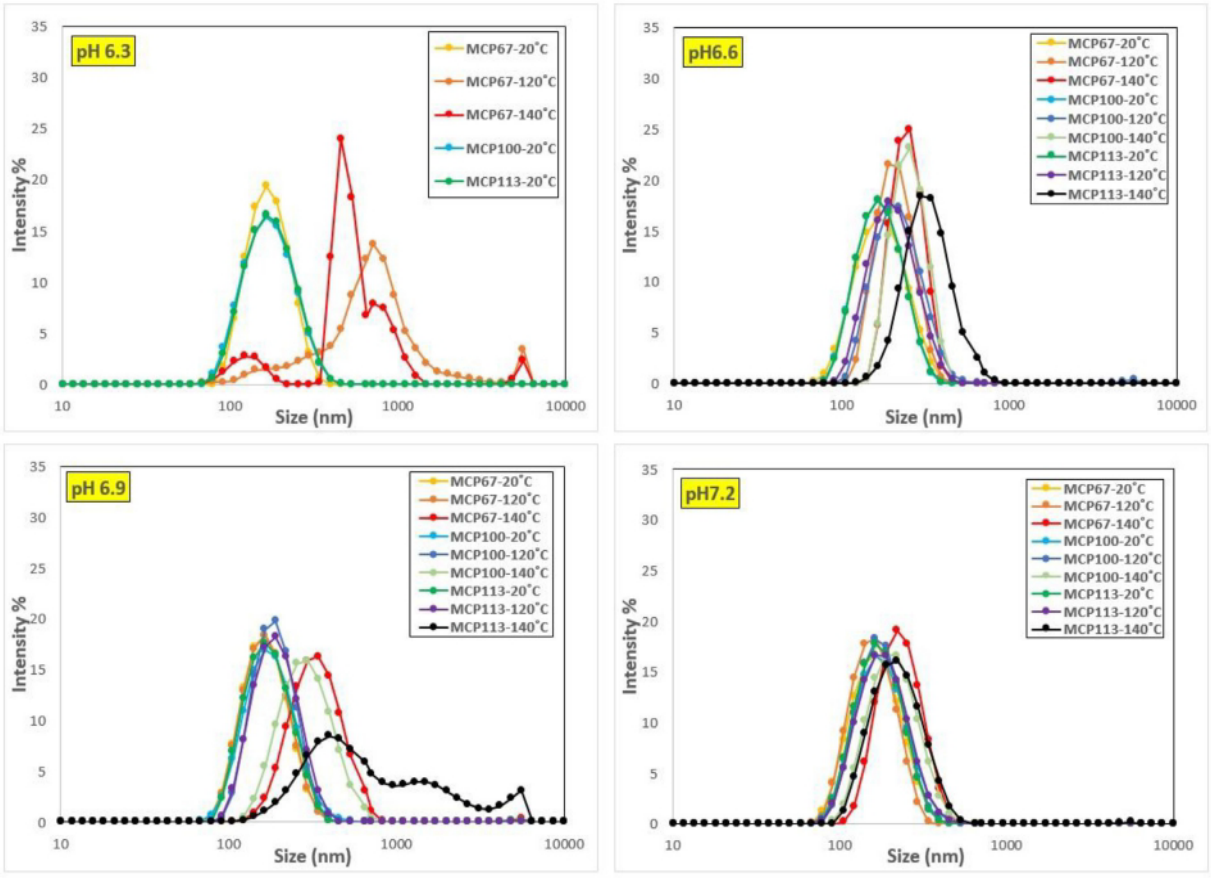

Fig. 2. Influence of heat treatment at 120 °C for 5 s or 140 °C for 1 s at pH 6.3, 6.6, 6.9 or 7.2 on the particle size distribution of pasteurized skim milk samples with their micellar calcium phosphate (MCP) content adjusted to 67% (MCP_{67}) to 113% (MCP_{113}) of control milk (MCP_{100}).

Table 2
Influence of heat treatment at 120 °C for 5 s or 140 °C for 1 s at pH 6.3, 6.6, 6.9 or 7.2 on the total calcium and non-sedimentable (100,000 × *g* for 60 min) calcium content of pasteurized skim milk samples with their micellar calcium phosphate (MCP) content adjusted to 67% (MCP_{67}) to 113% (MCP_{113}) of control milk (MCP_{100}). For sample details, see Fig. 1.

Samples	Adjustment pH	Calcium concentration (mmol L^{-1})					
		Total			Non-sedimentable		
		Unheated	120 °C	140 °C	Unheated	120 °C	140 °C
MCP_{67}	6.3	24.2^{Ac}	24.3^{Ac}	24.6^{Ac}	9.5^{Ab}	5.6^{Cc}	5.9^{Bb}
	6.6	25.4^{Ac}	24.2^{Ac}	25.0^{Ac}	7.5^{Ae}	5.2^{Bd}	5.0^{Ce}
	6.9	24.4^{Ac}	24.2^{Ac}	25.4^{Ac}	5.6^{Ai}	4.5^{Bf}	4.5^{Bf}
	7.2	24.1^{Ac}	23.6^{Ac}	23.9^{Ac}	5.5^{Aij}	4.8^{Ce}	5.0^{Be}
MCP_{100} (Control)	6.3	30.5^{Ab}	Coagulated	Coagulated	9.8^{Aa}	Coagulated	Coagulated
	6.6	30.7^{Ab}	30.2^{Ab}	29.4^{Ab}	7.9^{Ad}	5.5^{Bc}	5.7^{Cc}
	6.9	30.4^{Ab}	29.8^{Ab}	30.3^{Ab}	6.2^{Ag}	4.3^{Bf}	3.8^{Cg}
	7.2	29.8^{Ab}	31.2^{Ab}	30.3^{Ab}	5.4^{Aj}	4.4^{Cf}	5.1^{Bd}
MCP113	6.3	35.4^{Aa}	Coagulated	Coagulated	9.0^{Ac}	Coagulated	Coagulated
	6.6	35.3^{Aa}	34.9^{Aa}	34.5^{Aa}	7.3^{Af}	5.5^{Bc}	5.1^{Cd}
	6.9	35.0^{Aa}	34.7^{Aa}	35.2^{Aa}	5.9^{Ah}	4.4^{Bf}	3.5^{Ch}
	7.2	34.8^{Aa}	34.6^{Aa}	35.2^{Aa}	5.0^{Ak}	4.0^{Bg}	4.9^{Ae}

Lower- and upper-case superscript letters indicate significant difference ($P < 0.05$) within a row and a column, respectively.

turbidity were also observed for all other samples heated at 140 °C. However, for samples heated at 120 °C, no significant heat-induced increases in particle size were observed for all samples at pH 7.2 and for sample MCP_{67} at pH 6.9 (Table 1). Similar trends were observed in particle size distributions (Fig. 2). From the heat-induced changes in particle size and turbidity, it may thus be concluded that the stability of the casein micelles to heat-induced coagulation decreased with increasing MCP content and decreasing pH.

3.2. Heat-induced changes in the calcium distribution in MCP-adjusted skim milk

The effect of MCP-adjustment, pH and heat treatment on the levels of total and non-sedimentable Ca in skim milk is shown in Table 2. In the control sample (MCP_{100} at pH 6.6), total and non-sedimentable Ca content were 30.7 mmol L^{-1} and 7.9 mmol L^{-1}, respectively. Total Ca was ~5 mmol L^{-1} lower after MCP-depletion and ~5 mmol L^{-1} after MCP-enrichment (Table 2). As expected, pH adjustment after MCP adjustment did not affect total Ca content and neither did heat treatment at 120 or 140 °C (Table 2). Non-sedimentable Ca, in contrast, was comparable for all samples at pH 6.6 and decreased with increasing milk pH (Table 2). Heat treatment at 120 and 140 °C caused a significant ($P < 0.05$) reduction in the concentration of non-sedimentable Ca in all samples. In general, heat-induced reductions in non-sedimentable Ca were largest for samples that were heated at pH 6.3 and 6.6. Interestingly, in several instances, non-sedimentable calcium at pH 6.9 was lower than at pH 7.2 (Table 2). This observation may be linked to higher levels of non-sedimentable casein in samples heated at pH 7.2, as well be discussed later.

3.3. Heat-induced changes in the protein distribution of MCP-adjusted skim milk

The proportion of non-sedimentable caseins and whey proteins in the serum relative to total levels in the milk is shown in Table 3 and Table 4, respectively. In the unheated samples, reducing the MCP content significantly ($P < 0.05$) increased the concentrations of non-sedimentable αs_1-, αs_2-, β-, and κ-caseins, whereas MCP-enrichment decreased non-sedimentable levels of these caseins (Table 3). Adjusting the pH (6.3, 6.6, 6.9, or 7.2) had limited impact on the levels of non-sedimentable caseins in unheated samples (Table 3). After heating sample MCP_{100}, the concentration of most non-sedimentable caseins was not strongly ($P > 0.05$) affected, with a notable exception for κ-casein, which showed heat-induced dissociation at pH 6.9 and 7.2 (Table 3). For sample MCP_{67}, heat treatment caused reductions in levels of non-sedimentable α_{s1}-,

Table 3
Influence of heat treatment at 120 °C for 5 s or 140 °C for 1 s at pH 6.3, 6.6, 6.9 or 7.2 on the levels of non-sedimentable caseins (100,000 × *g* for 60 min) pasteurized skim milk samples with their micellar calcium phosphate (MCP) content adjusted to 67% (MCP_{67}) to 113% (MCP_{113}) of control milk (MCP_{100}). For sample details, see Fig. 1.

