Studies on heat-induced protein interaction and digestion behavior of sheep milk

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Food Technology

at Riddet Institute, Massey University,

Palmerston North, New Zealand

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2023

Riddet Institute
ADVANCING FRONTIERS IN FOOD SCIENCE

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Abstract

Sheep milk has low heat stability, which results in undesirable changes after heat treatment, such as separation of milk fat, sediment formation, and phase separation. However, the mechanism of low heat stability of sheep milk has not yet been elucidated. Additionally, the protein interactions in sheep milk during heating and the digestion behaviors of differently processed sheep milk are also unknown. Therefore, the aim of this thesis was to explore the protein interactions of sheep milk during heat treatment (67.5–90 °C and 140 °C), and the mechanism of heat coagulation of sheep milk. In addition, the effect of the commercial processing treatment [homogenization (200/50 bar) and thermal (75 °C/15 s and 95 °C/5 min) processing] on the digestion behavior of sheep milk were determined.

Sheep skim milk (SSM) was heated under various conditions (including temperatures, heating times and pH values) and the denaturation of whey protein and protein interactions occurring during heating were characterized using high-performance liquid chromatography. Casein micelle diameter increased upon heating, depending on the temperature and time. The association of whey protein with casein micelles and the aggregation of casein micelles occurred simultaneously and contributed to the increase in casein micelle size in SSM. SSM was stable to heat (140 °C) at pH 6.8–6.9 but became unstable at higher or lower pH. The low heat stability of sheep milk was attributed to the low proportion of κ-casein surrounding the casein micelles, high ionic calcium levels and ready dissociation of κ-casein from casein micelles upon heating at pH 7.0.

The Human Gastric Simulator was used for *in vitro* dynamic gastric digestion and pH-stat for simulated small intestinal digestion. Heat treatment of sheep milk resulted in the incorporation of MFGs into the curds through casein–whey protein or whey protein–

whey protein interactions; this hindered the formation of the closely knitted protein network and led to the formation of fragmented curds. Homogenization of sheep milk resulted in looser and more fragmented curd in comparison with unhomogenized sheep milk; this accelerated the protein hydrolysis and increased the rate of release of protein, fat, and calcium from the curds into the digesta. Processing treatments affected the lipolysis rate but not the lipolysis degree during small intestinal digestion.

In conclusion, the findings of this study have advanced our understanding of the heat-induced protein interactions in sheep milk and provided insights into the digestion behavior of differently processed sheep milk within the gastrointestinal tract. This may help to design and develop sheep milk-based products with desired digestive and functional properties.

Acknowledgments

Sincere thanks to my main supervisor Prof. Aiqian Ye for the many hours of stimulating discussion, helpful advice, encouragement, and friendship throughout the length of this project. I am also thankful for the support and guidance of my co-supervisors Dr. Anant Dave, Dr. Karl Fraser, and Dist. Prof. Harjinder Singh. Their constructive feedback throughout has challenged me to become more confident as a budding scientist. Thank you for the helpful advice and discussions which also encouraged me to think broadly but also stay on track and not lose sight of the end goal.

Thank you to the Riddet Institute, the School of Food and Advanced Technology at Massey as well as Te Ohu Rangahau Kai for providing the facilities to conduct the laboratory work and complete this project. The funding of this project by the New Zealand Milks Mean More-Ministry of Business, Innovation and Employment and Massey University Doctoral Scholarship is gratefully acknowledged.

Special thanks to Siqi Li for their friendship and advice in my PhD project; Jack Cui for his assistance in running the gastric digestion model; Peter Zhu for his assistance in running gas chromatography and confocal laser scanning microscopy; Maggie Zou for her assistance in purchasing my experimental consumables; Steve Glasgow for giving training on Mojonnier fat analysis; Gary Radford for assistance in running the pilot plant equipment; Yanyu He for preparing TEM samples and giving training on operating TEM.

Thank you also to Michelle Tamehana, Terri Palmer, Ansley Te Hiwi, John Henley-King and to all the postgrad students, especially Abhilasha, Le Deng, Debashree Roy, Anika Hoogeveen, Yangfan Ye, Lirong Cheng, Xin Wang and Mengxiao Yang. I would like to thank all of my friends who have given me so much support and made these four years enjoyable.

Last but not least, a special thanks to my family my mother Biyu Yan, my father Wenqu Pan and my extended family who have encouraged and supported me all this while. Thank you also to my dear wonderful wife Yiying Huang. My wonderful PhD journey started with meeting you in the same office PD Hut 103. I feel lucky that I have you as my life partner. Thanks for being the most amazing wife in the world and giving me countless happiness, constant love and encouragement.

Ngā mihi nui/感谢所有在博士期间帮助和陪伴我的家人，导师和朋友.

List of Publications

Pan, Z., Ye, A., Dave, A., Fraser, K., & Singh, H. (2022). Kinetics of heat-induced interactions among whey proteins and casein micelles in sheep skim milk and aggregation of the casein micelles. Journal of Dairy Science, 105(5), 3871-3882. doi:10.3168/jds.2021-21444

Pan, Z., Ye, A., Fraser, K., Dave, A., & Singh, H. (2023, 2023/09/01/). Heat stability of sheep's skim milk: Aggregation and interaction of proteins. International Dairy Journal, 144, 105689. https://doi.org/https://doi.org/10.1016/j.idairyj.2023.105689

Pan, Z.; Ye, A.; Dave, A.; Fraser, K.; Singh, H. (2022) pH-dependent sedimentation and protein interactions in ultra-high-temperature-treated sheep skim milk. Journal of Dairy Science. 106(3), 1626-1637. doi:10.3168/jds.2022-22637

Pan, Z., Ye, A., Li, S., Dave, A., Fraser, K., & Singh, H. (2021). Dynamic In Vitro Gastric Digestion of Sheep Milk: Influence of Homogenization and Heat Treatment. Foods, 10(8), 1938. doi:10.3390/foods10081938

Li, S., Pan, Z., Ye, A., Cui, J., Dave, A., & Singh, H. (2022). Structural and rheological properties of the clots formed by ruminant milks during dynamic in vitro gastric digestion: Effects of processing and species. Food Hydrocolloids, 126, 107465. doi: 10.1016/j.foodhyd.2021.107465

Manuscript in preparation

Pan, Z., Ye, A., Dave, A., Fraser, K., & Singh, H. Composition, processing properties, and digestion behaviors of sheep milk. Critical Reviews in Food Science and Nutrition. (to be submitted)

Pan, Z., Ye, A., Fraser, K., Li, S., Dave, A., & Singh, H. Comparative lipidomics analysis of different-sized fat globules in sheep and cow milks. Food Chemistry. (submitted)

Pan, Z., Ye, A., Fraser, K., Li, S., Dave, A., & Singh, H. Comparative lipidomics analysis of in vitro lipid digestion of sheep milk: Influence of homogenization and heat treatment. Journal of Dairy Science. (submitted)

Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

Milk is desired and valuable source of nutrients for humans. Milk from ruminant species (such as cow, goat, and sheep) is the most common source for producing various dairy products (such as fresh milk, cheeses, yogurt, and infant formula) (Park et al., 2017). Milk from different ruminant animals differ in the gross composition due to various factors (i.e., season, diet, environmental factors, stage of lactation, age, and fodder) (Wendorff & Haenlein, 2017). Generally, sheep milk has higher total solids, protein, fat, lactose and minerals than cow milk (Balthazar et al., 2017).

Sheep is traditionally used as the source of meat and wool. However, today sheep milk is popular for consuming in the form of various cheeses and yoghurt products because it contains a higher level of total solids and more nutrients than cow milk. The sheep milk constituents offer much more energy and the necessary nutrients for human growth and health. Furthermore, sheep milk is a good source of all essential amino acids; vitamins (except folate) and most minerals are higher in sheep milk than in cow milk (Park et al., 2017).

The sheep dairy industry has developed rapidly in recent years especially in New Zealand and Australia (Thomas & Haenlein, 2017), but the fresh sheep milk in liquid form is still not widely available in the market. One of the main reasons is sheep milk is highly sensitive to high temperature (Raynal & Remeuf, 1998). Numerous studies have investigated the kinetics of whey protein denaturation in cow milk across a wide range of heating temperatures and durations (Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Oldfield et al., 2005; Oldfield, Singh, Taylor, et al., 1998). Various models have been proposed to explain the mechanisms of whey protein denaturation and aggregation in cow milk, providing valuable insights into the denaturation processes and the interactions

between denatured whey proteins and casein micelles during heating. However, there is limited information regarding the kinetics of whey protein denaturation in sheep milk, and no research has been conducted on the association between denatured whey proteins and casein micelles in sheep milk. Since the high heat sensitivity of whey proteins in sheep milk, a lower temperature range (67.5−90 °C) was employed in this study compared to the typically utilized temperatures (75−130 °C) for whey proteins in cow milk (Anema & McKenna, 1996; Oldfield et al., 2005; Oldfield, Singh, Taylor, et al., 1998). In addition, the protein of sheep milk can be easily coagulated under ultra-high temperature (UHT) processing conditions, making it unable to be produced as a long shelf life liquid product (Martinez Alonso et al., 2009). To date, changes in proteins and lipids in cow and goat milk during the heat treatments have been extensively investigated and compared in-depth, however, these differences in sheep milk have only been superficially reported (Kalyankar et al., 2016). Considering the intricate nature of reactions taking place during heat treatment, and their association with milk composition, it is important to note that findings from studies conducted on cow milk cannot be directly applied to sheep milk. This is due to significant differences in composition (including variations in casein composition and calcium content) (Balthazar et al., 2017; Li, Delger, et al., 2022) and the difference in previously reported heat coagulation time-pH profiles (Fox & Hoynes, 1976; Muir & Tamime, 1993). Therefore, the heat stability testing on both lab and pilot scale was undertaken to examine the heat-induced alterations in sheep skim milk.

It is widely known that the composition of sheep milk is very different from cow milk, and the variance in composition could play an important role in the digestion behavior, nutrient release and absorption, and their influence on gut health (Ye, Cui, et al., 2019). The digestion behavior and absorption of nutrients from milks with different compositions from different species have been studied using *in vitro* digestion models (Dalziel et al., 2018;

Gallier et al., 2012a; Maqsood et al., 2019; Roy et al., 2021a, 2021b), but the effect of processing treatments (heat treatment and homogenization) on the digestion behavior of sheep milk is still unknown. Information on how processing treatments affect the digestive and functional properties of sheep milk should be established in order to develop a competitive dairy sheep industry.

So, the main objectives of this thesis were to:

- investigate the kinetics of denaturation of whey proteins and their association with the casein micelles in sheep skim milk (SSM), and to characterize the structural changes in whey proteins and casein micelles in SSM (Chapter 3),
- investigate the heat stability (examined at 140°C) of SSM and mechanism of heat-induced coagulation of SSM, and to understand the aggregation and dissociation behaviors of proteins under extra high heat treatments (Chapter 4),
- evaluate the effect of pH (6.6–7.0) on protein interactions during the pilot-scale manufacture of UHT SSM using a UHT plant with an indirect heating system (Chapter 5),
- investigate the effect of thermal processing (including pasteurization at 75 °C/15 s and heat treatment at 95 °C/5 min) and homogenization (200/50 bar) on the *in vitro* gastric digestion behavior of sheep milk using a dynamic gastric digestion model (human gastric simulator; Chapter 6).
- examine the effects of different heat treatments (pasteurization at 75 °C/15 s and heating at 95 °C/5 min) and homogenization (200/50 bar) on the lipid digestion behavior of sheep milk using a human gastric simulator (Chapter 7).

Chapter 2: Statement of Contribution – Doctorate with Publications/Manuscripts

DRC 16

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STATEMENT OF CONTRIBUTION
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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

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Chapter 2. Review of Literature

The contents of this chapter will be submitted to Critical Reviews in Food Science and Nutrition.

As this chapter was specifically written for publication, certain detailed information related to this PhD work has been excluded. However, a more comprehensive version of the literature review can be found in Appendix A, which is attached for reference.

2.1. Abstract

Sheep milk is a good source of human health because of higher major nutritional content than cow milk. The physicochemical properties of the proteins and fat in sheep milk differ from those in cow milk. This study reviews the properties of the proteins and fat of sheep milk with respect to their use in producing fresh liquid sheep milk products for human consumption. Technical aspects regarding processing-induced whey protein denaturation, protein interactions, changes in milk fat globules, and the digestion behavior of sheep milks are highlighted. The knowledge summarized in this review can be used as a basis for process design in the production of long-shelf-life sheep milk or for developing potential functional dairy products that possess the relevant dietary requirements for targeted consumers.

2.2. Introduction

Milk is an important daily food for human consumption, especially for infants; it is their primary source of nutrition. The main source of milk for producing various dairy products (i.e., fresh milk, cheeses, yogurt, and infant formulas) is ruminant milk (i.e., cow, goat, and sheep milks) (EFSA Panel on Dietetic Products & Allergies, 2014). Worldwide, the most commonly consumed milk is derived from cattle; around 81.6% of the world's milk production was derived from cow milk in 2019, followed by milk from other species

such as buffalo (14.5%), goat (2.2%), sheep (1.3%), and camel (0.3%) (FAO, 2021). However, the composition and the function of the milk components from different ruminant animals vary significantly because of various factors (i.e., diet, environmental factors, stage of lactation, age, and fodder) (Claeys et al., 2014). Cow milk allergy also occurs frequently in infants (Park, 1994). Therefore, sheep milk is promoted as a good alternative milk source for human consumption because some people who were unable to digest either cow milk or goat milk had no trouble in digesting sheep milk (Mills, 1989; Park et al., 2017).

Compared with cow milk products, the market for sheep milk products is small and undeveloped, suggesting great growth potential. Sheep milk products attract a premium over their cow milk equivalents because of their quality and nutritional value; the high nutritional value is attributed to the higher concentrations of proteins, fats, vitamins, and minerals (Park et al., 2017). Globally, sheep milk production is increasing, as consumer demand for specialty milks continues to grow. Sheep milk is consumed mainly as cheese and yogurt (Kalyankar et al., 2016), but its consumption in liquid form (i.e., fresh pasteurized sheep milk and infant formula) is now becoming popular in countries, such as China, USA, Malaysia, and Vietnam. However, fresh sheep milk in liquid form is not widely available because of its poor processing properties. One of the main reasons is that sheep milk is highly sensitive to high heating temperatures. For instance, the proteins of sheep milk coagulate under ultra-high temperature (UHT) processing conditions, because of its higher protein concentration or lower colloidal stability (Martinez Alonso et al., 2009; Raynal & Remeuf, 1998), which means that it cannot be produced as a long-shelf-life liquid product.

The digestibility and the function of milk are attracting increasing interest from consumers and the dairy industry with respect to their health benefits. The chemical composition and the physicochemical properties of sheep milk are known to be different

from those of cow milk, and these variances could play important roles in digestion behavior, release and absorption of nutrients, and gut health (Roy et al., 2020a; Ye, 2021; Ye, Cui, et al., 2019). Anecdotal reports indicate that sheep milk is perceived to be a better digestive, nutritional, and tolerable milk source for humans than cow milk; however, relatively few scientific reports on the digestive and nutritional benefits of sheep milk are available.

Recently, the growing interest in sheep dairy production in New Zealand, northern Europe, Australia, India, and the United States for infant nutrition has created new opportunities for developing high-value sheep milk (Claeys et al., 2014; Kalyankar et al., 2016). To develop a competitive sheep milk industry, the effect of processing treatments on the digestive and functional properties of sheep milk should be established; this could provide important information for the development of new milk products and could position sheep milk as a better food source for humans, particularly infants (Recio et al., 2009). Thus, this review aims to evaluate the scientific literature on the physicochemical and functional properties of sheep milk and the influence of processing treatments on the structure of sheep milk. Special attention is given to the effects of compositional differences and processing on the digestion behaviors of the proteins and fat of sheep milk.

2.3. Composition of sheep milk

Sheep milk is an ideal alternative to cow milk because of its higher levels of protein, fat, and calcium per casein unit, which gives sheep milk the advantage of a specific taste, a specific texture, and a healthy image (Anifantakis, 1986). As the comparative macronutrient features of sheep milk and cow milk have been extensively reviewed in previous studies (Balthazar et al., 2017; Claeys et al., 2014; Kalyankar et al., 2016; Park et al., 2007), they are not discussed extensively here. The components that play important roles in processing

and digestion are summarized in Table 2-1 and are discussed in more detail in the following sections.

Table 2-1. Chemical and physicochemical compositions of cow, goat, and sheep milks.

	Cow	Goat	Sheep
Total solids (wt/wt%)	12.1 ± 0.5	11.9 ± 0.5	17.5 ± 0.6
Protein (wt/wt%)	3.4 ± 0.1	3.2 ± 0.1	5.7 ± 0.3
Fat (wt/wt%)	3.3 ± 0.2	3.8 ± 0.4	6.0 ± 0.4
Lactose (wt/wt%)	4.7 ± 0.4	4.4 ± 0.1	4.8 ± 0.1
Casein (wt/wt%)	3.0 ± 0.1	2.6 ± 0.1	4.9 ± 0.2
κ-casein (% of total casein)	11.6	20.6	13.0
α_{S1}-casein (% of total casein)	39.7	11.0	39.6
α_{S2}-casein (% of total casein)	10.3	13.5	13.8
β-casein (% of total casein)	32.7	54.8	33.6
Whey protein (wt/wt%)	0.4 ± 0.0	0.6 ± 0.1	0.8 ± 0.1
β-Lactoglobulin (% of total whey protein)	58.2	40.5	63.7
α-Lactalbumin (% of total whey protein)	21.8	18.9	9.8
Milk fat globule size			
$D_{4,3}$ (μm)	4.4 ± 0.1	3.6 ± 0.0	4.3 ± 0.0
$D_{3,2}$ (μm)	3.8 ± 0.1	2.9 ± 0.0	3.5 ± 0.0
Casein micelle size (Z-average diameter, μm)	158.4 ± 1.7	190.0 ± 3.5	179.6 ± 0.7

Note: Data collected from: Balthazar et al. (2017); Li, Delger, et al. (2022); Roy et al. (2022); Roy et al. (2020a).

Abbreviations: $D_{4,3}$ volume-weighted mean diameter; $D_{3,2}$, surface-weighted mean diameter.

2.3.1. Protein composition

Selvaggi et al. (2014) reported that the casein fraction is approximately 80% of the total protein of sheep milk, consisting mainly of four fractions: β-casein (~ 61.6%), α_{S2}-casein (~ 22.8%), α_{S1}-casein (~ 6.7%), and κ-casein (~ 8.9%). However, a recent study

showed a different casein composition in sheep milk, i.e., with α_{S1}-casein (~ 39.6%) and β-casein (~ 33.6%) being the most abundant caseins, followed by α_{S2}-casein (~ 13.8%) and κ-casein (~ 13.0%) (Li, Delger, et al., 2022). The proportions of the casein fractions in sheep milk reported by Li, Delger, et al. (2022) are similar to those in cow milk but different from those in goat milk (Table 2-1). It has been reported that the protein composition in sheep milk could be greatly influenced by protein genotypes (Ha et al., 2014; Pirisi et al., 1999; Selvaggi et al., 2014), whereas the protein composition in cow milk tends to be more consistent (Ceballos et al., 2009; Martin et al., 2002).

The different proportions of the caseins in milk can affect its functional properties because of the different structures and degrees of phosphorylation of the different caseins (Fang et al., 2017). The phosphorylation of caseins plays an important role in constructing and stabilizing the casein micelles (De Kruif & Holt, 2003). All the major caseins are phosphoproteins; α_S-casein (α_{S1}- and α_{S2}-casein) is the most phosphorylated, and its phosphorylation profiles are more heterogeneous than those of β-casein and κ-casein. Additionally, the caseins have been categorized into calcium-sensitive casein (α_S-casein and β-casein) and calcium-insensitive casein (κ-casein); the latter is also one of the key factors that is responsible for stabilizing the former against precipitation when calcium is present (Dalgleish, 2011; Holt, 1992). The caseins function as carriers to deliver calcium phosphate and thus provide a source of calcium, phosphorus, and amino acids for the growth of infants (Selvaggi et al., 2014). Therefore, sheep milk could have different physicochemical and technological properties from those of the milks from other species (such as cow and goat).

The second major group of milk proteins are the whey proteins, which include mostly β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). The whey proteins of sheep milk are more sensitive to heating than those of cow milk and goat milk. Heat treatment (78–

100 °C/16 s and 95 °C/5 min) of sheep milk denatured β-LG and α-LA to a greater extent compared with goat milk (Li, Delger, et al., 2022; Moatsou et al., 2021). Heating sheep milk at 65 °C/30 min or 80–90 °C/0–5 min resulted in greater denaturation of the whey proteins compared with cow milk (Molik et al., 2012; Raynal & Remeuf, 1998). The extent of denaturation of the whey proteins has been associated with the rate of digestion of the milk proteins because denatured whey proteins could affect the formation and structure of the protein matrix in the stomach and thus its hydrolysis by pepsin (Li, Pan, et al., 2022; Li et al., 2021). Therefore, thermal processing of the milks from different species will affect their digestion behaviors within the gastrointestinal tract differently because of their different heat stabilities.

2.3.2. Genetic polymorphism of sheep milk proteins

A marked polymorphism in the genetic variants of sheep milk proteins has been reported, with 27 different variants being observed for six major sheep milk proteins: A, B, C, D, E, F, H, and I for α_{S1}-casein; A, B, C, D, E, F, and G for α_{S2}-casein; A, B, C, X, and Y for β-casein; A and B for κ-casein; A and B for α-LA; A, B, and C for β-LG (Amigo et al., 2000; Martin et al., 2002; Pirisi et al., 1999; Selvaggi et al., 2014). These differences in the genetic polymorphisms are attributed to the varied amino acid substitutions, phosphorylations, and glycosylations, resulting in different chain lengths and molecular weights, electrical charges, hydrophobicities of the proteins, and proportions of the four major caseins (especially α_{S1}-casein and α_{S2}-casein) between individuals and breeds (Park et al., 2017; Selvaggi et al., 2014).

The genetic polymorphisms of milk proteins are often associated with their physicochemical properties (hydration, gelation, emulsification, enzymatic reactions such as rennet coagulation time and curd firming time, curd firmness, and digestion), evidences

on the gene sequences, casein genotypes and structures have been given (Erhardt, 1989). For instance, Loch et al. (2014) showed different β-LG gene sequences and three-dimensional structures between sheep milk and cow milk, and demonstrated that those differences could influence the electrostatic potential on the molecular surface of β-LG. Genetic factors such as species, breed, and individual animal play key roles in affecting the coagulation properties of sheep milk (Bittante et al., 2012). Noce et al. (2016) explored the association between the gene polymorphisms of Sarda sheep milk proteins and the traits and coagulation properties of the milk and found that there were significant associations between the genotypes of α_{S1}-casein and the rennet coagulation time, curd firming time, and curd firmness, between the genotypes of β-casein and the curd firming time, and between the genotypes of α_{S1}-casein and κ-casein and the milk protein and casein contents. However, Gencheva et al. (2020) reported that the α_{S1}-casein genotype did not have a significant impact on the rennet coagulation time in the Sofia sheep population. These contradictory results may be due to the high genetic variability (such as varying protein genotypes and gene frequencies) in the milk proteins among sheep breeds. The different variants of proteins have differently phosphorylated forms with different numbers of phosphate groups (Selvaggi et al., 2014). This probably affects the protein interactions in sheep milk during thermal processing and cheese making because the phosphate groups of caseins are involved in the formation of colloidal calcium phosphate between caseins and play an important role in stabilizing and destabilizing the casein micelles in the milk system. However, the effect of the genetic variants and the gene frequencies of milk proteins on the protein interactions during thermal processing and clotting is still unknown. Further research in this field is needed to take advantage of the polymorphic differences of sheep milk proteins, to better understand how these genetic differences in milk proteins affect

protein interactions, and to select appropriate sheep populations that have the milk proteins that are needed for the development of sheep milk protein products.

2.3.3. Lipid composition

The total fat contents of goat milk and cow milk are relatively similar, whereas sheep milk has a markedly higher fat content (Table 2-1). Compared with cow milk, sheep milk and goat milk contain higher proportions of short-chain and medium-chain fatty acids but lower proportions of long-chain fatty acids; the proportion of saturated fatty acids is highest in sheep milk and lowest in cow milk; sheep milk fat contains lower proportions of monounsaturated fatty acids than cow and goat milk fats; the proportion of polyunsaturated fatty acids in sheep milk fat is lower than that in cow milk fat but higher than that in goat milk fat (Table 2-2). Short- or medium-chain triacylglycerols (TAGs) are considered to be more efficiently hydrolyzed by lipase (Park et al., 2017), and medium-chain fatty acids can be absorbed by the infant more easily than long-chain fatty acids (Lindquist & Hernell, 2010); this suggests that sheep milk and goat milk could be advantageous in developing infant formulas and that these differences in the fatty acid composition may contribute to the different digestion behaviors of the milk fats from different species. It has been reported the fatty acid compositions of cow, sheep, and goat milk fats are, to some degree, different from that of human milk fat (Zou et al., 2013). Therefore, the addition of vegetable oils to these milks can modify their fatty acid compositions to mimic that of human milk, and this method is commonly used in the production of infant formula.

Table 2-2. Fatty acid compositions of cow, goat, and sheep milks.

Fatty acid (g/100 g fat)	Cow	Goat	Sheep
C4:0	3.1 ± 0.3	2.6 ± 0.1	2.8 ± 0.1
C6:0	2.2 ± 0.3	2.8 ± 0.0	2.5 ± 0.1
C8:0	1.4 ± 0.2	3.3 ± 0.2	2.6 ± 0.1
C10:0	3.3 ± 0.5	11.3 ± 0.7	9.9 ± 0.4
C12:0	3.6 ± 0.5	5.6 ± 0.8	6.8 ± 0.3
C13:0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
C14:0	11.6 ± 1.2	11.4 ± 0.9	15.0 ± 0.4
C15:0	3.4 ± 0.1	1.4 ± 0.2	2.0 ± 0.2
C16:0	24.9 ± 1.4	27.7 ± 1.4	29.8 ± 0.5
C17:0	0.9 ± 0.0	0.8 ± 0.1	0.9 ± 0.0
C18:0	12.7 ± 1.6	8.3 ± 0.9	4.8 ± 0.3
C20:0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
C10:1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
C12:1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C14:1	0.8 ± 0.1	0.2 ± 0.3	0.4 ± 0.0
C16:1	1.0 ± 0.2	1.2 ± 0.1	2.1 ± 0.0
C17:1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.0
C18:1	24.8 ± 3.8	19.8 ± 0.6	15.7 ± 1.4
C20:1	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
C18:2	2.8 ± 0.4	2.2 ± 0.2	2.0 ± 0.6
CLA	1.6 ± 0.7	0.5 ± 0.1	1.1 ± 0.0
C18:3	0.9 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
SCFA	5.3 ± 0.3	5.4 ± 0.1	5.4 ± 0.1
MCFA	23.6 ± 0.4	33.2 ± 0.4	36.5 ± 0.2
LCFA	71.1 ± 0.7	61.4 ± 0.3	58.1 ± 0.2
SFA	67.7 ± 5.3	75.5 ± 0.7	77.5 ± 0.9
MUFA	27.3 ± 4.2	21.8 ± 0.5	19.0 ± 1.4
PUFA	5.3 ± 1.1	3.0 ± 0.3	3.9 ± 0.5

Note: Data collected and modified from: Pietrzak-Fiećko and Kamelska-Sadowska (2020).

Abbreviations: CLA, conjugated linoleic acid (% of total fatty acids); SCFA, sum of short-chain fatty acids (% of total fatty acids); MCFA, sum of medium-chain fatty acids (% of total fatty acids); LCFA, sum of long-chain fatty acids (% of total fatty acids); SFA, sum of

saturated fatty acids (% of total fatty acids); MUFA, sum of monounsaturated fatty acids (% of total fatty acids); PUFA, sum of polyunsaturated fatty acids (% of total fatty acids).

Teng et al. (2020) showed that sheep milk contained significantly lower proportions of saturated TAGs (saturated fatty acids esterified on the *sn*-1/3 positions of TAGs) but significantly higher proportions of polyunsaturated TAGs (polyunsaturated fatty acids esterified on the *sn*-1/3 positions of TAGs) than cow milk prior to and after homogenization, indicating that sheep milk fat could be more suitable for infant consumption than cow milk fat because the regiodistribution of the saturated and unsaturated fatty acids in sheep milk TAGs is closer to that in human milk TAGs than in cow milk TAGs. Additionally, the TAGs could be more digestible when the medium-chain fatty acids, rather than the long-chain fatty acids, are esterified on the *sn*-1/3 positions. Teng et al. (2020) reported that TAG_12:0/14:0/4:0 in sheep milk was nearly twice as digestible as its positional isomer TAG_14:0/12:0/4:0 in cow milk. Zou et al. (2013) compared the fatty acid compositions of the milks of five mammalian species with that of human milk and found that, for the content of saturated fatty acids at the *sn*-2 position, cow and sheep milk fats contained higher proportions of medium-chain saturated fatty acids, but significantly lower proportions of long-chain saturated fatty acids compared with human milk fat; the saturated fatty acids at the *sn*-2 position in human milk fat were mainly C16:0, whereas cow milk and sheep milk showed significantly lower contents and concentrations of C16:0 at the *sn*-2 position. This suggests that sheep and cow milk fats could be less digestible than human milk fat.

Milk fat globules (MFGs) are covered with the MFG membrane (MFGM), which is a natural milk emulsifier that stabilizes the lipid droplets in an aqueous phase and consists of a trilayer of polar lipids (phospholipids and sphingolipids), cholesterol, and proteins (glycoproteins and enzymes) (Lopez et al., 2011). Sheep milk and goat milk MFGMs have

been reported to contain more polar lipids than cow milk MFGM (Bitman & Wood, 1990; Sánchez-Juanes et al., 2009) but fewer than human milk MFGM (Morrison & Smith, 1967). In contrast, Zou et al. (2013) analyzed the concentrations of polar lipids in human, cow, and sheep milks and found no significant differences in the polar lipid contents among the three types of milk. However, the milks from different species vary in phospholipid composition. Zancada et al. (2013) determined the phospholipids in sheep milk and goat milk and reported that the profiles of the individual polar lipids [including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM)] of sheep milk and goat milk were similar. Rodríguez-Alcalá and Fontecha (2010) and Castro-Gómez et al. (2015) analyzed the lipid composition of sheep milk and found that the polar lipid content decreased in the following order: PE > PC > SM. Et-Thakafy et al. (2017) showed that the phospholipid content of sheep milk follows the order PE > SM > PC. In contrast, Zancada et al. (2013) showed that SM is the most abundant phospholipid in sheep milk, followed by PC and PE. These differences between studies in the concentration and the composition of sheep milk polar lipids have been attributed to the MFG size, genetics, breed, season, lactation stage, and diet choice (Argov-Argaman et al., 2016; Chilliard et al., 2007; Lopez et al., 2011; Lopez et al., 2008; Michalski, 2009), and may contribute to the different digestion processes of the milk fats from different species because the polar lipids affect the interactions between interfacial compounds of fat droplets and digestive enzymes (Golding & Wooster, 2010; Liu et al., 2021).

2.3.4. MFG characteristics

The MFGs are secreted in a diverse range of sizes (0.2–15 μm) depending on the species (Argov et al., 2008; Michalski et al., 2005), lactation stage (Mesilati-Stahy & Argov-Argaman, 2014), season (Briard et al., 2003), and nutrition (Lopez et al., 2008). The

size of the MFGs varies among the milks of different species and these variations may influence the digestion of their fat differently from various perspectives (such as interactions between lipase and TAGs or contact between pepsin and MFGM proteins). The MFGs of sheep milk are smaller than those of cow milk but larger than those of goat milk (Table 2-1) (Claeys et al., 2014; Crowley et al., 2017; Roy et al., 2020b). Size-dependent chemical and physical properties of MFGs have been reported by Mesilati-Stahy et al. (2011), who showed that the small MFGs in cow milk contained higher concentrations of polyunsaturated fatty acids, PI and SM but lower concentrations of PS and PE. However, the chemical and physical properties of the different-sized MFGs in sheep milk have not yet been reported.

The MFGM consists of a range of polar lipids, cholesterol, and membrane-specific proteins, which stabilize the fat globules in the serum phase of the milk (Heid & Keenan, 2005). From a structural point of view, the trilayer (from the inner face to the outer face of the membrane) is composed of a monolayer of polar lipids and proteins (an electron-dense layer covers the TAG-rich core lipids), a bilayer of phospholipids, proteins, and cholesterol, and a glycocalyx that consists of the sugar residues on glycoproteins and glycolipids (Gallier et al., 2017). The MFGM is secreted in a variety of sizes, ranging from 5 to 25 nm, because of the protruding glycosylated molecules of the MFGM glycocalyx (Gallier et al., 2015; Heid & Keenan, 2005), which could provide steric repulsion between the MFGs (Gallier et al., 2017).

It has been reported that the glycoproteins and glycolipids in the MFGM could hinder the interaction between digestive enzymes and lipids, thereby hindering the digestion of the MFGs (Ye et al., 2010; Zhao et al., 2019). The protein composition of the MFGM has been shown to be different between the milks from different species (Jia et al., 2022; Zancada et al., 2013) and the digestion and absorption of the lipids are affected by their

interfacial compositions (including the MFGM and the milk proteins) (Gallier et al., 2017; Roy et al., 2020a; Zaeim et al., 2022). Additionally, the commonly used processing treatments (homogenization and thermal processing) in the dairy industry will change the interfacial compositions and structures, and the size of the MFGs, making the functional properties of the MFGs more complex. Therefore, the differences in the characteristics of the MFGs are crucial for lipid digestion and can affect the digestion behaviors of the milks from different species differently (Pan et al., 2023b).

2.3.5. Minerals

Sheep milk has higher contents of macrominerals (calcium, magnesium, and phosphorus) and microminerals (copper, selenium, and zinc) than cow milk and goat milk (Li, Delger, et al., 2022; Park et al., 2007; Raynal-Ljutovac et al., 2008), which is probably due to the association of a considerable proportion of these minerals with the casein micelles (Gaucheron, 2005). However, the soluble calcium concentration is lower in sheep milk (19.6%) than in goat milk (29.7%) and cow milk (30%) (Gaucheron, 2005; Li, Delger, et al., 2022), whereas the ionic calcium concentration in sheep milk (2.70 m*M*) is higher than that in cow milk (1.8–2.3 m*M*) (Lewis, 2011) but lower than that in goat milk (3.18 m*M*) (De La Fuente et al., 1997; Li, Delger, et al., 2022).

Calcium plays an important role during the thermal processing and digestion of milk. Higher concentrations of ionic calcium can promote the association of the whey proteins with the casein micelles and micelle–micelle aggregation (Li et al., 2019; Singh, 2004). Li et al. (2019) and Li, Delger, et al. (2022) investigated the effects of compositional changes and heat treatment on cow, goat, and sheep milks and found that the extent of whey protein denaturation and the association of the denatured whey proteins with the casein micelles are positively correlated with the ionic calcium concentration, probably because of the

binding of ionic calcium to negatively charged proteins and thus the facilitation of association between the proteins. Moreover, the concentration of ionic calcium also significantly affects the heat stability of milk (Deeth & Lewis, 2017). Lewis et al. (2011) showed that there is a sharp boundary between milk instability (ionic calcium levels > 2.0 m*M*), which produces large amounts of sediment, and milk stability (ionic calcium levels < 2.0 m*M*), which produces little sediment. Additionally, Dumpler et al. (2020) reported that the low heat stability of milk at acidic pHs could be attributed to salt-induced coagulation because the colloidal stability of the casein micelles would be lowered by the reduced micellar surface charge, the reduced electrostatic repulsion, and the collapse of the hairy layer because of charge neutralization. The higher ionic calcium concentration in sheep milk probably partly contributes to its lower heat stability compared with cow milk and thus the ready aggregation of the proteins during thermal processing (Lewis, 2011; Lin, 2002; Silanikove et al., 2003). Pan et al. (2022b) investigated the effect of pH on the sedimentation and protein interactions in sheep skim milk and showed that UHT treatment (140 °C/5 s) at the natural pH (~ 6.6) resulted in a large amount of sediment because of the high ionic calcium concentration of ~ 2.2 m*M*. Increasing the pH of the sheep skim milk led to a decrease in the ionic calcium concentration, resulting in a significant decrease in the amount of sediment.

In an *in vitro* study (Zangenberg et al., 2001), multiple nutritionally relevant cations (such as ionic calcium, magnesium, zinc, and copper) were shown to interact with ionized free fatty acids to form fatty acid soaps; this could affect the digestion of lipids. However, the extent of soap formation is dependent on both the type and the availability of the cations and the fatty acids (Stroebinger, 2016). Calcium is the predominant cation that is involved in soap formation as the ionic calcium concentration in cow, goat, and sheep milks is significantly higher than that of the other cations (He et al., 2020). However, the majority

of the calcium in milk is associated with the casein micelles, and can be released only upon protein hydrolysis during digestion(B. Ingham et al., 2018). The ionized long-chain fatty acids of TAGs that are hydrolyzed by lipase are more likely to accumulate at the oil–water interface, because of their lower solubility compared with the short- and medium-chain fatty acids, and can complex with free ionic calcium in a 1:2 molar ratio to form a layer of crystalline calcium–fatty acid soap surrounding the lipid droplets (Torcello-Gómez et al., 2018). When the bile salt concentration is low, especially for infants, the accumulation of lipolytic products at the surface of the oil droplets can slow down the activity of lipase (Zangenberg et al., 2001), thereby hindering further lipid digestion. Therefore, the different calcium concentrations in cow, goat, and sheep milks may have different impacts on the lipolysis rate during the digestion of milk in the infant. Further study is needed to understand how the different calcium concentrations in the milks from different species affect the digestion and absorption of their lipids under infant gastrointestinal conditions.

2.4. Processing properties of sheep milk

2.4.1. Thermal processing

Pasteurization and UHT treatment are technologies that are commonly used in the dairy industry to produce liquid milk products that are of satisfactory organoleptic quality and shelf life. However, UHT treatment has significant disadvantages for sheep milk because it results in unstable proteins during storage. The phenomenon that occurs in UHT-treated sheep milk is a large amount of sediment, which can be attributed mainly to the formation of protein aggregates and precipitates during the heat treatment (Martinez Alonso et al., 2009).