MCP Adjusted sample	pH	Milk proteins concentration (%)[a]											
		α_{s1}-Casein			α_{s2}-Casein			β-Casein			κ-Casein		
		Unheated	120 °C	140 °C	Unheated	120 °C	140 °C	Unheated	120 °C	140 °C	Unheated	120 °C	140 °C
MCP_{67}	6.3	17.1^{Aab}	9.1^{Bbc}	5.4^{Cde}	11.2^{Ac}	3.6^{Bbc}	2.4^{Bbc}	31.6^{Ab}	16.6^{Bcd}	12.8^{Bf}	29.1^{Abc}	23.6^{Be}	23.5^{Bde}
	6.6	16.7^{Aab}	7.6^{Bcde}	7.4^{Bd}	11.2^{Ac}	3.4^{Bbc}	1.4^{Ccd}	32.8^{Ab}	18.8^{Ccd}	23.0^{Bd}	29.7^{abc}	23.6^{Be}	21.9^{Be}
	6.9	15.5^{Ab}	8.7^{Bbc}	21.8^{Cb}	13.4^{Ab}	3.6^{Bbc}	3.4^{Bb}	39.0^{Aa}	27.6^{Bab}	30.0^{Bbc}	31.6^{Bab}	65.6^{Aa}	69.4^{Aa}
	7.2	18.5^{Ba}	19.8^{Ba}	40.9^{Aa}	18.6^{Aa}	4.0^{Cb}	9.8^{Ba}	42.8^{Aa}	31.3^{Ba}	44.6^{Aa}	34.1^{Ca}	68.3^{Aa}	50.9^{Bb}
MCP_{100} (control)	6.3	10.6^{Ac}	Coagulated	Coagulated	6.4^{Ade}	Coagulated	Coagulated	26.0^{Acd}	Coagulated	Coagulated	27.6^{Abc}	Coagulated	Coagulated
	6.6	9.4^{Ac}	4.3^{Bfg}	2.8^{Bef}	6.3^{Ade}	4.6^{ABb}	3.5^{Bb}	24.1^{ABd}	27.1^{Ab}	22.4^{Bd}	30.0^{Aabc}	15.5^{Bf}	13.8^{Bf}
	6.9	10.3^{Ac}	5.2^{Bef}	3.4^{Bef}	7.1^{Ad}	1.2^{Bd}	1.6^{Bbc}	31.2^{Ab}	20.5^{Bc}	16.6^{Ce}	26.2^{Bbc}	53.9^{Ac}	49.8^{Ab}
	7.2	3.9^{Cd}	10.3^{Bb}	18.0^{Ac}	8.3^{Ad}	1.9^{Bcd}	2.3^{Bbc}	30.5^{Ab}	29.9^{Aab}	33.5^{Ab}	27.4^{Cbc}	61.0^{Ab}	32.0^{Bc}
MCP_{113}	6.3	4.9^{Ad}	Coagulated	Coagulated	5.3^{Ade}	Coagulated	Coagulated	17.2^{Af}	Coagulated	Coagulated	18.7^{Aef}	Coagulated	Coagulated
	6.6	5.8^{Ad}	4.0^{ABfg}	2.0^{Bfg}	5.0^{Be}	8.2^{Aa}	2.5^{Cbc}	20.0^{Bef}	19.6^{Bc}	28.0^{Ac}	20.3^{Aef}	15.3^{Bf}	13.2^{Bf}
	6.9	8.5^{Ac}	2.9^{Bg}	4.9^{Bde}	4.4^{Aef}	0.9^{Bd}	2.0^{Bbc}	23.3^{Bde}	15.4^{Cd}	31.3^{Abc}	22.6^{Bde}	53.5^{Ac}	49.4^{Ab}
	7.2	4.6^{Bd}	6.0^{Bdef}	15.5^{Ac}	5.3^{Ae}	0.6^{Bd}	2.6^{Bbc}	22.2^{Be}	27.7^{Aab}	27.0^{Ac}	17.3^{Cf}	51.9^{Acd}	27.9^{Bcd}

Lower- and upper-case superscript letters indicate significant difference ($P < 0.05$) within a row and a column, respectively.
[a] The percentage of caseins in the serum relative to the original milk.

Table 4
Influence of heat treatment at 120 °C for 5 s or 140 °C for 1 s at pH 6.3, 6.6, 6.9 or 7.2 on non-sedimentable whey proteins (100,000 × *g* for 60 min) in pasteurized skim milk samples with their micellar calcium phosphate (MCP) content adjusted to 67% (MCP_{67}) to 113% (MCP_{113}) of control milk (MCP_{100}).

MCP Adjusted sample	pH	Relative protein concentration (%)[a]					
		α-Lactalbumin			β-Lactoglobulin		
		Unheated	120 °C	140 °C	Unheated	120 °C	140 °C
MCP_{67}	6.3	97.9^{Aa}	34.5^{Bc}	12.7^{Cg}	91.2^{Aa}	34.2^{Bbc}	32.5^{Bd}
	6.6	91.6^{Aa}	52.5^{Ba}	29.3^{Ce}	90.4^{Aa}	35.8^{Bbc}	33.8^{Bd}
	6.9	95.4^{Aa}	39.9^{Cb}	53.1^{Bab}	93.9^{Aa}	59.2^{Ba}	51.8^{Bb}
	7.2	97.7^{Aa}	39.4^{Cb}	56.3^{Ba}	91.9^{Aa}	39.6^{Cb}	70.1^{Ba}
MCP_{100} (Control)	6.3	94.9^{Aa}	Coagulated	Coagulated	90.0^{Aa}	Coagulated	Coagulated
	6.6	96.7^{Aa}	27.0^{Bd}	19.6^{Cf}	98.1^{Aa}	29.3^{Bc}	18.2^{Ce}
	6.9	97.4^{Aa}	38.2^{Cb}	46.1^{Bc}	94.4^{Aa}	30.8^{Cbc}	49.6^{Bb}
	7.2	95.7^{Aa}	34.7^{Bc}	36.2^{Bd}	93.1^{Aa}	30.9^{Cbc}	51.6^{Bb}
MCP_{113}	6.3	94.9^{Aa}	Coagulated	Coagulated	93.1^{Aa}	Coagulated	Coagulated
	6.6	94.2^{Aa}	11.4^{Bf}	11.7^{Bg}	89.5^{Aa}	10.7^{Be}	15.7^{Be}
	6.9	95.6^{Aa}	18.9^{Ce}	42.8^{Bc}	89.7^{Aa}	36.9^{Cbc}	47.9^{Bbc}
	7.2	94.5^{Aa}	20.8^{Ce}	51.4^{Bb}	90.9^{Aa}	23.4^{Ccd}	43.7^{Bbc}

Lower- and upper-case superscript letters indicate significant difference ($P < 0.05$) within a row and a column, respectively.
[a] The proportion of whey proteins in the serum relative to the original milk.

α_{s2}-and β-caseins at pH 6.3–6.9, whereas for non-sedimentable κ-casein, notable heat-induced increases were observed at pH 6.9 and 7.2. For non-sedimentable α_{s1}-casein, a notable heat-induced increase was also observed at pH 7.2 after heat treatment at 140 °C (Table 3). For sample MCP_{113}, the main heat-induced increases in non-sedimentable casein were also observed for κ-casein at pH 6.9 and 7.2 and for α_{s1}-casein at pH 7.2 (Table 3). In the unheated samples, virtually all whey proteins was non-sedimentable (Table 4). In the heated samples, a parallel trend emerges between the non-sedimentable fractions of whey proteins and the pH level, mirroring observations made for κ-casein. As the pH increased, the proportion of non-sedimentable whey proteins also rose (Table 4).

4. Discussion

Heat treatment induces significant structural changes in milk, closely linked to influential factors such as calcium levels and pH (Dumpler et al., 2020; Lewis et al., 2011; Nieuwenhuijse & Huppertz, 2022). Continuing on our previous investigation (Ahmadi et al., 2023), this study expands understanding of the heat stability of three distinct MCP-adjusted skim milk samples: MCP_{67} (33% MCP-depleted), MCP_{113} (13% MCP-enriched), and the control, MCP_{100} to temperatures >100 °C. It is well known that pH plays an important role in the heat-induced changes in milk (Anema, 2021; Nieuwenhuijse & Huppertz, 2022). In the present study, heating at a reduced pH (6.3) resulted in reduced thermal stability of milk, as seen by visible coagulation as well as increase in turbidity and particle size (Table 1). These findings were in a good agreement with those reported previously (Fox & Hoynes, 1975; Singh & Fox, 1987). The reduced micellar charge below the natural milk pH (<6.7) appears to fail in counteracting the heat-induced alterations, leading to thermal instability (Singh & Fox, 1985).

Adjustment of MCP content within the casein micelle has been reported to affect the heat coagulation time (HCT) of skim milk at a set temperature (140 °C) (Fox & Hoynes, 1975; Singh & Fox, 1985). In present study, UHT treatment was applied for a set time (120 °C for 5 s and 140 °C for 1 s; Fig. 1). In line with our previous findings (Ahmadi et al., 2023), MCP reduction resulted in enhanced thermal stability, albeit accompanied by an increase in particle size at high high temperatures (Table 1), which aligns with previous findings (Fox & Hoynes, 1975; Singh & Fox, 1985). For instance, MCP-enhanced skim milk samples displayed a strong increase in particle size and turbidity (Table 1, Fig. 2), and a decrease in non-sedimentable individual caseins at high pH and coagulated at low pH (Table 3). On the other hand, in the MCP-depleted skim milk samples, the impact of UHT treatment was less pronounced, and visible coagulation did not occur, even at the lowest pH. The observed increases in particle size after heat treatment (Table 1, Fig. 2) went beyond the anticipated interactions between whey proteins and casein micelles, pointing to substantial micellar aggregation. In our previous study, conducted at a lower temperature, no significant aggregation or coagulation occurred (Ahmadi et al., 2023). In contrast, coagulation was observed at low pH in the current study; however, the reduction of MCP appeared to prevent heat induced coagulation. This highlights how minor fluctuations in MCP content significantly impact thermal stability of milk. Although variations in MCP content were process-induced in this study, it is important to note that notable variation also occurs naturally between milk from individual cows (Huppertz, Heck, Bijl, Poulsen, & Larsen, 2021). Although heat stability is known to vary widely between milk from individual cows (Davies & White, 1966), the influence of variation in MCP content has not been investigated as a contributing factor to date.