2.4.1.1. Thermal denaturation of whey protein

Several studies have reported on the denaturation of the whey proteins in sheep milk. Raynal and Remeuf (1998) reported that ~ 15–80% of the whey proteins were denatured under the conditions of 75–90 °C/0.5–10 min. A recent study also showed that ~ 5–99% of the whey proteins were denatured after heating sheep skim milk at 67.5–90 °C/0.5–30 min, with denaturation levels of ~ 3–99% and ~ 5–100% for α-LA and β-LG, respectively (Pan et al., 2022a). Pan et al. (2022a) showed that α-LA has higher heat stability than β-LG in sheep milk, which is in agreement with results for cow milk (Dumitraşcu et al., 2013; Oldfield, Singh, Taylor, et al., 1998; Pintado & Malcata, 1996).

Under the same heating conditions, sheep milk has a greater extent of whey protein denaturation than cow milk (Dumitraşcu et al., 2013; Law, 1995; Raynal & Remeuf, 1998). Under pasteurization conditions (65 °C/30 min), about 15% of the whey proteins in sheep milk but only 2.3% of whey proteins in cow milk were denatured (Molik et al., 2012). The higher ratio of β-LG to α-LA and the higher whey protein content in sheep milk are considered to be responsible for the greater extent of whey protein denaturation in sheep milk than in cow milk (Law & Leaver, 1997).

2.4.1.2. Interactions between whey proteins and caseins/casein micelles

Heat-induced denatured whey proteins (β-LG, α-LA, lactoferrin, and serum albumin) can interact with the caseins (especially κ-casein), mostly through sulfhydryl–disulfide interactions, and can form aggregates (Wang et al., 2017). Most of the denatured whey proteins (mainly β-LG) can be covalently bound to the κ-casein on the surface of casein micelles, leading to an increase in the casein micelle size (Anema & Li, 2003a). The interaction between whey proteins and κ-casein also occurs in the serum phase when the κ-casein dissociates from the casein micelles; this could create unstable hydrophobic areas so that the casein micelles come closer to each other and associate, and thus form large

aggregates (Van Hooydonk et al., 1987). The presence of β-LG during heating helps α-LA to interact with κ-casein; α-LA cannot interact directly with κ-casein (Baer et al., 1976; Elfagm & Wheelock, 1978).

The protein composition in milk is complex, containing numerous types of protein that could potentially interact with each other through thiol–disulfide exchange or noncovalent bonding upon heat treatment (Anema, 2014). For instance, α_{S2}-casein could participate in heat-induced interactions through thiol–disulfide exchange reactions with denatured thiol-bearing whey proteins (especially β-LG) because of its disulfide bonds (Anema, 2014; Farrell et al., 2009). Interactions between α_{S2}-casein and denatured whey proteins in pasteurized milk are limited by the low accessibility of α_{S2}-casein because of its location in the interior of the casein micelles, although some interactions occur under UHT conditions (Patel et al., 2006). Sheep milk contains higher proportions of α_{S2}-casein than cow milk (Table 2-1), probably resulting in the α_{S2}-casein of the casein micelles being more accessible for interaction with β-LG. Recent studies showed that sheep skim milk had a greater extent of association of denatured whey proteins with the casein micelles than cow milk after heat treatment (75–90 °C/30 min) (Anema & Li, 2003a; Pan et al., 2022a), and Pan et al. (2022a) assumed that the higher α_{S2}-casein content in sheep milk was responsible. However, the distribution of individual casein (in the presence of high proportions of α_{S2}-casein) of casein micelles in sheep milk is still unclear, which requires more studies to further investigate the structure of casein micelles of sheep milk.

The interaction between denatured whey proteins and casein micelles can be easily influenced by several factors, such as heating temperature, heating-up time, holding time, the concentration of milk components, and pH (Anema, 2018b; Anema & Li, 2003a, 2003b; Dumpler & Kulozik, 2015; J.E.O'Connell & P.F.Fox, 2011; Sutariya et al., 2017; Vasbinder & De Kruif, 2003). For example, milk needs a long heating-up time when subjected to a

laboratory thermal water bath, and this heating method (at 90 °C for 20 min) resulted in approximately 80% of the denatured β-LG being associated with the casein micelles (Smits & Brouwershaven, 1980). In contrast, milk needs a much shorter heating-up time when subjected to commercial heating systems (such as a UHT plant); only around 55% of the denatured β-LG and α-LA were associated with the casein micelles in the temperature range 75–130 °C; the remaining denatured whey proteins remained in the serum phase of milk as disulfide-bonded and hydrophobically associated aggregates (Oldfield, Singh, & Taylor, 1998; Singh & Creamer, 1991b). The negative charge of the micelles increased with increasing levels of association of whey proteins with the casein micelles at 80–100°C for 20 min, preventing aggregation of the milk (Singh & Fox, 1987a). The same hypothesis of stabilization of the β-LG in sheep milk in the pH range of 6.4–6.8 has also been given by Fox and Hoynes (1976).

The degree of interaction between denatured whey proteins and casein micelles can be dramatically affected by the pH of the milk at heating. When cow milk was heated at a high temperature (90–140 °C) for 30 min at a pH lower than 6.7, microscopy images showed that the surface of the casein micelles was complexed by denatured whey proteins in a filamentous form, whereas, when heated at a pH higher than 6.7, the denatured whey proteins were found in the serum phase (Creamer et al., 1978; Creamer & Matheson, 1980; Kudo, 1980). It has been shown that up to 90% of the denatured whey proteins can co-sediment with the casein micelles at low pH (< 6.7), whereas most of the denatured whey proteins and a high level of κ-casein are observed in the serum phase at high pH (> 6.8) (Anema & Klostermeyer, 1997; Kudo, 1980; Oldfield et al., 2000; Singh & Fox, 1985; Singh & Fox, 1987a, 1987b). The heat-induced dissociation of κ-casein is pH dependent in the pH range 6.5–7.1, with a linearly increasing level of κ-casein in the serum phase as the pH increases. Moreover, the dissociation of κ-casein can precede the denaturation of the

whey proteins. Therefore, the interaction between κ-casein and whey proteins occurs preferentially in the serum phase, resulting in greater formation of whey protein/κ-casein complexes in the serum phase when heated at higher pH values (Anema, 2007, 2008).

The extent of denaturation of the whey proteins and the degree of association of denatured whey proteins with the casein micelles significantly affect the physicochemical properties of the micelles. For example, denaturation of the whey proteins in sheep skim milk increased with increasing heating temperature (75–90 °C) and holding time (0.5–30 min), leading to an increase in the degree of association between the denatured whey proteins and the casein micelles and thus an increase in the casein micelle size of sheep milk of up to 75%, in comparison with unheated sheep skim milk (Figure 2-1A) (Pan et al., 2022a; Raynal-Ljutovac et al., 2007). The degree of association between denatured whey proteins and casein micelles and the casein micelle size were greater in sheep milk than in cow milk under the same heating conditions (Anema & Li, 2003a; Corredig & Dalgleish, 1996; Smits & Brouwershaven, 1980). As mentioned above, denatured whey proteins associated with the casein micelles could provide steric repulsion and a negative charge, preventing aggregation among the casein micelles in cow milk. It can be inferred that the association of whey proteins with the κ-casein on casein micelles may provide greater stabilizing effects for the casein micelles of sheep milk when subjected to high temperature (Calavia & Burgos, 1998). However, the impact of the degree of the association of denatured whey proteins with the casein micelles on the heat stability of sheep milk under UHT treatment has not yet been reported; further studies are needed to confirm the hypothesis.

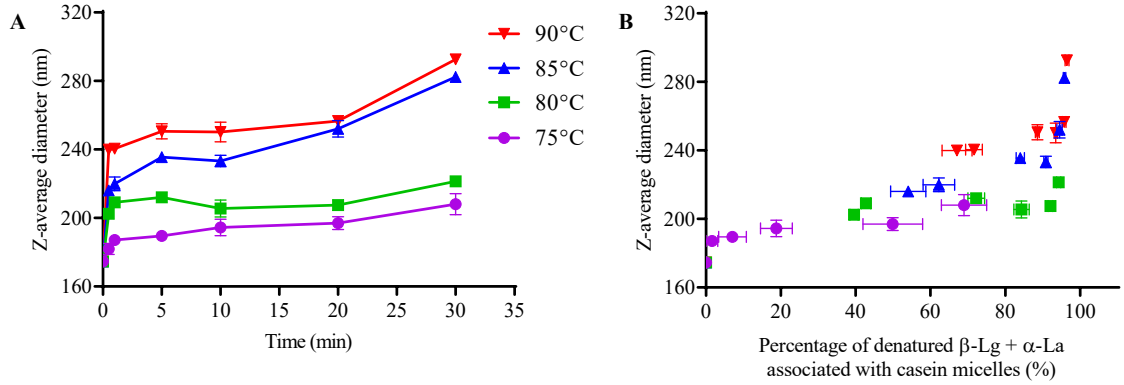

Figure 2-1. (A) Effect of heating temperature and time on the average size (Z-average diameter) of the particles in sheep skim milk; (B) relationship between the percentage of denatured whey proteins (β-LG + α-LA) associated with the casein micelles and the casein micelle size in sheep skim milk heated at different temperatures. Each data point represents the mean ± standard deviation of the results from three different batches of sheep milk. Adapted from Pan et al. (2022a).

2.4.1.3. Heat-induced aggregation of casein micelles

Sheep milk is known to have lower heat stability than cow milk, but this has not yet been extensively studied (Park et al., 2007; Raynal-Ljutovac et al., 2007). Unlike UHT-treated cow milk, which usually forms a thin layer of sediment that does not affect its organoleptic quality, UHT-treated sheep milk can produce a large amount of sediment (Martinez Alonso et al., 2009). It has been reported that the sediment formed during the UHT treatment of milk is composed mainly of milk proteins, specifically κ-casein-depleted casein micelles (Gaur et al., 2018). The recent study of Pan et al. (2022a) showed that protein aggregation was observed when sheep skim milk was heated at 90–95 °C; the particle size of the sheep skim milk increased markedly once the extent of association between denatured whey proteins and casein micelles had exceeded ~ 95% (Figure 2-1B).

These authors stated that aggregation of the casein micelles contributed to the markedly increased particle size as there was little association between the whey proteins and the casein micelles at that time point. Another recent study of Pan et al. (2022b) showed that UHT treatment (140 °C/5 s) of sheep skim milk at the natural pH (~ 6.6) and at pH 7.0 produced a large amount of sediment; it was composed mainly of κ-casein-depleted casein micelles with a low level of whey protein. The low heat stability of sheep milk proteins may have been responsible for the occurrence of protein aggregation under these heating conditions (Li, Delger, et al., 2022; Pan et al., 2022a).

pH, ionic calcium concentration, protein content, protein composition, and lactose content have been suggested to affect the heat stability of milk during UHT processing. The primary destabilizing effect has widely been reported to be a high ionic calcium concentration (> 2.0 m*M*) as the milk proteins usually aggregate via calcium bridging (Chen et al., 2015; Crowley et al., 2014; Deeth, 2020; Dumpler et al., 2020). The pH also markedly affects the heat stability of milk. Lewis et al. (2011) reported that the amount of sediment increased as the pH decreased despite a constant ionic calcium concentration. In addition, the heat-induced dissociation of κ-casein plays an important role in destabilizing the milk proteins, particularly at the point of decreasing heat stability on the alkaline side (pH > 6.7) of the pH–heat coagulation time profile (Dumpler, 2018). It has been reported that the low heat stability of milk with high total solids can be attributed primarily to the high ionic calcium concentration at pH < 6.7, whereas the initial reduction in the heat coagulation time with increasing pH > 6.7 has been linked to the fact that heat-induced dissociation of κ-casein from the casein micelles becomes more pronounced in this pH range and increases markedly with increasing pH (Dumpler, 2018). Therefore, the very rapid heat-induced coagulation of milk with high total solids could be attributed to a combination of high ionic calcium activity and sufficient heat-induced dissociation of κ-casein. Pan et al. (2022b)

reported sheep skim milk contains a high ionic calcium concentration and that UHT treatment of sheep skim milk resulted in a marked level of dissociation of κ-casein from the casein micelles, compared with cow skim milk. These differences could contribute to the formation of the large amount of sediment after the UHT treatment of sheep milk. However, the changes in the milk proteins during UHT treatment have been extensively focused on cow milk, and cannot be directly extrapolated to sheep milk as the physicochemical properties of sheep milk are different from those of cow milk and may affect the heat-induced protein interactions in a different manner (Balthazar et al., 2017; Roy et al., 2020a). Further studies are needed to investigate the mechanism of the low heat stability of sheep milk.

2.4.2. Homogenization

Homogenization is a standard treatment for commercial milk or milk products; whole milk or half-fat milk can be treated effectively and this increases the physical shelf life by preventing fat separation (Britz and Robinson, 2008). Homogenization of milk greatly reduces the MFG size (from 1–10 μm to 0.2–2 μm) and alters the MFG surface composition because of the adsorption of mainly casein micelles and whey proteins (Gallier et al., 2017; Singh & Gallier, 2017). The MFGs of raw sheep milk and raw cow milk vary in size (Roy et al., 2020a) and in MFGM composition (Nguyen et al., 2017); these factors are considered to have different influences on the digestion of the fat. Homogenization of the milk may narrow these differences in the digestion behaviors of the fat between sheep milk and cow milk as the size and the interfacial composition of the MFGs may be altered to similar degrees. Additionally, the homogenization of milk is often accompanied by thermal processing, which could further alter the MFG interfacial composition because of the interaction between denatured whey proteins and the proteins on the surface of the MFGs. However, there is relatively little scientific information on the impacts of

homogenization on the MFG composition of sheep milk compared with that of cow milk. The interfacial compositions of the MFGs before and after either homogenization or a combination of homogenization and heat treatment of sheep milk and the milks from other species should be compared; this will help to better understand how the homogenization-induced changes in the MFG interfacial composition (such as polar lipids, glycoproteins, and caseins) affect the digestion behavior of the fat within the gastrointestinal tract.

Teng et al. (2020) compared the fatty acid and TAG profiles of sheep milk and cow milk before and after homogenization and showed that there were no significant ($P > 0.05$) differences in the fatty acid regiodistribution between the raw milks and the homogenized milks from sheep and cow. This indicates that the homogenization of milk does not alter the TAG and fatty acid profiles in sheep milk and cow milk; the differences in the TAG and fatty acid profiles between sheep milk and cow milk remained unchanged before and after homogenization.

In general, the differences in the heat-induced protein interactions and the homogenized MFGs in sheep milk are considered to play important roles in their digestion behavior and nutrient delivery during gastrointestinal digestion; this is discussed in the following sections on the digestion of sheep milk.

2.5. Digestion of sheep milk

Milk as an important source of protein and fat for humans has been widely studied using both *in vitro* and *in vivo* digestion. The milk composition and the processing treatment have significant impacts on the digestion behavior (Boland & Singh, 2020). Sheep milk and cow milk differ in composition (Table 2-1), potentially affecting their digestion behaviors. The digestion behavior of cow milk has been extensively reviewed in previous studies (Li et al., 2021; Van Lieshout et al., 2020; Ye, 2021; Ye et al., 2020).

2.5.1. Curd formation and structure of sheep milk under gastric conditions

Milk contains mainly two types of protein: caseins and whey proteins. These proteins have different digestion behaviors, depending on their structure. Caseins have a loose, highly flexible, and disordered conformation, leading to more natural exposure to hydrolysis by pepsin during gastric digestion. Whey proteins have a globular and well-defined three-dimensional structure, resulting in extreme resistance to proteolysis. However, caseins are known as "slow proteins" in releasing amino acids into the plasma even though caseins are more sensitive to hydrolysis by pepsin. Unlike caseins, whey proteins rapidly increase the amino acid content in the plasma (Boirie et al., 1997). The difference can be attributed to the formation of casein curds in the stomach because of the combined effects of acid secretion and digestive enzymes. The casein curds can remain in the stomach for a longer time than the whey proteins, which remain soluble and are rapidly released into the small intestine (Ye, 2021).

Recent studies have confirmed that, similar to cow milk, the formation of sheep milk curds in the stomach is due to the hydrolysis by pepsin of the Phe105–Met106 bond of κ-casein to give para-κ-casein and caseinomacropeptide and not to the effect of low pH on the stability of the casein micelles because the curds are formed at pH higher than 6.0 (Z. Pan et al., 2021; Roy et al., 2022; Roy et al., 2021a). The formation of sheep milk curds under *in vivo* conditions has also been reported by Roy et al. (2022); they used a piglet model to investigate the digestion behaviors of sheep, goat, and cow milks and reported that the three types of milk formed a curd within 30 min of digestion (pH ~ 5.9) and that all curds became more compact, tighter, and smaller in size as the digestion progressed. These studies also showed that the formation of the curd in raw sheep milk and pasteurized sheep milk did not involve whey proteins, and that the whey proteins could be emptied out quickly from the stomach during digestion (Z. Pan et al., 2021; Roy et al., 2022; Roy et al., 2021a).

Roy et al. (2022) also reported differences in the physical properties of the curds formed from raw sheep milk and raw cow milk. In the early stages of digestion, the complex modulus (G*) values and the protein networks (observed in transmission electron micrographs) of the curds were similar between raw sheep milk and raw cow milk. As the digestion progressed, the curd was broken down because of protein hydrolysis by pepsin and the gastric contraction forces, leading to significant differences in the G* values and the curd structures. Compared with the raw cow milk curds, the raw sheep milk curds showed a lower extent of fusion and compaction of the protein network as the digestion time increased and had a lower G* value after 150 min of digestion, suggesting that the curds formed from raw sheep milk were softer than those formed from raw cow milk. The structural differences in the gastric curds were assumed to be due to the different α_{S1}-casein contents (sheep < cow, Table 2-1) along with the different casein micelle sizes (sheep > cow, Table 2-1).

Previous research showed that the fat content has an impact on curd formation during gastric digestion. Roy et al. (2021b) compared the *in vitro* gastric digestions of cow, goat, and sheep skim milks and found that sheep skim milk had firmer curds and a denser clustered protein network towards the end of digestion than cow and goat skim milks because the sheep skim milk curds had a higher total solids content and a lower moisture content. Similar results were also reported by Roy et al. (2020b), who showed that the gel formed from sheep skim milk after being treated with glucono-δ-lactone and porcine pepsin had a higher storage modulus and a less open protein network compared with goat and cow skim milks. These results differ from those reported for raw milk, which showed that the curd formed from raw sheep whole milk was softer than those formed from raw goat and cow whole milks at the end of gastric digestion. This indicates that the fat content has

different impacts on the curd structure for the milks from different species during gastric digestion.

Not only protein and fat, but also different processing treatments (such as pasteurization, intense heat treatment, and homogenization) may affect the formation and structure of the curds during gastric digestion and, in turn, the delivery of proteins and fat to the small intestine and their subsequent absorption. Milk processing has considerable impacts on the structure of proteins, MFGs, and MFGMs, such as the heat-induced association between denatured whey protein and casein micelles, homogenization-induced changes in the MFGM composition, and disruption of the MFGs into smaller droplets, which in turn lead to different digestion behaviors and bioavailabilities of the milk protein and fat (Gallier et al., 2012a; Gallier et al., 2013; Li et al., 2021). For instance, the curds formed from pasteurized and intensely heated sheep, goat, and cow milks appeared visually to be more fragmented and looser than those formed from their raw milk counterparts (Figure 2-2) (Li, Pan, et al., 2022; Li, Ye, et al., 2022; Z. Pan et al., 2021; Roy et al., 2021a). This may have been due to the heat-induced covalent interactions between the denatured whey proteins and the κ-caseins at the surface of the casein micelles in the milk during thermal processing, resulting in the involvement of whey proteins in the formation of curds during the gastric digestion of heated milk and thus hindering the formation of a strong protein network in the pasteurized milk curd compared with the raw milk curd (Ye, Liu, et al., 2019).

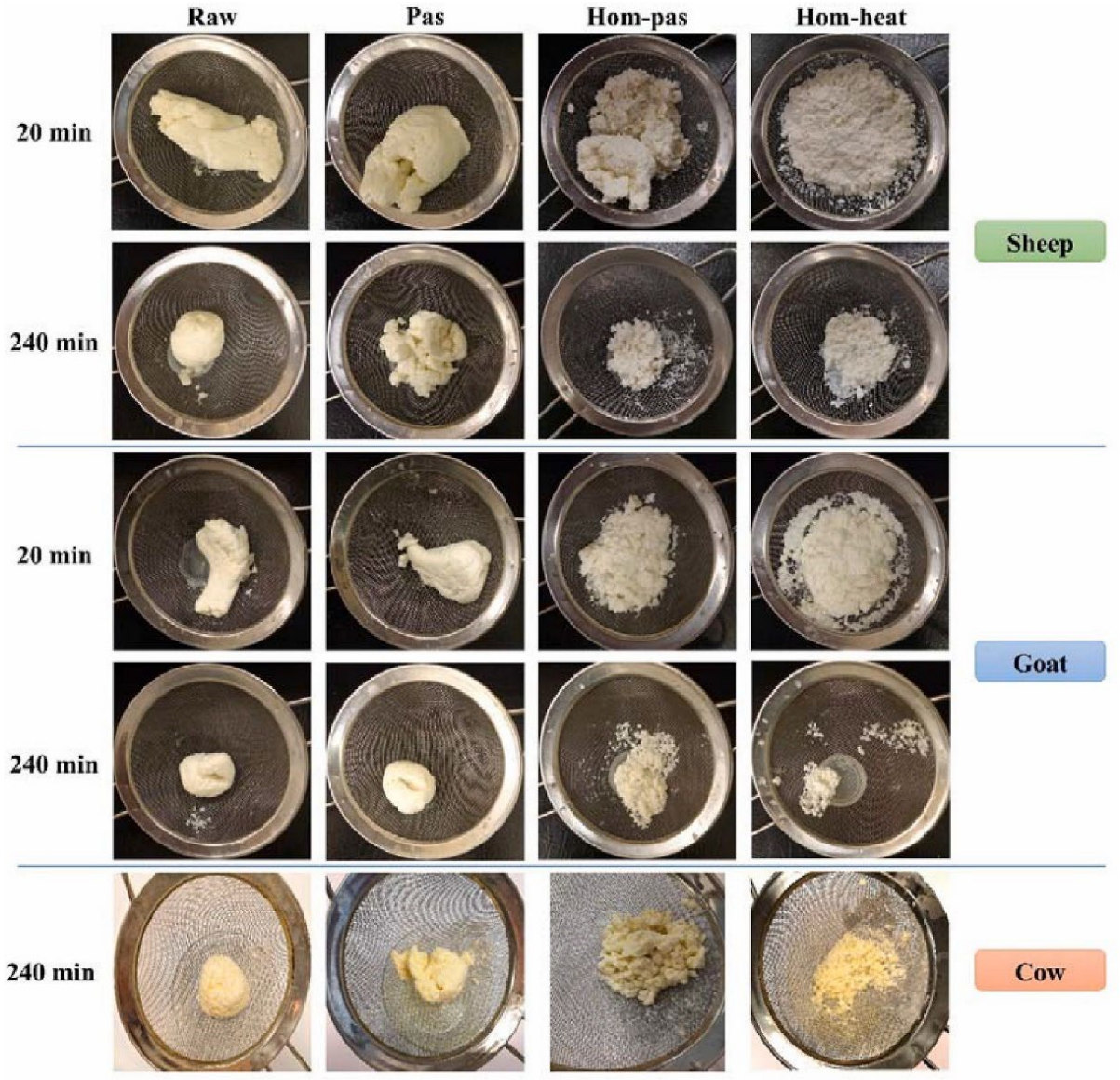

Figure 2-2. Appearance of the curds formed during the gastric digestion of sheep, goat and cow milks. Adapted from Li, Pan, et al. (2022).

Heat treatment is one of the most commonly used processes in dairy manufacturing. As mentioned above, the protein structures of the milks from different species can be differently affected by heat treatment, leading to different extents of structural changes in the proteins (such as casein micelle size and structure). Li, Pan, et al. (2022) studied the structural properties of the curds from sheep, goat, and cow milks and showed that the curd

structure of pasteurized sheep milk was more fragmented at the later stages of gastric digestion than those of pasteurized goat and cow milks (Figure 2-2). This may have been due to the greater denaturation of the whey proteins in sheep milk than in goat and cow milks during pasteurization (Dumitraşcu et al., 2013; Li, Delger, et al., 2022; Pan et al., 2022a), thereby hindering the formation of a strong protein network to a greater extent. Moreover, the denatured whey proteins are more susceptible to hydrolysis by pepsin (Wang et al., 2018). Consequently, the greater extent of whey protein denaturation may have contributed to the faster hydrolysis of the whey proteins and thus the faster disintegration of the sheep milk curds. Li, Pan, et al. (2022) also reported the impacts of intense heat treatment (95 °C/5 min) on the curd structures of homogenized sheep, goat, and cow milks; the homogenized and intensely heated milk curds from all species consisted of finer grains than the homogenized and pasteurized milk curds but the curd structures showed few differences among the species (Figure 2-2).

It should be noted that the whey proteins of the milks of all three species could have been fully denatured under the intense heat treatment (95 °C/5 min) (Dumitraşcu et al., 2013; Oldfield et al., 2005; Pan et al., 2022a). Therefore, the greater extent of whey protein denaturation in intensely heated milk could hinder the formation of a close-knit protein network to a greater degree compared with pasteurized milk, resulting in a looser and finer curd structure during gastric digestion. Additionally, the extent of whey protein denaturation could be similar among the milks from all species under the intense heat treatment, probably affecting the curd formation to similar extents and thus reducing the differences in the curd structures among the milks from different species. This can be further confirmed by testing the extent of denaturation of the whey proteins in the milks from different species under intense heat treatment (95 °C/5 min).

Homogenization of milk reduces the MFG size and the newly formed smaller MFGs are stabilized by the milk serum proteins that are adsorbed at the surface of the MFGs. The changes have been reported to influence the interactions of proteins during gastric digestion and therefore to affect the curd structure during *in vitro* gastric digestion (Mulet-Cabero et al., 2019; Ye et al., 2017). Z. Pan et al. (2021) and Li, Pan, et al. (2022) compared the curd structures of pasteurized and homogenized-pasteurized sheep and cow milks and found that the curds from the homogenized-pasteurized sheep and cow milks appeared to be more fragmented and looser than those from the pasteurized sheep and cow milks throughout the gastric digestion (Figure 2-2). Additionally, Li, Pan, et al. (2022) showed that homogenized and pasteurized sheep milk curds had a "couscous-like" grainy structure, which was different from the larger grains and large continuous structures of homogenized and pasteurized cow milk curds (Figure 2-2). Compared with the pasteurized milks, the combination of homogenization and pasteurization affected the macrostructures of the curds formed from the milks of all species to different extents in the order: goat > sheep > cow. These authors suggested that the greater impacts of homogenization on the macrostructure and the consistency of the sheep and goat milk curds may have lessened the apparent impact of heat intensity to some extent. A further study on the gastric digestion of whole or skim milk treated with different heat intensities and without homogenization should be carried out.

2.5.2. Protein digestion

A few studies have investigated the protein or fat digestion of raw sheep milk. Roy et al. (2022) determined the protein profile after the gastric digestion of raw sheep, goat, and cow milks in piglets and found that the changes in the protein profiles of the curds from raw sheep and cow milks were similar. As the digestion progressed, the caseins of both raw cow milk and raw sheep milk were gradually hydrolyzed, leading to increased peptides in

the emptied gastric digesta. However, there were differences in the gastric emptying rate of proteins from the stomach to the small intestine between the raw sheep and cow milks. Roy et al. (2022) showed that the protein was emptied out faster for raw sheep milk than for raw cow milk, which could be explained by the relatively open microstructure and softer consistency of the raw sheep milk curds.

Roy et al. (2021b) also compared the protein profiles of sheep, goat, and cow skim milks during gastric digestion and found that the pattern of protein hydrolysis of the raw sheep skim milk curds was similar to those of the raw cow and goat skim milk curds although they generated some peptide bands at different molecular weights. The band intensities for the whey proteins increased markedly in pasteurized skim milk curds compared with raw skim milk curds, especially for goat and sheep milks. The protein profiles of the pasteurized skim milk curds from all species showed a changing trend that was similar to that of their respective raw skim milk curds; the amount of peptides in the pasteurized sheep skim milk curds was lower than that in the pasteurized goat skim milk curds but similar to that in the pasteurized cow skim milk curds. For the gastric digesta samples, the protein hydrolysis profiles of the raw and pasteurized sheep and goat skim milks were similar to those of the raw and pasteurized cow skim milks.

Tagliazucchi et al. (2018) studied the biological activities and the products of the protein digestion of skim milks from different species and found that sheep milk generated more free amino groups than cow milk during gastric digestion and significantly more peptides than the milks from cow, camel, and goat, indicating that the protein hydrolysis rates were higher in sheep milk than in cow milk. The higher degree of hydrolysis of the sheep milk proteins was probably due to the higher susceptibility of sheep milk proteins to pepsin (El-Zahar et al., 2005; Roy et al., 2020b). El-Zahar et al. (2005) compared the hydrolysis of β-LG by pepsin between sheep milk and cow milk and reported that sheep

milk β-LG was hydrolyzed faster because of its slightly different tertiary structure and higher surface hydrophobicity. In contrast to the studies mentioned above, Mros et al. (2017) found no differences in protein hydrolysis between sheep milk and cow milk after a simulated *in vitro* digestion using pepsin and pancreatin.

Z. Pan et al. (2021) studied the impact of homogenization and heat treatment on the *in vitro* gastric digestion of sheep milk using a human gastric simulator and reported that the homogenized sheep milk had faster hydrolysis of the proteins by pepsin and generated more peptides than the unhomogenized sheep milk, which could have been caused by the looser and crumbly structure of the curds, allowing pepsin to diffuse into the curds rapidly and to hydrolyze the protein. Intense heat treatment (95 °C/5 min) of homogenized sheep milk also resulted in greater hydrolysis of the proteins compared with homogenized and pasteurized sheep milk, which was attributed to the looser and finer structure of the curds formed from the homogenized and intensely heated sheep milk. When the digestion time was greater than 60 min, the gastric emptying rate of proteins and peptides followed the order: homogenized and intensely heated > homogenized and pasteurized > pasteurized > raw sheep milk.

To date, no studies on the impact of different heating or processing conditions on the small intestinal digestion of sheep milk have been reported in the literature. It should be noted that, as the hydrolysis and emptying rates of the proteins in differently processed sheep milks showed differences in the gastric phase, they are likely to behave differently in the small intestinal phase, possibly affecting the absorption of the proteins.

2.5.3. Lipid digestion

2.5.3.1. Release of MFGs in the stomach

The MFGs are involved in the formation of the curd during the gastric digestion of whole milk (Ye et al., 2016b); they are evenly distributed in the protein matrix and are released from the curd structure when the curd is broken down (Ye et al., 2016a, 2017). The release of fat from the stomach to the small intestine can be affected by the structure and the breakdown of the curd during gastric digestion. On the other hand, the presence of fat globules with different structures and compositions can affect the curd structure and breakdown. For instance, Li, Pan, et al. (2022) used cryo-scanning electron microscopy to investigate the structural changes of the curds during the *in vitro* gastric digestions of sheep and goat milks and showed that the MFGs of raw sheep and goat milks could be involved in the curd only via entrapment as there was no association between the MFGM and the protein matrix. In contrast, when the milk was homogenized, the fat globules became smaller and were stabilized by caseins and whey proteins, consequently participating in the formation of the curd through either fusing into the protein network or presenting in the pores. This significantly decreased the firmness of the curds. In homogenized and intensely heated (95 °C/5 min) sheep milk, more denatured whey proteins bound on to the surface of the MFGs, resulting in slightly larger pores within the protein matrix and a decrease in firmness compared with homogenized and pasteurized sheep milk. This indicates that the participation of different-sized and different-structured fat in the formation of the curd changed the structure of the protein network, which may, in turn, have affected the digestion rate of the fat itself.

Roy et al. (2021a) investigated the impact of gastric coagulation on the release of MFGs from the milks of different species and reported that fat was proportionally released when the curds of raw and pasteurized whole sheep, goat, and cow milks were broken down

during gastric digestion. For all types of milk, there was a linear correlation between the release of MFGs from the curd and disintegration of the curd during gastric digestion, and the slope of the regression line was close to 1. In another gastric digestion study, Z. Pan et al. (2021) reported a similar result, i.e., that the slope of the regression line for the raw and pasteurized sheep milks was close to 1, suggesting a strong correlation between fat release and disintegration of the protein curd. These authors found a nonlinear correlation between the release of fat and the disintegration of the protein curds for homogenized (including homogenized and intensely heated, and homogenized and pasteurized) sheep milk, indicating that the release rate of fat and protein from the homogenized sheep milk curd was different. Homogenized sheep milk had a faster rate of protein hydrolysis as well as a faster release of fat during gastric digestion, compared with unhomogenized (raw and pasteurized) sheep milk (Z. Pan et al., 2021). These differences between homogenized and unhomogenized sheep milk were due to the crumbly and more fragmented protein aggregates formed from the homogenized sheep milk compared with the unhomogenized sheep milk (Z. Pan et al., 2021).

The *in vitro* digestion of sheep milk resulted in coalescence of MFGs that were entrapped within the protein matrix as well as present in the liquid phase of the gastric chyme (Z. Pan et al., 2021), which could be attributed to the hydrolysis of the proteins surrounding the MFGs, resulting in destabilization of the MFGs and coalescence (Roy et al., 2020a). Additionally, flocculation of the MFGs was also observed in the gastric digesta of homogenized sheep milk, in which the MFGs were incorporated into the protein/peptide particles (Z. Pan et al., 2021). It has been reported that flocculation of MFGs can occur via protein–peptide or peptide–peptide interactions as the peptides resulting from the hydrolysis of proteins by pepsin on the surface of MFGs cannot provide sufficient electrostatic repulsion and steric barriers (Ye et al., 2011). Consequently, the difference in

the structures of the emptied MFGs among the differently processed sheep milks potentially influences the rate of lipid digestion of the MFGs in the small intestine.

2.5.3.2. Gastrointestinal digestion of fat

Little information on the gastrointestinal digestion of sheep milk fat is available. Lipid digestion occurs primarily in the small intestine in adults, but also to a lesser extent (accounting for 5–30%) in the stomach (Golding & Wooster, 2010). Lipid digestion in the stomach is facilitated by gastric lipase, which is an acid-stable lipase with a broad pH activity range from 3 to 7 (Golding & Wooster, 2010). The relative importance of gastric lipolysis has been recognized only recently as it may facilitate the lipolysis by intestinal lipase, particularly for infants who have developmental pancreatic insufficiency (Fave et al., 2004).

Santillo et al. (2018) compared the fatty acid profiles of the milks from different species after *in vitro* gastrointestinal digestion without gastric lipase and reported that the total amount of free fatty acids in sheep milk was lower than that in human milk, similar to those in cow, goat, and donkey milks, and higher than that in formula milk. The differences observed in the free fatty acid profiles of the digested milks from different species could be due to several factors, such as the interaction between the milk substrate and the digestion process (presence of endogenous enzymes in the milk), the different structures of the MFGs, and the binding site of the fatty acids on TAGs. Teng et al. (2020) compared the gastric digestibilities of sheep and cow milk fats before and after homogenization and found that the homogenization of sheep and cow milks increased the gastric digestion rates of all TAGs by 50% compared with their respective raw milk samples, although fatty acid composition, TAG composition, and fatty acid regiodistribution showed few differences between the raw and homogenized sheep and cow milks. Similar results have also been reported for human milk; homogenized human milk favored lipolysis *in vitro* by releasing

~ 1.5 fold the total amount of fatty acids compared with raw human milk. Homogenization of sheep milk increased the degree of TAG lipolysis and the rate of lipid digestion but did not show impacts on the lipase regiospecificity towards TAGs. This is in agreement with the results reported for cow milk, which showed that, for the same amount of lipids, smaller droplets led to a faster rate of digestion compared with larger droplets (Gallier et al., 2017; Zhao et al., 2019). This can be explained by the smaller MFGs in homogenized milk, which provide a larger surface area and therefore a greater number of binding sites for lipases (Gallier et al., 2017; Golding & Wooster, 2010). Additionally, sheep milk had a faster TAG digestion rate than cow milk in both the raw milk (16.1 vs. 12.4%) and the homogenized milk (24.1% vs. 20.3), which has been attributed to the higher proportions of medium-chain fatty acids esterified on the *sn*-1 or *sn*-3 positions of sheep milk TAGs than cow milk TAGs before and after homogenization (Teng et al., 2020). Similar results were also reported by Saviard et al. (2022), who found that sheep milk had a higher degree of lipolysis and greater fatty acid release than cow milk.

Other studies have shown that not only the particle size but also the structural changes (such as reduced polar lipids and increased protein) at the interfacial layer of MFGs after processing treatments can affect the digestion of milk fat (Gallier et al., 2017; Gallier et al., 2013; Garcia et al., 2014). However, to date, no studies on the impact of different heat intensities or combinations of heat treatment with homogenization on the lipid digestion of sheep milk have been reported in the literature. Therefore, studies investigating the effects of interface changes during heat treatment or a combination of heat treatment with homogenization on lipid digestion are required to better understand this process.

2.6. Conclusions and Perspectives

This review has summarized the effects of thermal processing and homogenization on the physicochemical properties of the proteins and fat in sheep milk. These treatments can be applied as tools to manipulate the gastrointestinal digestion of protein and fat. Sheep milk is an excellent source of nutrients because of its high total solids content. However, the low heat stability of sheep milk limits its production in liquid form and thus its availability around the world. Modifications of the protein ratios, ionic equilibria, colloidal micellar structures, and dynamic dissociation of the casein micelles during heating play important roles in stabilizing sheep milk during heat treatment. More research on the effects of the serum environment (such as ionic calcium and serum proteins) and the protein compositions on the heat stability of sheep milk is needed. Furthermore, the protein interactions under intense heat treatment and the impact of heat intensity and homogenization on the small intestinal digestion of sheep milk have been little studied. A study of the small intestinal digestion of sheep milk after heat treatment and (or) homogenization could help to better understand how the changes that are induced by thermal processing or a combination of thermal processing with homogenization affect the gastrointestinal digestion of sheep milk. Furthermore, sheep milk has the potential to underpin a new and more effective functional milk for infant foods; health-enhancing substances may be added into the model infant formula to promote its nutritional value. Although some infant formulas made from sheep milk are available on the market, no studies on their digestion behavior under infant gastrointestinal conditions have been reported. There is also a need for more translational research and clinical trials to confirm the effect of dairy processing on the gastrointestinal outcomes of adult humans as, to date, most evidence is from *in vitro* or animal studies only.