Heat-induced dissociation of κ-casein has often been linked to thermal instability of milk (Anema, 2021; Dumpler et al., 2020; Huppertz, 2016). The heat-induced dissociation of k-casein occurs primarily at elevated pH levels following UHT-treatment, in line with previous findings (Anema & Li, 2000; Singh & Fox, 1985). Interestingly, MCP content had only a minor impact on the heat-induced dissociation of κ-casein (Table 3). Although heat-induced κ-casein dissociation is typically deemed a primary trigger for heat coagulation, our observations with MCP-depleted skim milk samples heated at high pH revealed substantial κ-casein dissociation following heating (Table 3). In addition, the distribution of whey proteins between the sedimentable and non-sedimentable phases closely followed that of κ-casein (Table 4). However, the MCP-depleted skim milk exhibited increased thermal stability, evident by the absence of visible coagulation and minimal impact on particle size and turbidity (Table 1), implying the involvement of other factors in the coagulation process. Considering other factors involved in heat-induced coagulation, MCP-enhanced skim milk, which displayed largest particle size and highest turbidity after heating, contains highest micellar calcium levels. Micellar calcium can be defined as the amount of Ca that sediments on ultracentrifugation (Huppertz & Lambers, 2020) and it can be estimated from the difference between total and non-sedimentable calcium (Table 2). During the heating, Ca and PO_4 tend to precipitate in the

casein micelle (Nieuwenhuijse & Huppertz, 2022). This can lead to the exceeding critical levels of MCP, thereby causing instability in the micelles. Consequently, the heat-induced changes observed in skim milk are influenced by fluctuations in MCP content.

5. Conclusion

The study highlighted the substantial impact of MCP adjustment on alterations induced by ultra-high-temperature treatment, significantly influencing the overall heat stability of milk. 33% MCP-depletion appeared to have the greatest thermal stability among all samples. MCP adjustment led to varying behaviour among individual caseins, influencing their interactions in the soluble and colloidal phases. The pH level of the milk was also observed to have a linear correlation with heat stability during UHT-treatment. Notably, this work provided for the first time an insight into the effect of MCP-adjustment on the impact of ultra-high temperature treatment on skim milk heated at different pH.

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CRediT authorship contribution statement

Elaheh Ahmadi: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Thom Huppertz:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Todor Vasiljevic:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

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

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Supplementary data

Figure S1. SDS-PAGE electropherograms of skim milk and supernatants. The proteins were resolved under non-reducing A1 (milk and pH6.3); A2(serum and pH6.3); C1 (milk and pH 6.6) and C2(serum and pH 6.6); D1 (milk and pH6.9); D2(serum and pH6.9); E1 (milk and pH7.2); E2(serum and pH7.2). **1:** MCP67-20˚C; **2:** MCP67-120˚C; **3**: MCP67-140˚C; **4**: MCP100-20˚C; **5:** MCP100-120˚C; **6:** MCP100-140˚C; **7:** MCP113-20˚C; **8:** MCP113-120˚C; **9:** MCP113-140˚C

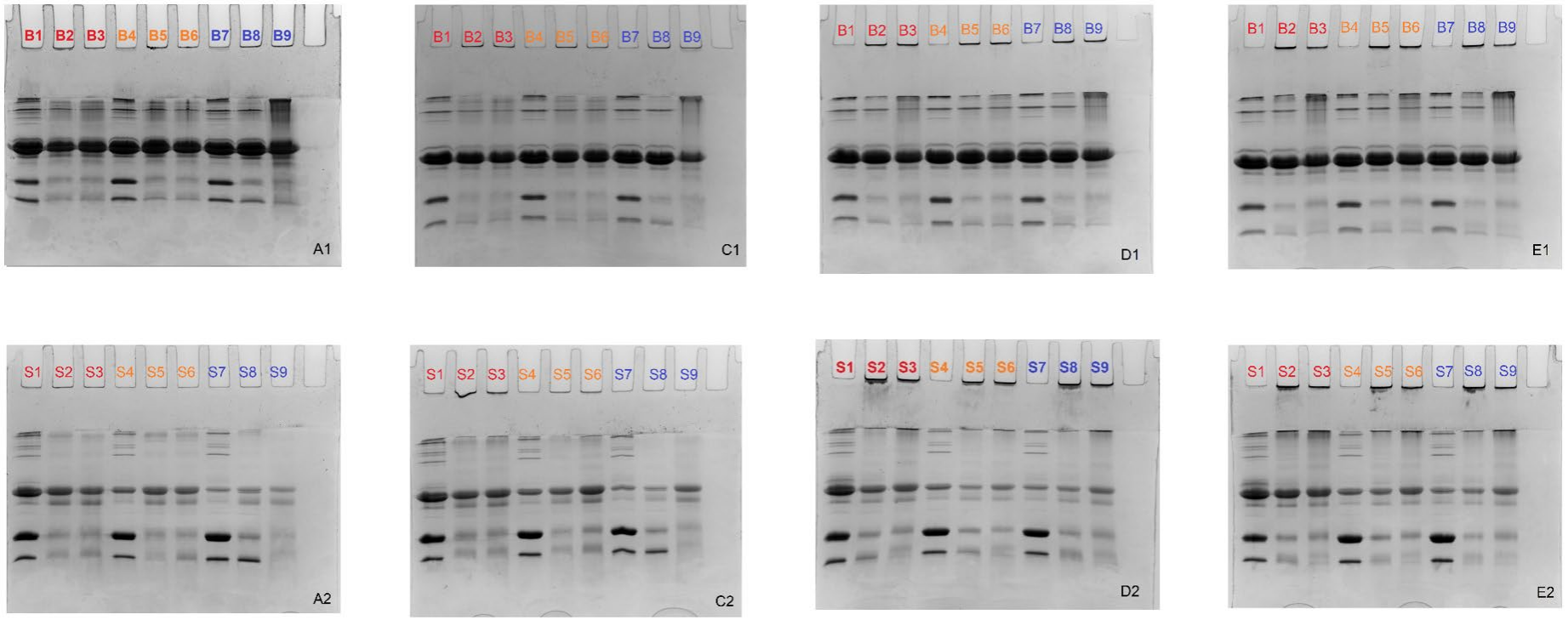
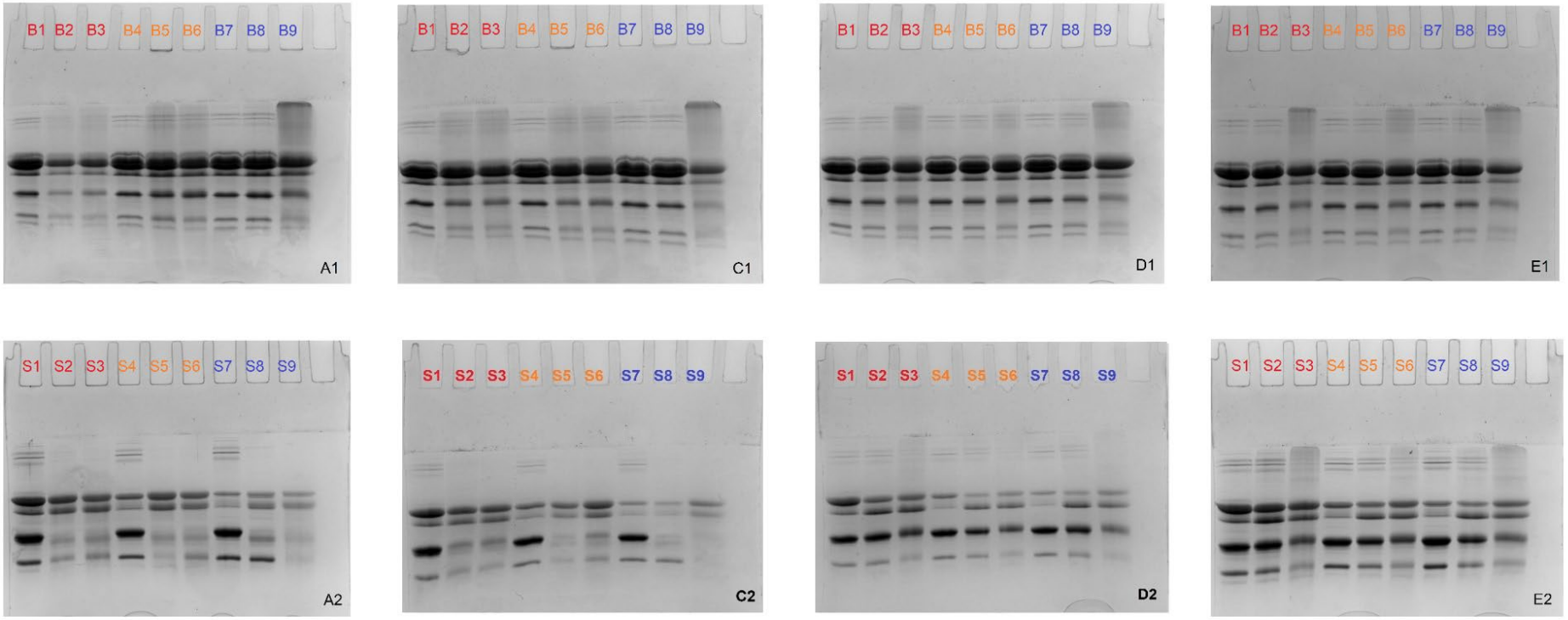


Figure S2. SDS-PAGE electropherograms of skim milk and supernatants. The proteins were resolved under reducing A1 (milk and pH6.3); A2(serum and pH6.3); C1 (milk and pH 6.6) and C2(serum and pH 6.6); D1 (milk and pH6.9); D2(serum and pH6.9); E1 (milk and pH7.2); E2(serum and pH7.2). **1:** MCP67-20˚C; **2:** MCP67-120˚C; **3**: MCP67-140˚C; **4**: MCP100-20˚C; **5:** MCP100-120˚C; **6:** MCP100-140˚C; **7:** MCP113-20˚C; **8:** MCP113-120˚C; **9:** MCP113-140˚C



Chapter 6

Chapter 6. Acid-induced gelation of MCP-adjusted skim milk

This chapter has been submitted as a research paper entitled “Influence of heating temperature and pH on acid gelation of MCP-adjusted skim milk” by Elaheh Ahmadi, Thom Huppertz and Todor Vasiljevic in the peer-reviewed. International Dairy Journal, 2024, submited.

Correction note: Caption Table 2 should read: **Table 2.** Proportion[1] of non-sedimentable caseins in the serum phase of the MPC-adjusted skim milk adjusted to pH 6.3, 6.6., 6.9, or 7.2 after heating at 80 or 90°C for 10 min

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This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

[redacted]	**07/03/2024**
Signature	**Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

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Elaheh Ahamdi	80	Designed and wrote the original draft, conceptualised, reviewed, edited, revised the manuscript, designed the experiments, the tables and the figures, and data analysis	[redacted]	07/03/2024
Thom Huppertz	10	Concept development, experimental design, manuscript editing and revision		07/03/2024
Todor Vasiljevic	10	Concept development, experimental design, manuscript editing, revision, submission, and supervision		07/03/2024

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Influence of Heating Temperature and pH on Acid Gelation of Micellar Calcium Phosphate-Adjusted Skim Milk

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Abstract: Micellar calcium phosphate (MCP) plays an important role in maintaining the structure and stability of the casein micelle and its properties during processing. The objective of this study was to investigate how heating (10 min at 80 or 90 °C) at different pH levels (6.3, 6.6, 6.9, or 7.2) impacted the acid-induced gelation of MCP-adjusted milk, containing 67 (MCP_{67}), 100 (MCP_{100}), or 113 (MCP_{113}) % of the original MCP content. The unheated sample MCP_{100} at pH 6.6 was considered the control. pH acidification to pH 4.5 at 30 °C was achieved with glucono delta-lactone while monitoring viscoelastic behaviour by small-amplitude oscillatory rheology. The partitioning of calcium and proteins between colloidal and soluble phases was also examined. In MCP-depleted skim milk samples, the concentrations of non-sedimentable caseins and whey proteins were higher compared to the control and MCP-enriched skim milk samples. The influence of MCP adjustment on gelation was dependent on pH. Acid gels from sample MCP_{67} exhibited the highest storage modulus (G′). At other pH levels, MCP_{100} resulted in the greatest G′. The pH of MCP-adjusted skim milk also impacted the gel properties after heating. Overall, this study highlights the substantial impact of MCP content on the acid gelation of milk, with a pronounced dependency of the MCP adjustment effect on pH variations.

Keywords: rheology; acid gelation; micellar calcium phosphate; pH adjustment; skim milk

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1. Introduction

Milk is a highly nutritious food containing various proteins, such as caseins and whey proteins, and micronutrients, such as calcium. The stability, nutritional properties, and technological and processing characteristics of milk are mainly influenced by the state of casein micelles and how caseins interact among themselves and with other components [1]. Micellar calcium phosphate (MCP) plays a pivotal role in the structure of casein micelles, acting as a cross-linking bridge and neutralising negatively charged phosphoseryl groups. MCP stands out as a major factor responsible for maintaining the integrity of the micelle [2,3]. Milk processing involves various techniques to stabilise or transform raw milk into a wide range of products [4]. Acid-induced gelation of milk is applied in the production of yogurt and various other fermented dairy products [5], but also takes place during the gastric digestion of milk [6]. The stability of casein micelles in the solution as the building blocks of acid milk gels is governed by steric stabilisation, provided by κ-casein protruding from the surface of the micelles. A reduction in pH leads to a loss of steric stabilisation and the solubilisation of MCP can lead to the coagulation of casein micelles [7].

In the production of fermented products like yogurt, milk is first subjected to heat treatment (e.g., at 80–90 °C for 5–30 min). The purpose of such a high heat treatment is to enhance the textural properties of acid gels, through the denaturation of whey proteins [8]. This denaturation facilitates interactions between whey proteins and caseins, and the heat

treatment also causes dissociation of some of the caseins, particularly κ-casein, from casein micelles. These processes contribute to an increased firmness and viscosity of acid-induced gels from heated milk [9]. The impact of composition and processing parameters on the textural properties of acid milk gels has been widely studied in previous studies [9–14]. Heat treatment temperature and pH have a significant impact on the texture and physical properties of gels [15]. The intensity of heat treatment influences the extent of whey protein denaturation, thereby affecting the firmness and viscosity of milk [16]. Furthermore, the pH of milk during heat treatment affects the balance between hydrophobic attractions and electrostatic repulsion. Lowering the pH, for instance, shifts the balance toward hydrophobic interactions [3] and it can be possible to produce milk with different levels of whey proteins associated with casein micelles [17].

MCP content can influence the functional properties of acid-induced milk gels [18,19]. Limited MCP removal has been noted to support casein micelle structure, potentially improving the formation of crosslinks between strands within the gel network [20]. Over the years, studies have adjusted the content of MCP in both skim milk retentates [21,22] and milk [6,23–30] and examined the impact of MCP adjustment on several important functional properties of milk. It has been found that the levels of individual caseins increase in the serum phase of milk almost linearly with an MCP reduction [31]. In addition, adjusting the MCP content to 67% of its original value results in a high level of intact casein micelles and the greatest thermal stability [30].

Previous studies have indicated that modifications in MCP levels within the casein micelle could affect the acid gelation of milk [7,20,32–34]. For example, modifying MCP levels by adding different levels of trisodium citrate (TSC) impacted gel stiffness and syneresis in yoghurt [20]. Improved stiffness and reduced syneresis were associated with a low depletion of MCP, which enhanced the rearrangement and molecular mobility of the micelle structure, potentially contributing to increased crosslinking among the strands in yogurt gel networks. Famelart et al. [32] found that a 30% calcium depletion increased the acid gelation pH of milk, while a more extensive depletion led to a decrease in gelation pH. Moreover, Anema [33] reported that partially removing MCP from milk before heat treatment and acidification had minimal impact on gelation pH but significantly reduced the storage modulus, G′. Ozcan et al. [34], on the other hand, elevated the MCP content of milk and noted that it resulted in a limited impact on G′.

All these findings suggest that the properties of acid-set milk gels are influenced by the level of MCP in the milk undergoing acidification. This study undertook a distinctly different approach, incorporating not only MCP adjustment but also the subsequent adjustment of pH before heating to various levels below and above the natural milk pH, along with exposure to different heating temperatures. The aim of this study was to investigate the influence of heat treatment and pH levels on the acid gelation properties, as well as the protein and mineral distributions, of milk with MCP content adjusted to three levels (67%, 100%, and 113%).

2. Materials and Methods

2.1. Sample Preparation

Freshly pasteurised skim milk was obtained from Warrnambool Cheese and Butter—Saputo (Warrnambool, Australia). To prevent bacterial growth, 0.02% (*w*/*w*) sodium azide was added. The MCP concentration was adjusted using glucono delta-lactone (GDL) or 1.0 M NaOH to lower or increase the pH of skim milk to 6.1 or 7.5, respectively, thereby adjusting MCP content to 67% (MCP_{67}) and 113% (MCP_{113}) of the initial MCP content. After stabilising the pH, the dialysis process was carried out as described previously [31]. After MCP content adjustment, the pH of the MCP-adjusted milk was adjusted to 6.3, 6.6, 6.9, or 7.2 through the addition of HCl or NaOH. pH-adjusted samples were heated in an oil bath set at 80 or 90 °C. The temperatures selected are reflective of those commonly used in the pre-heating of milk for yogurt production [9]. The time required to reach these temperatures was approximately 2.5 or 3 min, respectively. Subsequently, the samples

were held for a further 10 min at the required temperature, before being cooled to 20 °C by immersion in an ice bath.

Acid gelation of unheated and heated pH-adjusted MCP-adjusted skim milk samples was conducted by adding glucono-delta-lactone (GDL) and then incubating the mixture at 30 °C. As the buffering capacity of the milk was altered by changing the MCP levels and pH, the GDL level was varied so that a pH of 4.5 was achieved within a selected time frame; therefore, this GDL amount was established pre-experiments and a pre-determined amount of GDL was added [33]. Incubation was stopped when pH reached 4.5. The experimental design of this study is depicted in Figure 1.

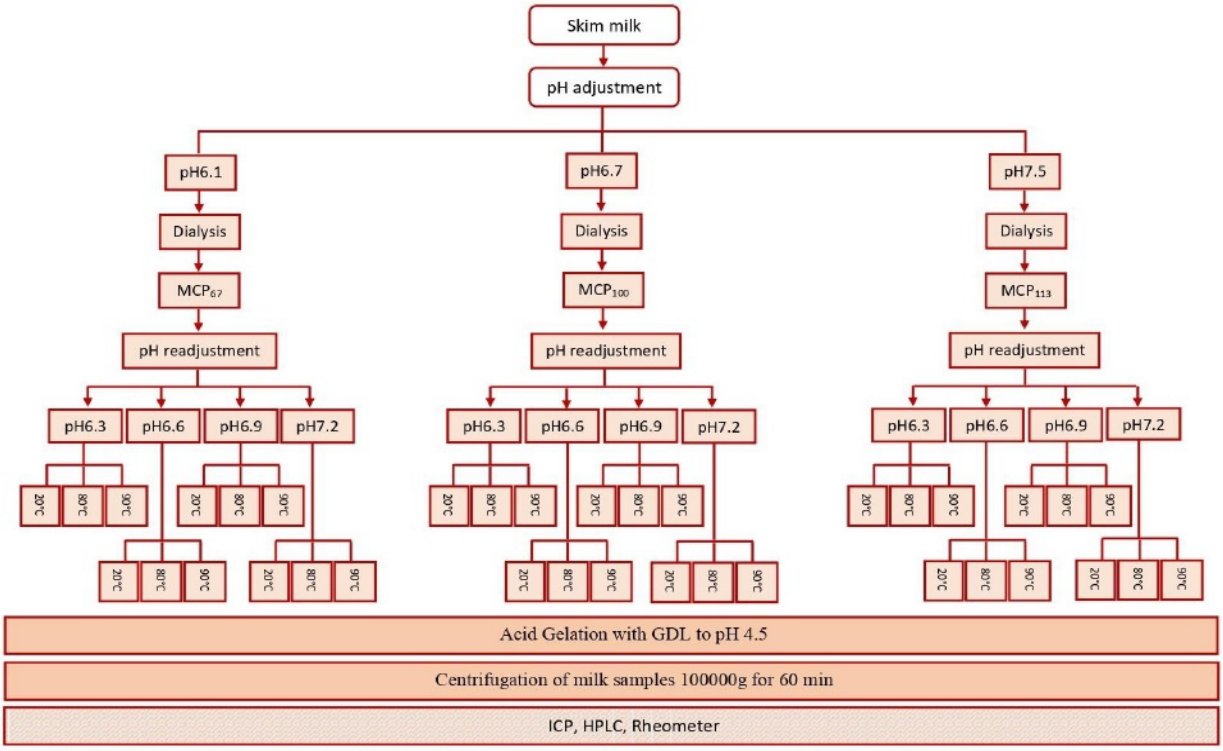

Figure 1. Experimental design of this study.