Chapter 3: Statement of Contribution – Doctorate with Publications/Manuscripts

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Zheng Pan
Name/title of Primary Supervisor:	Professor Aiqian Ye
In which chapter is the manuscript /published work:	Chapter 3

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Chapter 3. Kinetics of heat-induced interactions among whey proteins and casein micelles in sheep skim milk and aggregation of the casein micelles

The contents of this chapter have been published in Journal of Dairy Science (open access):

Pan, Z., Ye, A., Dave, A., Fraser, K., & Singh, H. (2022). Kinetics of heat-induced interactions among whey proteins and casein micelles in sheep skim milk and aggregation of the casein micelles. Journal of Dairy Science, 105(5), 3871-3882. doi:10.3168/jds.2021-21444

3.1. Abstract

The interactions among the proteins in sheep skim milk (SSM) during heat treatments (67.5–90°C for 0.5–30 min) were characterized by the kinetics of the denaturation of the whey proteins and of the association of the denatured whey proteins with casein micelles, and changes in the size and structure of casein micelles. The relationship between the size of the casein micelles and the association of whey proteins with the casein micelles is discussed. The level of denaturation and association with the casein micelles for β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) increased with increasing heating temperature and time; the rates of denaturation and association with the casein micelles were markedly higher for β-Lg than for α-La in the temperature range 80–90°C; the Arrhenius critical temperature was 80°C for the denaturation of both β-Lg and α-La. The casein micelle size increased by 7–120 nm, depending on the heating temperature and the holding time. For instance, the micelle size (about 293 nm) of SSM heated at 90°C for 30 min increased by about 70% compared with that (about 174.6 nm) of unheated SSM. The casein micelle size increased slowly by a maximum of about 65 nm until the level of

association of the denatured whey proteins with casein micelles reached 95%, and then increased markedly by a maximum of about 120 nm when the association level was greater than about 95%. The marked increases in casein micelle size in heated SSM were due to aggregation of the casein micelles. Aggregation of the casein micelles and association of whey protein with the micelles occurred simultaneously in SSM during heating.

3.2. Introduction

Milk is commonly exposed to commercial thermal processing, which induces some physicochemical changes, such as whey protein denaturation and an increase in casein micelle size (Dalgleish & Corredig, 2012; Raynal-Ljutovac et al., 2007). Most of the denatured whey proteins (especially β-Lg) bind covalently to the casein micelles via thiol-disulfide bond exchange with κ-casein (Donato & Dalgleish, 2006; Van Hooydonk et al., 1987). In addition, the presence of β-Lg during heating helps α-La to connect with κ-casein; α-La cannot associate directly with κ-casein because of the lack of the thiol group (Baer et al., 1976; Elfagm & Wheelock, 1978; Wijayanti et al., 2019). These interactions between whey proteins and caseins/casein micelles lead to structural changes in the casein micelles and an increase in the size of the casein micelles (Anema & Li, 2003a). However, these changes may vary to some degree in heated milks from different species because of different compositions and structures of the proteins. Studies have shown that sheep milk has lower heat stability than cow milk (Park et al., 2007; Raynal-Ljutovac et al., 2007). In comparison with cow milk, sheep milk showed a greater extent of whey protein denaturation after heating at 80–90 °C for 0.5–10 min (Law, 1995; Raynal & Remeuf, 1998).

Previous studies suggested that the casein micelle size increased from its initial value by 25–75% in sheep milk upon heating at 75–90 °C for 0.5–10 min but remained unchanged in cow milk (Raynal & Remeuf, 1998). The different effects of heating on the

casein micelle size between sheep milk and cow milk might be caused by the differences in protein content and the extents of dissociation or aggregation of the casein micelles (Raynal & Remeuf, 1998). Previous studies in cow milk have shown that heating can induce dissociation of caseins from casein micelles, leading to an increase in the proportion of small-sized protein particles (Anema & Li, 2000; Singh & Creamer, 1991b); the extent of casein dissociation may be different for milks from different species (Raynal & Remeuf, 1998). The casein micelles in sheep milk are more mineralized micelles than cow milk; this is responsible for the greater extent of micelle aggregation at high temperature (Muir et al., 1993; Van Hooydonk et al., 1987). In addition, sheep milk has higher protein content than cow milk, which probably increases the micelle–micelle interactions during heating and thus leads to aggregation of the casein micelles (Raynal & Remeuf, 1998). However, no studies to date demonstrate whether the changes in casein micelle size in heated sheep milk are caused exclusively by the denatured whey proteins associating with the casein micelles or are also caused by the partial aggregation of the casein micelles that accompanies this association behavior.

Many studies on the kinetics of the whey protein denaturation of cow milk over wide ranges of heating temperature and time have been conducted (Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Oldfield et al., 2005; Oldfield, Singh, Taylor, et al., 1998), and several models to describe the mechanisms of whey protein denaturation and aggregation in cow milk have been proposed. For instance, Oldfield, Singh, Taylor, et al. (1998) investigated the kinetics of denaturation and aggregation of the whey proteins in cow skim milk over a wide range of temperature–time (70–130°C for 3–1,800 s); they found that the aggregation of β-Lg involved the dissociation of the dimer, unfolding, and the formation of intermolecular disulfide linkages, whereas the aggregation of α-La appeared to involve hydrophobic interactions. These kinetic models provide useful

information for understanding the mechanisms of the denaturation of the whey proteins and the association of the denatured whey proteins with the casein micelles in heated milk. To date, little information on the kinetics of the denaturation of the whey proteins in sheep milk is available and no studies on the association of denatured whey proteins with the casein micelles in sheep milk have been reported.

The objective of this study was to investigate the kinetics of the irreversible denaturation of β-Lg and α-La and the association of denatured β-Lg and α-La with the casein micelles in sheep skim milk (SSM) at natural pH in the temperature range 67.5–90°C for 0.5–30 min, and to understand how interactions between whey proteins and casein micelles affect the casein micelle size. The structural changes in the casein micelles after heat treatment were also determined using transmission electron microscopy.

3.3. Materials and methods

3.3.1. Milk supply and heat treatment

Pooled mid-lactation sheep milk (pH 6.56 ± 0.01) was obtained from Neer Enterprises Limited (Carterton, New Zealand). The milking breed for sheep was predominantly East Friesian. The main composition of 3 batches of sheep milk was analyzed using a MilkoScan FT1 (FOSS, Hillerød, Denmark) and by SDS-PAGE as described by Ye et al. (2016a), and is shown in Table 3-1. A small amount of sodium azide (0.01%) was added to the unheated milk as a preservative. The whole sheep milk was skimmed at 3,000 × *g* for 15 min at 25 °C using a bench centrifuge (Heraeus Multifuge X3R; Thermo Fisher Scientific Inc., MA). The skimmed sheep milk (6 mL) was transferred into 10 mL sealable glass tubes and the well-sealed tubes were then heated at a range of temperatures (67.5–90 °C) and times (0.5–30 min) with continuous rocking in a thermostatically controlled water bath. After heat treatment, the milk samples were

immediately immersed in cold running water for cooling to room temperature. The heated milk samples were kept at room temperature for 6 h before further analyzes. The selection of temperature and time was based on our preliminary results of denaturation extents of both β-Lg and α-La in SSM heated at 60–100 °C for 0.5–60 min, and the detectable denaturation of β-Lg and α-La in SSM was found at temperature greater than 67.5 °C and holding time greater than 0.5 min. Temperature greater than 90 °C would result in a too fast denaturation of whey proteins to detect the changes in denaturation of whey proteins in a reasonable holding time.

Table 3-1 Composition of raw sheep milk.

Parameter	Sheep milk[1]
Total solids (g·100 g^{-1})	17.7 ± 0.3
Fat (g·100 g^{-1})	6.0 ± 0.2
Ash (g·100 g^{-1})	1.0 ± 0.1
Protein (g·100 g^{-1})	6.1 ± 0.2
Casein (g·100 g^{-1})	4.8 ± 0.2
α_{S2}-casein (%)[2]	19.2
α_{S1}-casein (%)[2]	7.2
β-casein (%)[2]	64.6
κ-casein (%)[2]	9.0
Whey protein (g·100 g^{-1})	1.3 ± 0.0
α-La (%)[3]	26.1
β-Lg (%)[3]	62.3

[1]*Values are reported as mean ± SD from 3 batches of sheep milk.*

[2]*Percentage of total caseins.*

[3]*Percentage of total whey proteins.*

3.3.2. Determination of denaturation of whey proteins

The native whey proteins were obtained by removing the caseins and denatured whey proteins from the skim milk samples using acetic acid precipitation as described by Vasbinder et al. (2003). A 0.4 mL subsample of SSM was mixed well with 0.8 mL of MilliQ water and 0.1 mL of acetic acid (10%) in an Eppendorf tube (2 mL) and left to stand for 10 min. Then, 0.6 mL of MilliQ water and 0.1 mL of sodium acetate (1 M) were added into the solution, which was mixed again. The mixed solution reached an equilibrium pH of 4.6 and was kept in an ambient environment for 1 h. The caseins and denatured whey proteins were precipitated after centrifugation at 3,000 × *g* for 5 min. The amount of native whey proteins in the supernatant was determined by HPLC. The percentage of denatured whey proteins was calculated by subtracting the percentage of native whey proteins in heated samples from that in unheated samples.

3.3.3. Determination of denatured whey proteins associated with casein micelles

To determine the association of the denatured whey proteins with the casein micelles, the unheated and heated SSMs were ultracentrifuged at 63,000 × *g* for 1 h at 20 °C using a Sorval WX 80+ Ultracentrifuge (Thermo Fisher Scientific Inc., MA) to remove all denatured whey proteins that had associated with casein micelles and all casein micelles. The serum-phase whey proteins were defined as those whey proteins that did not sediment after the ultracentrifugation. The resultant supernatant (serum phase) containing nonsedimentable proteins was carefully collected and analyzed by HPLC. The quantity of each protein in the supernatant of the heated SSM was presented as a percentage of that in the supernatant of the unheated SSM. The level of denatured whey proteins that had associated with casein micelles was calculated by subtracting the level of serum-phase whey proteins in heated samples from that in unheated samples. This centrifugal method

had been proved to be the minimum centrifugation speed required to effectively sediment the whey proteins associated with casein micelles and to reduce the possibility of centrifuging down soluble whey protein aggregates (Anema & Li, 2003a).

3.3.4. Analysis of protein composition

Milks and the supernatants obtained from acid-precipitated and ultracentrifuged milk samples were analyzed by reversed-phase HPLC using a reversed-phase C18 column (Aeris Widepore 3.6 μm XB-C18 RP; Phenomenex, Torrance, CA) to determine the protein composition, as described by Bobe et al. (1998). The quantity of native whey proteins (β-Lg and α-La) in heated SSM was calculated by comparing the relative peak areas of the heated SSM with the original unheated SSM. The quantity of whey proteins in the ultracentrifugal supernatants was determined by comparing the relative peak areas of the supernatant fractions of the heated SSM with the original unheated SSM. All peak areas of these chromatograms were determined using peak integration algorithm LabSolutions software (Shimadzu Corporation, Kyoto, Japan).

3.3.5. Kinetic analysis for whey protein denaturation

The order of thermal denaturation of the whey proteins was determined using a general rate equation:

$$-\frac{dC_t}{dt} = k_n C_t^{\,n} \qquad \textbf{Equation 3-1}$$

For $n \neq 1$, this equation yields

$$\left(\frac{C_t}{C_0}\right)^{1-n} = 1 + (n-1)\, k_n C_0^{\,n-1} t \qquad \textbf{Equation 3-2}$$

When $n = 1$, this equation yields

$$\ln\left(\frac{C_t}{C_0}\right) = -k_n C_0^{n-1} t \qquad \textbf{Equation 3-3}$$

where n is reaction order, C_0 ($g \cdot L^{-1}$) is concentration of native protein before heat treatment, C_t ($g \cdot L^{-1}$) is concentration of native protein at time t (s), and k_n ($g^{1-n} \cdot L^{n-1} \cdot s^{-1}$) is rate constant. Equations 3-2 and 3-3 were used to calculate the reaction order n at different temperatures for different whey proteins. The rate constant k_n was calculated when $\left(\frac{C_t}{C_0}\right)^{1-n}$ was plotted against t.

The temperature dependence of the rate constant k_n can be defined using the Arrhenius equation (Equation 3-4):

$$\ln(k_n) = \ln(k_{n,0}) - \frac{E_a}{RT} \qquad \textbf{Equation 3-4}$$

where $k_{n,0}$ ($g^{1-n} \cdot L^{n-1} \cdot s^{-1}$) is frequency factor, E_a ($J \cdot mol^{-1}$) is activation energy, R (8.314 $J \cdot mol^{-1} \cdot K^{-1}$) is universal gas constant, and T (K) is absolute temperature. The activation energy E_a was obtained as the logarithm of the rate constant ($\ln k_n$) resulting from Equation 3-2 or 3-3 versus the reciprocal of the absolute temperature.

3.3.6. Kinetic analysis for association of whey proteins with casein micelles.

The reaction kinetics of β-Lg and α-La associating with the casein micelles were fitted using a site-filling model (Equations 3-5 and 3-6) (Sharma & Dalgleish, 1994). Presumably, the reactive sites available for the association of β-Lg and α-La are κ-casein and α_{S2}-casein.

When $n \neq 1$,

$$\left(\frac{C_{max} - C_t}{C_{max}}\right)^{1-n} = 1 + (n-1) k_n C_{max}^{n-1} t \qquad \textbf{Equation 3-5}$$

where C_{max} ($g \cdot L^{-1}$) is the maximum amount of β-Lg or α-La, which is the total concentration of β-Lg or α-La, that could associate with the casein micelles, C_t ($g \cdot L^{-1}$) is the amount of β-Lg or α-La associated with the casein micelles at time t (s), and k_n ($g^{1-n} \cdot L^{n-1} \cdot s^{-1}$) is the apparent reaction rate constant for β-Lg or α-La associating with the casein micelles.

When $n = 1$,

$$\ln\left(\frac{C_{max} - C_t}{C_{max}}\right) = -k_1 t \qquad \textbf{Equation 3-6}$$

where k_1 (s^{-1}) is the first-order reaction rate constant.

3.3.7. Determination of casein micelle diameter

The method for casein micelle diameter measurement was based on the method described by Anema (2018b). Briefly, the unheated and heated SSMs were diluted 1:50 in Ca–imidazole buffer (20 mM imidazole, 5 mM $CaCl_2$, and 30 mM NaCl, pH 7.0) and measured by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK).

3.3.8. Microstructural changes in casein micelles

The microstructural changes in SSM after heat treatment were observed using transmission electron microscopy, as has been described previously by Mittal et al. (2015). Briefly, milk samples were mixed in tubes with warm melted (30–40°C) 3% low-temperature-gelling agarose at a ratio of 1:1. The mixture was fixed primarily with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 5°C for 24 h. Secondary fixation (overnight at room temperature) was done in 1% osmium tetroxide cacodylate buffer. The samples were rinsed with cacodylate buffer after each fixation. Dehydration of the samples

was performed in a series of acetone before embedding in resin (Procure 812). Ultrathin sections (100 nm) were obtained using an ultramicrotome (Leica, Vienna, Austria), followed by staining with uranyl acetate (2%, wt/vol) and lead citrate (2.5%, wt/vol). Samples were examined in a transmission electron microscope (FEI Tecnai G2 Biotwin, Hillsboro, OR), operated at 60 kV.

3.3.9. Statistical analysis

All experiments reported were fully triplicated on 3 batches of sheep milk, and the results are presented as the mean ± standard deviation (SD). Although there were some variations between different batches, the same trends and relationships as reported here have been observed for all samples examined to date. The data were plotted using GraphPad Prism 8.4.0 (GraphPad Software, San Diego, CA). Statistical analysis was performed using one-way and two-way ANOVA and Tukey's multiple comparison test at a significance level of $P < 0.05$.

3.4. Results and discussion

3.4.1. Denaturation of whey protein in heated SSM

Figure 3-1 shows the percentages of native β-Lg and native α-La in the supernatants obtained from differently heat-treated SSMs after isoelectric precipitation of the denatured whey proteins and the caseins at pH 4.6. The percentages of native β-Lg and native α-La decreased as both the heating temperature and the holding time increased. At heating temperatures from 67.5 to 75 °C, the percentage of native β-Lg decreased relatively slowly to a maximum of about 24% as the heating time increased up to 30 min (Figure 3-1A). In the high-temperature range (80–90 °C), the percentage of native β-Lg decreased rapidly during the first 5 min of heating and tended to plateau with further heating. After heat treatment at 90 °C for 10 min, the percentage of native β-Lg had decreased to 0.4%,

indicating that almost all of the β-Lg in the SSM had been denatured. A similar pattern was also found for native α-La (Figure 3-1B). The percentage of native α-La decreased slowly in the temperature range 67.5–75 °C, and decreased more rapidly at 80–90 °C. The percentages of native α-La in the SSMs heated for 10 min at 80, 85, and 90 °C were 16.3, 6.4, and 3.8%, respectively. These results indicate that nearly all the whey proteins (β-Lg and α-La) were denatured when heated at 85–90 °C for 10 min.

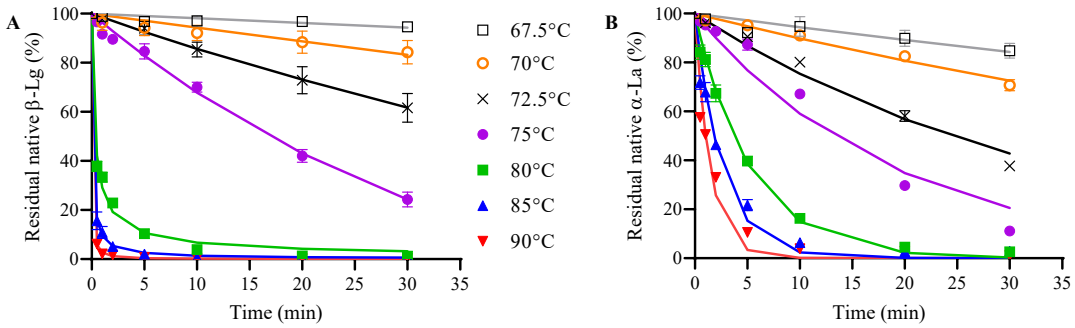

Figure 3-1. Percentage of residual native β-Lg (A) and α-La (B) in sheep skim milk heated at different temperatures (□, 67.5 °C; ○, 70 °C; ×, 72.5 °C; ●, 75 °C; ■, 80 °C; ▲, 85 °C; ▼, 90 °C) for different holding times. The lines represent the predicted denaturation from the model. Each data point represents the mean ± SD of the results from 3 different batches of sheep milk.

3.4.2. Kinetics of denaturation of whey proteins

3.4.2.1. Order of reactions

The reaction order n and the rate constant k_n for the thermal denaturation of β-Lg and α-La were calculated from the best fittings of the experimental data using Equations 3-2 and 3-3 (Table 3-2). For β-Lg, a reaction order of 1.7 was found when the SSM was heated at 67.5–90 °C (Table 3-2), and a linear relationship was obtained when $(C_t/C_0)^{-0.7}$

was plotted against the heating time (data not shown). A linear relationship for α-La in the heated SSM was also observed when ln (C_t/C_0) was plotted against the heating time (data not shown), suggesting that the denaturation of α-La followed first-order reaction kinetics. The reaction orders for the denaturation of β-Lg and α-La that were determined for the heated SSM are essentially in agreement with those from previous research on cow milk, which showed reaction orders of 1.5 and 1 for the denaturation of β-Lg and α-La, respectively determined by linear regression (Anema et al., 2006; Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Kessler & Beyer, 1991) and reaction orders of 1.3 ± 0.3 and 1.0 ± 0.4 for the denaturation of β-Lg and α-La, respectively determined by non-linear regression (Oldfield, Singh, Taylor, et al., 1998). For instance, Anema et al. (2006) investigated the influence of the concentrations of protein, nonprotein-soluble components, and lactose on the irreversible denaturation of β-Lg and α-La in reconstituted skim milk heated at temperatures ranging from 75 to 100 °C, and showed that the reaction orders for the irreversible thermal denaturation of β-Lg and α-La were 1.5 and 1, respectively, in all systems and under all conditions.

3.4.2.2. Rate constants for whey protein denaturation

The rate constants for the denaturation of β-Lg and α-La in SSM heated at different temperatures are shown in Table 3-2. The values of the rate constant k_n for β-Lg denaturation at 67.5–90 °C were between 0.01 and 4.208 10^{-3} $g^{1-n}\cdot L^{n-1}\cdot s^{-1}$, and those for α-La denaturation at 67.5–90 °C were between 0.006 and 0.680 10^{-3} $g^{1-n}\cdot L^{n-1}\cdot s^{-1}$. The calculated k_n values for β-Lg were markedly higher than those for α-La in SSM heated at 80–90 °C but were comparable with those for α-La at 67.5–75 °C, indicating a faster denaturation rate for β-Lg than for α-La at 80–90 °C and comparable denaturation rates for both β-Lg and α-La at 67.5–75 °C in SSM. This finding is in agreement with previous

reports that β-Lg was less heat stable than α-La in cow milk when the heating temperature was higher than 80 °C (Anema, 2020; Oldfield, Singh, & Taylor, 1998).

The Arrhenius plots for the denaturation of β-Lg and α-La were drawn using logarithms of the rate constants (ln k_n) resulting from Equations 3-2 and 3-3 at different temperatures (the reciprocal of the absolute temperature, 1/T) (Figure 3-2). The activation energies E_a and the frequency factor logarithms ln ($k_{n,0}$) for the denaturation of β-Lg and α-La were calculated using the equation resulting from the Arrhenius plot obtained by least-squares linear regression. Linear relationships in two temperature regions were found for both β-Lg and α-La; the activation energies (E_a) and the ln($k_{n,0}$) values were different in each region (Table 3-2). There was a break in the Arrhenius plot at 80 °C for both β-Lg and α-La. These findings are in agreement with previous findings in cow milk reported by Dannenberg and Kessler (1988) and Anema et al. (2006), who showed that abrupt changes in the temperature dependence of the rate constants were observed for the denaturation of both β-Lg and α-La in the temperature range 75–100 °C. The abrupt changes observed in the Arrhenius plots were probably due to changes in the rate-determining step from the denaturation process in the low-temperature range to association reactions in the high-temperature range (Anema et al., 2006). The temperature for the abrupt change for the denaturation of α-La (80 °C) reported here is in agreement with that reported previously in cow skim milk; that is, the break was observed at 80 °C for the denaturation of α-La (Anema & McKenna, 1996; Oldfield, Singh, Taylor, et al., 1998). However, the findings for the denaturation of β-Lg in SSM do not support the previous research on cow skim milk, which showed that the break was found at 90 °C for the denaturation of β-Lg (Dannenberg & Kessler, 1988; Oldfield, Singh, & Taylor, 1998). These differences indicated that the rate-determining step for the denaturation of β-Lg changed at a lower temperature in sheep milk than in cow milk; however, the mechanism for these differences between sheep milk and

cow milk is unclear. Previous studies on cow skim milk showed that the critical Arrhenius temperature of 90 °C for the denaturation of β-Lg was independent of the total protein concentration (Law & Leaver, 1997), the pH (Anema & McKenna, 1996; Kessler & Beyer, 1991; Law & Leaver, 2000), and the milk composition (i.e., nonprotein-colloidal/soluble components) (Anema et al., 2006), despite the rate constants k_n for the denaturation of β-Lg being affected by these factors. Several reports indicated that the physicochemical properties of β-Lg could be affected by its gene sequences and structures (Erhardt et al., 1989; Loch et al., 2014). Loch et al. (2014) showed different β-Lg gene sequences and three-dimensional structure between sheep milk and cow milk, and demonstrated that those differences could influence the physicochemical properties of β-Lg. Therefore, it is hypothesized that the lower Arrhenius critical temperature for β-Lg observed in sheep milk compared with cow milk may be related to the different gene sequences and structures of β-Lg.

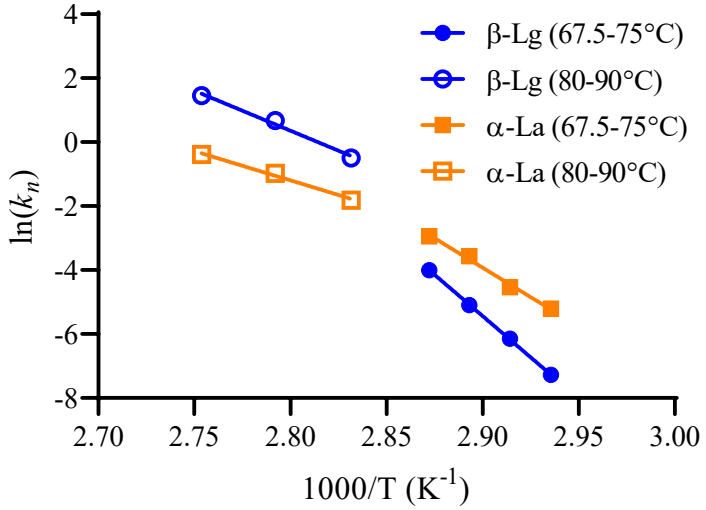

Figure 3-2 Arrhenius plots for the denaturation of β-Lg (●, 67.5–75 °C; ○, 80–90 °C) and α-La (■, 67.5–75 °C; □, 80–90 °C) in heated sheep skim milk. k_n has units of $g^{1-n} \cdot L^{n-1} \cdot s^{-1}$.

The activation energies E_a were calculated in two temperature regions using the Arrhenius plots, with values of 429.3 and 306.3 kJ·mol^{-1} at 67.5–75 °C for β-Lg and α-La, respectively, and values of 207.7 and 152.0 kJ·mol^{-1} at 80–90 °C for β-Lg and α-La, respectively (Table 3-2). The results presented here were higher than those reported previously by Dumitraşcu et al. (2013), who showed single values of 137 and 156 kJ·mol^{-1} in the whole heating temperature range of 72.5–90 °C for the activation energies E_a for the denaturation of β-Lg (based on a reaction order of 1.5) and α-La (based on a first-reaction order), respectively. The differences in the activation energies E_a may have been due to the different milk sources used. Dumitraşcu et al. (2013) used milk from Merino sheep, whereas this study used milk from East Friesian sheep; the gene sequences and structures for β-Lg of the two breeds could be different (Mastrangelo et al., 2012; Staiger et al., 2010), which may result in different kinetic parameters for the denaturation of β-Lg. Additionally, no break was observed in the Arrhenius plots for the denaturation of both β-Lg and α-La in the temperature range 72.5–90 °C in their results. A possible explanation for the discrepancy between the present results and their results might be that they worked with relatively fewer temperature and time points, resulting in the kinetic parameters being somewhat uncertain.

Table 3-2 Kinetic parameters for denaturation of β-Lg and α-La in heated sheep skim milk.

Whey protein	Temperature (°C)	k_n (10^{-3} $g^{1-n}\cdot L^{n-1}\cdot s^{-1}$)	E_a (kJ·mol^{-1})	ln($k_{n,0}$)	R^2
β-Lg	67.5	0.012			
n = 1.7	70	0.039	429.3	144.3	0.999
	72.5	0.104			
	75	0.306			
	80	10.238			
	85	33.256	207.7	70.3	0.990
	90	71.664			
α-La	67.5	0.096			
n = 1.0	70	0.179	306.3	102.9	0.993
	72.5	0.472			
	75	0.880			
	80	2.728			
	85	6.303	152.0	50.0	0.992
	90	11.335			

3.4.3. Thermodynamics of whey protein denaturation

The average thermodynamic parameters for the denaturation of β-Lg and α-La were calculated based on the Eyring equation described by Anema and McKenna (1996) and are shown in Table 3-3. The $\Delta H^{\ddagger}$ values were about 428 and 305 kJ·mol^{-1} for β-Lg and α-La, respectively, in the low-temperature range (67.5–75 °C), which were higher than those (about 206 and 150 kJ·mol^{-1} for β-Lg and α-La, respectively) in the high-temperature range (80–90 °C). In addition, the $\Delta H^{\ddagger}$ values for β-Lg and α-La in both temperature ranges were higher than those reported previously for cow skim milk, i.e., $\Delta H^{\ddagger}$ values of 260–300 kJ·mol^{-1} (70–90 °C) for β-Lg, and of 190–270 kJ·mol^{-1} (70–80 °C) and 50–70 kJ·mol^{-1} (85–115 °C) for α-La (Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Oldfield, Singh, Taylor, et al., 1998). The discrepancy between the present study and the data reported previously for cow skim milk can be attributed to the different Arrhenius critical

temperatures (Figure 3-2) and the different milk compositions (Akkerman et al., 2016), as discussed above.

All the entropy $\Delta S^{\ddagger}$ values for both β-Lg and α-La were positive and were higher in the 67.5–75 °C range than in the 80–90 °C range. This is consistent with the findings on cow skim milk reported by Anema et al. (2006), who showed a decrease in the value of the entropy $\Delta S^{\ddagger}$ from the low-temperature range (75–90 °C) to the high-temperature range (90–100 °C). The reduced $\Delta S^{\ddagger}$ values at higher temperatures suggested a decrease in disorder, indicating that association reactions were becoming the rate-determining step. In comparison, irreversible denaturation reactions were the rate-limiting step in the low-temperature range (Oldfield, Singh, Taylor, et al., 1998).

At all heating temperatures, the values of the free energy $\Delta G^{\ddagger}$ were relatively constant, at 85–100 kJ·mol^{-1} for β-Lg and 90–100 kJ·mol^{-1} for α-La. These results match the data reported in earlier studies on both sheep milk and cow milk, and what is expected for protein unfolding (Anema & McKenna, 1996; Dannenberg & Kessler, 1988; Dumitraşcu et al., 2013).

Table 3-3 Enthalpy ($\Delta H^{\ddagger}$), free energy ($\Delta G^{\ddagger}$), and entropy ($\Delta S^{\ddagger}$) obtained for denaturation of β-Lg and α-La in heated sheep skim milk.

Whey protein	Temperature (°C)	$\Delta H^{\ddagger}$ (kJ·mol^{-1})	$\Delta G^{\ddagger}$ (kJ·mol^{-1})	$\Delta S^{\ddagger}$ (kJ·mol^{-1}·K^{-1})
β-Lg	67.5–75	428.1	100.9	0.950
	80–90	205.7	86.6	0.333
α-La	67.5–75	305.1	96.4	0.606
	80–90	150.0	91.7	0.164

3.4.4. Association of denatured whey proteins with casein micelles

The percentages of serum-phase β-Lg and α-La decreased with increases in both the temperature and the duration of heat treatment (Figure 3-3). At 75 °C, the percentage of serum-phase β-Lg decreased relatively slowly throughout the heating time, reaching about 34% after 30 min of heating (Figure 3-3A). At higher heating temperatures (80–90 °C), the decrease in serum-phase β-Lg was more rapid and reached a plateau at a longer holding time. After 10 min of heating at 85–90 °C, only about 3% of the β-Lg remained in the serum phase. Serum-phase α-La (Figure 3-3B) showed a similar pattern to serum-phase β-Lg. When SSM was heated for 30 min, the percentages of serum-phase α-La decreased to around 23, 11, 9, and 7% at 75, 80, 85, and 90 °C, respectively. These results suggested that nearly all the whey proteins had associated with the casein micelles after heating at 80–90 °C for 30 min.

Previous studies have shown that not all the denatured whey proteins associate with the casein micelles in cow milk; a maximum of about 70–80% of the denatured whey proteins associated with the micelles in the heating temperature range 75–90 °C (Anema & Li, 2003a). However, in sheep milk, it appeared that nearly all the denatured whey proteins associated with the casein micelles after heat treatments of 80–90 °C for 30 min. This was probably due to the higher content of α_{S2}-casein in sheep milk. It has been reported that pressure treatment of cow skim milk resulted in the exposure of α_{S2}-casein in casein micelles, and α_{S2}-casein can provide two cysteines for β-Lg to interact with via thiol–disulfide exchange reactions (Patel et al., 2006). As sheep milk contains higher content of α_{S2}-casein than cow milk, partial α_{S2}-casein of SSM may distribute at the out layer of casein micelles for denatured β-Lg to interact with without the requirement of pressure treatment. Thus, the higher content of α_{S2}-casein in SSM may result in more denatured whey proteins

associating with the casein micelles (Patel et al., 2006; Rasmussen et al., 1992). Further studies are required to investigate the interactions between α_{S2}-casein and β-Lg in heated sheep milk and 2-dimentional SDS-PAGE could be used to verify the interactions.

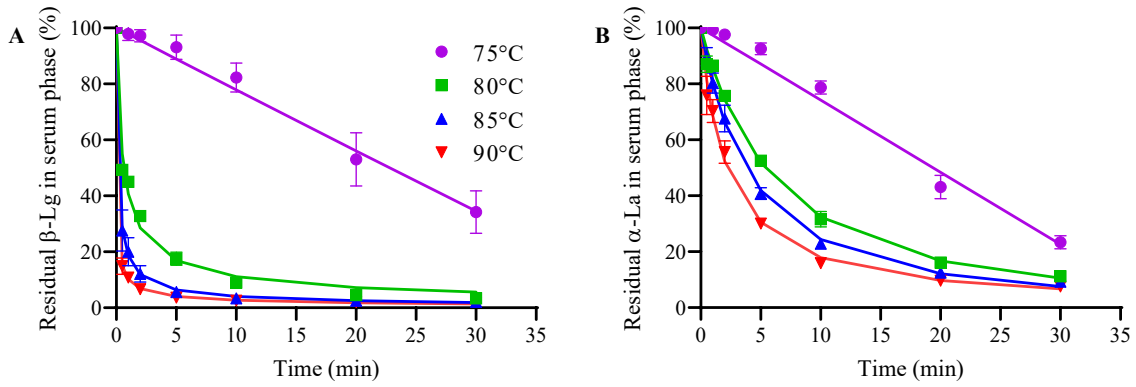

Figure 3-3 Percentages of residual β-Lg (A) and α-La (B) in the serum phase of sheep skim milk heated at different temperatures (●, 75 °C; ■, 80 °C; ▲, 85 °C; ▼, 90 °C) for different holding times. The lines represent the predicted associations of denatured β-Lg and α-La with the casein micelles from the model. Each data point represents the mean ± SD of the results from 3 different batches of sheep milk.

3.4.5. Kinetics of association of denatured whey proteins with casein micelles

The kinetic parameters for the association of denatured β-Lg and α-La with the casein micelles were calculated using Equations 3-5 and 3-6 and are shown in Table 3-4. The associations of β-Lg and α-La with the casein micelles at 80–90 °C had reaction orders of 2.6 and 1.8, respectively, which were higher than the reaction orders for denaturation of 1.7 for β-Lg and first-order for α-La. The results suggested that a more complex reaction occurred for the association process during heating, which may have been due to the involvement of the association between β-Lg and α_{S2}-casein. Additionally, sheep milk has

a higher content of α_{S2}-casein (19.2% of total caseins, Table 3-1) than cow milk (10.3% of total caseins) (Balthazar et al., 2017), which may affect the association process of β-Lg during heating and thus lead to changes in the kinetic parameters for SSM compared with cow milk.

Table 3-4 Kinetic parameters for associations of β-Lg and α-La with the casein micelles in heated sheep skim milk.

Whey protein	Temperature (°C)	k_n (10^{-3} $g^{1-n}\cdot L^{n-1}\cdot s^{-1}$)	E_a (kJ·mol^{-1})	ln($k_{n,0}$)	R^2
β-Lg	80	1.275			
n = 2.6	85	6.516	241.73	80.0	0.980
	90	14.412			
α-La	80	1.299			
n = 1.8	85	1.743	61.77	21.3	0.999
	90	2.319			

The rate constants k_n at 80–90 °C for β-Lg and α-La association were lower than those for β-Lg and α-La denaturation (Table 3-2). Additionally, the value of the rate constant k_n for association with the casein micelles was lower for α-La than for β-Lg, indicating a slower association with the casein micelles for α-La than for β-Lg during heating. The findings of the slower association rates for α-La than for β-Lg are in agreement with previous findings in cow milk, which showed that a great amount of β-Lg was denatured and associated with the casein micelles in skim milk in the temperature range 80–130 °C before α-La began to denature and complex with β-Lg to any significant extent (Oldfield, Singh, & Taylor, 1998). It has been reported that α-La associates with casein micelles by forming a complex with β-Lg, as α-La cannot associate with casein micelles on its own (Elfagm & Wheelock, 1978). Therefore, the slower association rates of α-La with

the casein micelles were probably caused by the less accessible thiol group of denatured β-Lg that had already associated with the casein micelles (Oldfield, Singh, & Taylor, 1998).

3.4.6. Casein micelle size

The Z-average diameters of the casein micelles in unheated and heated SSM are shown in Figure 3-4A. The Z-average diameters of the casein micelles in SSM heated at 75 and 80 °C had a sharp increase in the first 2 min and then increased slowly to about 208 and 221 nm, respectively, with further heating to 30 min. For SSM heated at 85 and 90 °C, the diameters of the casein micelles showed similar patterns to those for SSM heated at 75 and 80 °C in the first 20 min, but the size increased markedly at 30 min to about 290 nm, which was about 1.7 times higher than that in the unheated milk (174.6 nm). The increases in the casein micelle size in heated SSM, especially at 85 and 90 °C, were generally in agreement with the result reported by Raynal and Remeuf (1998), who showed that the casein micelle size increased from its initial value by 75% in SSM heated for 1 min at 85 and 90 °C.

3.4.7. Relationship between casein micelle size and association of denatured whey proteins with casein micelles

The relationship between the casein micelle diameter and the percentage of denatured whey proteins (β-Lg and α-La) associated with the casein micelles in heated SSM is shown in Figure 3-4B. For all heating temperatures, the diameter of the casein micelles in the SSM increased slowly and steadily as the percentage of denatured whey proteins associated with the casein micelles increased until the association level reached nearly 95%, with a maximum increase of about 25–30% for the average casein micelle diameter compared with that in unheated SSM. However, different diameters of the casein micelles were observed when the heated SSMs had similar percentages of denatured whey proteins

associated with the casein micelles (Figure 3-4B). For instance, when the association level was about 70%, the casein micelle size was significantly higher ($P < 0.01$) for the SSM heated at 90 °C (about 240 nm) than for that heated at 75 °C (about 208 nm). One possible explanation is that the aggregation of the casein micelles and the association of the whey proteins with the casein micelles occurred simultaneously during heating. Previous studies have stated that it is unclear whether the changes in the casein micelle size of cow milk during heating are due exclusively to the association of the denatured whey proteins with the casein micelles or are also due to the partial aggregation of the casein micelles that occurs at the same time as the whey proteins associating with the casein micelles (Anema, 2007; Anema & Li, 2003a). Additionally, aggregation of the casein micelles might occur to different extents at different temperatures. Therefore, the different sizes of the casein micelles with the similar association level found in the present study may be a consequence of aggregation of the casein micelles to different extents under different heating conditions. Another possible explanation is that the denatured whey proteins formed larger aggregates at higher heating temperatures and subsequently associated with the casein micelles (Oldfield, Singh, Taylor, et al., 1998), resulting in different sizes of the casein micelles with the similar association level.