2.2. Sample Fractionation

To separate the sedimentable and non-sedimentable phases of skim milk samples before and after heat treatment, ultracentrifugation was performed at 100,000× *g* for 1 h at 20 °C using a Beckman Ultra L-70 centrifuge (Beckman Coulter, Australia Pty., Ltd., Gladesville, Australia). After ultracentrifugation, the clear supernatant from each tube was carefully collected using a syringe [35].

2.3. Sample Analysis

2.3.1. Calcium Content

The calcium (Ca) concentrations in the whole samples and serum phases were examined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) with an ICPE-9000 system provided by Shimadzu Corporation in Kyoto, Japan, as outlined in a previous paper [30].

2.3.2. High-Performance Liquid Chromatography

Protein levels in milk samples and ultracentrifugal supernatants were examined using reversed-phase high-performance liquid chromatography (RP-HPLC). The RP-HPLC analysis was performed using a Shimadzu HPLC system (Model Prominence-i, LC-2030

C, Shimadzu Corporation, Kyoto, Japan) with a Varian 9012 system controller (Agilent Technologies Inc., Santa Clara, CA, USA). The system was equipped with a refractive index (RI) detector (Varian, 9050) and utilised a C_4 column (Aeris Widepore, 150 mm × 4.6 mm, 3.6 µm particle size, 300 Å pore size, Phenomenex, Torrance, CA, USA). The analysis was carried out at room temperature [30].

2.3.3. Rheological Measurement

Acid gelation was performed using a controlled-stress rheometer (Physica MCR 301, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with a cup (27.11 mm diameter) and bob (25 mm diameter) configuration (CC 25/PR-SN, Anton Paar). The samples were mixed with the required amount of GDL and immediately transferred into the cup. All measurements were performed at a temperature of 30 °C. During the evaluation, the storage modulus (G′) was measured at a strain of 0.5% and a frequency of 1 Hz [36]. The gelation point was identified as the point at which G′ reached a value of 1 Pa [17,37]. Simultaneously, the pH was measured throughout the entire gelation process using a calibrated pH meter equipped with a combined pH electrode featuring a temperature sensor and fixed cable (Model H1131, Hanna Instruments, Woonsocket, RI, USA). These pH measurements were concurrently recorded alongside the rheological measurements [37] to enable the evaluation of G′ as a function of pH.

2.4. Statistical Analysis

A randomised, split-plot blocked design was employed for statistical analysis, treated as a General Linear Model with the MCP level serving as the main plot. Subplots included pH adjustment and temperature as factors. The replications were considered as blocks within the design. The dataset underwent analysis using SAS statistical software (version 9.1, SAS Institute, Cary, NC, USA). The predetermined level of significance was set at $p < 0.05$. To enhance reliability, the experimental setup was replicated three times, ensuring robustness and consistency in the obtained results.

3. Results

3.1. Calcium Distribution

The effect of MCP adjustment, pH, and heat treatment on total calcium and serum calcium is shown in Table 1. Sample MCP_{100} at pH 6.6 and 20 °C had a calcium content of 31.9 mmol L^{-1} and a serum calcium content 10.0 mmol L^{-1} (Table 1). Adjustment of MCP content had a clear influence on the total calcium, as anticipated, with the lowest total calcium level in sample MCP_{67} and the highest in sample MCP_{113} (Table 1). Adjusting pH to 6.3, 6.6, 6.9, or 7.2 did not affect total calcium content before heating all milk samples, while an inverse relation was observed between pH and the amount of soluble calcium (Table 1).

Heating the milk samples at 80 or 90 °C did not impact total calcium but had a substantial impact on the levels of soluble calcium (Table 1). Soluble calcium concentration decreased significantly ($p < 0.05$), by approximately 20–25%, on heating the samples with pH 6.3–6.9 at 80 °C and further decreased ($p < 0.05$) on heating at 90 °C. At pH 6.9, sample MCP_{113} behaved differently compared to other MCP contents, initially following a similar trend—experiencing a decline in soluble calcium after heating at 80 °C but subsequently showing an increase after heating at 90 °C (Table 1). On the contrary, samples adjusted to pH 7.2 exhibited a significant increase ($p < 0.05$) in soluble calcium concentration after heating at 80 °C. However, after heating at 90 °C, the concentration of soluble Ca was either similar to or lower than the initial values. The substantial decrease in the soluble calcium concentration indicated a likely involvement of calcium in complexation with sedimentable proteins.

Table 1. Calcium concentration of the bulk and serum phase of pasteurised skim milk with their MCP adjusted to 67% (MCP_{67}) or 113% (MCP_{113}) of their original level (MCP_{100}) by either acidification or alkalisation followed by exhaustive dialysis against bulk milk followed by pH readjustment to 6.3, 6.6, 6.9, or 7.2 before and after heat treatment at 80 or 90 °C for 10 min. For sample details, see Figure 2.

Sample	pH	Calcium Concentration (mmol L^{-1})					
		Milk			Serum		
		20 °C	80 °C	90 °C	20 °C	80 °C	90 °C
MCP_{67}	6.3	24.8 ^{Ad}	24.2 ^{Ad}	24.7 ^{Ad}	11.5 ^{Ab}	9.1 ^{Bb}	8.8 ^{Ba}
	6.6	25.5 ^{Ad}	25.2 ^{Ad}	24.8 ^{Ad}	10.0 ^{Ac}	7.8 ^{Bd}	6.9 ^{Cd}
	6.9	26.7 ^{Ac}	26.2 ^{Ac}	26.2 ^{Ac}	8.8 ^{Ae}	6.7 ^{Bg}	5.1 ^{Ci}
	7.2	25.7 ^{Ac}	26.0 ^{Ac}	25.6 ^{Ac}	5.5 ^{Bi}	6.8 ^{Ag}	5.4 ^{Bh}
MCP_{100}	6.3	31.9 ^{Ab}	31.8 ^{Ab}	31.6 ^{Ab}	13.4 ^{Aa}	10.6 ^{Ba}	7.9 ^{Cc}
	6.6	31.9 ^{Ab}	31.9 ^{Ab}	31.2 ^{Ab}	10.0 ^{Ac}	8.3 ^{Bc}	8.7 ^{Ba}
	6.9	30.9 ^{Ab}	31.2 ^{Ab}	31.6 ^{Ab}	8.4 ^{Af}	7.2 ^{Be}	6.9 ^{Bd}
	7.2	31.4 ^{Ab}	31.7 ^{Ab}	31.0 ^{Ab}	7.2 ^{Ah}	7.8 ^{Ad}	6.2 ^{Bf}
MCP_{113}	6.3	34.2 ^{Aa}	34.1 ^{Aa}	34.4 ^{Aa}	9.6 ^{Ad}	8.2 ^{Bc}	8.3 ^{Bb}
	6.6	35.1 ^{Aa}	34.5 ^{Aa}	34.9 ^{Aa}	8.0 ^{Ag}	7.0 ^{Bf}	6.6 ^{Ce}
	6.9	35.3 ^{Aa}	34.3 ^{Aa}	34.5 ^{Aa}	5.6 ^{Bi}	4.9 ^{Bh}	6.3 ^{Af}
	7.2	34.6 ^{Aa}	34.2 ^{Aa}	34.6 ^{Aa}	5.3 ^{Bj}	7.8 ^{Ad}	5.8 ^{Bg}

Lowercase superscript letters indicate significant differences in columns ($p < 0.05$). Uppercase superscript letters signify the differences in the rows for the milk and for the serum separately ($p < 0.05$).

3.2. The Protein Distribution of MCP-Adjusted Skim Milk

The proportions of non-sedimentable caseins and whey proteins in the supernatant of MCP-adjusted skim milk samples relative to the corresponding milk are shown in Tables 2 and 3, respectively. In sample MCP_{100} at pH 6.6, the proportion of non-sedimentable αs_1-, αs_2-, β-, and κ-caseins; α-lactalbumin; and β-lactoglobulin were 3, 13, 18, 13, 95, and 98%, respectively. In unheated samples, a reduction in MCP content resulted in a noticeable increase in the concentrations of non-sedimentable α_{s1}-, α_{s2}-, β-, and κ-casein (Table 2), while non-sedimentable α-lactalbumin and β-lactoglobulin were not affected (Table 3). In contrast, MCP enrichment significantly ($p < 0.05$) decreased the non-sedimentable levels of αs_2- and β-caseins, with no observed change in αs_1- and κ-caseins (Table 2). The pH adjustment to lower pH (6.3, 6.6) slightly elevated the levels of non-sedimentable α_{s1}-, α_{s2}-, and κ-caseins before heating (Table 2), whereas for non-sedimentable α-lactalbumin and β-lactoglobulin, no change was observed (Table 3).

Heating the samples led to decreased levels of non-sedimentable α_{s1}-, α_{s2}-, and β-casein (Table 2), as well as non-sedimentable α-lactalbumin and β-lactoglobulin (Table 3), while the level of non-sedimentable κ-casein significantly ($p < 0.05$) increased (Table 2). The changes in the levels of non-sedimentable α_{s1}-, α_{s2}-, and β-caseins were dependent on both pH and the temperature of heating.

Non-sedimentable αs_1-casein decreased after heating at 80 and 90 °C, while α_{s2}- and β-caseins showed a pH-dependent trend—inverse relationship—with substantial decreases after heating at 80 and 90 °C. In contrast, κ-casein exhibited opposite behaviour from other caseins, with its concentration increasing directly with pH and temperature. The level of non-sedimentable κ-casein in MCP_{100} at elevated pH (6.9 or 7.2) was greater than at lower pH; however, it was lower ($p < 0.05$) than in samples MCP_{67} and MCP_{113} (Table 2).

As expected, heating significantly impacted the whey proteins, particularly at pH 6.9 and 7.2 (Table 3). At pH 6.6 and 6.3, α-lactalbumin appeared more affected than β-lactoglobulin, irrespective of the MCP content (Table 3). A higher temperature (90 °C versus 80 °C) generally led to greater aggregation and lower non-sedimentable α-lactalbumin, except at pH 6.9, where it increased. Non-sedimentable β-lactoglobulin remained consistent across factors, with more

retained at lower MCP contents and high pH levels (Table 3). The temperature effect on non-sedimentable β-lactoglobulin might have been confounded by the pH, as it declined at pH 6.3 and 6.6 but increased at pH 6.9 and 7.2 after heating at 90 °C compared to 80 °C (Table 3).