The micelle diameter increased markedly by 70% compared with unheated SSM when the association level exceeded about 95% (Figure 3-4B), indicating that the casein micelle size increased significantly with little further association of the denatured whey proteins with the casein micelles. Previous studies on cow milk have noted that moderate heat-induced increases in casein micelle size are caused mainly by the association of denatured whey proteins with the casein micelles (Anema, 2007), but that the casein micelle size of milk samples heated at 75–90 °C for 0.5–30 min would not increase by more than 30% compared with that in unheated cow milk at natural pH (Anema, 2018b; Li et al., 2019;

Raynal & Remeuf, 1998). For example, Anema and Li (2003a) reported that heat treatment (75–100 °C for up to 60 min) of cow skim milk resulted in a maximum of about 80% of the whey proteins associating with the casein micelles, leading to a maximum of only about a 15% increase in the casein micelle size. These reports on heat-treated cow skim milk suggest that, even if all the whey proteins associated with the casein micelles, the increase in the casein micelle size of heated milk would not exceed 30% compared with unheated cow milk (Anema, 2008; Raynal & Remeuf, 1998). Moreover, the ratio of caseins to whey proteins (3.7:1) in SSM, presented here (Table 3-1), is close to that in cow milk (4:1) (Anema, 2021). It can thus be concluded that the denatured whey proteins associating with the casein micelles cannot induce such marked increases in the size of the casein micelles in heated SSM. Therefore, the significant increases in the casein micelle size in heated SSM when the extent of association exceeded about 95% could have been caused by aggregation of the casein micelles. The mechanism of casein micelle aggregation in this temperature range (75–90 °C) is not clear. It is possible that heating SSM at 85 and 90 °C might induce casein (particularly κ-casein) dissociation from the micelles, creating unstable hydrophobic areas on the surface of the casein micelles so that other casein micelles might be able to get closer and aggregate (Van Hooydonk et al., 1987). Additionally, the protein content was higher in the SSM (Table 3-1) than in cow skim milk, which may result in more micelle–micelle interactions during heating and thus more aggregation of the casein micelles (Singh & Creamer, 1991b). An alternative explanation is that the large whey protein aggregates at the surface of the casein micelles at high temperatures tend to form bridges between the casein micelles at higher protein content. However, further studies to investigate how aggregation of the casein micelles occurs during the heating of SSM are needed.

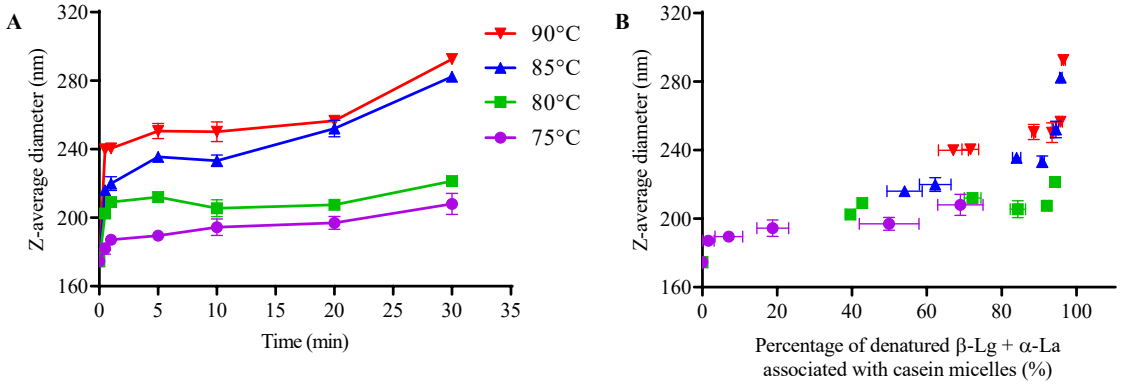

Figure 3-4 (A) Effect of heating temperature and time on the average size (Z-average diameter) of the casein micelles in sheep skim milk; (B) relationship between the percentage of denatured whey proteins (β-Lg + α-La) associated with the casein micelles and the casein micelle size in heated sheep skim milk: ●, 75 °C; ■, 80 °C; ▲, 85 °C; ▼, 90 °C. Each data point represents the mean ± SD of the results from 3 different batches of sheep milk.

3.4.8. Microstructural changes in casein micelles

Figure 3-5 presents the microstructures of the casein micelles in unheated SSM and SSM heated at 75–90 °C for 30 min. In unheated samples, the casein micelles had mainly a spherical structure with a smooth outline and were separated from neighboring micelles (Figure 3-5A). When the SSM was heated at 75 °C for 30 min, the casein micelle structure showed a similar appearance to that in the unheated SSM, but some irregular projections associated with the surface of the casein micelles were observed (Figure 3-5B). This suggested that denatured whey proteins had associated with the casein micelles. At 80 °C, a greatly increased number of irregular projections surrounded the surface of the micelles (Figure 3-5C). Similar changes were also found in the SSMs heated at 85 and 90 °C for 30

min (Figures 3-5D and 3-5E). In addition, large numbers of casein micelles with different sizes appeared to aggregate together when the SSM was heated at 85–90 °C for 30 min (Figures 3-5D1 and 3-5E1). This observation is in line with the findings presented above; that is, aggregation of the casein micelles occurred synchronously with association of the whey proteins with the casein micelles. Therefore, this observation further supports that the marked increase in the casein micelle size of SSM at 85–90 °C is due to both aggregation among the casein micelles and association of denatured whey proteins with the casein micelles.

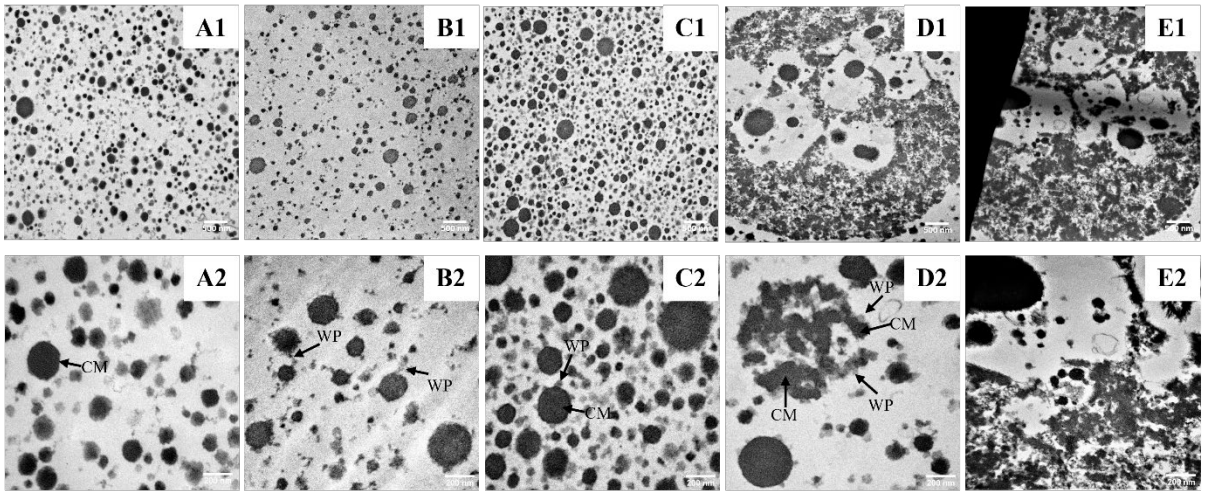

Figure 3-5 Transmission electron micrographs of the proteins in unheated sheep skim milk (A1 and A2) and sheep skim milk heated for 30 min at 75 °C (B1 and B2), 80 °C (C1 and C2), 85 °C (D1 and D2), and 90 °C (E1 and E2). The scale bars represent 500 nm (upper row) and 200 nm (lower row). CM, casein micelle; WP, whey protein.

3.5. Conclusions

In general, this study confirmed that the casein micelle size in SSM increased markedly under heat treatment (75–90 °C/0.5–30 min); the kinetics of the denaturation of

β-Lg and α-La and of the associations of β-Lg and α-La with the casein micelles were explored. Kinetic analysis showed that the denaturation processes for both β-Lg and α-La in SSM were similar to those reported for cow milk. However, a break in the Arrhenius plot was observed at the lower temperature of 80 °C for the denaturation of β-Lg in SSM, compared with those reported for cow milk, which may have been due to the different gene sequences and structures of β-Lg. The kinetic parameters showed that the β-Lg in SSM denatured and associated with the casein micelles at a faster rate than α-La in the temperature range 80–90 °C. The microstructural changes in the casein micelles and the relationship between the casein micelle size and the level of denatured whey proteins associated with the casein micelles confirmed that the increases in casein micelle size could be attributed to the denatured whey proteins associating with the casein micelles and to aggregation of the casein micelles. Further study to validate how the casein micelles form large aggregates and their relationship with the heat stability of sheep milk should be carried out.

Chapter 4: Statement of Contribution – Doctorate with Publications/Manuscripts

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Zheng Pan
Name/title of Primary Supervisor:	Professor Aiqian Ye
In which chapter is the manuscript /published work:	Chapter 4

Please select one of the following three options:

○ The manuscript/published work is published or in press

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International Dairy Journal

- The percentage of the manuscript/published work that was contributed by the candidate: 90.00
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The candidate contributes to the conceptualization, designed and conducted the experiment, analyzed all the experimental data with assistance from Professor Aiqian Ye in developing an appropriate method. The candidate wrote the original draft of the manuscript, with changes and corrections completed with input from all supervisors.

○ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Zheng Pan Digitally signed by Zheng Pan Date: 2023.02.21 16:58:53 +13'00'
Date:	21-Feb-2023
Primary Supervisor's Signature:	Aiqian Ye Digitally signed by Aiqian Ye DN: cn=Aiqian Ye, c=NZ, o=Massey University, ou=SF&AT, email=a.m.ye@massey.ac.nz Date: 2023.02.22 12:40:36 +13'00'
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GRS Version 5 – 13 December 2019
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Chapter 4. Heat-induced coagulation of sheep skim milk

The contents of this chapter have been published in Journal of Dairy Science (open access):

Pan, Z., Ye, A., Fraser, K., Dave, A., & Singh, H. (2023, 2023/09/01/). Heat stability of sheep's skim milk: Aggregation and interaction of proteins. International Dairy Journal, 144, 105689. https://doi.org/https://doi.org/10.1016/j.idairyj.2023.105689

4.1. Abstract

Sheep milk proteins are susceptible to heat-induced coagulation but the protein interactions under high heat treatment have not been determined. Heat stability and protein interactions of sheep skim milk (SSM) at pH 6.2–7.2 were examined at 140 °C. SSM had the longest heat coagulation time at pH 6.9 but became very unstable at higher or lower pH. The protein aggregates formed consisted mainly of whey proteins and κ-casein-depleted casein micelles. Modification of pH in SSM alters the ionic calcium concentration, dissociation of caseins and electrostatic interactions, resulting in different extents of protein interactions. The extent of dissociation of κ-casein from casein micelles increased with increasing pH (from pH ~6.6 to 7.0) before and after heat treatment, contributing to the aggregation of κ-casein-depleted casein micelles. High ionic calcium concentrations, low levels of κ-casein on casein micelles and ready dissociation of κ-casein from casein micelles may be responsible for the low heat stability of sheep milk.

4.2. Introduction

Sheep milk has lower heat stability than cow milk. Sheep milk can be effectively pasteurized via either vat or high-temperature short-time treatment but cannot be easily processed by UHT treatment. Unlike cow milk, which can be stored for 6 months after UHT treatment without the addition of a stabilizer (Gaur et al., 2018), UHT treatment of sheep milk can produce a large amount of sediment immediately after packaging (Martinez

Alonso et al., 2009). The sediment that is formed during UHT treatment is composed mainly of proteins (O'Connell & Fox, 2003; Singh, 2004). Therefore, it is important to understand how proteins in sheep milk aggregate at high temperatures in order to improve its heat stability.

The heat stability of sheep milk has not been investigated in depth. In their heat coagulation time (HCT)–pH profile, Fox and Hoynes (1976) showed that sheep milk had a marked stability maximum at ~ pH 6.8 (measured at 140 °C) but became very unstable at higher and lower pH values. Muir and Tamime (1993) also reported a similar HCT–pH profile for sheep milk, but its heat stability was not further studied. A negative correlation between the heat stability and the non- protein nitrogen fraction of sheep milk was reported in the study of Muir and Tamime (1993).

The heat-induced changes in cow milk have been extensively investigated. It has been reported that the important factors affecting the colloidal stability of cow milk are calcium ions and pH, which affect the attractive forces between micelles, facilitate the aggregation of micelles, and possibly change the conformation of κ-casein at the micelle surface (indirectly reducing steric repulsions) (Singh, 2004). Additionally, heat treatment of cow milk significantly alters the serum-phase environment around the casein micelles (such as pH, soluble calcium ions and breakdown of lactose) and the casein micelles themselves (association of whey proteins, dephosphorylation and casein dissociation), and these changes contribute to the coagulation of milk proteins. For instance, heating cow milk at 140 °C resulted in dissociation of κ-casein and the formation of κ-casein/whey protein complexes, thereby reducing the protective effects of the hairy layers of casein micelles (Singh & Latham, 1993). Increasing the pH of cow milk before heating further increased the dissociation of κ-casein after heat treatment and thus the quantity of κ-casein/whey protein complexes in the serum phase (Anema, 2008; Singh & Latham, 1993), leading to

the aggregation of unstable casein micelles. However, it has been reported that the composition of the milk could have an impact on these heat-induced changes (Deeth & Lewis, 2016; Pan et al., 2022a). Sheep milk has considerable differences in its composition compared with cow milk; for instance, the main fractions of sheep milk versus cow milk are 5.5% and 3.4% for protein content, 197.5 mg/100 g and 112.0 mg/ 100 g for calcium contents, 61.6% and 32.7% for β-CN (of total casein), 22.8% and 10.3% for α_{S2}-CN (of total casein), 6.7% and 39.7% for α_{S1}-CN (of total casein), and 8.9% and 11.6% for κ-CN (of total casein) (Balthazar, et al., 2017; Li, Delger, Dave, Singh, & Ye, 2022); this can affect the stability and interaction of sheep milk proteins during heating. Given the complexity of the reactions that occur during heat treatment, which correlate with the milk composition, the result obtained from cow milk cannot be directly extrapolated to sheep milk as sheep milk has considerable differences in its composition compared with cow milk (such as casein composition and calcium content) (Balthazar et al., 2017; Li, Delger, et al., 2022). Therefore, these heat-induced changes in sheep skim milk (SSM) were investigated in this study.

This study investigated the aggregation of proteins, the dissociation of caseins and the association of whey proteins in SSM at 140 °C and in the pH range 6.2–7.2. The HCT–pH profile of SSM was used as an indicator to select the pH and the heating time for further analysis. The ionic calcium concentration, the particle size distribution and the protein composition in unheated and heated samples were determined to develop an understanding of the differences observed in the HCT–pH profiles of SSM.

4.3. Materials and methods

4.3.1. Materials and pH adjustment

The raw sheep milk was purchased from Fernglen Ltd (Masterton, New Zealand). A small amount of sodium azide (0.01%, w/w) was added to the raw milk as a preservative. The raw sheep milk samples were skimmed at 3000 × *g* and 20 °C for 15 min using a bench centrifuge (Heraeus Multifuge X3R; Thermo Fisher Scientific Inc., Waltham, MA, USA). The composition of the SSM was analyzed using a MilkoScan FT1 (FOSS, Hillerød, Denmark) and is shown in Table 4-1. The pH of the SSM was adjusted to values between 6.2 and 7.2 by slowly adding 3 *M* HCl or 3 *M* NaOH to well-stirred solutions. The natural pH of the SSM was 6.59 ± 0.02. The milk samples were kept at ambient temperature for at least 2 h before the final pH reading and minor readjustment.

Table 4-1. Composition of sheep skim milk.

Component	Composition
Protein (g 100 mL^{-1})	6.68 ± 0.40
Casein (g 100 mL^{-1})	5.12 ± 0.33
Fat (g 100 mL^{-1})	0.62 ± 0.27
Lactose (g 100 mL^{-1})	4.82 ± 0.10
Total solids (g 100 mL^{-1})	13.29 ± 0.63
Casein/Protein (%)	76.51 ± 0.51

4.3.2. Heat stability testing

The HCT of the SSM was examined at 140 °C as a function of pH (6.2–7.2), as described by Davies and White (1966). A 2 mL subsample of pH-adjusted SSM was transferred into a glass vial (capacity, 8 mL; height, 63 mm; diameter, 17 mm) and heated at 140 °C with continuous rocking (8 cycles min^{-1}) in a thermostatically controlled oil bath.

The HCT was defined as the time that elapsed between placing the vial in the oil bath and the first visible onset of coagulation. The heat-up time for the milk to reach 140 °C was around 2 min.

4.3.3. Heat treatment

SSM at pHs 6.8, 6.9 and 7.0 was heated at 140 °C for 120, 150, 200, 300 and 500 s using the same method as described above. After heat treatment, these milk samples were immediately immersed in cold running water and cooled to room temperature. The heated milk samples were kept at room temperature for 6 h before further determination. The selection of heating time was based on the minimum HCT observed in section 4.3.2.

4.3.4. pH and ionic calcium concentration

Unheated and heated SSM samples at different pH values were preheated in a water bath at 20 °C for 1 h to equilibrate the temperature. The pHs of these samples were then determined using a pH 700 Benchtop Meter (OAKTON Instruments, Vernon Hills, IL, USA). The concentration of ionic calcium in these milk samples was determined as described in Li et al. (2019), using an Orion calcium-selective electrode (9720BNWP, Thermo Fisher Scientific Inc., Beverly, MA, USA) coupled with the pH 700 pH/mV Benchtop Meter. Calibration was conducted using standard (0.5–5 m*M*) $CaCl_2$ in 80 mM $CaCl_2$–KCl solution. The millivolt value was measured and recorded by dipping the electrode into the milk samples. The recorded millivolt value was converted to the ionic calcium concentration using the calibration curve obtained from the standard $CaCl_2$–KCl solution.

4.3.5. Separation of milk protein fractions

Unheated and heated SSM samples were centrifuged to obtain different fractions of soluble protein, as described by Dumpler et al. (2017). After centrifugation, the protein

composition of the resultant supernatants was analyzed by RP-HPLC. The fractions obtained are summarized below.

Fraction A: Large aggregates (> 3 μm) sedimented at 3000 × *g* for 10 min at 20 °C using a 50 mL centrifuge tube (Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China)) and a bench centrifuge Heraeus Multifuge X3R coupled with a swing bucket rotor (TX-750; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Fraction B: Colloidally stable micelles (100–1000 nm) sedimented at 48,800 × *g* for 26 min at 20 °C using a Sorval™ WX 80+ Ultracentrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA). This centrifugation condition has been proven to efficiently remove all casein micelles while κ-casein/whey protein complexes and soluble proteins remain in the supernatant (defined as the serum phase) (Dumpler et al., 2017). The level of individual proteins associated with sedimentable casein micelles under 48,800 × g for 26 min was calculated by subtracting the level of protein in the supernatant of fraction B from that of fraction A.

Fraction C: Submicellar particles (20–100 nm) sedimented at 70,000 × *g* for 60 min. The particles in this size range consist mainly of κ-casein/whey protein complexes with comparably small amounts of calcium-sensitive caseins (Dumpler et al., 2017; Morand et al., 2011). Therefore, the submicellar particles obtained are referred to as κ-casein/whey protein complexes in the following sections.

Fraction D: Soluble proteins (< 20 nm).

4.3.6. Particle size analysis

Particle size measurements of sheep skim milk heated at different conditions were performed on a MasterSizer 2000S (Malvern Instruments GmbH, Herrenberg, Germany), as described by Pan , Ye, Dave, Fraser, and Singh, (2022b).

4.3.7. Protein composition analysis

Milk and the supernatants obtained from centrifuged milk samples were analyzed by RP-HPLC using a reversed-phase C18 column (Aeris Widepore 3.6 μm XB-C18 RP; Phenomenex, Torrance, CA, USA) to determine the protein composition, as described by Bobe et al. (1998). As the intensive heating could result in different extent of glycosylation of proteins, this could overlap the individual peaks and decrease the separation efficiency of chromatographic peaks. Therefore, the integration of peak area could be affected and reduced the accuracy of comparison between peaks. Previous studies show that peak height is better than area especially if peaks are poorly resolved (Dyson & Smith, 1998; Grant & Clarke, 1971; Kadjo et al., 2017); it is less affected by asymmetry and overlap (Meyer, 1995; Snyder, 1972). In this study, therefore, the quantity of whey proteins and caseins in the ultracentrifugal supernatants was determined by comparing the relative peak height of the supernatant fraction in the heated SSM with that in the original unheated SSM. All peak heights of these chromatograms were analyzed using peak integration algorithm LabSolutions software (Shimadzu Corporation, Kyoto, Japan).

4.3.8. Statistical analysis

All experiments reported were repeated three times using freshly collected sheep milk samples, and the results are shown as the mean ± standard deviation. Although there were some variations between individual milks, the same trends and relationships as reported here have been found for all samples examined to date. Graphs and analysis of variance tests were produced using GraphPad 8.4.0 (GraphPad Software).

4.4. Results and discussion

4.4.1. Heat coagulation time as a function of pH

The HCT of the SSM as a function of pH from pH 6.2 to pH 7.2 is shown in Figure 4-1. The HCT increased as the pH of the SSM was increased, reaching a maximum at pH 6.9. However, a further increase in the pH led to a decrease in the HCT. The results are in agreement with a previous report, which showed that sheep milk had the longest HCT at pH 6.8 and that the HCT decreased when the pH value was higher or lower than pH 6.8 (Fox & Hoynes, 1976). However, the maximum HCT (~ 10 min) of the SSM observed in this study was markedly lower than that of cow skim milk reported previously (Fox & Hoynes, 1976); that is, the maximum HCT of cow milk was ~ 20–30 min (measured at 140 °C) at ~ pH 6.7. The lower heat stability of sheep milk has been attributed to its higher protein content and lower level of κ-casein than cow milk (Fox & Hoynes, 1976; Raynal-Ljutovac et al., 2007). Other possible factors, such as different pH-induced changes in the surface charge of the casein micelles, electrostatic repulsion and ionic calcium activity prior to heat treatment, have also been reported to affect milk protein interactions (such as aggregation of casein micelles and dissociation of caseins) during heating. However, how these parameters result in the low heat stability of sheep milk has not been clarified. Based on the HCT–pH profile, the samples with pH 6.8–7.0 and heating times of 120–500 s were selected to further investigate the mechanisms of heat coagulation in SSM, and the results are shown in the following sections.

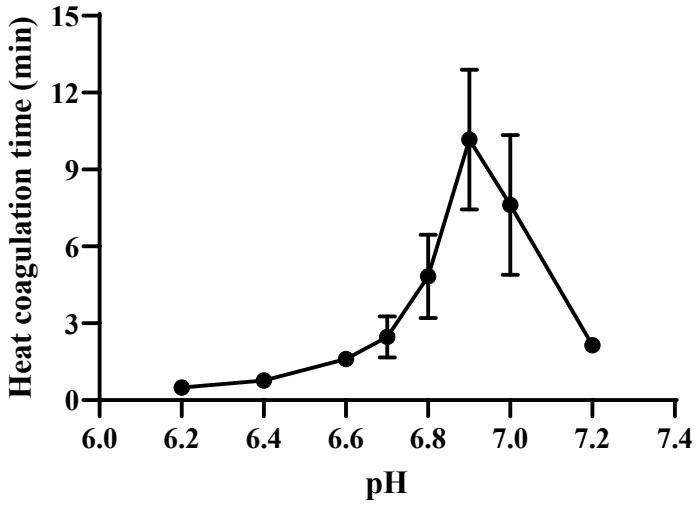

Figure 4-1. Heat coagulation time–pH profile of sheep skim milk at 140 °C.

4.4.2. Particle size

The average particle size (D [4, 3]) of the SSM before and after heat treatment was determined and is shown in Figure 4-2A. After heat treatment, the particle size of the SSM at all pH values increased with an increase in the heating time. This may have been due to the association of whey proteins with casein micelles and protein aggregation during heating (Pan et al., 2022a). However, the changes in particle size of the heated SSM occurred to different extents and showed significant ($P < 0.0001$) differences among the different pH values. For example, at the heating time of 300 s, the particle size of the SSM at pH 6.9 (~ 0.90 μm) was significantly ($P < 0.0001$) lower than those at pH 6.8 (~ 9.13 μm) and pH 7.0 (~ 13.81 μm). These results suggested that the proteins of the SSM were relatively more stable at 140 °C and pH 6.9, and were more susceptible to aggregation during heating and larger protein particles were formed at pH 6.8 and pH 7.0.

4.4.3. pH and ionic calcium concentration

The ionic calcium concentration–pH profile of unheated SSM is shown in Figure 4-2B. The ionic calcium concentration decreased with increasing pH, which was in line with results found previously for cow milk (Lewis, 2011). Previous studies on cow skim milk have shown there is a strong negative correlation between the ionic calcium concentration and the pH of cow milk (Gaur et al., 2018; Ho et al., 2018), and the ionic calcium concentration has been reported to be an important factor that alters the heat stability of milk (Deeth & Lewis, 2017). Lewis et al. (2011) showed there is a sharp boundary between the instability of milk, which produces large amounts of sediment, and stability of milk, which creates small amounts of sediment; a small shift in ionic calcium concentration at the boundary could convert a stable milk system to an unstable milk system or vice versa. In addition, Dumpler et al. (2020) reported that the low heat stability at the acidic pHs of milk can be attributed to salt-induced coagulation because the colloidal stability of casein micelles might be lowered by the reduced micellar surface charge, the reduced electrostatic repulsion and the collapse of the hairy layer because of charge neutralization.

The ionic calcium concentration of the SSM (~ 2.7 m*M*) at the natural pH (~ 6.6) in the present study was higher than that of cow milk (1.8–2.3 m*M*) at the natural pH, as reported previously by Lewis (2011), which is in agreement with previous reports that sheep milk has a higher concentration of ionic calcium than cow milk (Li, Delger, et al., 2022; Lin, 2002; Silanikove et al., 2003). Therefore, the lower heat stability of the SSM at pH < 6.9 was assumed to be due to the charge neutralization that was induced by the lowered pH and the increased ionic calcium concentration. In contrast, the destabilization of casein micelles at the more basic pHs of the milk was correlated with the heat-induced dissociation of κ-casein from the casein micelles, which results in an increased tendency towards aggregation (Singh, 2004).

The changes in pH of the SSM after heating are shown in Figure 4-2C. The pH after heating decreased in all samples and the decrease rate of the pH was similar among samples with different pHs when the heating time was less than 300 s. This is in agreement with earlier studies, which found a decreasing trend in the milk pH as the heating time was increased (Fox, 1981). The decline in pH of the SSM after heat treatment may have been due to the production of organic acids from lactose decomposition, precipitation of tertiary calcium phosphate with the concomitant release of H^+ ions and the heat-induced dephosphorylation of casein with the subsequent precipitation of the released phosphate as tertiary calcium phosphate (Fox, 1981; Pyne & McHenry, 1955).

The changes in the ionic calcium concentration of the SSM after heating are shown in Figure 4-2D. The ionic calcium concentration decreased at the initial stage of heating but increased upon prolonged heating. The heat treatment of cow milk has been reported to reduce the ionic calcium concentration because of the precipitation of calcium phosphate during thermal processing (Chandrapala et al., 2010; Geerts et al., 1983). The decreased ionic calcium concentration in the heated SSM is in accordance with the observation in cow milk that the calcium and phosphate are transferred to the colloidal phase upon heating (De La Fuente et al., 2002; Singh, 2004; Zhang & Aoki, 1996). However, the precipitation of calcium phosphate could lead to a re-equilibration between HPO_4^{2-} and $H_2PO_4^-/PO_4^{3-}$ in the serum phase, producing more H^+ ions and resulting in a decrease in pH (Chandrapala et al., 2010). Therefore, the increasing trend of the ionic calcium concentration in the SSM at the longer heating times was attributed to the further decrease in pH, which could in turn increase the concentration of ionic calcium (Chen et al., 2015). Additionally, the dephosphorylation of the caseins upon heating at 140 °C could reduce the binding of ionic calcium to caseins, which could also contribute to the increase in the ionic calcium concentration (Fox, 1981; Singh, 2004). With the further increase in the ionic calcium

concentration at prolonged heating, the SSM may have formed protein aggregates when the ionic calcium concentration reached a critical level, thus increasing the particle size, as shown in Figure 4-2A.

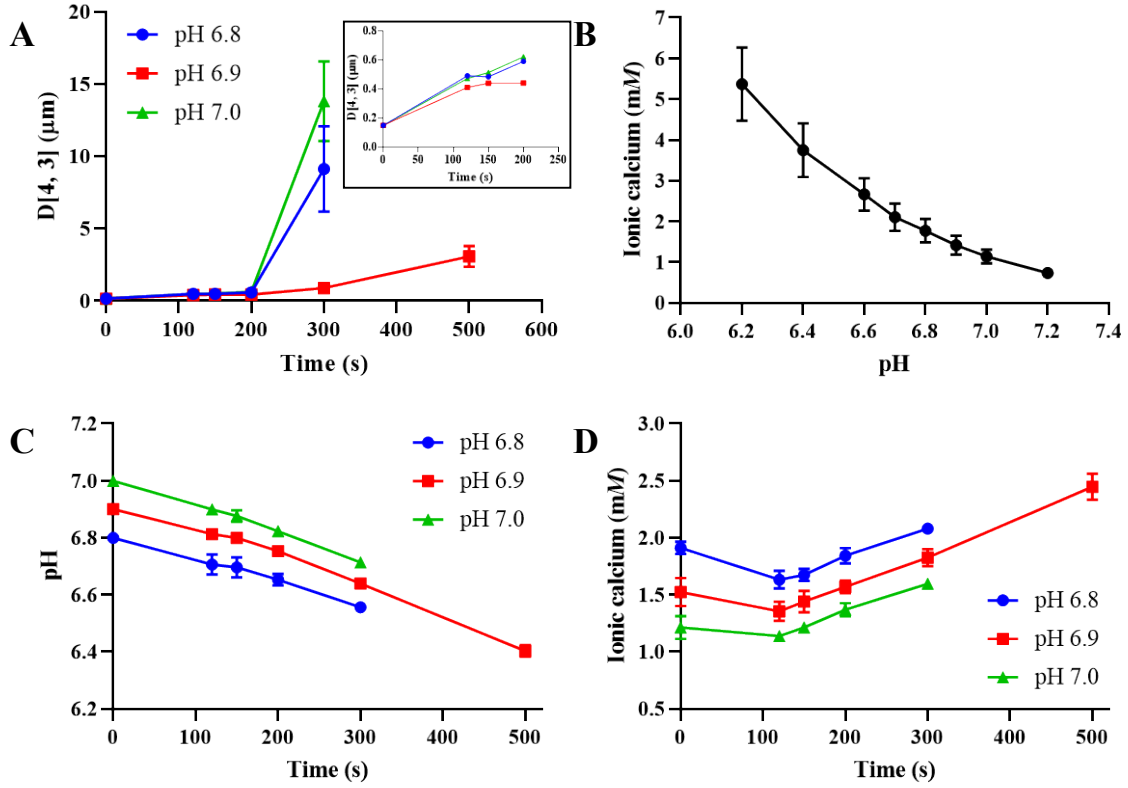

Figure 4-2. (A) Changes in particle size of heated sheep skim milk, (B) ionic calcium–pH profile of unheated sheep skim milk, (C) pH and (D) ionic calcium concentration of heated sheep skim milk at 140 °C as a function of time. pH 6.8 (●), pH 6.9 (■) and pH 7.0 (▲).

4.4.4. Heat-induced protein aggregation

The changes in the relative amounts of non-sedimentable proteins in the SSM after centrifugation at 3000 × *g* for 10 min are presented in Figure 4-3. At all pHs, there was a decreasing trend for all caseins and whey proteins as the heating time increased, but κ-casein decreased to a lesser extent than the other proteins. This indicated that heat treatment

of the milk resulted in the formation of large protein aggregates, which were composed mainly of whey proteins and casein micelles that were depleted in κ-casein, as observed in a previous report (Pan et al., 2022). When the heating time exceeded 200 s, there is an indication the levels of non-sedimentable β-casein, α_{S2}-casein, α_{S1}-casein and β-LG in the SSM at pH 6.9 decreased at a slower rate compared with those at pH 6.8 and pH 7.0, whereas their κ-casein and α-lactalbumin (α-LA) counterparts showed few differences among the pH values. The results suggested that the casein micelles of the SSM were more stable at pH 6.9 than at pH 6.8 and pH 7.0 during heating, which is in line with the particle size results (Figure 4-2A).

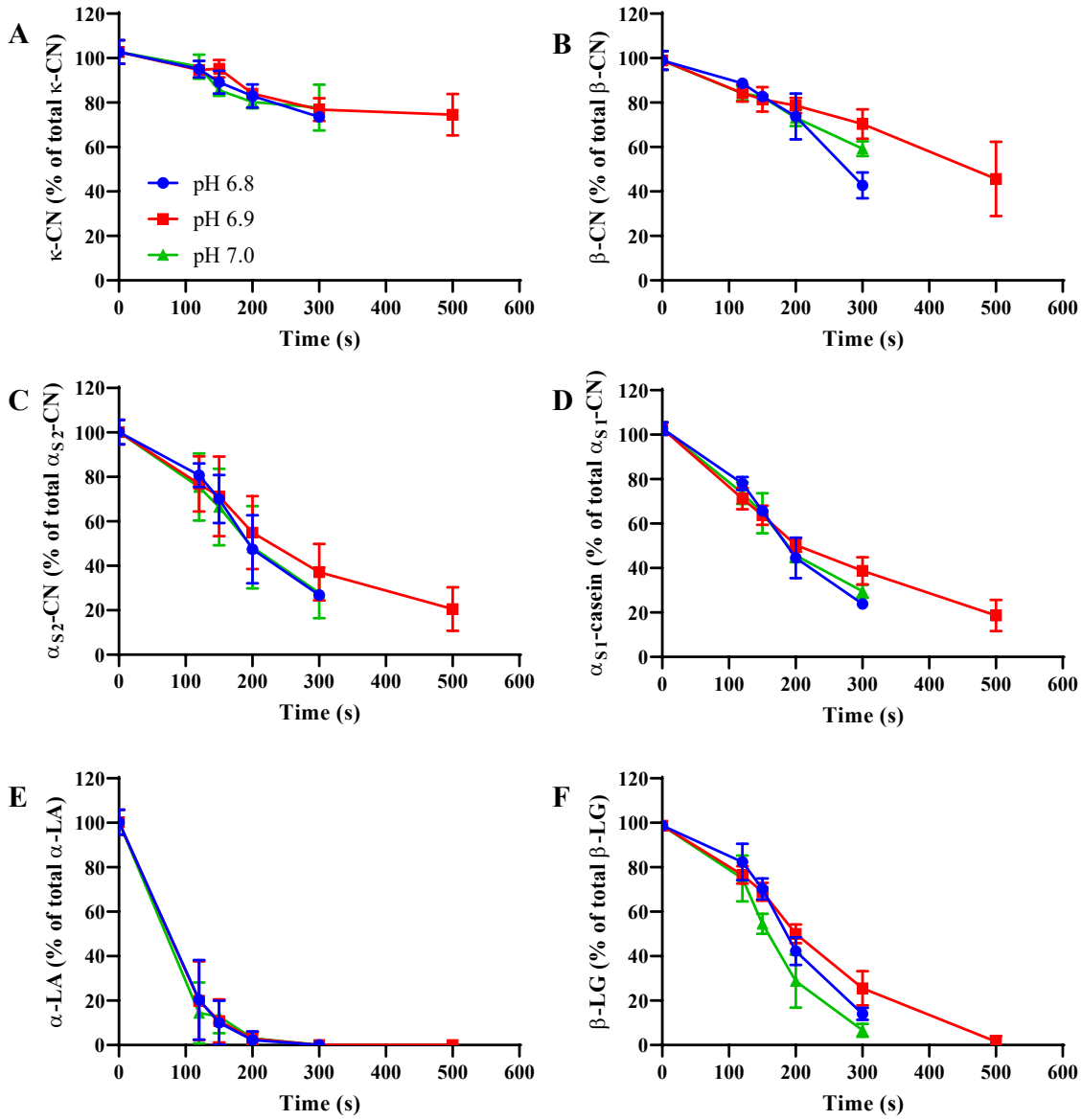

Figure 4-3. Changes in non-sedimentable (A) κ-casein (κ-CN), (B) β-casein (β-CN), (C) α_{S2}-casein (α_{S2}-CN), (D) α_{S1}-casein (α_{S1}-CN), (E) α-lactalbumin (α-LA) and (F) β-lactoglobulin (β-LG) in sheep skim milk at pH 6.8 (●), pH 6.9 (■) and pH 7.0 (▲), centrifuged at 3000 × g for 10 min and heated at 140 °C for different times.

4.4.5. Protein dissociation from casein micelles

The levels of individual proteins in the supernatant obtained by centrifuging unheated SSM at 48,800 × *g* for 26 min are shown in Figure 4-4; the supernatant obtained with this centrifugation condition is termed the serum phase in the following description. The level of κ-casein was found to increase significantly ($P < 0.05$) with increasing pH compared with that at the natural pH (~ 6.6), whereas the other serum-phase caseins changed little ($P > 0.05$). This indicated that increasing the pH of the unheated SSM resulted in the dissociation of κ-casein from the casein micelles before heat treatment, which is different from previous findings in cow milk; that is, increasing the pH of unheated cow skim milk from 6.3 to 7.1 did not induce obvious dissociation of κ-casein from the casein micelles (Anema & Klostermeyer, 1997; Anema & Li, 2000). It is possible that the connection between κ-casein and the rest of the casein micelle could be more susceptible to pH changes in sheep milk than in cow milk because of its higher concentration of proteins. The different protein compositions and total solids between sheep milk and cow milk (Balthazar et al., 2017) may be responsible for the different dissociation behavior of κ-casein as induced by pH changes. Different proportions of caseins could have different physical properties (such as hydrophobic attraction and electrostatic repulsion) and thus could affect the connection between κ-casein and other caseins within the casein micelles. Additionally, the level of soluble κ-casein in the serum phase of unheated SSM (~ 18.1% of total κ-casein, Figure 4-4) at the natural pH was higher than that of unheated cow skim milk (~ 7–10% of total κ-casein) at the natural pH (Anema, 1998, 2008; Dumpler et al., 2017; Li et al., 2019). This may suggest less κ-casein on the casein micelles of sheep milk than of cow milk. As the remarkable stability of casein micelles relies on the κ-casein at the surface of the micelle (Anema, 2018a; Dalgleish, 1992; Huppertz et al., 2018), the lower level of κ-casein attached to the casein micelles and the vulnerable connection of κ-casein

with casein micelles in the SSM may contribute to the low heat stability of sheep milk and thus prompt the aggregation of the casein micelles during high heat treatment.

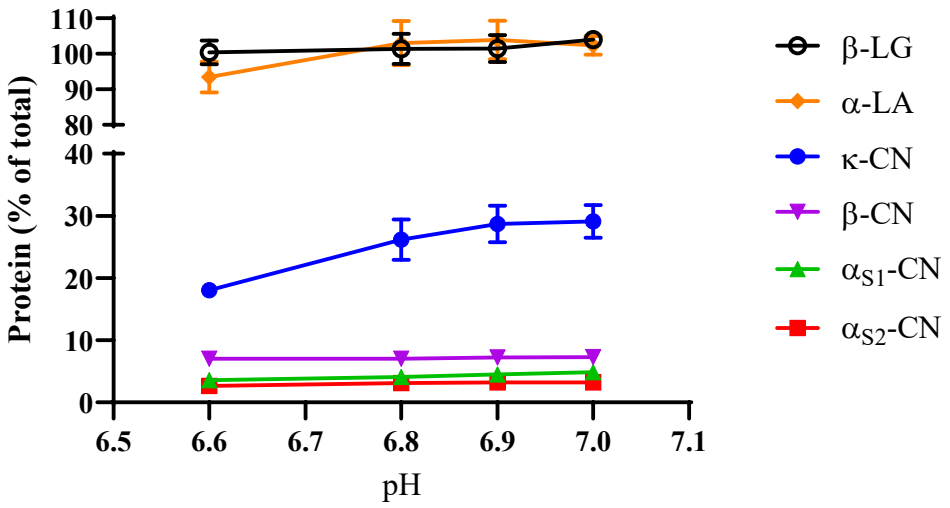

Figure 4-4. Changes in the levels of non-sedimentable κ-casein (κ-CN) (●), β-casein (β-CN) (▼), α_{S2}-casein (α_{S2}-CN) (■), α_{S1}-casein (α_{S1}-CN) (▲), α-lactalbumin (α-LA) (♦) and β-lactoglobulin (β-LG) (○) in unheated sheep skim milk at different pH values and centrifuged at 48,800 × g for 26 min.

Figure 4-5 shows the changes in the levels of κ-casein, α-LA and β-LG in the serum phase obtained from the SSM at pH 6.8–7.0 after centrifugation at 48,800 × *g* for 26 min and in the colloidal-stable micelle phase of the SSM after heat treatment. The level of serum-phase κ-casein in the SSM at all pHs increased in the first 150 s of heating, followed by a decrease with a longer heating time. The level of serum-phase κ-casein was highest in the SSM at pH 7.0 and lowest in the SSM at pH 6.8 throughout the heating period (Figure 4-5A1). This indicated that the dissociation of κ-casein occurred during the early stage of heating and that raising the pH of the SSM increased the dissociation level of κ-casein. The greater level of dissociation of κ-casein at higher pH has been attributed to the enhanced

electrostatic repulsion between the individual sub-micelles at a more alkaline pH (Anema & Klostermeyer, 1997; Anema & Li, 2000; Dumpler, 2018; Pan et al., 2022b). In contrast, the level of κ-casein in the colloidally stable micelle phase showed a decreasing trend when the heating time was less than 200 s, followed by an increasing trend with further heating (Figure 4-5B1). This suggested that a longer heating time led to the reassociation of κ-casein with the casein micelles probably because of a heat-induced decrease in pH (Deeth & Lewis, 2016; Singh, 2004).

Serum-phase α-LA and β-LG decreased markedly in the pH range 6.8–7.0 and ~ 95% of the α-LA and β-LG were lost in the serum phase when the heating time exceeded 200 s (Figures 4-5A2 and 4-5A3). The loss of serum-phase α-LA and β-LG could be attributed to either the formation of large whey protein aggregates or the association of denatured whey proteins with the casein micelles (Pan et al., 2022a). In contrast, whey proteins in micelle phase showed a different pattern; that is, α-LA and β-LG increased during the first 120 s and then decreased upon further heating (Figures 4-5B2 and 4-5B3). This indicated that a proportion of whey proteins were associated with the casein micelles initially, and those casein micelles with associated whey proteins formed sufficiently large aggregates to sediment on ultracentrifugation when the heating time was further extended. These results match previous studies of Pan et al. (2022a) and Singh (2004), who reported that the aggregation of casein micelles in SSM could be attributed to the bridging effect of the whey protein on the surface of the casein micelles. Further studies are required to verify the bridging effect of whey proteins that are coated on the surface of casein micelles.

Interestingly, by comparing the whey proteins between the serum phase (Figures 4-5A2 and 4-5A3) and the micelle phase (Figures 4-5B2 and 4-5B3), not all the serum-phase whey proteins associated with the casein micelles after heat treatment, but the unassociated whey proteins were still centrifuged down by ultracentrifugation. This confirmed that parts

of denatured whey proteins might form large aggregates by themselves during heating and that the whey protein aggregates were sufficiently large to sediment on ultracentrifugation. This finding is consistent with other research on cow milk, which showed that the sedimentable whey protein is either associated with casein micelles or in whey protein aggregates that are sufficiently large to sediment on ultracentrifugation (Crowley et al., 2015; Oldfield et al., 2000).

Although the SSM at pH 6.9 had intermediate values for ionic calcium concentration, the proportion of κ-casein on the casein micelles and the level of dissociation of κ-casein from the casein micelles compared with the SSMs at pH 6.8 and pH 7.0, the SSM at pH 6.9 still showed the maximum heat stability (Figure 4-1). Previous studies have stated that the heat instability at more acidic pHs of milk could be due to the bridging effect of the high concentration of ionic calcium among the casein micelles and that the heat instability at more basic pHs of milk could be due to the greater dissociation of κ-casein (Dumpler, 2018; Singh, 2004). The maximum heat stability of the SSM at pH 6.9 might be linked to a critical level of ionic calcium in the serum phase and a critical level of dissociation of κ-casein that alter the steric and electrostatic interactions of micelles and maintain the stability of casein micelles.

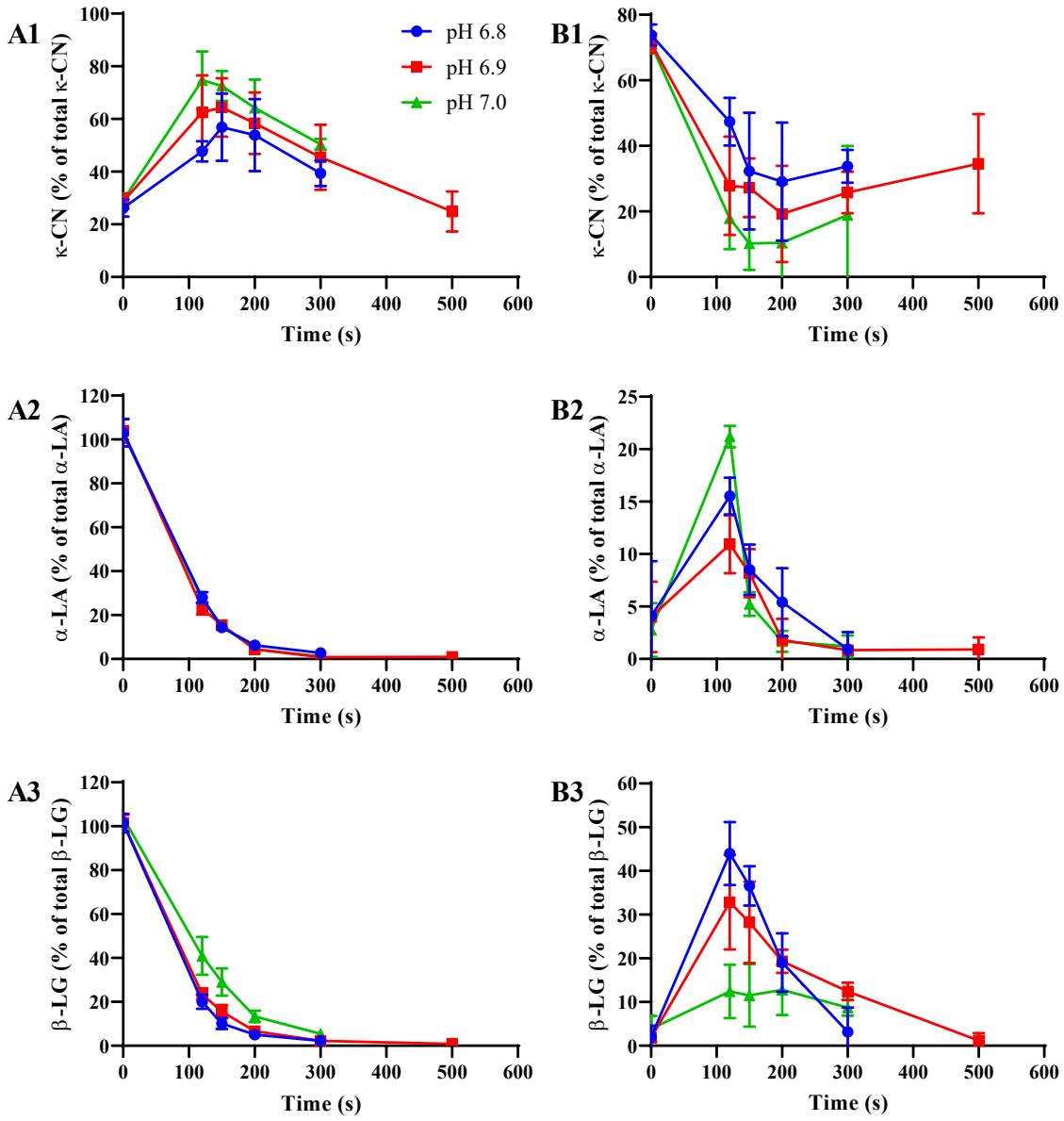

Figure 4-5. (A) Changes in non-sedimentable individual proteins in heated (140 °C) sheep skim milk centrifuged at 48,800 × g for 26 min. (B) Changes in individual proteins in the colloidal-stable micelles of heated (140 °C) sheep skim milk. A1 and B1, κ-casein (κ-CN); A2 and B2, α-lactalbumin (α-LA); A3 and B3, β-lactoglobulin (β-LG). pH 6.8 (●), pH 6.9 (■), and pH 7.0 (▲).

4.4.6. Formation of κ-casein/whey protein complexes

The levels of κ-casein, α-LA and β-LG in the κ-casein/whey protein complexes are shown in Figure 4-6. The levels of κ-casein, α-LA and β-LG increased significantly ($P < 0.05$) at all pH values when the SSM was heated for 120 s, followed by a decrease with longer heating. The levels of κ-casein, α-LA and β-LG in the SSM were highest at pH 7.0 and lowest at pH 6.8, and followed the same order throughout the period of heating. This indicated that increasing amounts of κ-casein/whey protein complexes formed in the SSM at the early stage of heating; these newly formed complexes might either reassociate with the casein micelles or form larger κ-casein/whey protein complex aggregates upon further heating; raising the pH of the SSM increased the proportion of κ-casein/whey protein complexes in the serum phase. Previous studies have shown that the most denatured β-LG is observed in the serum phase and is associated with κ-casein when the pH of the milk is higher than pH 6.8; in contrast, at pH lower than 6.8, the denatured β-LG is present on the surface of casein micelles (Deeth & Lewis, 2017; Kudo, 1980; Singh & Latham, 1993). These findings suggested that the preferential association of whey proteins with the casein micelles at lower pH may have been due to the higher level of κ-casein at the surface of the casein micelles, whereas more whey proteins in the serum phase at higher pH could be attributed to the increased amount of dissociated κ-casein in the serum phase during heating.

Based on the results for colloidally-stable micelle phase (Figures 4-5B1, 4-5B2 and 4-5B3), only κ-casein showed an increasing trend on prolonged heating; the levels of α-LA and β-LG decreased. We can infer that the newly formed κ-casein/whey protein complexes aggregated with either other complexes or whey proteins instead of reassociating with the casein micelles; only a small proportion of dissociated κ-casein that was not complexed with whey proteins might reassociate with the casein micelles at a longer heating time. It is therefore likely that the complexation of κ-casein with denatured whey proteins in the

serum phase sensitized the casein micelles to the destabilizing effect of heat-induced calcium phosphate precipitation or the exposure of hydrophobic groups and promoting the aggregation of casein micelles.

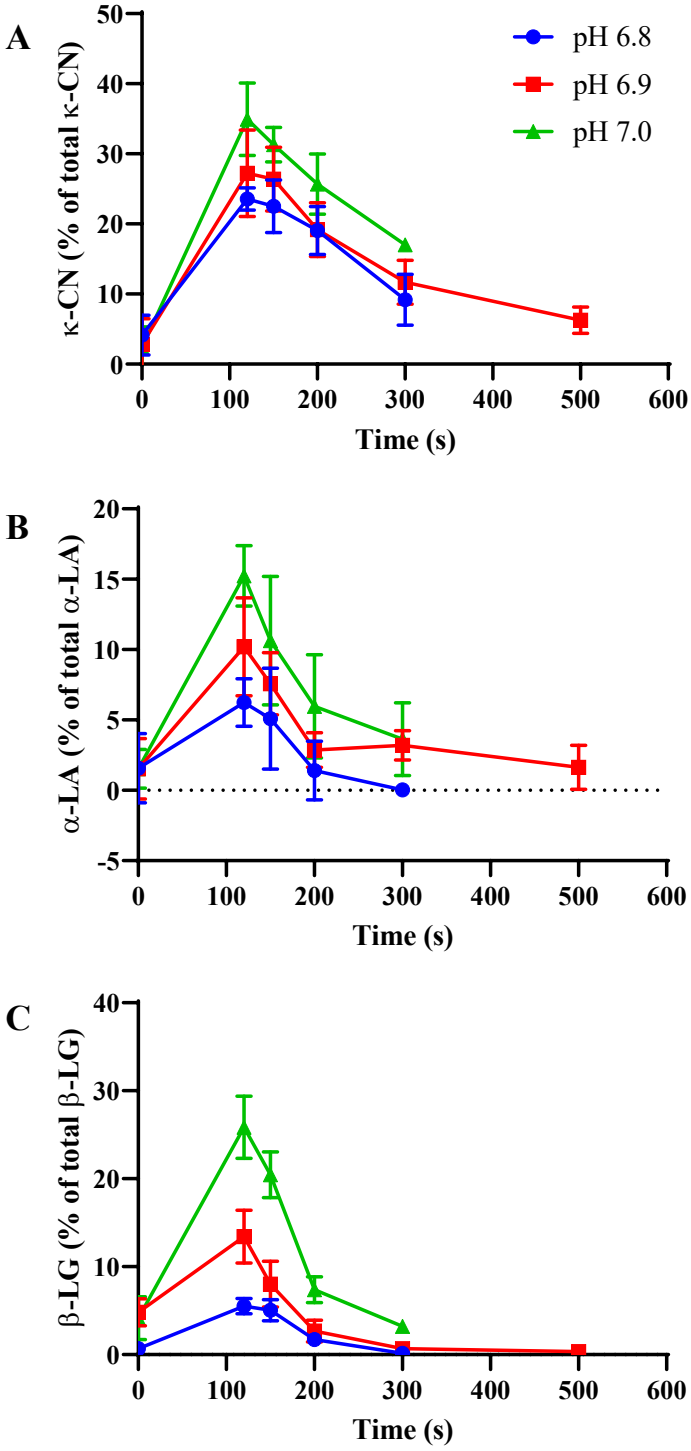

Figure 4-6. Changes in the levels of κ-casein (κ-CN), α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) in κ-casein/whey protein complexes of sheep skim milk at pH 6.8 (●), pH 6.9 (■), and pH 7.0 (▲) and heated at 140 °C for different times.

4.5. Conclusions

This study examined the stability of SSM at 140 °C and discussed the protein interactions in SSM at pHs 6.8–7.0 at different heating times ranging from 0 to 500 s. The results showed that SSM had maximum heat stability at pH 6.9 and became increasingly unstable at higher and lower pH values, and confirmed that sheep milk has lower heat stability than cow milk, by comparison with the HCT–pH profile reported previously for cow milk. The aggregates formed in the SSM during heating were composed mainly of κ-casein-depleted casein micelles and whey proteins. The lower heat stability of SSM at low pH (< 6.9) could be due to salt-induced coagulation because of its high ionic calcium concentration, the low surface charge of the casein micelles, a reduced electrostatic repulsion and a collapse of the hairy layer because of charge neutralization. The dissociation of κ-casein increased with increasing pH of the SSM before and after heat treatment. A large proportion of the dissociated κ-casein complexed with whey proteins and presented in the serum phase, which might inhibit the reassociation of κ-casein with casein micelles and promote the aggregation of κ-casein-depleted micelles. The occurrence of maximum heat stability at pH 6.9 is probably due to the critical level of ionic calcium concentration, κ-casein dissociation and electrostatic repulsion, which keep the casein micelle relatively stable.

In comparison with previously reported results on cow milk, lower proportions of κ-casein in the casein micelles and higher ionic calcium concentrations were observed in the SSM, and the κ-casein of the SSM dissociated from the casein micelles to a greater extent under the same heating conditions. These differences observed in SSM could contribute to the easy aggregation of casein micelles. These findings suggest that the casein micelles with a low level of κ-casein, the vulnerable connection of κ-casein with the casein

micelles and the high ionic calcium concentration may be responsible for the lower heat stability of sheep milk than of cow milk.

Chapter 5: Statement of Contribution – Doctorate with Publications/Manuscripts

DRC 16

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Zheng Pan
Name/title of Primary Supervisor:	Professor Aiqian Ye
In which chapter is the manuscript /published work:	Chapter 5

Please select one of the following three options:

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- Please provide the full reference of the Research Output:

Pan, Z.; Ye, A.; Dave, A.; Fraser, K.; Singh, H., pH-dependent sedimentation and protein interactions in ultra-high-temperature-treated sheep skim milk. J. Dairy Sci. 2022. In press. Doi:10.3168/jds.2022-22637

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- The name of the journal:
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Candidate's Signature:	Zheng Pan Digitally signed by Zheng Pan Date: 2023.02.21 16:59:52 +13'00'
Date:	21-Feb-2023
Primary Supervisor's Signature:	Aiqian Ye Digitally signed by Aiqian Ye DN: cn=Aiqian Ye, c=NZ, o=Massey University, ou=SF&AT, email=a.m.ye@massey.ac.nz Date: 2023.02.22 12:41:31 +13'00'
Date:	22-Feb-2023

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DRC 19/09/10

Chapter 5. pH-dependent sedimentation and protein interactions in UHT-treated sheep skim milk

The contents of this chapter have been published in Journal of Dairy Science.

Pan, Z.; Ye, A.; Dave, A.; Fraser, K.; Singh, H. (2022) pH-dependent sedimentation and protein interactions in ultra-high-temperature-treated sheep skim milk. J. Dairy Sci. (In press). doi:10.3168/jds.2022-22637

5.1. Abstract

Sheep milk is considered to be unstable to UHT processing, but the instability mechanism has not been investigated. This study assessed the effect of UHT treatment (140 °C/5 s) and milk pH values from 6.6 to 7.0 on the physical properties of sheep skim milk (SSM), including heat coagulation time, particle size, sedimentation, ionic calcium level, and changes in the protein composition. Significant amounts of sediment were found in UHT-treated SSM at the natural pH (~ 6.6) and pH 7.0, whereas lower amounts of sediment were observed at pHs 6.7–6.9. The proteins in the sediment were mainly κ-casein–depleted casein micelles with low levels of whey proteins regardless of the pH. Both the pH and the ionic calcium level of the SSM at all pH values decreased after UHT treatment. The dissociation levels of κ-, β-, and α_{S2}-casein increased with increasing pH of the SSM before and after heating. The protein content, ionic calcium level, and dissociation level of κ-casein in the SSM were higher than those in cow skim milk reported previously. These differences may contribute to the high amounts of sediment in the UHT-treated SSM at the natural pH (~ 6.6). Significantly higher levels of κ-, β-, and α_{S2}-casein were detected in the serum phase after heating the SSM at pH 7.0, suggesting that less κ-casein was attached to the casein micelles and that more internal structures of the casein micelles may have been

exposed during heating. This could, in turn, have destabilized the casein micelles, resulting in the formation of protein aggregates and thus high amounts of sediment after UHT treatment of the SSM at pH 7.0.

5.2. Introduction

Sheep milk is promoted as a good alternative to cow milk for humans because of its high nutritional content, such as higher concentrations of proteins, fats, vitamins, and minerals, compared with cow milk (Park et al., 2017). Unlike the commonly seen cheeses and yogurt products made from sheep milk, sheep milk products in liquid form are not widely available in most markets and exist only in some small farms or rural areas such as the mid-east Asian and Mediterranean basin (Kapaj & Deci, 2017; Tamang & Kailasapathy, 2010).

A combination of heating temperature and heating time is frequently used to treat milk to produce a product with a significantly extended shelf life. For sheep milk, it is difficult to produce liquid products with a long shelf life using commercial thermal treatments (such as UHT treatment) because of its low heat stability (Raynal and Remeuf, 1998). For instance, sheep milk coagulates under UHT processing conditions, leading to a high amount of sediments (mainly protein aggregates) during storage (Martinez Alonso et al., 2009). The instability of the milk can lead to sedimentation after UHT treatment, with aggregated material settling at the bottom of the container during storage. In general, the sediment consists of milk proteins, specifically caseins. Gaur et al. (2018) reported that all sediments were composed mostly of κ-casein-depleted casein micelles (~ 85%) with low levels of β-LG and α-LA compared with those in the bulk milk; similar observations were also reported by Malmgren et al. (2017), who showed a predominant content of β- and α_S-caseins but no whey proteins or κ-casein in the sediment after the UHT processing of milk.

The sedimentation has been attributed to the dissociation of κ-casein from the casein micelles during UHT processing, resulting in the aggregation of κ-casein-depleted micelles. The aggregated casein micelles settle at the bottom of the container to form sediment. However, as the κ-casein-depleted casein micelles do not always aggregate to form a sediment, there must be other factors that induce the aggregation (Gaur et al., 2018). Higher pH values of the milk could result in a lower level of ionic calcium and a higher charge on the casein micelles, which could prevent the aggregation of casein micelles that are depleted in κ-casein. In contrast, the decreased charge on the casein micelles and the higher levels of ionic calcium at low pH could induce aggregation of the casein micelles (Dumpler et al., 2020). In fact, no sheep milk products that have been subjected to UHT treatment without stabilizers can be preserved at room temperature with satisfactory organoleptic quality for as long as UHT-treated cow milk. In contrast, almost all UHT-treated cow milks can be stored for at least 6 months without the addition of stabilizers, although they may form a layer of sediment during storage, which is usually thin and does not affect the quality of the milk (Gaur et al., 2018).

Several factors, including pH, ionic calcium level, protein concentration, and protein composition, have been suggested to affect the heat stability of milk during UHT processing. The negative effects of increased ionic calcium on the heat stability of milk have been widely reported (Crowley et al., 2014, Chen et al., 2015, Deeth, 2020, Dumpler et al., 2020). As the milk proteins usually aggregate via calcium bridging (Deeth, 2020), high ionic calcium can be considered to be a primary factor in the low heat stability of milk. The pH of milk also plays an important role in stabilizing it during heating. Lewis et al. (2011) investigated the mechanism for the sedimentation in UHT-treated cow milk using a centrifugation method and showed that the amount of sediment increased when the pH was lowered, regardless of an increased or a constant level of ionic calcium. This clearly showed

that there was a strong relationship between the amount of sediment and the ionic calcium level, and between the amount of sediment and the pH in UHT milk. Additionally, the dissociation of κ-casein from the casein micelles that occurs as a consequence of the UHT treatment also contributes to the instability of milk (Anema, 2017). Extensive studies showed that dissociation of κ-casein from the casein micelles in milk occurred during heating (McMahon, 1996, Hillbrick et al., 1999, Malmgren et al., 2017). Anema (2017) investigated the age gelation of reconstituted UHT-treated cow skim milk in the absence of proteolysis by plasmin and found that the gelled materials consisted mainly of κ-casein-depleted casein micelles with very low levels of associated whey proteins; κ-casein dissociated from the micelles to a significant extent during UHT processing but changed little on further storage. Therefore, it was suggested that the sediment formation was initiated by the κ-casein-depleted casein micelles via calcium bridging (Anema, 2017). Furthermore, the heat stability of milk is known to be dependent on the protein concentration. Milks with higher protein content had lower heat stability than those with lower protein content (Crowley et al., 2015, Anema, 2017).

It is well known that sheep milk has higher concentrations of proteins and minerals than cow milk, and that their protein compositions differ (Balthazar et al., 2017). A recent study showed that a marked increase in casein micelle size and aggregation of the casein micelles were observed in sheep skim milk (SSM) heated at 85–90 °C (Pan et al., 2022a). However, these changes occurred to a significantly lower extent in cow milk than in sheep milk heated in the same temperature range (80–90 °C) in the study of Raynal and Remeuf (1998), which might be attributed to the higher protein content, different casein composition, or both in sheep milk. Therefore, sheep milk can be expected to react differently under severe heat treatment. Only limited studies indicating the mechanism behind the high sedimentation in UHT-treated sheep milk have been published. Fox and Hoynes (1976)

reported that sheep milk showed low heat stability (measured at 140 °C using oil bath methods) than cow milk, which could be attributed to a lower level of κ-casein in sheep milk than in cow milk. However, the protein interactions in sheep milk during heating in a UHT plant were not illustrated. Therefore, the objective of the present study was to investigate the sedimentation and protein interactions in UHT-treated SSM at different pH values.

5.3. Materials and methods

5.3.1. Materials and pH adjustment

Fresh sheep milk was purchased from Fernglen Ltd (Masterton, New Zealand). A small amount of sodium azide (0.01%) was added to the raw milk as a preservative. The raw sheep milk samples were skimmed using a milk skimmer. The composition of the skimmed sheep milk (SSM) was analyzed using a MilkoScan FT1 (FOSS, Hillerød, Denmark) and is shown in Table 5-1. The pH of the SSM (at 25 °C) was adjusted to values between 6.2 and 7.2 by slowly adding 2 M HCl (food grade) or 2 M NaOH (food grade) to well-stirred SSM. The milk samples were kept at ambient temperature for 2 h before the final pH reading and minor readjustment. The natural pH of the SSM was ~ 6.62 ± 0.02.

Table 5-1. Composition of sheep skim milk (SSM).

Component	g/100 mL SSM
Fat	0.27 ± 0.02
Lactose	5.04 ± 0.09
Protein	5.94 ± 0.01
Ash	1.03 ± 0.01
Total solids	12.27 ± 0.12

5.3.2. UHT treatment

SSMs at the natural pH (~ 6.6) and pHs 6.7–7.0 were heated at 140 °C for 5 s in an indirect UHT plant (Massey University pilot plant, Palmerston North, New Zealand). Steam was used to heat up the tubular-type UHT equipment. After heat treatment, these milk samples were immediately cooled to room temperature and packaged in aseptic milk bottles (capacity 2,000 mL). The UHT-treated SSM was kept at room temperature for 6 h before further analysis.

For the justification of pH selection for UHT treatment, the heat coagulation time (HCT) of the SSM was determined at different pH values before UHT treatment, to make sure that the SSMs in a certain pH range could withstand the UHT treatment without causing severe fouling in the UHT plant. The HCT of the SSM as a function of pH (6.2–7.2) was examined at 140 °C, as described by Davies and White (1966). The preliminary results showed that the HCT was highest at pH 6.8 and decreased when the pH value was lower or higher than pH 6.8 (data not shown). The HCTs of the SSMs at pH 6.2 and pH 6.4 were lower than 120 s, whereas the SSMs at other pH values had HCTs greater than 200 s. In our previous experiments, the required heat-up time for SSM to reach 140 °C was ~ 120 s, which meant that the SSMs at pH 6.2 and pH 6.4 coagulated before reaching 140 °C. The SSMs at the natural pH (~ 6.6) and pHs 6.7–7.2 had HCTs that were longer than the heat-up time. Therefore, SSMs at the natural pH (~ 6.6) and pHs 6.7–7.0 were selected for further investigation on the UHT plant.

5.3.3. pH and ionic calcium level

Unheated and heated SSMs at different pH values were prewarmed in a water bath at 20 °C for 1 h to equilibrate the temperature. The pH was then measured using a pH 700 Benchtop Meter (Oakton Instruments, Vernon Hills, IL). The ionic calcium level in these

milk samples was determined using an Orion calcium-selective electrode (9720BNWP; Thermo Fisher Scientific Inc., Beverly, MA) coupled with the pH 700 pH Benchtop Meter. Calibration was conducted using standard (0.5–5 mM) $CaCl_2$ in 80 mM KCl solution. The millivolt value of all milk samples was measured and recorded by dipping the electrode into the milk sample. The recorded millivolt value was converted to the ionic calcium level using a calibration curve obtained from the standard $CaCl_2$–KCl solution.

5.3.4. Size distribution of SSM

The particle size distributions of the SSMs before and after UHT treatment were determined using a MasterSizer 2000S (Malvern Instruments, Malvern, Worcestershire, England). The refractive index of the dispersant (reverse osmosis water) was set to 1.33 and the refractive index of the skim milk was set to 1.50. The particle absorption index was set to 0.001. The SSM was shaken well before measurement. The milk sample was added to the dispersion unit until an obscuration between 10 and 20% had been reached. Each sample was measured in triplicate at 20 °C. The results are shown as the average value of 3 measurements.

5.3.5. Separation of milk protein fractions

Unheated and heated SSMs were centrifuged to obtain different fractions of soluble protein. The large aggregates formed during heating were centrifuged at 3,000 × g for 10 min at 20 °C and the weight of wet sediment obtained after centrifugation was measured and analyzed by reversed-phase HPLC. To determine the amount of sediment, the liquid phase of centrifuged milk was decanted into a beaker and the centrifuge tube containing sediments was placed on the paper towel for 1 min to drain off the excess liquid. The container, including any sediment, was weighed and recorded.

Protein particles smaller than 100 nm were obtained by ultracentrifuging the milk samples at 48,800 × g for 26 min at 20 °C using a Sorval WX 80+ Ultracentrifuge (Thermo Fisher Scientific Inc.; Waltham, MA). This centrifugation condition has been proven to efficiently remove colloidal stable casein micelles while retaining soluble proteins (< 20 nm) and submicellar particles (20–100 nm, mainly consisting of κ-casein/whey protein complexes with comparably small amounts of calcium-sensitive caseins) in the supernatant (Dumpler et al., 2017). After ultracentrifugation, the resultant supernatants, which are defined as the serum phase in this study, were analyzed by reversed-phase HPLC.

5.3.6. Protein composition analysis

Milk and the supernatants obtained from centrifuged milk samples were analyzed by reversed-phase HPLC using a reversed-phase C18 column (Aeris Widepore 3.6 µm XB-C18 RP, Phenomenex, Torrance, CA) to determine the protein composition, as described by Bobe et al. (1998). The quantities of whey proteins and caseins in the supernatants were determined by comparing the relative peak area of the supernatant fraction in the heated SSM with that in the original unheated SSM. All peak areas of these chromatograms were obtained using peak integration algorithm LabSolutions software (Shimadzu Corporation, Kyoto, Japan).

5.3.7. Statistical analysis

All experiments reported were repeated 3 times using freshly collected sheep milk samples, and the results are given as the mean ± standard deviation. GraphPad Prism 8.4.0 was used to plot the data (GraphPad Software, San Diego, CA). At a significance level of $P < 0.05$, one-way and two-way ANOVAs, as well as Tukey's multiple comparison test, were used in the statistical analysis.

5.4. Results and discussion

5.4.1. Stability of UHT-treated SSM

5.4.1.1. Heat-induced protein aggregation in SSM

Figure 5-1A shows the macrostructures of the SSMs at different pH values before and after UHT treatment. Large amounts of coagulated material were found immediately in the SSM at the natural pH (~ 6.6) after the UHT treatment. However, there was no visible coagulation in the UHT-treated SSMs at pHs 6.7, 6.8, and 6.9. The UHT-treated SSM at pH 7.0 had visible coagulation but the size of those coagulates appeared to be much smaller than that formed in the UHT-treated SSM at the natural pH (~ 6.6).

5.4.1.2. Particle size distribution of SSM

Figure 5-1B shows the particle size distributions of SSMs at different pH values before and after UHT treatment. For unheated SSM, only one peak in the particle size range 0.02–0.30 μm was observed. Two major peaks were observed in UHT-treated SSM: the left-hand peak (peak 1) ranged from 0.02 to 1 μm and the right-hand peak (peak 2) ranged from ~ 10 to 2,000 μm. After UHT treatment, peak 2 of the SSM at the natural pH (~ 6.6) appeared at the rightmost position and peak 1 was significantly smaller, indicating that the proportion of small protein particles was reduced, and that the protein had formed large aggregates (ranging from ~ 10 to 1,445 μm), which is in agreement with the changes in appearance observed in Figure 5-1A. The UHT-treated SSM at pH 7.0 showed a similar pattern to the UHT-treated SSM at the natural pH (~ 6.6) but with less peak-shifting (the large aggregate particle size ranged from ~ 20 to 180 μm). The UHT-treated SSMs at pHs 6.7–6.9 had similar particle size distributions after UHT treatment, showing that peak 1 was increased and that peak 2 (large aggregates ranging from ~ 10 to 100 μm) was reduced, compared with the UHT-treated SSMs at the natural pH (~ 6.6) and pH 7.0. The results of

the particle size distribution are in agreement with the visual appearances presented in Figure 5-1A.

5.4.1.3. Ionic calcium level–pH profile in SSM

The ionic calcium level–pH profiles of the SSMs before and after UHT treatment are shown in Figure 5-1C. The highest ionic calcium level (~ 2.2 mM) of the SSMs was found at the natural pH (~ 6.6). After adjustment of the pH, the ionic calcium level decreased with increasing pH, with levels of ~ 1.8, 1.6, 1.4, and 1.2 mM for pHs 6.7, 6.8, 6.9, and 7.0, respectively. There was a negative linear relationship between the ionic calcium level and the pH for the SSMs, as found for cow milk (Lewis et al., 2011, Gaur et al., 2018). Previous studies have shown that ionic calcium can be complexed with inorganic or organic phosphate with increasing pH because of reduced solubility of calcium phosphate, thereby decreasing the ionic calcium level in cow milk (Ho et al., 2018; Vaia et al., 2006). The complexation of ionic calcium with phosphate could be accompanied by the drawing casein-bound calcium and the increased negative charge of proteins, consequently weakening the internal micellar structure and affecting the stability of casein micelles during heating (Horne, 2016).

After UHT treatment of the SSMs at pHs ranging between 6.6 and 7.0, the ionic calcium levels decreased by ~ 4–7% (Figure 5-1C). This was similar to previous studies on cow milk, which showed that the UHT treatment of cow milk decreased the ionic calcium level by 5% (Chen et al., 2015). However, the pH of the SSM decreased by around 0.03–0.1 units in the pH range 6.6–7.0 after UHT treatment, which was different from the reports on cow milk that UHT treatment has little effect on its pH (Chen et al., 2015, Deeth and Lewis, 2016). There is little information on the reduction in the pH of UHT milk. Pyne and McHenry (1955) and Van Boekel et al. (1989) presumed that heat-induced acidity of the

milk in the temperature range 100–130 °C was due to the thermal decomposition of lactose, displacement of the calcium phosphate equilibrium, and the liberation of phosphate from the casein micelles with subsequent precipitation of the released phosphate as tertiary calcium phosphate. As the lactose contents of SSM (Table 5-1) and cow milk (Balthazar et al., 2017) are similar, the breakdown of lactose should not be the main reason for the pH decrease in UHT-treated SSM. Belec and Jenness (1962) investigated the dephosphorization of caseins in skim milk heated at 140 °C and showed that a higher protein content increased the rate of dephosphorization. This indicates that the higher protein content of SSM might produce more phosphate and thus result in more precipitation of tertiary calcium phosphate during UHT treatment, leading to a decrease in its pH.

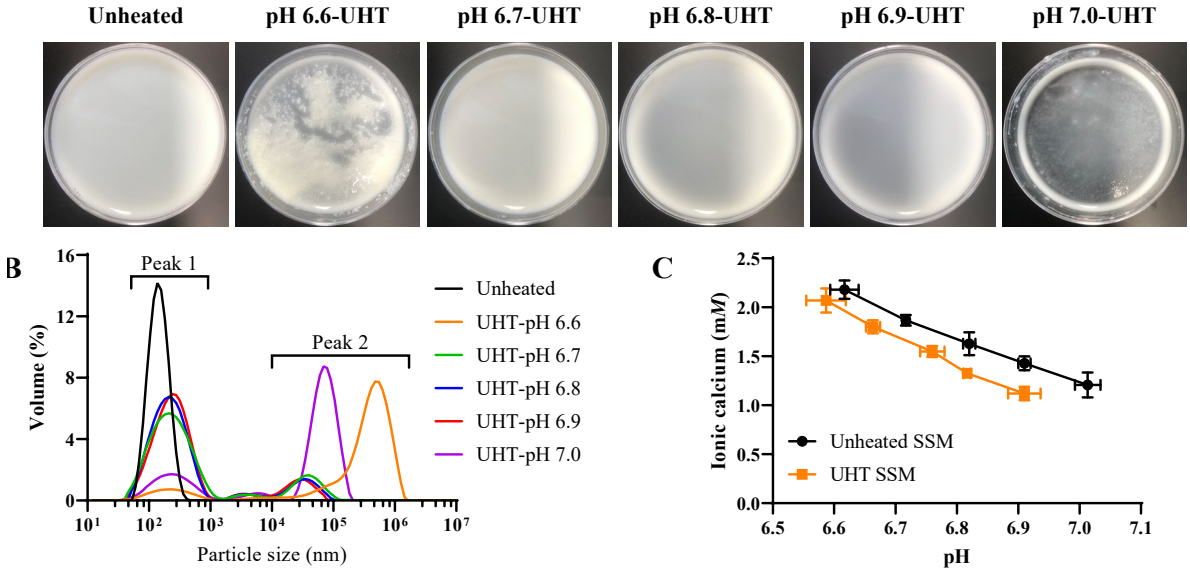

Figure 5-1. (A) Appearances of unheated sheep skim milk (SSM) at the natural pH (~ 6.6) and UHT-treated SSMs at different pH values. (B) Particle size distributions of unheated SSM and UHT-treated SSMs at different pH values. (C) Ionic calcium level as a function of the pH profile in SSM before and after UHT treatment. Error bars represent standard deviations.