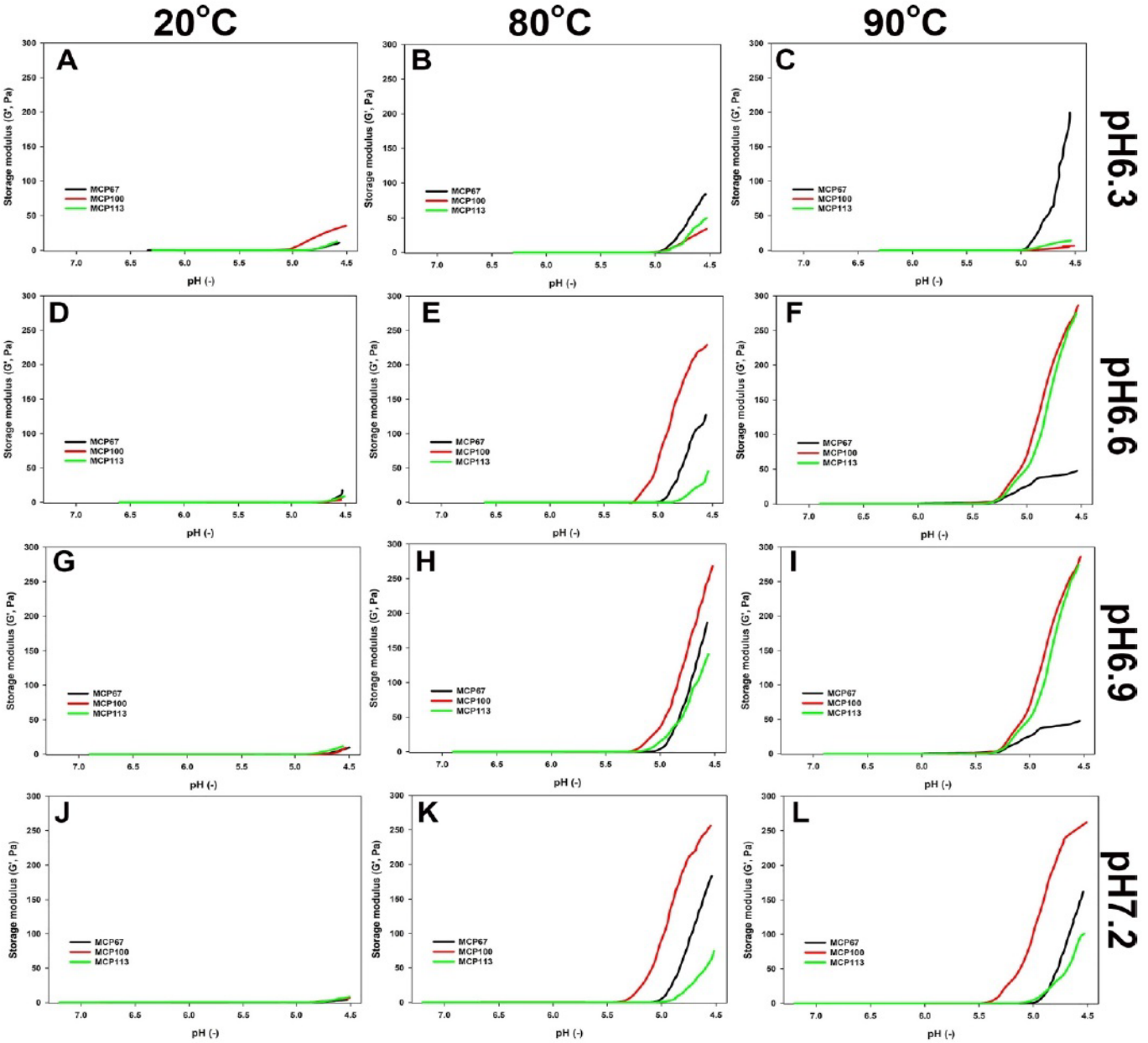

Figure 2. Elastic modulus (G′) as a function of pH during acidification of the skim milk samples with the MCP content adjusted to either 67% (MCP_{67}) or 113% (MCP_{113}) of its original level (MCP_{100}) and pH readjusted to 6.3 (**A**–**C**), 6.6 (**D**–**F**)., 6.9 (**G**–**I**), or 7.2 (**J**–**L**) before (**A**,**D**,**G**,**J**) or after heating 80 (**B**,**E**,**H**,**K**) or 90 (**C**,**F**,**I**,**L**) °C for 10 min. Acidification was achieved by the addition of glucono delta-lactone followed by incubation at 30 °C until pH reached 4.5.

Table 2. The proportion of non-sedimentable caseins, expressed as a percentage of caseins in the bulk sample, of MPC-adjusted skim milk adjusted to pH 6.3, 6.6., 6.9, or 7.2 before heating at 80 or 90 °C for 10 min.

Sample	pH	Milk Protein Concentration (%)											
		α_{s1}-Casein			α_{s2}-Casein			β-Casein			κ-Casein		
		20 °C	80 °C	90 °C	20 °C	80 °C	90 °C	20 °C	80 °C	90 °C	20 °C	80 °C	90 °C
MCP_{67}	6.3	11.3 ^{Aa}	5.6 ^{Bc}	5.7 ^{Ba}	21.2 ^{Ab}	5.7 ^{Bd}	3.7 ^{Cf}	36.9 ^{Aa}	21.3 ^{Ba}	18.9 ^{Ba}	26.0 ^{Aa}	23.1 ^{Bd}	26.9 ^{Ad}
	6.6	10.6 ^{Aa}	6.7 ^{Bb}	5.8 ^{Ba}	23.4 ^{Aa}	5.8 ^{Bd}	5.0 ^{Be}	36.4 ^{Aa}	19.3 ^{Bb}	15.2 ^{Cb}	15.4 ^{Bb}	24.1 ^{Ad}	24.9 ^{Ae}
	6.9	8.0 ^{Ab}	5.0 ^{Bc}	5.8 ^{Ba}	17.1 ^{Ac}	1.3 ^{Bf}	1.3 ^{Bg}	36.6 ^{Aa}	12.3 ^{Bd}	10.5 ^{Ccd}	13.6 ^{Cbc}	29.5 ^{Bbc}	40.8 ^{Ab}
	7.2	8.8 ^{Ab}	7.8 ^{Ba}	5.8 ^{Ca}	10.1 ^{Af}	3.3 ^{Be}	2.0 ^{Cg}	36.3 ^{Aa}	12.9 ^{Bd}	11.4 ^{Cc}	12.5 ^{Ccd}	28.1 ^{Bc}	37.0 ^{Ac}
MCP_{100}	6.3	5.5 ^{Ad}	2.1 ^{Cf}	3.8 ^{Bb}	14.8 ^{Ad}	9.3 ^{Bb}	8.4 ^{Bc}	18.0 ^{Ac}	12.8 ^{Bd}	10.0 ^{Cd}	10.2 ^{Be}	20.0 ^{Ae}	19.7 ^{Af}
	6.6	2.9 ^{Be}	2.0 ^{Cf}	4.0 ^{Ab}	12.9 ^{Ae}	7.5 ^{Bc}	6.6 ^{Bd}	17.9 ^{Ac}	13.1 ^{Bd}	10.0 ^{Cd}	12.6 ^{Bc}	17.7 ^{Af}	19.4 ^{Af}
	6.9	3.1 ^{Ae}	2.9 ^{Aef}	2.5 ^{Ac}	15.6 ^{Acd}	7.2 ^{Bcd}	1.7 ^{Cg}	19.9 ^{Ab}	7.7 ^{Be}	7.7 ^{Be}	10.9 ^{Ccd}	30.2 ^{Bb}	35.8 ^{Ac}
	7.2	2.2 ^{Ce}	6.7 ^{Ab}	3.7 ^{Bb}	8.3 ^{Ag}	4.4 ^{Bde}	1.0 ^{Cgh}	12.1 ^{Ae}	10.1 ^{Bd}	8.5 ^{Cde}	10.2 ^{Ce}	28.9 ^{Bbc}	35.8 ^{Ac}
MCP_{113}	6.3	5.7 ^{Ad}	4.1 ^{Bcd}	3.2 ^{Bbc}	11.9 ^{Ce}	15.8 ^{Aa}	13.6 ^{Ba}	13.4 ^{Bde}	15.1 ^{Ac}	11.4 ^{Cc}	11.1 ^{Ccd}	18.5 ^{Bef}	23.9 ^{Ae}
	6.6	6.7 ^{Ac}	5.2 ^{Bc}	4.0 ^{Cb}	7.1 ^{Bg}	9.6 ^{Ab}	11.0 ^{Ab}	14.9 ^{Ad}	13.5 ^{Bcd}	10.4 ^{Ccd}	12.7 ^{Cc}	19.8 ^{Be}	23.9 ^{Ae}
	6.9	2.3 ^{Ae}	2.3 ^{Af}	1.5 ^{Ad}	2.5 ^{Ah}	2.2 ^{ABef}	0.7 ^{Bgh}	10.2 ^{Af}	7.0 ^{Be}	6.3 ^{Bef}	10.5 ^{Ce}	33.1 ^{Ba}	44.4 ^{Aa}
	7.2	3.6 ^{Ae}	3.4 ^{Ade}	1.4 ^{Bd}	9.5 ^{Afg}	1.1 ^{Bf}	0.0 ^{Bh}	17.3 ^{Ac}	8.0 ^{Be}	7.3 ^{Be}	10.5 ^{Ce}	34.0 ^{Ba}	43.7 ^{Aa}

Lowercase superscript letters indicate significant differences in columns ($p < 0.05$). Uppercase superscript letters signify the differences in the rows for each casein separately ($p < 0.05$).

Table 3. The proportion of non-sedimentable whey proteins, expressed as a percentage of whey proteins in the bulk sample, in MPC-adjusted skim milk adjusted to pH 6.3, 6.6., 6.9, or 7.2 before heating at 80 or 90 °C for 10 min.