5.4.2. Characterization of sediments

5.4.2.1. Weight of sediments of UHT-treated SSM

The weights of the sediments obtained from centrifuging the SSMs at 3,000 × g for 10 min are shown in Figure 5-2A. The UHT-treated SSM at the natural pH (~ 6.6) had the greatest sediment weight, and there was a significant decrease ($P < 0.0001$) in the sediment weights as the pH increased to pH 6.8. However, upon further increase in the pH of the SSMs, the sediment weights increased significantly ($P < 0.0001$). There was a remarkable increase in the sediment weight for the SSM at pH 7.0. The results suggested that the SSMs at the natural pH (~ 6.6) and pH 7.0 formed a large amount of aggregate during UHT treatment, and that the amount of aggregate could be reduced by adjusting the pH of the SSM to a range from pH 6.7 to pH 6.9.

Previous studies on the UHT treatment of cow milk demonstrated that the sedimentation increased when the pH was lowered, or the ionic calcium level was increased, or both (Lewis et al., 2011, Gaur et al., 2018). The results of this study showed a similar pattern when the pH of the SSM was increased from the natural pH (~ 6.6) to pH 6.7, which decreased the ionic calcium level from ~ 2.2 to 1.8 mM (Figure 5-1C), leading to a marked decrease in sedimentation (Figure 5-2A). Coagulation of proteins has been reported to occur during UHT processing when the pH of cow milk is lower than ~ 6.5, and this is believed to be due to high ionic calcium levels in the milk (Beliciu et al., 2012; Deeth & Lewis, 2015). The present study showed that the ionic calcium level in the SSM at the natural pH (~ 6.6) was higher than 2.0 mM and could cause instability of the caseins in the UHT milk, which is in agreement with previous results on cow milk (Lewis et al., 2011). However, a further increase in the pH from 6.9 to 7.0 significantly ($P < 0.01$) decreased the ionic calcium level to ~ 1.2 mM (Figure 5-1C) and markedly increased the sedimentation (Figure

5-2A). This is different from the previous findings for cow milk, which showed no increase in sedimentation when the pH was further increased or the ionic calcium level was decreased (Lewis et al., 2011, Gaur et al., 2018). It is unclear why sheep milk produced significant amounts of sediment at a more basic pH. A possible explanation is that the protein content (5.9%, Table 5-1) and the ionic calcium level (1.2 mM) at pH 7.0 of the SSM were higher than those (3.3% for protein content, < 0.7 mM for ionic calcium level at pH ~ 7.0) of cow milk, as reported previously (Gaur et al., 2018). The higher protein content in the SSM could give the proteins a greater probability of contacting each other and the ionic calcium level of the SSM at pH 7 might be still high enough for the formation of large protein aggregates during heating (Singh, 2004). An alternative explanation is that the casein composition of sheep milk is different from that of cow milk, which may affect protein interactions during UHT treatment (Amigo et al., 2000; Li, Delger, et al., 2022). The protein interactions are further discussed in the following sections.

These results suggested that there was a boundary between UHT-treated SSMs that produced high amounts of sediment and those that produced very low amounts of sediment. A small alteration in the pH values at the boundary region could convert SSMs from those that generate low amounts of sediment to those that generate high amounts of sediment or vice versa. This is generally in agreement with the previous reports on cow milk, which showed that there was a sharp boundary between stability (pH > 6.55 or ionic calcium level < 2.0 mM) and instability (pH < 6.55 or ionic calcium level > 2.0 mM) towards sedimentation (Lewis et al., 2011). It is possible that the attractive force and electrostatic repulsion of SSM casein micelles at pH 6.7–6.9 reach a critical level that prevents the aggregation of casein micelles as the pH adjustment, heat-induced deposition of calcium phosphate onto the micelles and association of whey proteins with casein micelles can alter the surface charge or provide steric repulsion for micelles; when the attractive and repulsive

forces exceed the critical level (possibly at pH < 6.7 or > 6.9), protein aggregation would occur (Dumpler et al., 2020).

5.4.2.2. Protein composition of sediments

The protein compositions of the sediments obtained from UHT-treated SSM are shown in Figure 5-2B. The protein composition of the SSM was ~ 78.8% α_S-casein (α_{S1}- + α_{S2}-casein) and β-casein, ~ 5.2% κ-casein, and ~ 16.0% whey proteins (α-LA + β-LG). After UHT treatment, the sediment of the SSM at all pH values showed similar protein composition with higher concentrations of α_S-casein and β-casein (89.0–93.1% combined) and lower concentrations of α-LA, β-LG (5.0–9.1% combined), and κ-casein (1.6–2.5% κ-casein) compared with the unheated SSM. These results suggested that the sediments of the UHT-treated SSMs were composed mainly of α_S-casein and β-casein with low levels of κ-casein and whey proteins regardless of the pH values, although significant differences in the amounts of sediment were observed between samples with different pHs (Figure 5-2A). The protein composition of sediment is in line with previous reports for cow milk, which showed that the sediment formed from UHT-treated cow milk was composed of κ-casein–depleted casein micelles with low concentrations of denatured whey proteins (Malmgren et al., 2017, Gaur et al., 2018). κ-casein at the surface of casein micelles is thought to limit their self-association, contributing to their remarkable stability (De Kruif et al., 2012). Therefore, casein micelles depleted in κ-casein could be less stable when the SSM pH is lower or the ionic calcium level is higher than a critical level, resulting in casein micelle aggregation and sedimentation (Anema, 2018a).

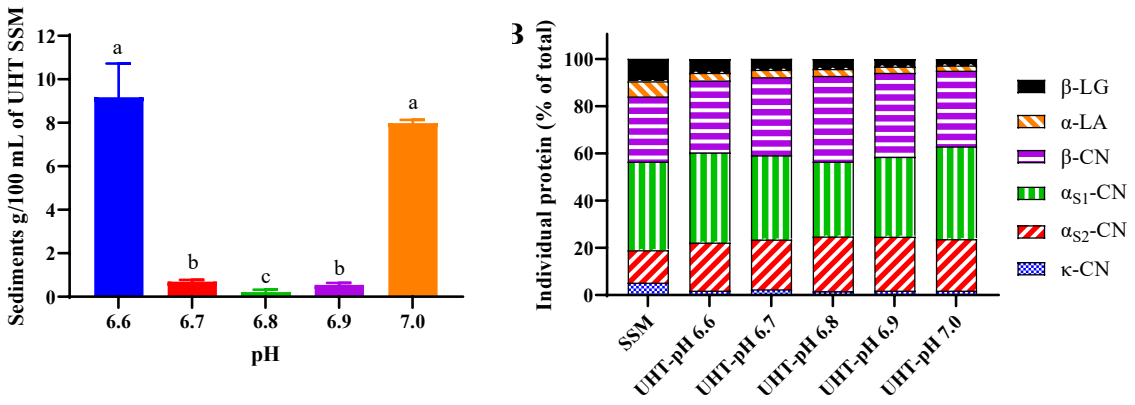

Figure 5-2. Weights of sediments obtained from UHT-treated SSMs after centrifugation at 3,000 × g for 10 min (A). Percentages of β-LG (black), α-LA (orange), β-casein (purple), α_{S1}-casein (green), α_{S2}-casein (red), and κ-casein (blue) in sheep skim milk (SSM) and sediments of UHT-treated SSMs at the natural pH (~ 6.6) and pHs 6.7–7.0 (B). Different lowercase letters above the column indicate significant differences ($P < 0.05$). Error bars represent standard deviations.

5.4.3. Characterization of serum proteins

5.4.3.1. Protein content of SSM serum phase

Figure 5-3 shows the protein contents of the serum phases obtained by centrifuging the SSMs at 48,000 × g for 26 min before and after UHT treatment. The protein contents of the serum samples of the unheated SSMs did not show significant differences ($P > 0.05$) between different pHs. In contrast, the serum-phase protein content of the UHT-treated SSMs increased with increasing pH, and the UHT-treated SSMs at pH 6.9 and pH 7.0 had significantly ($P < 0.05$) higher serum protein content than the UHT-treated SSM at the natural pH (~ 6.6). This is in agreement with previous results for cow milk, which showed that the concentration of protein in the serum phase increased as the pH of cow skim milk was increased after heat treatment in the pH range of 6.3–7.1 (Anema and Klostermeyer,

1997). Therefore, the increased serum-phase protein content in the UHT-treated SSMs might be attributed to the increasing dissociation of caseins from the casein micelles during heating (Anema and Klostermeyer, 1997).

The serum-phase protein contents of the SSMs at the natural pH (~ 6.6) and pH 6.7–6.8 decreased significantly ($P < 0.05$) after UHT treatment, whereas those of the UHT-treated SSMs at pH 6.9–7.0 were not significantly different ($P > 0.05$), compared with those of the unheated SSM (Figure 5-3). The decreased protein contents of the UHT-treated SSM serums could be attributed to the cosedimentation of denatured whey proteins with casein micelles on ultracentrifugation (Anema, 2020). In contrast, an increasing proportion of denatured whey proteins would remain soluble at higher pH values. More κ-casein would dissociate from the casein micelles during heating milk at higher pHs and still complex with the denatured whey proteins, and these denatured whey proteins complexed with κ-casein would still remain soluble in the serum phase (Anema and Klostermeyer, 1997) and thus increase the protein content of the SSM serum after UHT treatment. This probably narrows the differences in the protein contents of the serums between unheated and UHT-treated SSMs at pH 6.9 and pH 7.0. The detailed changes in the individual proteins of the serum are discussed in the following sections.

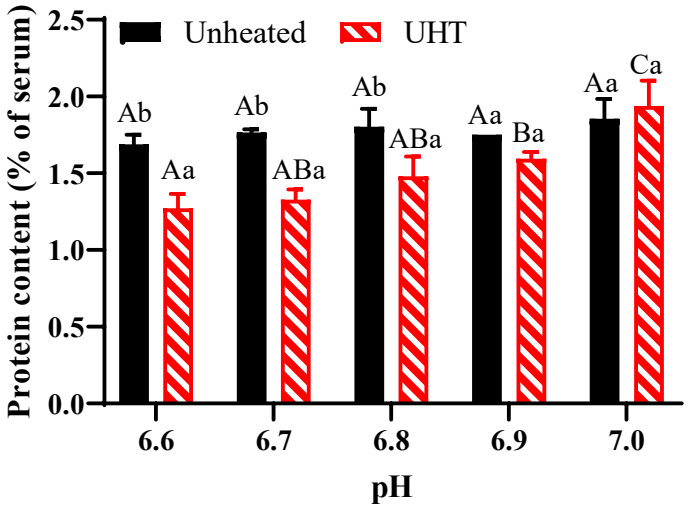

Figure 5-3. Protein contents of the supernatant in unheated (solid black bars) and UHT-treated (red striped) sheep skim milks (SSMs) obtained from centrifugation at 48,800 × g for 26 min. Different capital and lowercase letters represent significant differences ($P < 0.05$) among different pHs and between unheated and UHT-treated SSMs, respectively. Error bars represent standard deviations.

5.4.3.2. Dissociation of caseins from casein micelles

The changes in individual proteins of the supernatants obtained from the centrifugation of the SSMs at 48,800 × g for 26 min were quantitatively analyzed before and after UHT treatment and are shown in Figure 5-4. For both unheated and UHT-treated SSM, increasing the pH of the SSM significantly increased the levels of serum-phase κ-, β-, and α_{S2}-casein. This indicated that increasing the pH of the SSM resulted in the dissociation of κ-, β-, and α_{S2}-casein from the casein micelles into the serum phase in all SSMs.

The percentages of serum-phase κ-casein, β-casein, and α_{S2}-casein were significantly higher in UHT-treated SSMs than in the untreated SSMs at all pHs. However,

the percentage of α_{S1}-casein in the serum phase did not show significant differences between unheated and UHT-treated SSM regardless of pH values (Figure 5-4C). These results indicated that UHT treatment of SSM resulted in the dissociation of κ-, β-, and α_{S2}-casein from the casein micelles into the serum phase at all pHs but had little impact on the dissociation of α_{S1}-casein.

There is no detailed information on compositional changes in the individual caseins of heated sheep milk. The results observed in the current study are generally in agreement with previous reports on cow skim milk, which showed that the dissociation levels of α_S-casein, β-casein, and κ-casein increased with increasing pH (from pH 6.5 to pH 6.9) in the temperature range 20–120 °C (Anema and Klostermeyer, 1997, Anema, 1998). Additionally, caseins are more negatively charged at alkaline pH, which would enhance the electrostatic repulsion between the individual submicelles (Sinaga et al., 2017). The enhanced repulsive forces could lead to a looser casein micelle structure, which could contribute to the easy dissociation of caseins from the micelles (Liu & Guo, 2008; Madadlou et al., 2009). Therefore, the greater dissociation of α_S-casein, β-casein, and κ-casein at higher pH could have been due to the enhanced electrostatic repulsion between the individual caseins at alkaline pH.

Although there are extensive reports on the pH-dependent dissociation of micellar casein in heated cow milk (Anema et al., 1993; Anema & Li, 2000; Anema & Stanley, 1998; Kudo, 1980; Singh & Creamer, 1991a, 1991b; Singh & Fox, 1985), comparisons with the present study are limited as these previous studies examined the dissociation behavior in cow milk mainly at lower heating temperature (< 120 °C). Only a few publications have reported the dissociation behavior of micellar caseins in cow milk after UHT treatment, but these reports focused on the dissociation behavior of the caseins in cow milk at the natural

pH. Li et al. (2019) showed that ~ 30% of κ-casein dissociated from the casein micelles after heating cow skim milk at 140 °C for 5 s. Liu et al. (2019) reported that ~ 31% of κ-casein, ~ 1.1% of β-casein, ~ 12% of α_{S2}-casein, and ~ 6% of α_{S1}-casein dissociated from the casein micelles in cow milk after indirect UHT treatment at 141 °C for 2 s. Akkerman et al. (2021) showed that UHT treatment (141 °C/4 s) of cow skim milk at the natural pH resulted in the dissociation of ~ 9% of κ-casein, ~ 15% of β-casein, and ~ 5% of α_{S1}-casein from the micelles. In the current study, ~ 55% κ-casein (of total κ-casein) in UHT-treated SSM at natural pH (~ 6.6) was presented the the serum phase, indicating that ~ 39% κ-casein (by comparing with ~ 16% κ-casein in the serum of unheated SSM at natural pH) was dissociated from casein micelles after UHT treatment (Figure 5-4A). In comparison with the previous studies cited above, the heat-induced dissociation of κ-casein in UHT SSM at natural pH presented here is much higher than those in UHT-treated cow milk. It has been proven that a higher concentration of total solids increases the extent of dissociation of κ-casein (Singh and Creamer, 1991b, Anema, 1998). Therefore, the greater dissociation of κ-casein in the UHT-treated SSM can probably be attributed to the higher total solids and protein contents in sheep milk.

As the remarkable stability of the casein micelles relies on the κ-casein at the surface of the micelle, the removal of κ-casein from the micelles can induce their aggregation via calcium bridging when the ionic calcium level is higher than a critical level (Dalgleish, 1992, Anema, 2018a, Huppertz et al., 2018). Hence, the greater dissociation levels of κ-casein observed in the UHT-treated SSMs than in UHT-treated cow skim milk might reduce the protective effects of the κ-casein hairy layers on the casein micelles, leading to the increased aggregation of the casein micelles in SSM during UHT treatment. Noteworthy, significantly ($P < 0.05$) higher levels of κ-casein dissociated from the casein micelles after UHT treatment for the SSM at pH 7.0 than for the UHT-treated SSMs at the natural pH (~

6.6) and pH 6.8 (Figure 5-4A). The higher proportions of β- and α_{S2}-casein dissociated from the casein micelles (Figures 5-4B and 5-4D) in the UHT-treated SSM at pH 7.0 would expose more internal structure of the casein micelles so that other proteins could interact with during heating, contributing to the aggregation of the micelles. It could conceivably be hypothesized that the high amount of sediment for the UHT-treated SSM at pH 7.0 (Figure 5-2A) was induced by the greater extent of dissociation of caseins and thus more interactions between the casein micelles. The different appearances and sizes of the aggregates of the UHT-treated SSMs at the natural pH (~ 6.6) and pH 7.0 (Figures 5-1A and 5-1B) might be explained by the different aggregation pathways (mainly calcium-bridging micelles because of higher concentration of ionic calcium for natural pH ~ 6.6 and more interactions among micelles because of casein dissociation for pH 7.0) among the casein micelles. More studies are needed to verify whether the aggregates in UHT-treated SSMs are formed in different ways at different pHs.

5.4.3.3. Interactions of whey proteins with caseins/casein micelles

The contents of both β-LG and α-LA in the serums of the UHT-treated SSMs decreased significantly ($P < 0.0001$) at all pHs compared with the unheated SSM (Figures 5-4E and 5-4F), indicating that a proportion of the serum-phase β-LG and α-LA were denatured and precipitated with the casein micelles on ultracentrifugation, probably by associating with the casein micelles (Pan et al., 2022), or by forming large whey protein aggregates, or both (Deeth and Lewis, 2017). This confirmed that the decreased protein content of the serum after UHT treatment (Figure 5-3) could be attributed to the precipitation of the denatured whey proteins on ultracentrifugation.

The content of serum α-LA and β-LG remained unchanged in the unheated SSM, despite the changes in the pH (Figures 5-4E and 5-4F). For the UHT-treated SSMs, the

level of serum-phase α-LA remained nearly unchanged with increasing pH, indicating that increasing the pH of the SSM had little impact on the denaturation and interaction of serum-phase α-LA during UHT treatment. In contrast, the serum-phase β-LG of the UHT-treated SSMs showed an increasing trend as the pH was increased. Similar results have also been reported for cow milk, which showed that the level of whey protein/κ-casein complexes was found to increase in heated cow skim milk when its pH was raised in the range pH 6.3–7.3 (Kudo, 1980, Singh and Fox, 1985, Singh and Latham, 1993). It has been shown that pH changes in milk (pH 6.0–9.0) have little impact on the denaturation rate of α-LA and β-LG (Hillier & Lyster, 1979); therefore, in this study, similar proportions of α-LA and β-LG would be expected to be denatured at all pH values and still complexed with κ-casein (Anema and Klostermeyer, 1997). However, as an increased proportion of κ-casein dissociated from the casein micelles at higher pH (Figure 5-4A), the denatured whey proteins complexed with dissociated κ-casein would also remain soluble in the serum.

It is not clear whether the whey proteins of the complexes in UHT-treated milk samples are composed mainly of β-LG or β-LG plus α-LA. The present study showed that only the serum-phase β-LG levels increased with increasing pH in the UHT-treated SSMs, which does not support the results reported by Anema and Klostermeyer (1997), who showed that increasing the pH (pH 6.3–7.2) of cow skim milk increased the levels of both α-LA and β-LG in the serum phase after heating at 80–90 °C for 15 min. The differences between sheep milk and cow milk might be due to the different heating methods used or to the different structural properties of the whey proteins. Previous studies reported that the dissociation of κ-casein preceded the denaturation of β-LG and that the denatured whey proteins favored interactions with serum-phase κ-casein (Anema, 2008). Additionally, β-LG was denatured and associated with the casein micelles at a faster rate than α-LA in both sheep skim milk and cow skim milk heated at 80–130 °C (Oldfield, Singh, Taylor, et al.,

1998; Pan et al., 2022a). It is possible that a large amount of denatured β-LG complexed with the dissociated κ-casein during the initial stages of UHT treatment before α-LA began to denature to any significant extent. In the meantime, as β-LG complexed with κ-casein became less accessible for the interaction with denatured α-LA, the lately denatured α-LA might be able to associate with other proteins (remaining unassociated denatured β-LG, caseins, or casein micelles) only via either sulfhydryl–disulfide or hydrophobic interactions. Moreover, at higher pH of the SSM, a greater amount of dissociated κ-casein might result in higher levels of complexation between β-LG and κ-casein in the serum phase, leading to less β-LG being available for α-LA to interact with (Anema, 2008). The denatured α-LA thus presumably interacted with other proteins mainly via hydrophobic bonds and formed large aggregates during heating. Further investigations are required to verify this hypothesis.

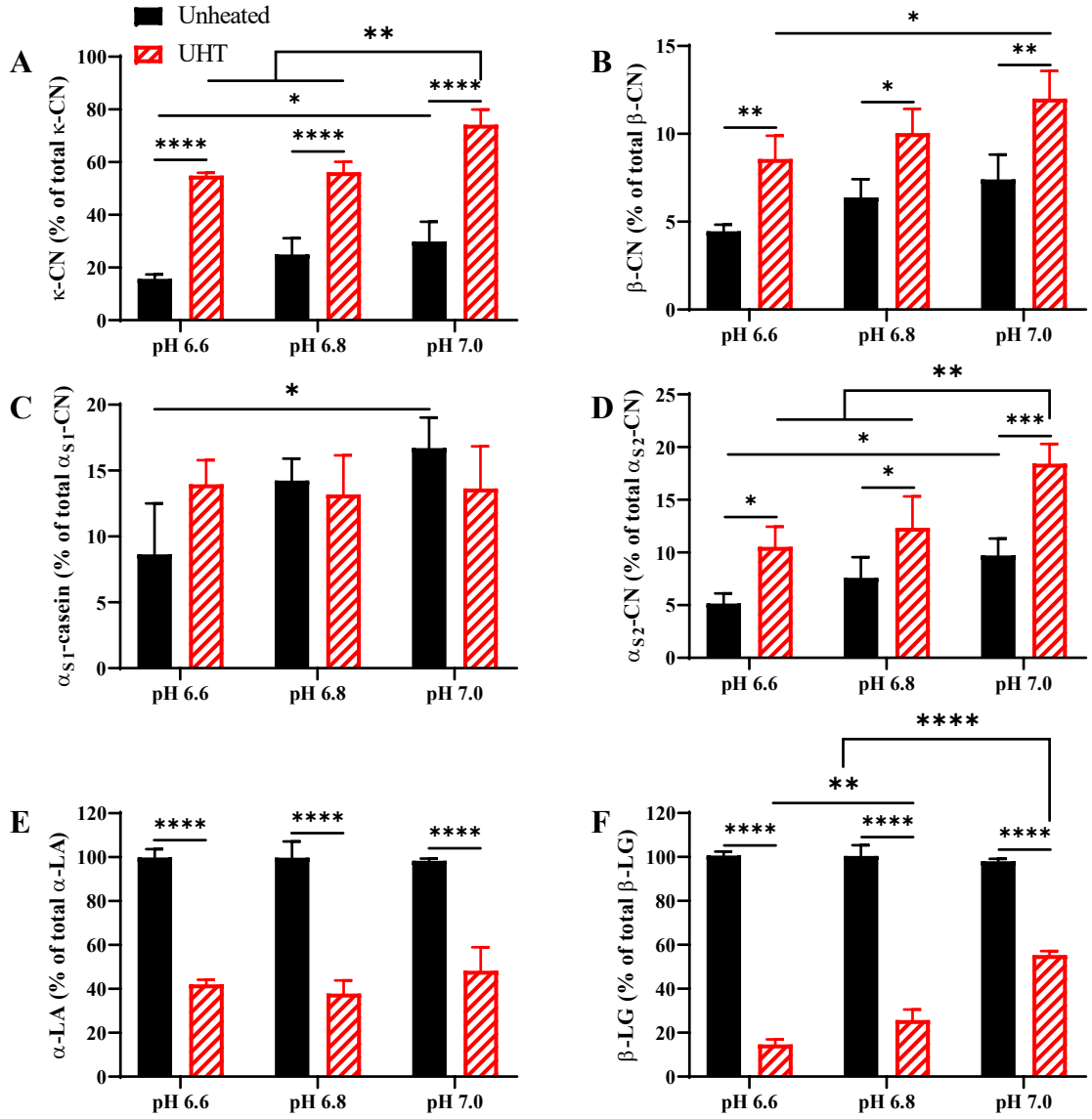

*Figure 5-4. Levels of individual proteins [κ-casein (κ-CN), A; β-casein (β-CN), B; αS1-casein (αS1-CN), C; αS2-casein (αS2-CN), D; α-lactalbumin (α-LA), E; β-lactoglobulin (β-LG), F] in the supernatants obtained by centrifuging unheated (solid black bars) and UHT-treated (red striped) sheep skim milks at 48,800 × g for 26 min. Error bars represent standard deviations. *, **, ***, and **** represent P values smaller than 0.05, 0.01, 0.001, and 0.0001, respectively.*

5.5. Conclusions

This study demonstrated hat the instability of sheep milk during UHT treatment was pH dependent; a large amount of aggregates formed during UHT treatment of the SSM at the natural pH (~ 6.6). The instability of SSM was pH dependent and the amount of sedimentations for UHT-treated SSM was significantly higher at the natural pH (~ 6.6) and pH 7.0 than at pH 6.7–6.9. There was a sharp boundary between stability (pH 6.7–6.9) and instability (pH < 6.6 and pH > 7.0) of the UHT-treated SSMs towards sedimentation. The sediment was composed mainly of κ-casein-depleted casein micelles with very low levels of whey proteins, regardless of the SSM pH. The high sedimentation in the UHT-treated SSM at the natural pH (~ 6.6) could probably be attributed to the high ionic calcium level and the significant dissociation of κ-casein from the casein micelles, whereas the high amount of sedimentation formed at pH 7.0 might be due to the greater extent of dissociation of κ-, β-, and α_{S2}-casein from the casein micelles, leading to reduced protection by the κ-casein hairy layer and the exposure of the internal structure of the casein micelles, and thus their aggregation. The greater dissociation of caseins from the casein micelles could be attributed to the higher protein content of the SSM, enhanced electrostatic repulsive forces with increasing pH, and the intense heating temperature that overcomes the forces maintaining micellar integrity and ensures dissociation. As adjusting the pH of milk may not be permissible in the industry, the selection of sheep milk samples with naturally more basic pH, or less ionic calcium, or both, or the addition of food stabilizers to the sheep milk may be necessary to produce UHT-treated sheep milk products with low sedimentation levels.

Chapter 6: Statement of Contribution – Doctorate with Publications/Manuscripts

DRC 16

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Zheng Pan
Name/title of Primary Supervisor:	Professor Aiqian Ye
In which chapter is the manuscript /published work:	Chapter 6

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Pan, Z.; Ye, A.; Li, S.; Dave, A.; Fraser, K.; Singh, H., Dynamic in vitro gastric digestion of sheep milk: Influence of homogenization and heat treatment. Foods 2021, 10 (8), 1938. DOI: https://doi.org/10.3390/foods10081938
Li, S., Pan, Z., Ye, A., Cui, J., Dave, A., & Singh, H. (2022). Structural and rheological properties of

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Candidate's Signature:	Zheng Pan Digitally signed by Zheng Pan Date: 2023.02.21 17:04:14 +13'00'
Date:	21-Feb-2023
Primary Supervisor's Signature:	Aiqian Ye Digitally signed by Aiqian Ye DN: cn=Aiqian Ye, c=NZ, o=Massey University, ou=SF&AT, email=a.m.ye@massey.ac.nz Date: 2023.02.22 12:42:19 +13'00'
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GRS Version 5 – 13 December 2019
DRC 19/09/10

Chapter 6. Dynamic *in vitro* gastric digestion of sheep milk: Influence of homogenization and heat treatment

The contents of this chapter have been published in Foods and Food Hydrocolloids (open access):

Pan, Z., Ye, A., Li, S., Dave, A., Fraser, K., & Singh, H. (2021). Dynamic In Vitro Gastric Digestion of Sheep Milk: Influence of Homogenization and Heat Treatment. Foods, 10(8), 1938. doi:10.3390/foods10081938

Li, S., Pan, Z., Ye, A., Cui, J., Dave, A., & Singh, H. (2022). Structural and rheological properties of the clots formed by ruminant milks during dynamic in vitro gastric digestion: Effects of processing and species. Food Hydrocolloids, 126, 107465. doi: 10.1016/j.foodhyd.2021.107465

6.1. Abstract

Milk is commonly exposed to processing including homogenization and thermal treatment before consumption, and this processing could have an impact on its digestion behavior in the stomach. In this study, we investigated the *in vitro* gastric digestion behavior of differently processed sheep milks. The samples were raw, pasteurized (75 °C/15 s), homogenized–pasteurized, and homogenized–heated (95 °C/5 min) milks. The digestion was performed using a dynamic *in vitro* gastric digestion system, the human gastric simulator. The pH, structure, and composition of the milks in the stomach and the emptied digesta, and the rate of protein hydrolysis were examined. Curds formed from homogenized and heated milk had much looser and more fragmented structures than those formed from unhomogenized milk; this accelerated the curd breakdown and protein digestion and promoted the release of protein, fat, and calcium from the curds into the digesta. Coalescence and flocculation of fat globules were observed during gastric digestion, and

most of the fat globules were incorporated into the emptied protein/peptide particles in the homogenized milks; this affected the gastric emptying rate of fat into the small intestine.

6.2. Introduction

Sheep milk is of high nutritional value and has potential for the development of nutritional and functional milk products, attracting a growing number of consumers worldwide (Wendorff & Haenlein, 2017). Milk, as an important source of protein for humans, has been widely examined for its digestion behavior in both *in vivo* and *in vitro* studies (Gallier et al., 2013; Mulet-Cabero et al., 2019; Ye et al., 2016b). The digestion of cow milk has been investigated extensively, whereas the digestion of non-cow milk (i.e., sheep milk) is less studied.

Sheep milk and cow milk vary significantly in composition, physicochemical properties, and structures, which may potentially lead to different digestion behaviors within the gastrointestinal tract and the bioavailability of nutrients (Wendorff & Haenlein, 2017). Jasińska (1995) conducted a study to examine the hydrolysis of the casein micelles in the raw milks from 4 species (human, goat, mare, and two breeds of cow) and showed that the degrees of hydrolysis of the caseins by pepsin were 80%, 65%, 45%, 42%, and 23% for human, goat, mare, black and white cow, and red polish cow milk, respectively. Jasińska (1995) attributed the differences in casein hydrolysis in the milks from different species to the different physicochemical properties and compositions of the caseins such as micellar structure and different levels of β-casein. Previous studies have also shown that the different compositions of milk proteins can result in different digestion behaviors (Roy et al., 2021b; Shen et al., 1996; Ye, Cui, et al., 2019). For instance, goat milk has lower α_{S1}-casein content and higher β-casein content than cow milk, and infant formulas made from goat milk formed smaller flocs of aggregated proteins and fat globules during *in vitro* gastric digestion,

resulting in faster protein digestion in the infant formula made with goat milk than in that made with cow milk (Claeys et al., 2014; Ye, Cui, et al., 2019). Sheep milk has markedly higher levels of β- and α_{S2}-casein but lower levels of α_{S1}-casein than cow milk, which may potentially affect its coagulation behavior and protein hydrolysis in the stomach (Balthazar et al., 2017). Previous research comparing the *in vitro* gastric digestions of cow, goat, and sheep milks found that the curds formed from sheep skim milk had higher total solids and lower moisture contents than those formed from cow and goat skim milks because of their different chemical compositions, resulting in a firmer curd from the sheep skim milk (Roy et al., 2021b).

Milk is commonly exposed to different processing treatments (i.e., pasteurization and homogenization), which leads to structural changes in its components (i.e., protein and fat). For instance, the heat treatment of milk could result in a series of protein–protein and protein–lipid interactions and changes, depending on the heat intensity level (Anema, 2007; Anema & Li, 2003a; Remeuf et al., 1989; Ye, Singh, Taylor, et al., 2004). The homogenization of milk increases the stability of the milk fat globules because of a decrease in fat globule size and the adsorption of caseins and whey proteins onto the surface of the newly formed milk fat globules (Ye et al., 2008). Additionally, homogenization coupled with the heat treatment of milk increases the association of denatured whey proteins with the adsorbed caseins and milk fat globule membrane (MFGM) proteins via disulfide bonds, leading to alteration of the interfacial composition of the fat globules (Michalski & Januel, 2006). In turn, these changes in the milk components could have an impact on the digestion behavior of milk within the gastrointestinal tract.

Roy et al. (2021a) investigated the effect of pasteurization on the *in vitro* gastric digestion of milks from cow, goat, and sheep and found that all pasteurized milks formed less integrated curds than their raw milk counterparts, resulting in a greater extent of

deformation and thus higher levels of fat release into the liquid phase. However, the effect of homogenization and intensive heat treatment on sheep milk has not been investigated. There has been extensive research on the *in vitro* digestion of cow milk treated with more intense heat treatment. The curds formed from intensively heated cow milk were more fragmented and crumbly compared with the more cohesive curds formed from unheated or pasteurized milk, which was attributed to the differences in the structural changes in the milk components that were induced by the different processing treatments (Mulet-Cabero et al., 2019; Ye, Liu, et al., 2019). The content and the structure of the curds formed from homogenized cow milk during gastric digestion also showed differences compared with those formed from raw cow milk. Ye et al. (2017) reported that homogenized milk formed an integrated curd but with a more porous structure than that formed from untreated whole milk in the early stage of *in vitro* gastric digestion, and that the curd became less integrated and was separated into several small pieces at longer digestion times. Milks treated with a combination of homogenization and heat treatment were digested more effectively than those treated with either heat treatment or homogenization alone (Mulet-Cabero et al., 2019; Ye et al., 2017). For example, Ye et al. (2017) investigated the effects of homogenization and heat treatment on the formation of curds during the *in vitro* gastric digestion of whole cow milk and observed that homogenization of the milk followed by heat treatment resulted in the formation of curds with more fragmented and crumbly structures than those formed from raw and singly homogenized whole milk. These differences in the digestion behaviors of differently processed milks suggest that they are likely to have different physiological effects within the gastrointestinal tract. Therefore, the effect of homogenization and intensive heat treatment on the digestion behavior of sheep milk was investigated.

In this study, both untreated and processed (homogenization, pasteurization, and heat treatment at 90 °C for 5 min) sheep milks were digested using a dynamic *in vitro* gastric

digestion model (a human gastric simulator, HGS) to investigate the effects of homogenization, heat treatment, and the combination of homogenization and heat treatment on their gastric digestion behaviors, including coagulation, curd structure, protein digestion, and the release of protein and fat from the stomach.

6.3. Materials and methods

6.3.1. Milk supply and processing treatments

Fresh sheep milk was collected from Spring Sheep Milk Co. and Maui Milk Co., Ltd, Waikato, New Zealand, during mid-lactation; the milks collected from the two companies were mixed at a ratio of 1:1. Pasteurization of the sheep milk was carried out at 75 °C for 15 s in a pilot-scale indirect UHT plant (Alfa-Laval, Huntingwood, NSW, Australia). The homogenized milk was obtained by homogenizing raw sheep milk at 200/50 bar and 65 °C in a 2-stage valve homogenizer in the Massey University pilot plant. In the experiments, the homogenized sheep milk was pasteurized at 75 °C for 15 s to make homogenized–pasteurized (homo–past) sheep milk; the homogenized and heated (homo–heat) sheep milk was obtained by heating to reach 95 °C in the UHT plant and then transferred to a water bath for holding for 5 min (the heating condition at 95 °C/5 min is commonly used for manufacturing yoghurt in dairy industry). After heat treatment, these milk samples were immediately cooled to 20 °C. The average fat globule sizes (d_{43}) of the milk samples, which were determined using a Mastersizer 2000 (Malvern Instruments Ltd, Malvern, UK), were 4.52 ± 0.14 μm and 0.62 ± 0.07 μm for the unhomogenized and homogenized sheep milks, respectively.

6.3.2. Chemicals for in vitro gastric digestion

Pepsin from porcine gastric mucosa (EC 3.4.23.1; Catalogue No. P7000, Sigma Chemical Co., St. Louis, MO, USA) had an enzymatic activity of 550 units/mg solid, as

tested in preliminary experiments. All other chemicals were of analytical grade and were purchased from BDH Chemicals (BDH Ltd, Poole, UK) and Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified. All solutions were prepared using Milli-Q water purified by treatment with a Milli-Q apparatus (Millipore Corp., Bedford, MA, USA).

Simulated salivary fluid (SSF) and simulated gastric fluid (SGF) were prepared at 1.25× concentration according to the method described by Brodkorb et al. (2019) with slight modifications. SSF was prepared based on the salt composition only, as described in Brodkorb et al. (2019) because milk contains no starch. The SGF (pH 1.5) did not include gastric lipase because this study focused on protein digestion, and its pH was changed in order to drop the pH of whole sheep milk to pH 2 in 4 h (Brodkorb et al., 2019). $CaCl_2(H_2O)_2$ was added into the SSF and the SGF immediately before the digestion experiment to achieve final concentrations of 1.5 mM and 0.15 mM, respectively. Furthermore, the SSF and SGF were supplemented with water to achieve a 1× concentration before addition into the stomach chamber.

6.3.3. In vitro gastric digestion

The HGS developed by Kong and Singh (2010) was employed for the *in vitro* gastric digestion of the sheep milks. The method described by Ye et al. (2017) was used for the gastric digestion with slight modifications. A 200 g sheep milk sample pre-warmed at 37 °C was firstly mixed with an amount of SSF that equaled the total solids content of the milk samples (i.e., 18.5 g of solids in the sheep milk to 18.5 g of SSF), and then transferred and warmed in the HGS at 37 °C for 2 min; a 20 mL fasting solution containing SGF (16 mL) and pepsin solution (4 mL, 10,000 units/mL, prepared in water) was added into the mixture of milk sample and SSF (Lydon et al., 1999; Vertzoni et al., 2005); the SGF and the pepsin solution (10,000 units/mL) were then added using two separate pumps at flow rates of 2.4

mL/min and 0.6 mL/min, respectively, to achieve a 1× concentration of SGF and a pepsin activity of 2,000 units/mL; the gastric emptying rate was 3.6 mL/min; the emptied digesta were removed from the bottom of the stomach chamber at 20 min intervals for accurate control of the gastric emptying. To mimic the contraction and temperature of the stomach, the contraction frequency of the HGS was set at 3 times/min and the temperature was maintained at 37 °C using a heater and a thermostat. The gastric digestion time was up to 240 min, but most of the experiments were stopped at different times to collect the coagulated milk curds for further analysis. The digesta removed from the HGS at each time interval were filtered through a mesh with a pore size diameter of 1 mm for further analysis, and the solid mass with size greater than 1 mm was put back into the HGS for further digestion. The processing and the digestion experiments were triplicated with 3 different batches of raw sheep milk.

6.3.4. pH measurement

The pH of the mixture of milk and SSF was defined as the initial pH in the HGS. The pHs of the emptied digesta at different digestion time points refer to the pH in the HGS as the simulated gastric contraction prevented easy access into the gastric chamber for direct determination using the pH meter.

6.3.5. Weight of curds

The content within the HGS at different digestion time points was collected and filtered through a sieve mesh with a 1-mm pore size to separate the curd and the aqueous phase. Curd larger than 1 mm was then immediately rinsed with pepsin-free SGF and weighed to obtain the weight of the curds. Subsequently, these curds were heated at 90 °C for 5 min to inactivate the pepsin and then dried at 105 °C for 24 h in a vacuum oven, so that the weight of total solids of the curd could be determined.

6.3.6. Calcium, protein, and fat content analysis

The total calcium content of the dried curds was determined by inductively coupled plasma optical emission spectroscopy after the curd powder had been dissolved and digested by nitric and hydrochloric acids. The protein and fat contents of the curds and digesta obtained at different digestion times from different milk samples were determined using the Dumas method (AOAC 968.06) and the Mojonnier method (AACC 30-10) (Standard, 1987). The protein content was calculated using a conversion factor of 6.25 for multiplication of the nitrogen content of the curds and digesta.

6.3.7. Protein hydrolysis

The protein compositions of the curds and the emptied digesta were determined using tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE). The sample buffer (containing 40% glycerol, 2% SDS, 0.04% Coomassie Brilliant Blue G-250, 5% β-mercaptoethanol, and 20% 0.2 M Tris-HCl, pH 6.8) was mixed with liquid digesta to achieve a protein concentration of 1 mg/mL. For the solid curd samples, lyophilized and ground curd powder was dissolved in 1 mL of sample buffer to achieve a protein concentration of 1 mg/mL. These mixtures were then heated at 90 °C for 5 min, and 7 μL of these heated mixtures was loaded in the wells of an electrophoresis gel that was pre-made using a Mini-PROTEAN electrophoresis system. The electrophoresis gel was composed of resolving gel (16% acrylamide, glycerol, 3 M Tris-HCl buffer, pH 8.45) and 4% stacking gel (4% acrylamide, 3 M Tris-HCl buffer, pH 6.8). Two running buffers were employed to separate low-molecular-mass proteins/peptides with high resolution: an anode buffer (0.2 M Tris-HCl, pH 8.9) and a cathode buffer (0.1 M Tris base, 0.1 M tricine, and 0.1% SDS, pH 8.25). After running at a constant voltage of 120 V for around 2 h, the gel was fixed using 5% glutaraldehyde with constant gentle shaking for 25 min. The fixed gel was stained for 20 min using 0.03% Coomassie Brilliant Blue G-250 solution (0.3 g of

Coomassie Brilliant Blue G-250 in 1 L of 10% glacial acetic acid solution) and then destained with destaining solution (10% glacial acetic acid solution) for 2 h to remove excess dye. The protein patterns of the gels were imaged and analyzed using a Molecular Dynamics Model PD-SI computing densitometer (Molecular Dynamics Inc., Sunnyvale, CA, USA).

6.3.8. Microstructure of curds and digesta

A confocal laser scanning microscope (Leica Microsystems Pty Ltd, Heidelberg, Germany) was used to study the microstructure of the digesta obtained from the digestion of the milks. The curd and digesta samples were stained and observed immediately without inactivating the pepsin after collection, according to the method described by Wang et al. (2019). The fluorescent dyes Nile Red and Fast Green were used to stain the fat (argon laser with an excitation line at 488 nm) and the protein (He–Ne laser with excitation line at 633 nm), respectively. A 200 μL aliquot of digesta was transferred into an Eppendorf tube and then mixed with 5 μL of 1.0% (w/v) Fast Green and 10 μL of 0.1% (w/v) Nile Red. The samples were stained for at least 5 min. For curd samples, an aliquot was taken using a blade and stained with 1.0% Fast Green and 0.1% Nile Red for at least 10 min. The stained samples were placed on concave confocal microscope slides (Sail; Sailing Medical-Lab Industries Co. Ltd, Suzhou, China), covered with a coverslip, and examined with ×40 and ×100 oil immersion lenses.

6.3.9. Statistical analysis

Each experiment was performed at least twice using freshly prepared milk samples. Data plotting and statistical analysis were performed using GraphPad Prism 8.4.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed using one-way analysis of variance and Tukey's multiple comparison test at a significance level of *P*

< 0.05. Correlations between protein release and fat release from the curds were determined using nonlinear regression analysis. The results are presented as the mean ± standard deviation.

6.4. Results and discussion

6.4.1. Gastric pH profile

The pH profiles of the milk samples in the HGS are shown in Figure 6-1. The pHs of all milk samples decreased progressively, reaching a pH etween 2 and 3 at 240 min of digestion. However, the pHs at 80–200 min of digestion showed statistically significant differences ($P < 0.05$) among the milk samples, except for the pHs of the homo–past and homo–heat milks ($P > 0.05$). The homogenized milk samples (homo–past and homo–heat) had a markedly slower decrease in pH with the digestion time, reaching about pH 2.6 at 240 min of digestion, compared with the unhomogenized milk samples (pH 2.0 and pH 2.3 for the raw and pasteurized milks, respectively). There was also a difference in the pH-decrease patterns between the unheated and heated milk samples; the effect of stronger heating conditions on the pH profiles was more marked, resulting in a slower decrease in pH. These differences were probably related to the formation of curds with different structures in the differently treated milk samples, which might lead to different diffusion rates of molecules and ions from the SGF into and out of the curds.

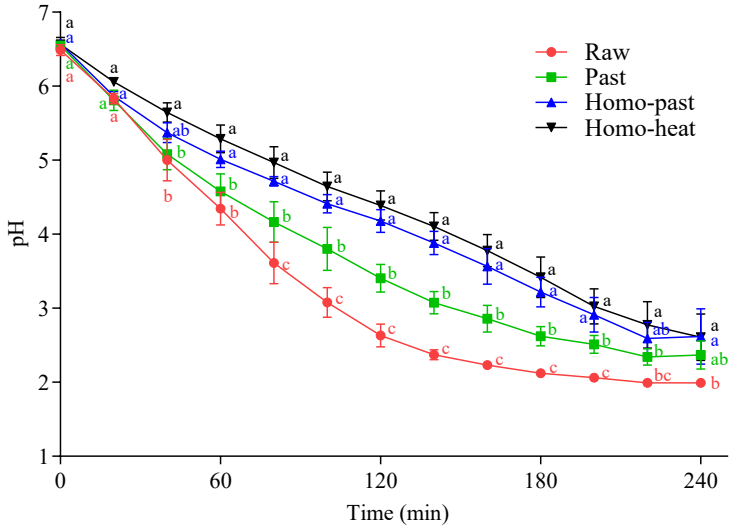

Figure 6-1. pH changes during the in vitro gastric digestion of differently processed sheep milks: ●, raw milk; ■, pasteurized (Past) milk; ▲, homogenized and pasteurized (homo–past) milk; ▼, homogenized and heated (homo–heat) milk. Error bars represent standard deviations.

6.4.2. Gastric coagulation of sheep milk

The appearance of the curds formed at different digestion times from differently processed sheep milks is shown in Figure 6-2. In all milk samples, protein coagulation was visible immediately after the addition of the milk into the SGF (20 mL of SGF with a pepsin activity of 2,000 units/mL). After 60 min of digestion, a firm curd with a smooth surface was observed in both the raw milk and the pasteurized milk; at this stage, the serum phase became clear, indicating that most of the casein micelles and fat globules were incorporated into the curd. With further digestion to 180 min, the firm curds formed from the raw milk samples became smaller but remained integrated, whereas the curds formed from the pasteurized milk samples became less integrated and were broken into several small pieces with various sizes (Figure 6-2). However, the curds from the homo–past and homo–heat

milks appeared to be more fragmented and looser than those from the raw and pasteurized milks throughout the gastric digestion. In the homo–heat sheep milk, the curd crumbles were much looser, smaller, and more evenly fragmented than those from the homo–past milk. These results indicated that homogenization and different levels of heat treatment of sheep milk could lead to the formation of differently structured curds.

Previous studies have suggested that the formation of curds in the stomach is initially driven by the enzymatic action of pepsin on κ-casein (Delfour et al., 1966; Mulvihill & Fox, 1979; Ye et al., 2016b). The present study showed that the coagulation of sheep milk occurred immediately after the mixing with SGF, in which the pH in the system was greater than 6. The result was in agreement with previous findings for cow milk (Ye et al., 2016b, 2017).

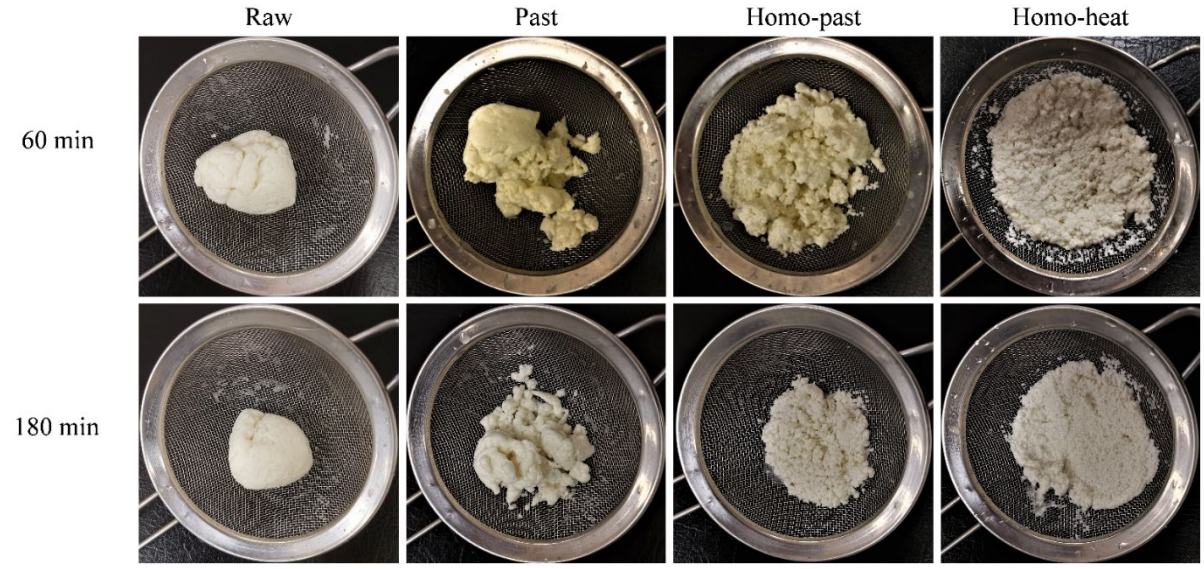

Figure 6-2 Appearance of curds collected at 60 and 180 min during the in vitro gastric digestion of raw, pasteurized (Past), homogenized and pasteurized (homo–past), and homogenized and heated (homo–heat) sheep milks.

6.4.3. Microstructure of curds

The structures of the milk curds formed from the raw, pasteurized, homo–past, and homo–heat sheep milks at different digestion times were determined using confocal microscopy (Figure 6-3). For all milk samples before digestion, the fat globules were evenly distributed in the protein aqueous phase, and the fat globule size in the homogenized milks appeared to be much smaller than that in the unhomogenized milks. At 60 min of digestion, a closely-knit network of protein matrix was observed in all milk samples, and many fat globules were also found within the protein matrix. Additionally, the size of the fat globules within the curd became larger during the digestion progress, compared with that in undigested milk samples, suggesting that aggregation and/or coalescence of the fat globules occurred because of the effect of hydrolysis by pepsin on the membrane proteins surrounding the fat globules (Gallier et al., 2012a; Ye et al., 2011).

The small fat globules of the homo–past and homo–heat sheep milks appeared to be well embedded within the protein matrix, whereas the fat globules of the raw and pasteurized milks seemed to be entrapped within the curd without much contact with the protein network (Figure 6-3). The difference between the homogenized and unhomogenized sheep milk samples was probably related to the changes in the structure of the casein micelles and the interfacial protein composition of the fat globules that were caused by homogenization and heat treatment. Homogenization of milk leads to increases in the total surface area of the fat globules and the adsorption of caseins and whey proteins (Kelly et al., 2008; Ye et al., 2008). These proteins that coat the smaller globules may interact with the protein network within the curd, leading to the structural changes in the curds during the gastric digestion (Mulet-Cabero et al., 2019). Further, more intense heat treatment of the homogenized sheep milk could result in a higher level of association of denatured whey proteins with the casein micelles and with MFGM proteins, which may

reduce the casein–casein interactions and casein–fat interactions and thus hinder the aggregation and coagulation of milk proteins (Ye, Liu, et al., 2019). It appears that these structural changes of the fat globules and proteins induced by homogenization and more severe heat treatment were responsible for the formation of the differently structured curds, as observed in Figure 6-2.

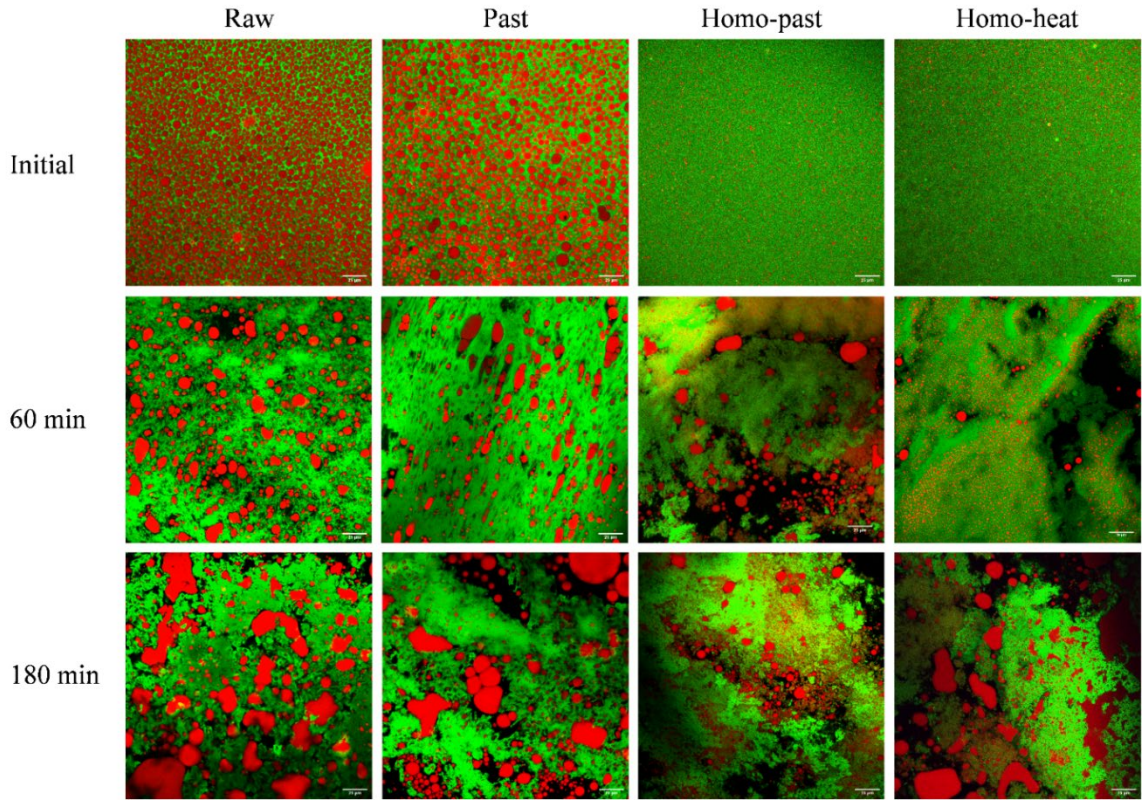

Figure 6-3 Confocal micrographs of curds obtained at 60 and 180 min during in vitro gastric digestion of raw, pasteurized (Past), homogenized and pasteurized (homo–past), and homogenized and heated (homo–heat) sheep milks. Red shows the fat and green shows the protein. The scale bar in all images is 25 μm.

6.4.4. Disintegration of curds

6.4.4.1. Weight of curds

The changes in the content of the curds formed from the different sheep milk samples are shown in Figure 6-4. The weights of the curd and the total solids of the curds in all milk samples decreased gradually throughout the gastric digestion (Figures 6-4A and 6-4B). The initial (20 min) weights of the curds and the total solids of the curds followed the order raw < pasteurized < homo–past < homo–heat, indicating that homogenization and more intense heating resulted in the incorporation of more milk components into the curds. This was attributed to the whey proteins that associated with the casein micelles during the heat treatment (Ye, Liu, et al., 2019). Statistical analysis showed that there were significant differences in the weights of the curds between the homogenized (homo–past and homo–heat) milks and the unhomogenized (raw and pasteurized) milks ($P < 0.01$) and between the homo–past and homo–heat milks at 20 min ($P < 0.05$), whereas there were no significant differences in the weights of the total solids of the curds ($P > 0.05$), suggesting that more moisture might be retained in the curds formed from the homogenized and heated sheep milks. This result was in agreement with previous results, which showed that curds with a looser structure contained more liquid (Ye et al., 2016a; Ye, Liu, et al., 2019). Thus, a curd with higher moisture content might contain more SGF and pepsin. With further digestion, the weights of the curds and the total solids of the curds decreased much faster in the homo–past and homo–heat sheep milks than in the raw and pasteurized sheep milks. Significant differences between each time point in the weights of the curds and the total solids were observed in the homo–past and homo–heat sheep milks ($P < 0.05$) but not in the raw and pasteurized sheep milks. At 240 min of digestion, both the weight of the curds and the weight of the total solids showed a reverse order compared with their initial weights: raw > pasteurized > homo–past > homo–heat. These results suggested that pasteurization alone of

sheep milk did not have much impact on the breakdown of the curd compared with the raw sheep milk; pasteurization combined with homogenization significantly accelerated the disintegration of the sheep milk curds; more intense heat treatment of the homogenized sheep milk did not further impact the breakdown of the curds compared with the homo–past sheep milk but resulted in a significantly higher weight of the curds at 20 min.

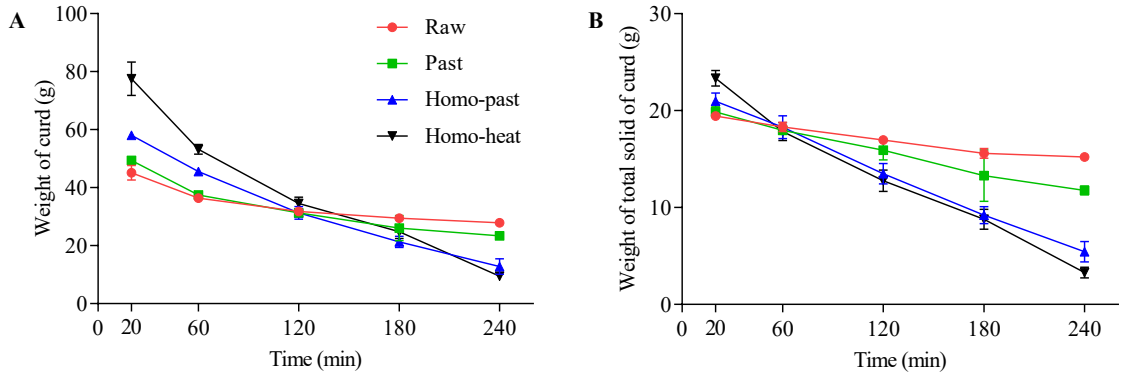

Figure 6-4 Changes in (A) the weight of the curds and (B) the weight of the total solids of the curds at different time points (20–240 min) during the in vitro gastric digestion of differently processed sheep milks: ●, raw milk; ■, pasteurized (Past) milk; ▲, homogenized and pasteurized (homo–past) milk; ▼, homogenized and heated (homo–heat) milk. Error bars represent standard deviations.

6.4.4.2. Changes in protein, fat, and calcium contents in the curd

Figure 6-5A shows the protein content of the dried curds as the function of digestion time in the 4 different sheep milks. The protein contents of the raw and pasteurized milks remained nearly constant at around 43% during the digestion period, whereas those of the homo–past and homo–heat sheep milks showed a decreasing trend as the digestion progressed, especially after 60 min. For the homo–past milk, the protein content of the curds

decreased in the first 120 min, after which it remained roughly unchanged at around 36% until the end of digestion. The decreasing trend for the protein content of the homo–heat milk continued to 180 min (from ~ 40% to ~ 30%). The fat content of the curds is shown in Figure 6-5B. Similar to the protein content, there were no obvious changes in the fat content of the curds of the unhomogenized milks throughout the gastric digestion, with a fat content of ~ 45% for the raw milk and of ~ 46% for the pasteurized milk. However, the fat contents of the curds of the homogenized milks showed an opposite trend compared with their protein counterparts. The fat contents of the curds increased for 120 min and 180 min of digestion for the homo–past milk (from 47.8% to 55.6%) and the homo–heat milk (from 43% to 63.8%), respectively, after which the level remained almost unchanged.

The relationships between the amounts of protein and fat in the curds are shown in Figure 6-5C. The slope of the regression line for the raw and pasteurized sheep milks was close to 1, indicating that the amount of fat in the curds was nearly the same as the amount of protein in the curds at different time points. Similar results have been reported for the gastric digestion of unheated and heated cow milks (Ye et al., 2016a); the slopes of the regression lines for the fat–protein profiles of the curds formed from these milk samples were close to 1. This indicates that the fat was evenly distributed in the curds and was released from the curds in equal proportion to the protein. However, a nonlinear correlation between the fat and protein in the curds was found for the homo–past and homo–heat sheep milks, suggesting that fat and protein had different rates of release from the curd. The amounts of fat and protein in the homo–past and homo–heat sheep milks were significantly lower than those in the raw and pasteurized sheep milks at 240 min of digestion (the leftmost points in Figure 6-5C) ($P < 0.05$), indicating that the release of fat and protein occurred more rapidly in homogenized sheep milk than in unhomogenized sheep milk.

The homo–past and homo–heat sheep milks had higher initial weights of the fat in the curds than the raw and pasteurized sheep milks (the rightmost point in Figure 6-5C). Previous studies have suggested that newly formed fat globules in milk after homogenization are covered by caseins and whey proteins and are able to act as pseudo-protein particles that can interact with the protein phase during coagulation, becoming an integral part of the protein matrix and thus resulting in higher fat contents of the curds (Lopez, 2005; Ong et al., 2011). The microstructures of the curds (Figure 6-3) also confirmed these findings. Further, the inclusion of smaller fat globules in the protein matrix can soften the casein network because the homogeneously distributed small fat globules in the protein network result in a larger intermicellar distance and thus hinder the fusion and syneresis of the casein matrix (Guinee & O'Brien, 2010; Kelly et al., 2008).

The calcium contents of the dried curds obtained from the 4 different types of sheep milk are presented in Figure 6-5D. The calcium content in all curds decreased with increasing digestion time, but the rates of decrease were different between the unhomogenized and homogenized milks. The calcium contents of the dried curds in the raw and pasteurized milks decreased steadily over the digestion time, whereas those in the homo–past and homo–heat milks decreased rapidly in the first 60 min and then slowly in the following 120 min. These results suggest that calcium was gradually released from the curd to the liquid phase as the digestion time increased. It has been suggested that bound calcium solubilizes when the pH of the curds decreases to below 5.6, resulting in the release of the solubilized calcium into the liquid phase with the progress of the digestion (Everett & Auty, 2008). The fast release of calcium from the curds of the homo–past and homo–heat sheep milks could be attributed to the faster diffusion of the acid (SGF) into the curds because of their fractured and looser structure (Figure 6-2). In comparison, the lower rate of calcium release from the curds of the raw and pasteurized sheep milks was due to the

formation of an integrated curd surface barrier (Figure 6-2) that probably impeded the flowing SGF.

These results suggest that the release of the curd contents (protein, fat, and calcium) was markedly dependent on the structure of the curds. The curd formed from the raw and pasteurized sheep milks was more integrated and firmer, which may have impeded the diffusion of the SGF and pepsin into the curds, resulting in a slower breakdown of the curd structure and a slower release of the curd contents. In contrast, the looser and crumbled structures of the curds of the homo–past and homo–heat sheep milks were easily deformed by the physical forces of gastric contraction, leading to a faster release from the curds. These results are in line with previous results on cow milk reported by Ye, Liu, et al. (2019), who showed that the homogenization of cow milk followed by heat treatment made the structure of the curds more open, leading to rapid loss of total solids and rapid release of fat from the curds during gastric digestion.

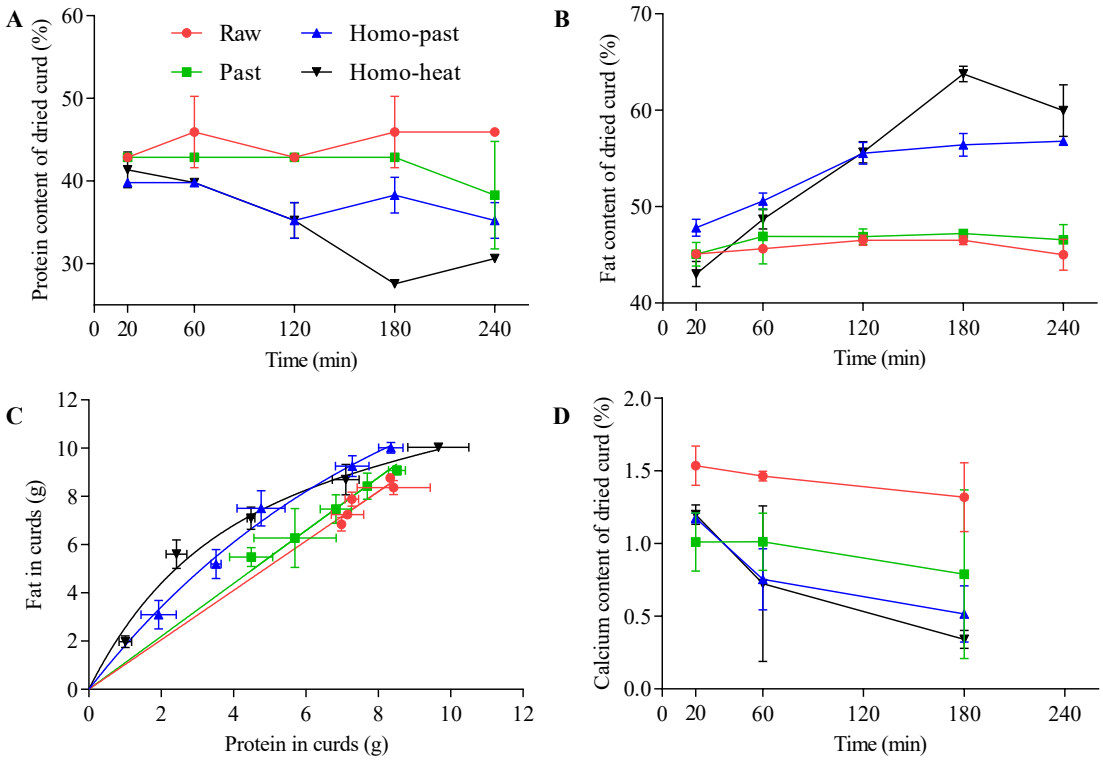

Figure 6-5 Changes in (A) the protein content of the dried curds and (B) the fat content of the dried curds, (C) the relationship between the amounts of fat and protein in the curds, and (D) the calcium content of the dried curds at different time points (20–240 min) during the in vitro gastric digestion of differently processed sheep milks: ●, raw milk; ■, pasteurized (Past) milk; ▲, homogenized and pasteurized (homo–past) milk; ▼, homogenized and heated (homo–heat) milk. Error bars represent standard deviations.

6.4.5. SDS-PAGE protein patterns of curds

The protein hydrolysis by pepsin in the curds was determined using tricine SDS-PAGE (Figure 6-6A). The amount of each individual protein in the curds is shown in Figure 6-6B. The κ-casein band disappeared and 2 new bands at ~ 23 kDa (macropeptides) and 14 kDa (para-κ-casein) appeared in all samples. Interestingly, unlike the α-lactalbumin (α-La)

band, which disappeared in all curd samples, a band corresponding to β-lactoglobulin (β-Lg) was observed throughout the digestion in all types of sheep milk. At 20 min, the amounts of β-Lg in the curds were raw (~ 0.26 g) < pasteurized (~ 0.31 g) < homo–past (~ 0.4 g) < homo–heat (~ 1.03 g) (Figure 6-6B), indicating that greater heat treatment incorporated more β-Lg into the curds. The small amount of whey proteins observed in the curds of the raw, pasteurized, and homo–past milk samples could have been caused by the entrapment of whey proteins during the formation of the curds; they could be expelled from the curds and hydrolyzed gradually as the digestion progressed (Roy et al., 2021a; Ye et al., 2016a). The markedly higher β-Lg content observed in the curd formed from the homo–heat milk was due to the higher level of denatured whey proteins associated with the casein micelles and the MFGM proteins (Anema & Li, 2003a; Ye et al., 2008).

The changes in the intensities of all intact protein bands, including α_S-casein, β-casein, and β-Lg, showed marked differences among the sheep milk samples. The intensities of these intact protein bands decreased slowly in the unhomogenized milks with increasing digestion time but faded away much faster in the homogenized milks (Figure 6-6A). Bands corresponding to peptides were detected in all milk samples after 60 min of digestion, with the homogenized milks having more intense peptide bands than the unhomogenized milks. This indicates that the homogenization of sheep milk followed by heat processing led to a greater digestibility of proteins during gastric digestion. Faster degradation of proteins has been shown to be caused by the looser and crumbly structure of curds, which allows pepsin to diffuse into the curds rapidly and to hydrolyze the proteins (Ye et al., 2017). These findings further support the faster breakdown of the curds and the release of fat and calcium from the curds in the homogenized and heated sheep milk because of the faster hydrolysis of proteins by pepsin, as observed earlier (Figures 6-4 and 6-5).

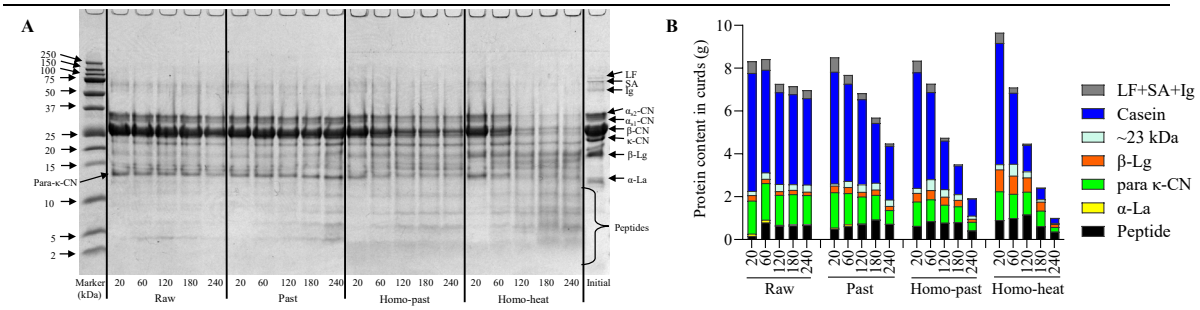

Figure 6-6 (A) Reducing tricine SDS-PAGE patterns and (B) protein contents of curds obtained from raw, pasteurized (Past), homogenized and pasteurized (homo–past), and homogenized and heated (homo–heat) sheep milks. The numbers (20, 60, 120, 180, 240) refer to different gastric emptying times (min). Initial refers to before digestion. The protein concentration in each sample for the tricine SDS-PAGE was 1 mg/mL.

6.4.6. Protein and fat contents of emptied digesta

The protein contents of the digesta emptied from the stomach at different digestion times from the different milk samples are shown in Figure 6-7A. The protein contents of the unhomogenized milks decreased gradually during 120 min of digestion, after which they remained roughly constant at ~ 0.41% for raw milk and ~ 0.69% for pasteurized milk towards the end of the digestion. However, the homo–past and homo–heat sheep milks showed the opposite trend, with the protein contents of the digesta initially increasing in the first 120 min of digestion, with little change on prolonged digestion. At digestion times of longer than 120 min, the protein contents of the digesta of the homo–past and homo–heat sheep milks were significantly higher than those of the raw and pasteurized milks ($P < 0.05$). The fat contents of the digesta are shown in Figure 6-7B. Those in the raw and pasteurized sheep milks decreased slightly during the first 120 min of digestion and then stayed almost unchanged on prolonged digestion, whereas the homo–past and homo–heat sheep milks showed an increasing trend over the whole digestion.

The changes in the protein contents of the digesta were in reasonable agreement with the release of protein from the curds (Figure 6-5). The rapid release of protein from the curds of the homo–past and homo–heat sheep milks (Figure 6-5) resulted in higher protein contents in the gastric chyme, thus leading to increased protein contents in the emptied digesta. In contrast, the slowly digested protein in the curds of the raw and pasteurized sheep milks could have resulted in lower levels of protein in the emptied digesta because of the dilution caused by the continuous addition of SGF. For the fat content, the trends were similar for the curds (Figure 6-5B) and the digesta (Figure 6-7B), which showed that the fat content increased throughout the digestion progress in the homo–past and homo–heat sheep milks, whereas it changed little in the raw and pasteurized sheep milks. The continuous increases in the fat contents of the emptied digesta for the homo–past and homo–heat sheep milks may have been caused by the phase separation between the water and oil phases in the stomach. Mulet-Cabero et al. (2019) found that the fat content at the end of digestion was generally higher in homogenized milk than in unhomogenized milk, as a cream layer formed at longer digestion times. Thus, the cream layer could remain in the stomach until the end of digestion, resulting in increases in the fat contents of both the curds and the digesta of the homo–past and homo–heat sheep milks at longer digestion times.

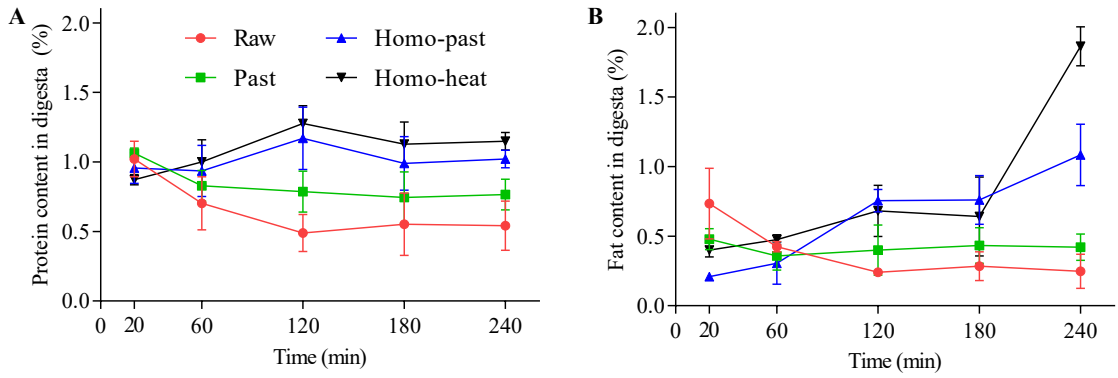

Figure 6-7 (A) Protein and (B) fat contents in the digesta emptied at different time points (0–240 min) during the in vitro gastric digestion of differently processed sheep milks: ●, raw milk; ■, pasteurized (Past) milk; ▲, homogenized and pasteurized (homo–past) milk; ▼, homogenized and heated (homo–heat) milk. Error bars represent standard deviations.

6.4.7. SDS-PAGE protein patterns of digesta

Figure 6-8A shows the protein patterns of the digesta obtained from the differently processed sheep milks. Faint intact casein bands were present at 20 min of digestion but disappeared after 60 min of digestion in the raw, pasteurized, and homo–past sheep milk samples, and bands corresponding to peptides (between 2 and 10 kDa) appeared concomitantly. This finding is consistent with previous studies, which reported that the casein bands in the digesta could be due to the delivery of very fine casein particles to the digesta when the curd lost its mass, and that increasing amounts of peptides were evacuated from the stomach because of hydrolysis of the caseins (Awad et al., 1998; Roy et al., 2021a; Ye et al., 2017). In contrast, the homo–heat milk samples showed more conspicuous intact casein bands in the digesta, the intensities of which were significantly higher than for the raw, pasteurized, and homo–past milk samples ($P < 0.01$) (Figure 6-8B). The difference

can be attributed to the looser and crumblier structure of the curds formed from the homo–heat milk, leading to the delivery of crumbly particles (containing mostly caseins) smaller than 1 mm to the small intestine.

Differences were also found for the bands at ~ 14, 18, and 23 kDa, which correspond to α-La, β-Lg, and macropeptides, respectively. Within the first 120 min of digestion, there were low intensities of these bands (especially β-Lg) in the homo–heat sheep milk, whereas markedly higher intensities of these bands were observed in the other three types of sheep milk (Figure 6-8A). About 0.32, 0.34, 0.28, and 0.08 g of β-Lg were detected in the digesta obtained at 20 min for the raw, pasteurized, homo–past, and homo–heat milks, respectively (Figure 6-8B). The lower β-Lg content in the digesta of the homo–heat milk was attributed to the higher amounts of β-Lg that were incorporated in the curds (Figure 6-6) because of their association with the casein micelles after more intense heat treatment (Anema, 2020). The β-Lg and α-La bands in all types of milk faded away gradually during the digestion and disappeared in the digesta emptied at 240 and 120 min, respectively, which is in agreement with the previous findings of Roy et al. (2021a). The decreased intensities of the β-Lg and α-La bands could have been due to the dilution by the continuous addition of SGF and the hydrolysis by pepsin when the pH was less than 4 (Wang et al., 2018).

These results suggested that, in the raw, pasteurized, and homo–past sheep milks, the protein emptied from the stomach was composed mainly of whey proteins in the early stages of digestion and consisted mainly of peptides at longer digestion times because of the digestion of protein by pepsin. In comparison, the protein in the homo–heat sheep milk was digested faster, leading to a predominant content of peptides in the digesta from the beginning of digestion.