Sample	pH	Whey Protein Concentration (%)					
		α-Lactalbumin			β-Lactoglobulin		
		20 °C	80 °C	90 °C	20 °C	80 °C	90 °C
MCP_{67}	6.3	86.8 ^{Ac}	21.6 ^{Be}	20.3 ^{Bf}	91.4 ^{Ab}	45.2 ^{Bcd}	42.9 ^{Bde}
	6.6	87.7 ^{Abc}	30.6 ^{Bd}	24.2 ^{Be}	91.7 ^{Ab}	54.2 ^{Bb}	39.4 ^{Cef}
	6.9	84.5 ^{Acd}	76.6 ^{Ba}	79.3 ^{Ba}	97.8 ^{Aa}	60.8 ^{Ba}	56.1 ^{Bb}
	7.2	81.9 ^{Ae}	67.6 ^{Bb}	60.4 ^{Bd}	98.4 ^{Aa}	53.7 ^{Bb}	56.4 ^{Bb}
MCP_{100}	6.3	89.3 ^{Ab}	10.7 ^{Bg}	10.5 ^{Bh}	97.8 ^{Aa}	39.9 ^{Ce}	61.8 ^{Ba}
	6.6	94.6 ^{Aa}	23.6 ^{Be}	17.1 ^{Cg}	98.4 ^{Aa}	37.1 ^{Cf}	59.4 ^{Ba}
	6.9	95.4 ^{Aa}	62.5 ^{Cc}	67.0 ^{Bb}	95.3 ^{Aa}	43.9 ^{Bd}	44.6 ^{Bd}
	7.2	91.3 ^{Ab}	60.4 ^{Cc}	63.4 ^{Bc}	97.9 ^{Aa}	32.7 ^{Cg}	41.5 ^{Be}
MCP_{113}	6.3	91.1 ^{Ab}	18.1 ^{Bf}	10.2 ^{Ch}	83.2 ^{Ac}	39.0 ^{Bef}	37.0 ^{Bf}
	6.6	85.6 ^{Ac}	36.0 ^{Bd}	25.2 ^{Ce}	78.8 ^{Ad}	34.6 ^{Bfg}	22.6 ^{Cg}
	6.9	82.3 ^{Ade}	68.3 ^{Bb}	63.7 ^{Cc}	97.1 ^{Aa}	41.5 ^{Cde}	48.6 ^{Bc}
	7.2	85.0 ^{Ac}	68.6 ^{Bb}	60.6 ^{Cd}	79.7 ^{Ad}	47.4 ^{Bc}	44.7 ^{Bd}

Lowercase superscript letters indicate significant differences in columns ($p < 0.05$). Uppercase superscript letters signify the differences in the rows for each whey protein separately ($p < 0.05$).

3.3. Influence of pH and Heating on Acid Gelation Behaviour of MCP-Adjusted Milk Samples

The acid gelation properties of MCP-adjusted skim milk are shown in Table 4. Additionally, Figure 2 shows the evolution of G′ as a function of pH during the GDL-induced acidification of MCP-adjusted milk samples. For unheated milk samples, the MCP content did not affect gelation pH, whereas heat treatment significantly increased gelation pH for most samples (Table 4). The effect of heating temperature showed dependence on the initial pH of the adjusted skim milk in relation to the pH at the gelation point (Table 4). The effects were direct as the samples adjusted to higher pH and heated at the higher temperature started to gel at a higher pH. For example, the samples heated at 90 °C at pH 6.9 or 7.2 began to gel at pH 5.3–5.4, whereas those heated at pH 6.3 started to gel just below pH 5.0 (Table 4).

Table 4. Time and pH at gelation point during acid-induced gelation of skim milk with MCP content adjusted to either 67 (MCP_{67}) or 113% (MCP_{113}) of its original level (MCP_{100}) with the pH readjusted to 6.3, 6.6., 6.9, or 7.2 before and after heat treatment at 80 or 90 °C for 10 min.

Sample	pH	pH at Gelation Point (-)		
		Temperature (°C)		
		20	80	90
MCP_{67}	6.3	4.81 ^{Ab}	4.96 ^{Abc}	4.97 ^{Abc}
	6.6	4.71 ^{Bb}	4.98 ^{Abc}	4.92 ^{ABbc}
	6.9	4.70 ^{Cb}	5.01 ^{Bbc}	5.29 ^{Aab}
	7.2	4.76 ^{Cb}	5.02 ^{Bbc}	5.40 ^{Aa}
MCP_{100}	6.3	5.05 ^{Aa}	4.98 ^{Abc}	4.79 ^{Bc}
	6.6	4.66 ^{Bb}	5.13 ^{Aab}	5.22 ^{Aab}
	6.9	4.77 ^{Bb}	5.25 ^{Aab}	5.43 ^{Aa}
	7.2	4.79 ^{Bb}	5.36 ^{Aa}	5.44 ^{Aa}
MCP_{113}	6.3	4.81 ^{Ab}	4.95 ^{Abc}	4.95 ^{Abc}
	6.6	4.70 ^{Bb}	5.06 ^{Ab}	4.96 ^{Abc}
	6.9	4.82 ^{Bb}	5.16 ^{Aab}	5.33 ^{Aab}
	7.2	4.77 ^{Bb}	4.96 ^{ABbc}	5.09 ^{Ab}

Lowercase superscript letters indicate significant differences in columns ($p < 0.05$). Uppercase superscript letters show differences in the rows for time and pH at the gelation point separately ($p < 0.05$).

As shown in Figure 2, the unheated samples gelled less strongly in comparison to the heated samples. The evolution of G′ during acidification was affected by the MCP content, the adjusted pH, and the extent of heat treatment. The acid-induced gels produced by MCP_{100} appeared to have the highest G′. The gel formation of samples MCP_{100} and MCP_{113} was greatly affected at pH 6.3 compared to other pH levels before and after heating. Interestingly, when MCP_{100} was heated at 90 °C with low pH, it formed a gel with a G′ even lower than the sample acidified after heating at 80 °C. After heating at 80 °C, the MCP_{67} samples produced a gel with an appreciable high G′, which was lower than that of the control but greater than that of MCP_{113}. The reverse was observed when samples had a pH of 6.6 or 6.9, with MCP_{67} showing impaired gelling behaviour compared to other MCP levels and even to the same MCP level heated at 80 °C (Figure 2E,F,H,I). Gel-forming ability improved somewhat when the pH was adjusted to 6.3 or 7.2 (Figure 2C,L).

4. Discussion

Acid-induced gelation of milk is closely linked to pH levels and mineral composition, in particular calcium content and fat and protein content [19,38]. To explore the role of MCP adjustment on acid-induced gelation, three distinct MCP-adjusted skim milk samples—MCP_{67} (33% MCP-depleted), MCP_{113} (13% MCP-enriched), and the control, MCP_{100}—were subjected to pH adjustment and heating followed by acidification with GDL at 30 °C in this study. Adjusting the MCP content in skim milk altered the mineral equilibria, significantly impacting the protein distribution between the phases and the properties of caseins in these MCP-adjusted skim milks. Previous studies have investigated the impact of MCP adjustment on milk gelation properties [33,34]. In these studies, only the impact of MCP depletion or the enrichment of skim milk on acid-induced gelation was examined. In the present work, however, a different approach was employed; in addition to MCP adjustment, it involved the adjustment of pH to various levels below and above that of the natural milk pH and subjecting such milk to different heating temperatures.

The adjustment of MCP content changes the calcium equilibrium in milk (Table 1), resulting in a significant alteration in the amount of non-sedimentable caseins (Table 2), which likely depicts a partial dissociation of individual caseins from the micelle, which

confirmed previous findings [6,25,29–31]. Variations in MCP content also influenced the properties of the casein micelles. The stability of the casein micelle primarily relies on the steric stabilisation by a layer of κ-casein, often described as a salted polyelectrolyte brush [39]. The κ-casein level in the serum phase of the milk sample (MCP_{100}) from the control group was determined to be minimal. However, as the MCP content decreased, the level of κ-casein in the serum phase increased (Table 2). Reducing MCP content increases the concentration of non-micellar caseins in the serum phase, whereas an increase in the MCP content induces a transition of individual caseins from the soluble phase into the micelles (Table 2) [31]. A consistent proportion of non-sedimentable κ-casein in the MCP-enhanced skim milk across the whole pH range compared to MCP_{100} (Table 2) suggests that its concentration may not be entirely dependent on the MCP content. pH adjustment before heating has also affected the distribution of soluble calcium and caseins. Lowering the pH to 6.3 and 6.6 increased the dissociation of calcium and non-sedimentable caseins from the casein micelle into the serum phase, which is illustrated in Tables 1 and 2, and supported by our previous findings [29,30].

The initial pH of skim milk during heating has a crucial impact on the acid-induced gel. Anema et al. [17] and Lucey et al. [15] demonstrated that, at pH levels equal to or greater than 7.0, only minimal amounts of denatured whey proteins are associated with the casein micelles, which is supported by a higher level of κ-casein, α-lactalbumin, and β-lactoglobulin in the serum phase (Tables 2 and 4) and a slightly lower G′ of acid gels from all samples made from milk samples adjusted to pH 7.2 (Figure 2) in our study. Heating at pH 6.3 led to a lower value of G′ in these acid gels (Figure 2), indicating weaker intramolecular interactions. As the pH decreases, MCP progressively solubilises, causing the removal of residual MCP linkages from the gel structure during its formation [33]. Interestingly, the content of MCP had only a slight effect on the dissociation of k-casein following heating (Table 2). In addition, the distribution of whey proteins between the sedimentable and non-sedimentable phases closely followed that of k-casein (Table 3).

Moreover, the partial removal of MCP from the milk (MCP-depleted skim milk) before heat treatment and acidification significantly decreased the G′. The G′ modulus of gels correlates with the quantity, strength, or number of bonds among casein particles, as well as the arrangement of casein strands in the network [40]. In addition, dissolving MCP within casein particles leads to a decrease in MCP crosslinks and potentially heightens electrostatic repulsion among exposed phosphoserine residues [16]. Both of these factors could significantly contribute to the decrease in G′ values observed in MCP-depleted acidified milk. Ozcan-Yilsay et al. [20] reported findings that agree with the current observations, outlining that the removal of MCP from the milk before heat treatment and acidification significantly decreased the G′. In contrast, Anema [33] reported that reducing the MCP level of milk increased the G′ of acid gels therefrom. While these outcomes might be conflicting, they could also be attributed to the differing methods used to alter MCP levels.

Furthermore, surprisingly, the effects of MCP adjustment demonstrated a notable dependence on pH in cases where heat treatment was applied. The acidification of MCP_{67} at pH 6.3 exhibited a pronounced rise in G′, particularly after heating at 90 °C compared to other MCP-adjusted samples (Figure 2B,C). The possible reason for this observation is that the solubilisation of MCP during MCP adjustment results in a reduction in MCP crosslinks, prompting the dissociation of caseins from casein micelles (Table 2) [2,20]. The key distinction lies in the increased presence of non-sedimentable caseins, which are a consequence of reduced MCP content and may influence the aggregation behaviour of whey proteins. For instance, when samples with lower MCP content were heated at pH 6.3, there was less incorporation of β-lactoglobulin and α-lactalbumin into sedimentable aggregates (Table 3). As whey proteins require interaction with κ-casein to form bridges with the casein micelle, this leads to a greater G′ in a lower MCP content. However, the acidification of heated MCP-depleted skim milk at higher pH levels (6.6, 6.9, 7.2) indicated minimal gel formation in MCP-depleted skim milk samples.

Nevertheless, at most other pH levels, samples containing 100% MCP demonstrated the highest G′ increase and firmer gels. This observation highlights the nuanced influence of MCP concentration on pH variations, emphasising the critical interplay between these factors in determining the gelation properties. Conflicting views exist regarding the impact of MCP enhancement on acid gelation properties. Anema [33] reported that higher MCP levels contribute to the formation of a more elastic gel, whereas Ozcan et al. [34] proposed that an increase in MCP content had a limited impact on G′. The present study suggested that the effect of MCP enhancement depends on the initial pH of skim milk and the heat treatment temperature; after heat treatment at 90° for MCP-enhanced skim milk at pH 6.6 and 6.9, G′ showed the highest increase.

5. Conclusions

Adjusting MCP levels alters the total calcium content in skim milk, while pH adjustment impacts the soluble calcium. Both non-sedimentable caseins and whey proteins are influenced by changes in MCP levels and pH. However, the behaviour of non-sedimentable κ-caseins may not be solely dependent on MCP content and might be affected by other electrostatic interactions. Additionally, heat treatment significantly influences the protein and calcium balance, leading to different properties in acid-induced gels. Higher temperatures cause calcium phosphate solubilisation, whey protein denaturation, and increased gel firmness. The initial pH of skim milk during heating is crucial as it affects the solubilisation of micellar calcium phosphate and, consequently, gel strength. This study highlights the nuanced effects of MCP concentration and pH variations on gelation properties. Overall, this comprehensive investigation sheds light on the intricate interplay of factors affecting acid-induced gelation, contributing valuable insights for the dairy industry.

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Chapter 7

Chapter 7. Conclusions and Future directions

This chapter provides the overall project conclusions and the scope for future work.

7.1 Conclusions

The overall aim of this study was to understand the impact of micellar calcium phosphate concentration on the casein micelle structure, stability and functionality and the work was subdivided into four objectives. Under the first objective, the impact of adjusting the micellar calcium phosphate (MCP) content in skim milk on the structural characteristics of casein micelles, with a specific focus on evaluating its effect on micellar integrity using FTIR and NMR spectroscopies was investigated. Several important physico-chemical changes in milk proteins took place when the content of micellar calcium phosphate (MCP) in the casein micelle was altered. The smallest size of the casein micelle and the greatest MCP depletion was observed at MCP7. Turbidity decreased progressively by decreasing the MCP content. However, increasing the MCP to 129% did not have any effects on the turbidity. The integrity of the casein micelle did not appear to be substantially affected, even with a decline in MCP concentration down to 42% of its original level, although there was an observed increase in the level of individual caseins in the serum phase of milk. A MCP content of 42% of the initial level appeared to be a minimum threshold to maintain the integrity of the casein micelle. Although there were no significant physical changes observed beyond this threshold, the adjustment notably influenced the conformational changes of the casein micelle. One of the key findings of this project was that the MCP-depleted skim milk sample, with 67% of the initial MCP content, exhibited the minimum MCP amount necessary to maintain a high level of intact casein micelles. Moreover, adjusting the MCP levels at elevated pH resulted in an increase in MCP content, although there seemed to be a limit to the additional calcium that could be incorporated into the nanoclusters. This study provided insights into the conformational and physico-chemical changes in pasteurized milk with adjusted MCP.

The knowledge generated about the physicochemical and conformational changes resulting from MCP adjustment in skim milk samples, along with the kinetics of changes in protein interactions from the initial study, were employed to enhance the understanding of the thermal behavior and conformational alterations in MCP-adjusted skim milk during heating at 90°C for 10 min. This extended to exploring varying levels of MCP (ranging from 67% to 113% of the original) and varying pH levels, ranging from low (pH 6.3) to high (pH 7.2). A significant impact of MCP adjustment on the thermal behavior of milk proteins and, consequently, on the heat stability of milk was found. There is a direct relationship observed between the pH level of the milk and its heat stability, with lower pH levels correlating with reduced heat stability. MCP adjustment induces varying behavior of individual caseins; they dissociate to some extent

at lower MCP content while maintaining higher levels at an MCP level above the initial. This leads to varied interactions among proteins in the soluble and colloidal phases, leading to different outcomes. Interactions among milk proteins at low MCP levels heated at low pH exhibit distinct characteristics. Although an increase in particle size was anticipated, attributed to the minimization of surface charge, the observed rise was not as pronounced as that observed in the enhanced MCP sample.

Continuing on our previous investigations, the third study further explores the heat stability of three distinct MCP-adjusted skim milk samples: MCP67 (33% MCP-depleted), MCP113 (13% MCP-enriched), and the control, MCP100, subjected to temperatures of 120°C for 5 seconds and 140°C for 1 second. The study underscores the significant influence of MCP adjustment on alterations induced by ultra-high-temperature treatment, impacting the overall heat stability of milk. Notably, the 33% MCP-depletion exhibited the highest thermal stability among all samples. MCP adjustment resulted in varied behavior among individual caseins, influencing their interactions in the soluble and colloidal phases. The milk's pH level demonstrated a linear correlation with heat stability during UHT treatment. The observed increase in particle size after UHT treatment went beyond anticipated interactions between whey proteins and casein micelles, indicating substantial micellar aggregation. Importantly, this work offers the first insight into the effect of MCP-adjustment on the impact of ultra-high-temperature treatment on skim milk heated at different pH levels.

In the last study, exploring the role of MCP adjustment on acid-induced gelation, , three distinct MCP-adjusted skim milk samples—MCP_{67} (33% MCP-depleted), MCP_{113} (13% MCP-enriched), and the control MCP_{100} subjected to acidification with GDL at 30˚C in this study. MCP-adjustment induced changes in the concentration of total calcium and calcium content of supernatant of acidified skim milk gels, while heating temperature and pH showed no correlation with them. However, MCP-adjustment, readjustment of pH, and heating temperature influenced the proportion of non-sedimentable caseins and whey proteins. Regarding the impact of MCP adjustment on acid-gelation, this study highlighted a pronounced dependence of MCP content to pH variations. At low pH, the heated MCP-depleted skim milk samples appeared to have higher G' and the firmer gel formation, while at other pH levels in both heating temperature (80 and 90C) exhibited the low G' and weaker gel, likely beneficial gel quality for better milk digestion. Original skim milk displayed lower whey protein proportion in skim milk serum relative to original milk after heating, and the highest G' surge and firmer gel formation at various pH levels (6.6, 6.9, 7.2) in both heating temperature. MCP-

enhanced skim milk samples showed low G' in most samples and weaker gels. This study for the first time provided the effect of MCP-adjustment on the acid induced gelation properties of heated skim milk at different pH.

Overall, this work for the first time has provided insights into the effect of MCP-adjustment on the conformational changes and secondary structure of MCP-depleted and enriched skim milk using FTIR and NMR spectroscopy. This study illustrates how modifying MCP content without dissociating the micelle can enhance the thermal stability of skim milk in various temperatures and acid gelation properties of skim milk, contributing to a more profound understanding of the conformational and compositional alterations of the casein micelle during MCP adjustment, pH modification, heat treatment and acid gelation. The results can contribute to improving the functional properties of various ratios of MCP-modified skim milk under industrial-scale dairy processing conditions, aiming to achieve highly stable skim milk. This newfound knowledge obtained from this study can contribute to a better understanding of the functional properties of MCP-adjusted skim milk in industrial-scale dairy processing, aiming to achieve skim milk with improved thermal and structural stability.

7.2 Future research directions

In this study, the impact of adjusting the micellar calcium phosphate (MCP) content in skim milk on the structural characteristics of casein micelles, using FTIR and NMR spectroscopies. When skim milk was adjusted to 67% of its initial MCP content, the casein micelle integrity seemed to be maintained even with a 33% decrease in MCP content. Exploring the potential alterations and sedimentation of this MCP-adjusted skim milk during storage could offer valuable insights. Investigating storage instabilities in MCP-adjusted milk and similar fluids could be further enhanced by integrating FTIR spectroscopy with complementary techniques such as Raman spectroscopy.

It was interesting observation that 33% MCP-depleted skim milk has a highest thermal stability at 90°C for 10 minutes and at ultra-high temperature processing at 120°C for 5 second or 140°C for 1 second. It would be more informative if in future a sensory evaluation could be done to know whether the levels of taste, texture and the mouthfeel of this MCP-adjusted skim milk after heating is appropriate in compared to original native milk. In addition, NMR spectroscopy measurement could also add valuable insight. It would also be interesting if this UHT treated

MCP-adjusted milk analysed for maillard reaction and browning in milk. In addition, it would be fascinating to investigate the influence of MCP-depleted skim milk samples on fouling during UHT treatment in a pilot-scale experiment.
Moreover, acid-induced gelation of MCP-adjusted skim milk at different pH and various heat treatment temperature were analysed in this study. Exploring the possibility of using the FTIR technique to identify and forecast early gelation in MCP-adjusted skim milk would be an interesting path to explore. Moreover, it would be valuable to investigate how the adjustment of MCP (micellar casein phosphate) influences the Rennet-induced gelation of skim milk.

The analysis of acid-induced gelation of MCP-adjusted skim milk demonstrated, MCP-depleted skim milk produced the weaker gels. Given that milk proteins undergo a complex digestion process in the human digestive system, leading to the hydrolysis of proteins into various peptides upon ingestion, it is valuable to explore how MCP-adjustment in milk influences the digestion process under both in vitro and in vivo conditions.