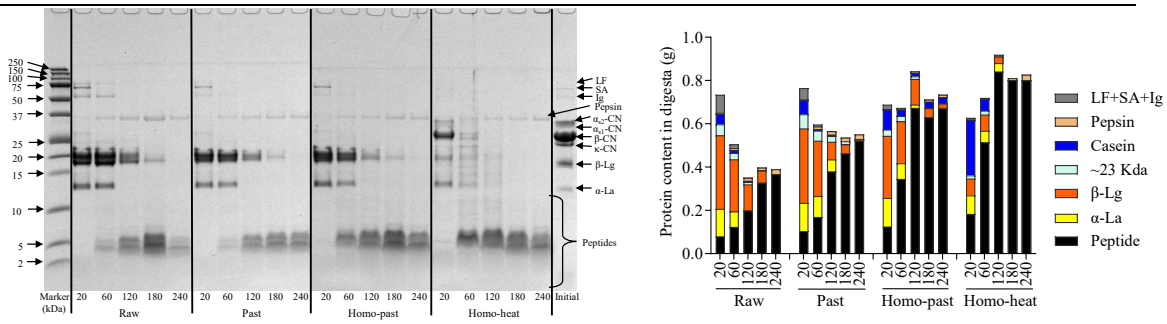

Figure 6-8 (A) Reducing tricine SDS-PAGE patterns and (B) protein contents of the digesta obtained from raw, pasteurized (Past), homogenized and pasteurized (homo–past), and homogenized and heated (homo–heat) sheep milks. The numbers (20, 60, 120, 180, 240) refer to different gastric emptying times (min). Initial refers to before digestion. The protein concentration in each sample for tricine SDS-PAGE was 1 mg/mL.

6.4.8. Microstructure of digesta

The microstructures of the fat and protein in the digesta were observed using confocal microscopy (Figure 6-9). There were large amounts of fat globules in the digesta of the raw and pasteurized sheep milks throughout the gastric digestion; the fat globule size appeared to remain unchanged during the first 120 min of gastric digestion but increased slightly at 240 min of gastric digestion. This is consistent with the previous report of Roy et al. (2021a), who also found that there were a few larger fat globules in the digesta of raw and pasteurized sheep milks at 90–240 min, which was caused by their coalescence after the MFGM proteins had been hydrolyzed by pepsin. Small-sized fat globules were found in the digesta of the homo–past and homo–heat sheep milks at 60 min of digestion, accompanied by some protein/peptide particles. There was an increasing number of protein/peptide particles with various sizes in the digesta of the homo–past and homo–heat sheep milks when the gastric digestion time was extended beyond 120 min. Interestingly,

the small fat globules were still well embedded in the protein/peptide particles in the digesta of the homo–past and homo–heat sheep milks at digestion times of longer than 120 min. In agreement with the present results, a previous study demonstrated that the peptides resulting from the hydrolysis of protein by pepsin formed a new surface layer on the fat globules, which was unable to create strong interfacial layers with sufficient electrostatic repulsion and steric barriers, thus resulting in flocculation of fat globules via protein–peptide or peptide–peptide interactions (Ye et al., 2011). Moreover, no individual fat globules were observed in the digesta of the homo–past and homo–heat sheep milks at digestion times longer than 120 min, suggesting that all fat globules had been incorporated into the protein/peptide particles. However, the incorporation of fat globules into protein/peptide particles may change the density of the particles, leading to the creaming of the less dense particles. This may support the occurrence of phase separation in the stomach, as mentioned earlier.

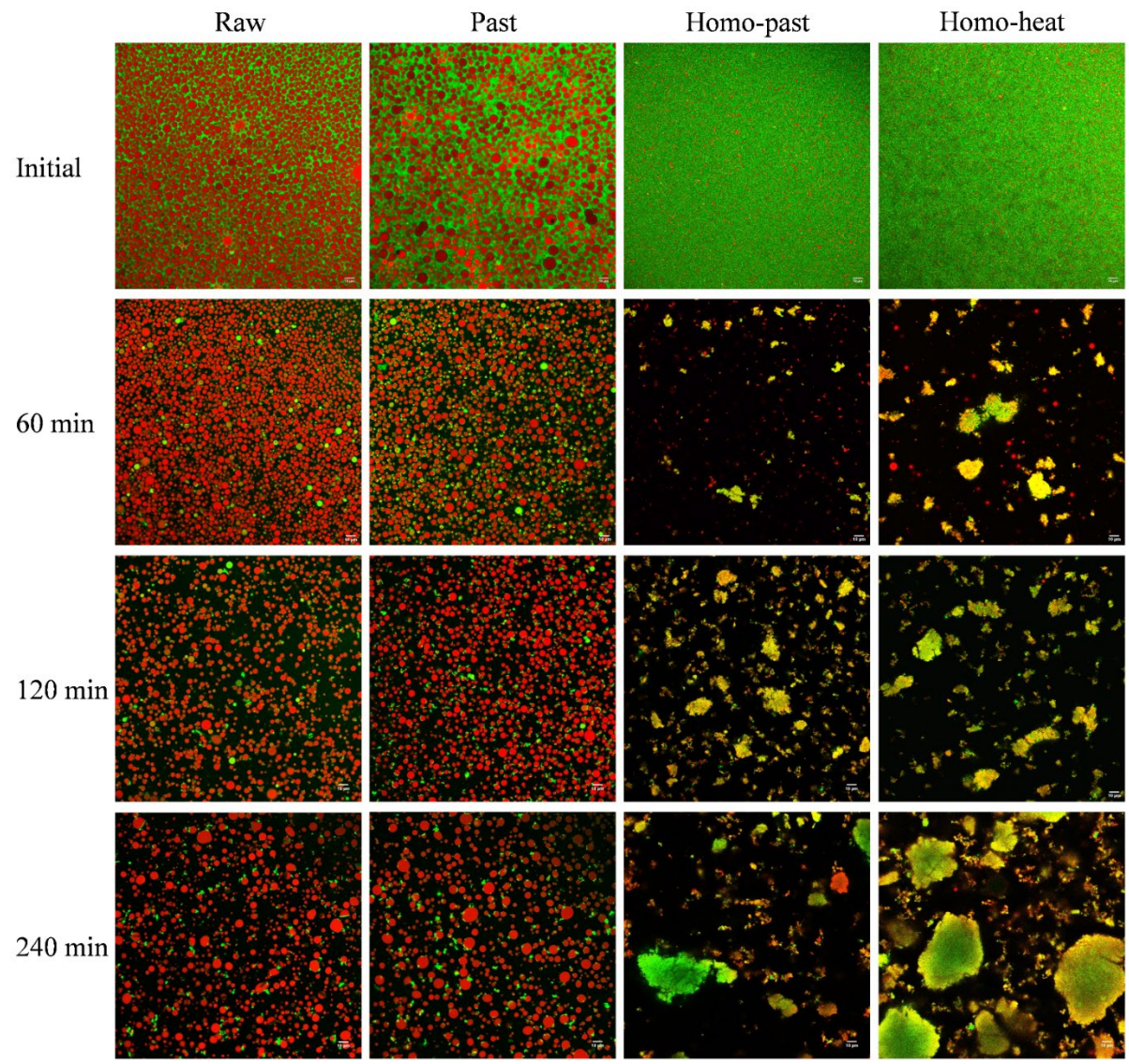

Figure 6-9 Confocal micrographs of digesta obtained at different time points (0–240 min) during in vitro gastric digestion of raw, pasteurized (Past), homogenized and pasteurized (homo–past), and homogenized and heated (homo–heat) sheep milks. Red shows the fat and green shows the protein. The scale bar in all images is 10 μm.

6.5. Conclusions

This study demonstrated the effect of heat treatment and homogenization on the dynamic *in vitro* gastric digestion of sheep milk. All milk samples formed structured curds

in the stomach. The curds formed from the homogenized milks had a much looser and fractured structure than those formed from the unhomogenized milks because of the inclusion of smaller fat globules into the curd; the homogenization of sheep milk followed by heating at 90 °C for 5 min resulted in crumblier curds compared with homogenization coupled with pasteurization because of the incorporation of more whey proteins into the curd. The differently structured curds led to different rates of disintegration of the curds and of protein hydrolysis by pepsin, and thus impacted the gastric emptying rate. The relative rates of fat release from the curds to the emptied digesta were dependent on whether or not the sheep milk was homogenized. The release of protein, fat, and calcium from the curds occurred at much faster rates in the homogenized sheep milks than in the unhomogenized sheep milks, leading to higher fat and protein contents in the emptied digesta of the homogenized sheep milks. Flocculation of the fat globules was observed in the digesta of the homogenized sheep milks, and most of the fat globules were incorporated into the protein/peptide particles. The findings reported here have extended our knowledge of how homogenization and heat treatment affect the formation of structured curds during the gastric digestion of sheep milk.

Chapter 7: Statement of Contribution – Doctorate with Publications/Manuscripts

DRC 16

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Zheng Pan
Name/title of Primary Supervisor:	Professor Aiqian Ye
In which chapter is the manuscript /published work:	Chapter 7

Please select one of the following three options:

○ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

◉ The manuscript is currently under review for publication – please indicate:

- The name of the journal:

Journal of Dairy Science

- The percentage of the manuscript/published work that was contributed by the candidate: 80.00
- Describe the contribution that the candidate has made to the manuscript/published work:

The candidate contributes to the conceptualization, designed and conducted the experiment, analyzed all the experimental data with assistance from Professor Aiqian Ye in developing an appropriate method and Dr Karl Fraser in analyzing lipidomics data. The candidate wrote the original draft of the manuscript, with changes and corrections completed with input from all supervisors.

○ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Zheng Pan Digitally signed by Zheng Pan Date: 2023.02.21 17:04:59 +13'00'
Date:	21-Feb-2023
Primary Supervisor's Signature:	Aiqian Ye Digitally signed by Aiqian Ye DN: cn=Aiqian Ye, c=NZ, o=Massey University, ou=SF&AT, email=a.m.ye@massey.ac.nz Date: 2023.02.22 14:09:04 +13'00'
Date:	22-Feb-2023

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GRS Version 5 – 13 December 2019
DRC 19/09/10

Chapter 7. Comparative lipidomics analysis of *in vitro* lipid digestion of sheep milk: Influence of homogenization and heat treatment

The contents of this chapter have been submitted to Journal of Dairy Science.

A separated work but connected to this Chapter has been established and the research article has been submitted to Food Chemistry and is attached in the Appendix B.

7.1. Abstract

This study investigated the changes in sheep milk lipids during *in vitro* gastrointestinal digestion in response to heat treatment (75 °C/15 s and 95 °C/5 min) and homogenization (200/50 bar) using lipidomics. Homogenized and pasteurized sheep milk had higher levels of polar lipids in gastric digesta emptied at 20 min than raw sheep milk. Heat treatment of homogenized sheep milk resulted in a reduced level of polar lipids compared with homogenized–pasteurized sheep milk. The release rate of free fatty acids during small intestinal digestion for gastric digesta emptied at 20 min followed the order: raw ≤ pasteurized < homogenized–pasteurized ≤ homogenized–heated sheep milk; the rate for gastric digesta emptied at 180 min showed a reverse order. No differences in the lipolysis degree were observed among differently processed sheep milks. These results indicated that processing treatments affect the lipid composition of digesta and the lipolysis rate but not the lipolysis degree during small intestinal digestion.

7.2. Introduction

The lipolysis of milk fat is an interfacial process that is dependent on the milk fat globule (MFG) surface composition, the MFG size, and the adsorption of lipolytic enzymes to the surface. The surface of native MFGs is composed of milk fat globule membrane (MFGM), which is a trilayer of phospholipids naturally encapsulating MFGs and contains

a variety of lipids, proteins, and carbohydrate moieties (Brink et al., 2020; Zhao et al., 2019). The surface composition and the size of MFGs can be altered by processing treatments (such as heat treatment and homogenization). The changes in the surface composition and the size of MFGs can affect their interactions with digestive enzymes within the gastrointestinal tract, thereby influencing the digestion of MFGs (Thum et al., 2023).

Previous studies have shown that the surface structure of MFGs released from the stomach can be affected by the processing treatment (such as heat treatment, homogenization, and their combination) (Z. Pan et al., 2021; Zhao et al., 2019). Z. Pan et al. (2021) showed that the MFGs of raw and pasteurized sheep milks released from the gastric phase remained almost unchanged at an early stage of gastric digestion but appeared to coalesce and become larger at longer digestion times. However, the MFGs of homogenized and pasteurized/heated sheep milks remained small and were well embedded within protein/peptide particles towards the end of gastric digestion when released to the small intestine. Therefore, the surface layer of the MFGs released from the stomach could be different in differently treated milks, which might in turn have an impact on lipid digestion in the small intestinal phase.

The effect of processing treatments on the digestion of MFGs by pancreatic lipase has been studied extensively (Islam et al., 2017; Liang et al., 2017; Ye et al., 2010; Zhao et al., 2019; Zhao et al., 2022). The homogenization of cow milk has been found to increase the initial digestion rate of the MFGs compared with raw cow milk but not to affect the final extent of lipid digestion; heat treatment of the homogenized milk decreased the initial lipid digestion rate and the final extent of lipid digestion (Liang et al., 2017; Zhao et al., 2019). The effect of homogenization on lipid digestion has been attributed to the reduced MFG size, the increased surface area of the MFGs, and the reduced MFGM area on the MFG surface, which allows more pancreatic lipase to bind with the interface of MFGs and

to easily hydrolyze the core fat (Ye et al., 2010). The subsequent heat treatment might alter the interfacial structure of the MFGs (such as the binding of denatured whey proteins with the MFG surface), thereby influencing the adsorption of lipase on to the surface of the MFGs and thus the lipolysis rate (Liang et al., 2017). These studies confirm that homogenization- and heat-induced alterations in the size and the surface composition of MFGs could affect lipid digestion in the small intestinal phase to different extents (Michalski, 2009; Zhao et al., 2019).

The lipid digestion of cow milk has attracted extensive research; however, in comparison, knowledge of the lipid digestion of sheep milk is quite limited. Y. Pan et al. (2021) investigated the *in vitro* lipid digestion characteristics of human, cow, and goat milks and found different lipolysis degrees and released fatty acid compositions among the different milks. This was attributed to different MFG sizes and fatty acid compositions (Y. Pan et al., 2021). Previous studies have shown that sheep milk has different MFG sizes and lipid compositions from cow, goat, and human milks (Balthazar et al., 2017; Roy et al., 2020a), which probably leads to different lipid digestion behaviors of sheep milk. Therefore, in-depth identification of the lipid constituents in the milk lipidome during gastrointestinal digestion is necessary for a more detailed understanding of the lipid digestion behavior in sheep milk.

The present study aimed to provide a better understanding of how the lipid digestion behavior of sheep milk is affected by different processing treatments. It examined the effects of different heat treatments (pasteurization at 75 °C/15 s and heating at 95 °C/5 min) and homogenization (200/50 bar) on the lipid digestion behavior of sheep milk using a human gastric simulator for the gastric phase and a pH-stat for the small intestinal phase. The lipid composition, the lipolysis rate, and the lipolysis degree among the differently

processed sheep milks during *in vitro* gastrointestinal digestion were compared; this would provide information for the formulation of sheep milk products.

7.3. Materials and methods

7.3.1. Sample preparation and chemicals

Fresh sheep milk was collected from two companies (Spring Sheep Milk Co. and Maui Milk Co., Ltd, Waikato, New Zealand). The fresh sheep milk was pasteurized at 75 °C for 15 s. Homogenized milk was obtained by homogenizing at 200/50 bar and 65 °C in a two-stage valve homogenizer in the Massey University pilot plant. In the experiments, the homogenized milk was pasteurized at 75 °C for 15 s in a pilot-scale indirect UHT plant (Alfa-Laval, Australia) to make homogenized and pasteurized (homo–past) milk, and homogenized and heated (homo–heat) milk was obtained by heating the homogenized milk to 95 °C in the UHT plant and then transferring to a water bath and holding for 5 min. After heat treatment, the milks were immediately cooled to 20 °C in cold running water. The average fat globule sizes, which were determined using a Mastersizer 2000 (Malvern Instruments Ltd, Malvern, UK), were d_{43} = 4.52 ± 0.14 μm and d_{43} = 0.62 ± 0.07 μm for the unhomogenized sheep milk and the homogenized sheep milk, respectively.

Pancreatin from porcine pancreas (EC 232-468-9; Catalogue No. P7545, Sigma Chemical Co., St. Louis, MO) had a trypsin activity of 6.1 ± 0.2 units/mg solid, as tested in preliminary experiments. All other chemicals were of analytical grade and were purchased from BDH Chemicals (BDH Ltd, Poole, UK) or Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. All solutions were prepared using Milli-Q water purified by treatment with a Milli-Q apparatus (Millipore Corp., Bedford, MA).

Simulated intestinal fluid (SIF) was prepared at a 1.25× concentration according to the method described by Brodkorb et al. (2019) The SIF consisted of 6.8 m*M* KCl, 0.8 m*M*

KH_2PO_4, 123.4 m*M* NaCl, and 0.33 m*M* $MgCl_2(H_2O)_6$, and its pH was adjusted to 7 using a 6 *M* HCl solution. $CaCl_2(H_2O)_2$ was added into the SIF immediately before the digestion experiment to achieve a final concentration of 0.6 m*M* to avoid precipitation during storage at –20 °C. The SIF was then supplemented with water to achieve a 1× concentration.

7.3.2. In vitro gastric digestion

As this study is a follow-up to our previously published study on the gastric digestion of differently processed sheep milks, the chemicals for the gastric digestion are as described in our previous publication (Z. Pan et al., 2021). The *in vitro* gastric digestion was performed using a human gastric simulator (HGS), as developed by Kong and Singh (2010). The gastric digestion procedure is as described in our previous publication. The gastric emptying rate was 3.6 mL/min and the emptied digesta was removed from the bottom of the stomach chamber at 20-min intervals to accurately control the gastric emptying. The gastric digestion time was up to 240 min, and the digesta removed from the HGS at each time interval was filtered through a mesh with a pore size diameter of 1 mm for further analysis; the solid mass with size greater than 1 mm was put back into the HGS for further digestion. The gastric digesta emptied at 20 (G20) and 180 (G180) min were used for the *in vitro* small intestinal digestion.

7.3.3. In vitro small intestinal digestion

The *in vitro* small intestinal digestion of the sheep milks was carried out using a pH-stat (SI Analytics Titroline 7000 Titrator, Xylem Inc., Rye Brook, NY). Before digestion, the digesta emptied from the HGS at 20 and 180 min of gastric digestion were adjusted to pH 7.5 using 1 *M* NaOH solution. The digestion procedure for the intestinal phase followed the method of Brodkorb et al. (2019). An aliquot (25 mL) of the gastric digesta was mixed with prewarmed (37 °C) SIF electrolyte (25 mL) to reach a ratio of 1:1, and the pH of the

mixture was adjusted to 7.0. The digestion was conducted for 2 h, with the pH being maintained at 7.0 by automatic pH-stat titration with a 0.05 *M* NaOH solution. To better estimate the volume of NaOH consumed during the lipid digestion, simulated intestinal digestions of bovine skim milk containing 0–4% protein were carried out to build a standard curve. The volume of NaOH consumed because of the presence of SIF components and proteins was estimated using the standard curve. The volume of NaOH solution required to neutralize the pH of the digesta was recorded and was used to calculate the release of free fatty acids (FFAs) during the lipid digestion using the following equation:

$$\frac{\mu mol_{fatty\ acids}}{ml_{gastic\ digesta}} = \frac{[V_{NaOH\ for\ sample}(t) - V_{NaOH\ for\ blank}(t)] \times C_{NaOH} \times 1000}{V_{gastric\ digesta}} \quad \textbf{Equation 7-1}$$

where $V_{NaOH}(t)$ is the volume of NaOH solution added into the reaction mixture at digestion time t, C_{NaOH} is the molar concentration of the NaOH solution (0.05 *M*), and $V_{gastric\ digesta}$ is the volume of the gastric digesta used (25 mL).

A site-filling model described by Ye et al. (2013) was used to calculate the rate constant of FFA release, as shown below:

$$Ln\left(\frac{C_{max} - C_t}{C_{max}}\right) = -k_1 t \qquad \textbf{Equation 7-2}$$

where C_t is the molar concentration of released FFAs at time t (min), C_{max} is the molar concentration of released FFAs at the end of the digestion (120 min), and k (min^{-1}) is the rate constant.

7.3.4. Lipidomics

The milk and digesta samples (stored at -180 °C) were thawed and well shaken for 1 min using a vortex mixer before aliquoting. A biphasic liquid–liquid extraction method was used for untargeted metabolomics, and the lower organic phase containing extracted lipids was obtained for a lipidomic profile using liquid chromatography coupled to mass

spectrometry (LCMS). Briefly, 300 µL of milk sample and 800 µL of prechilled (–20 °C) chloroform:methanol (50:50, v/v) were added into a 2 mL Eppendorf tube and vortex mixed for 30 s; the tube was then placed in a freezer (–20 °C) to precipitate proteins. After 60 min, this mixture was mixed with 400 µL of water, agitated for 30 s, and centrifuged at 11,000 rev/min and 4 °C for 10 min using an Eppendorf centrifuge 5427 R (Eppendorf AG, Germany). A 200 µL aliquot of the lower organic layer was evaporated to dryness under a stream of nitrogen and stored at –80 °C before analysis. Pooled quality control (QC) samples for the differently processed sheep milks were prepared by combining 50 µL of the lower organic phase from each sample of raw, pasteurized, homo–past, or homo–heat sheep milk in a new tube. The pooled QC samples were well mixed and dried under a stream of nitrogen, and these dried samples were stored at –80 °C until analysis.

The lipid extracts were analyzed using a Shimadzu Nexera-x2 Ultra Performance Liquid Chromatography® system coupled to a Shimadzu LCMS-9030 mass spectrometer. A 2 mL sample was injected into a Waters CSH-C18 column (2.1 mm × 100 mm, 1.7 µm particle size) and the temperature of the column oven was 60 °C. The chromatographic conditions were as follows: total run time, 15 min; flow rate, 400 µL/min; solvent A, 10 m*M* ammonium formate and a mixture of water, acetonitrile, and isopropanol in a ratio of 5:3:2 (v/v/v); solvent B, 10 m*M* ammonium formate and a mixture of water, acetonitrile, and isopropanol in a ratio of 1:9:90 (v/v/v). The solvent gradient program was as follows: 10–45% solvent B (0–2.7 min), 45–53% solvent B (2.7–2.8 min), 53–65% solvent B (2.8–9.0 min), 65–89% solvent B (9.0–9.1 min), 89–92% solvent B (9.1–11.0 min), and 92–100% solvent B (11.0–11.1 min); the sample was held for 0.8 min (11.1–11.9 min) before being returned to the starting conditions of 10% solvent B in 0.1 min (11.9–12.0 min); before injection of the next sample, the column was re-equilibrated under the starting conditions for 15 min (Abshirini et al., 2021). Mass spectrometry analysis was performed in positive

ion mode. The following mass spectrometer conditions were used: gas temperature, 150 °C; nebulizing gas flow rate, 2.0 L/min; heater gas flow rate, 10 L/min; interface temperature, 300 °C; drying gas flow rate, 10 L/min; desolvation line temperature, 250 °C; heater block temperature, 400 °C; source voltage, +4.0 kV; sheath gas flow rate, 10 L/min. Spectra were obtained over the range 250–1250 *m/z* and the data-independent acquisition data were collected in 20 *m/z* windows from 300 to 1100 *m/z*. High-purity nitrogen was used for the drying and collision gases.

7.3.5. Data processing

Data processing of the untargeted LCMS lipidomics data was performed using the untargeted data processing software package MSDIAL (v. 4.90; http://prime.psc.riken.jp/compms/msdial/main.html), which contains the LipidBlast database internally (v. 2022, https://fiehnlab.ucdavis.edu/projects/LipidBlast) (Tsugawa et al., 2015). The data-independent acquisition spectra were used to identify the aligned peaks. The lipidomic features were searched against the built-in lipid library in silico-generated lipid fragmentation spectra. The locally weighted scatterplot smoother (LOWESS) regression analysis and the pooled QC samples were used to correct per-feature run-order and normalize the resultant peak intensity table. Features within the pooled QC samples with an average QC-to-blank sample ratio of less than 5 and a CV of 30% were removed. In total, the full datasets for 21 sheep milk samples and 21 cow milk samples were included in the lipidomic analysis.

7.3.6. FFA analysis

The individual FFAs released from all lipids [including FFAs, monoglycerides (MGs), diglycerides (DGs), and triglycerides (TGs)] in the small intestinal digesta were extracted using the Bligh and Dyer method (Bligh & Dyer, 1959). Briefly, 30 mL of the

digesta was mixed with 15 mL of chloroform:methanol (1:2, v/v) and then vortexed for 30 s. The mixture was subsequently centrifuged at 3,500 × *g* for 5 min using a bench centrifuge Heraeus Multifuge X3R (Thermo Fisher Scientific Inc., Waltham, MA) for complete separation. After collecting the organic phase, the water phase was re-extracted twice following the same procedure. The organic phases of three extractions were pooled together and dried using an evaporator (Thermo Fisher Scientific, Rockford, IL). The dried lipids were dissolved by adding 4 mL of methanol. The lipid extracts from triplicate samples were pooled before analysis.

The total amount of fatty acids (TFA) was analyzed using the method described by Zhu et al. (2013) with slight modifications. Briefly, 200 μL of the lipid extract was transferred into a 10 mL screw-cap glass tube and then mixed with 0.5 mL of internal standard (100 mg nonadecanoic acid/mL heptane), 0.7 mL of 10 *M* NaOH, and 5 mL of methanol. The tube was incubated in a water bath at 55 °C for 1.5 h with handshaking for 5 s every 20 min. It was cooled to room temperature by immersion in tape water after incubation, and 0.58 mL of 12 *M* H_2SO_4 was added to the tube. The tube was well mixed and incubated in a water bath for another 1.5 h at 55 °C, with vigorous handshaking for 5 s every 20 min. The sample was then centrifuged at 3,500 × *g* for 10 min at 20 °C. The heptane layer containing fatty acid methyl esters was transferred into a 350 μL glass insert fitted into an autosampler vial. The vial was capped and stored at –18 °C before gas chromatographic analysis.

The ester form of fatty acids (EFA), including MG, DG, and TG forms, of the lipid extracts was determined using the sodium methoxide transesterification method. Briefly, 200 μL of the lipid extract was mixed with 0.5 mL of internal standard and 0.5 mL of sodium methoxide (1% in methanol) in a glass tube. The capped tube was then incubated in a 50 °C water bath for 60 min with handshaking for 5 s every 20 min. The sample was

cooled to room temperature and then mixed with 5 mL of 2% glacial acetic acid solution using a vortex mixer. After 10 min, it was centrifuged at 1000 × *g* for 10 min at 20 °C. The upper heptane layer was transferred into a 350 μL glass insert fitted into an autosampler vial and stored at –18 °C before gas chromatographic analysis. Using this method, which excluded all FFAs from the lipid extracts, the FFA content was obtained by subtracting the EFA content from the TFA content of the same extract. All analyses were done in triplicate.

The amount of each individual FFA was calculated as

$$FFA_i = TFA_i - EFA_i \qquad \textbf{Equation 7-3}$$

where FFA_i is the amount (mol) of the individual FFA, TFA_i is the total amount (mol) of the individual fatty acid, and EFA_i is the amount (mol) of the ester form of the individual fatty acid.

7.3.7. Gas chromatographic analysis of fatty acid methyl esters

The compositions of the fatty acids extracted were determined by gas chromatography using an Agilent 7890 system equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA) and a capillary column (Supelco Park, Bellefonte, PA). The helium carrier gas flow rate was 20 cm/s and the column head pressure was 76 kPa. The oven temperature program was as follows: level 1, 180 °C held for 5 min; level 2, 210 °C at 1 °C/min and then held for 25 min. The injector and detector temperatures were set at 270 °C. The fatty acids were represented as the percentage of the total fatty acid weight within each sample.

7.3.8. Statistical analysis

The peak intensity of each individual fatty acid was converted to a relative proportion of the total lipids. A two-way analysis of variance (ANOVA) test followed by

multiple comparisons were used to verify differences in the abundances of the fatty acids in sheep milk and its digesta. These analyses were carried out using GraphPad Prism v. 8.4.0 software (GraphPad Software). The lipidomics data were transformed by generalized log-transformation and auto-scaling to correct for heteroscedasticity, to reduce the skewness of the data, and to reduce mask effects. Principal component analysis (PCA) identified differential lipid metabolites among the differently treated sheep milks. The heatmap was clustered by Euclidean distance and Ward's minimum variance method. The PCA and the heatmap analysis were produced using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

7.4. Results and discussion

7.4.1. Comparison of lipidome among differently processed sheep milk

7.4.1.1. Effect of processing treatments on the lipidome of sheep milk

In the milk samples, 126 lipids from two lipid classes were identified, i.e., 125 TGs and one phosphatidylcholine (PC). Differences in the lipidomic composition after different processing treatments of the sheep milk are shown in Figure 7-1. The PCA of the lipidomics data provided evidence of compositional differences among the differently treated sheep milks. Notably, the unprocessed (raw sheep milk) and the most processed (homo–heat sheep milk) samples were on opposite sides of the component 1 axis (PC1), with the other (pasteurized and homo–past) sheep milks sitting more in the center of PC1 (Figure 7-1A). There appeared to be minor differences among the differently processed sheep milks but these were not statistically significant ($P > 0.05$ from ANOVA of inter-types comparison). The heatmap showed the top 60 lipids of the differently processed sheep milks. The raw and the processed (i.e., pasteurized, homo–past, and homo–heat) sheep milks were grouped into two different clusters; 37 lipids were more abundant in the raw sheep milk than in the processed sheep milks, and ~95% of these lipids were TGs with a carbon number less than

50. In contrast, the abundances of the remaining 23 lipids were higher in the pasteurized, homo–past, and homo–heat sheep milks than in the raw sheep milk; ~ 65% of these lipids were TGs with a carbon number greater than 50. Previous studies showed that heat treatment of milk promoted the hydrolysis of TGs and produced more DGs and FFAs (Xu et al., 2020), and that a higher intensity of heat treatment could increase the hydrolysis of the TGs (Zhang et al., 2022). This could have been due to the hydrolysis of the TGs by heat-resistant lipases from contaminating bacteria and heat damage to the MFGM that enhanced the exposure of TGs to lipase and aggravated the hydrolysis of the TGs (Jukkola et al., 2018; Zhang et al., 2022). Therefore, the lipid compositions of the differently processed sheep milks could have been slightly altered by the heat-induced hydrolysis of the TGs.

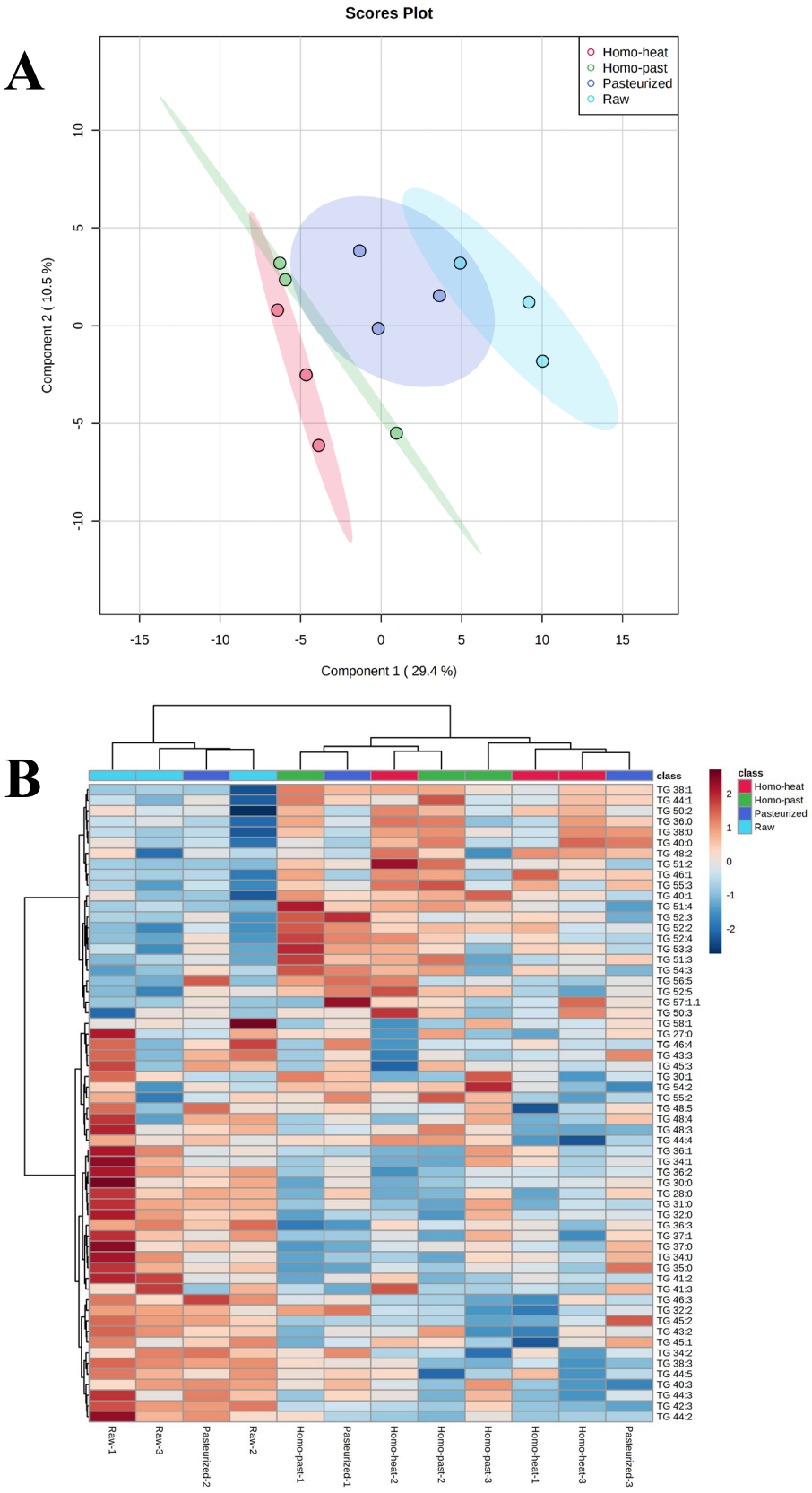

Figure. 7-1. Lipidome differences among raw, pasteurized, homogenized and pasteurized (homo–past), and homogenized and heated (homo–heat) sheep milks. (A) Principal

component analysis and (B) heatmap of all lipids in the differently processed sheep milks. The heatmap colors reflect the abundance of milk lipids (mean-centered and divided by the standard deviation of each variable).

7.4.1.2. Comparison of lipidome among gastric digesta

Lipidomics analysis detected 150 lipid species from six different lipid classes, i.e., 131 TGs, six PCs, five DGs, four lysophosphatidylcholines (LPCs), three sphingomyelins (SMs), and one phosphatidylethanolamine (PE). Figure 7-2 shows the differences in the lipidomes of the gastric digesta obtained from the differently processed sheep milks and at the different emptying times of 20 and 180 min. The G20 and the G180 were fully separated from each other in the raw and homo–past sheep milks but were partially separated in the homo–heat sheep milk (Figure 7-2A), suggesting that the lipid composition of the gastric digesta was different at the different emptying times. The heatmap shows the 65 significantly ($P < 0.05$ from an ANOVA of inter-types comparison) different lipids of the gastric digesta (Figure 7-2B). In the raw sheep milk, the G20 contained higher abundances of 56 lipids (43 TGs and 13 polar lipids) but lower abundances of seven lipids (all TGs) than the G180; the homo–past sheep milk showed lower abundances of 43 lipids (all TGs) but higher abundances of the remaining 22 lipids (13 polar lipids, seven TGs, and two DGs) in the G20 than in the G180; the homo–heat sheep milk showed lower levels of 43 lipids (all TGs) and higher levels of seven lipids (all TGs) in the G20 than in the G180. These results suggested that the gastric digestion time had a significant impact on the lipid composition of the gastric digesta, which could in turn have influenced the lipid digestion in the small intestinal phase.

In the homo–past sheep milk, the lower levels of TGs but the higher levels of DGs and polar lipids in the G20 than in the G180 may have been a consequence of the

homogenization of the sheep milk, which changed the surface structure of the MFGs. Homogenization of sheep milk reduced the MFG size and enhanced the adsorption of milk proteins (mainly casein micelles) at the MFG surface. As a result, an increasing number of MFGs were incorporated into the protein matrix through casein–casein interactions at an early stage of digestion, because pepsin cleaves the κ-casein of casein micelles and results in coagulation of the micelles (Li, Ye, et al., 2022; Z. Pan et al., 2021; Roy et al., 2021b). The homogenized MFGs that were incorporated into the protein matrix may have been less accessible for the enzymes. With increasing digestion time, the pH of the gastric mixture decreased and the pepsin activity gradually increased; thus the proteins on the surface of the MFGs would be hydrolyzed to a greater extent by pepsin (Z. Pan et al., 2021). Consequently, the entrapped homogenized MFGs would be released from the protein matrix into the liquid phase at a long digestion time, increasing the level of TGs in the G180. This was evidenced by the result that the G20 of the raw sheep milk and the G180 of the homo–past and homo–heat sheep milks were clustered together (Figure 7-2B), indicating that the lipid compositions of these three gastric digesta samples were similar. Additionally, a proportion of small native MFGs that were not influenced by homogenization may have been present in the liquid phase in the stomach. A previous study on the lipid composition of different-sized MFGs showed that, in raw sheep milk, the smaller MFGs contained significantly higher levels of DGs, polar lipids, and some TGs than the larger MFGs (Mesilati-Stahy et al., 2011). The species of lipids (13 polar lipids, seven TGs, and two DGs, Figure 7-2B) that were abundant in the homo–past sheep milk were aligned with those abundant in the small MFGs of raw sheep milk reported by Pan et al. (2023a). Small native MFGs are less likely to be incorporated into the curds because of their relatively unaltered interfacial composition. Further, the proportion of small native MFGs in the liquid phase would be reduced with increasing digestion time because of gastric emptying. Therefore,

the differences in lipid composition between the G20 and the G180 of the homo–past sheep milk can be attributed to the different proportions of small native MFGs being retained in the liquid phase (Figure 7-2B).

For the homo–heat sheep milk, no differences in polar lipids and DGs between the G20 and the G180 were found, suggesting that most small MFGs were incorporated into the protein matrix. The intense heat treatment (95 °C/5 min) of sheep milk resulted in greater denaturation of the whey proteins (Pan et al., 2022a) and greater association of denatured whey proteins with the surface of small native MFGs, compared with pasteurization (Ye, Singh, James Oldfield, et al., 2004). The small native MFGs were probably coated with a mixture of denatured whey proteins and caseins and thus incorporated into the protein matrix through casein–whey protein or whey protein–whey protein interactions during digestion; this reduced the differences in polar lipids between the G20 and the G180.

When the lipid compositions of the G20 among the differently processed sheep milks were compared, the raw, homo–past, and homo–heat sheep milks were separated from each other (Figure 7-2A). This suggested that the lipid compositions of the G20 were significantly different among the raw, homo–past, and homo–heat sheep milks. For the G20, the raw sheep milk had higher abundances of 43 lipids (all TGs) than the homogenized (homo–past and homo–heat) sheep milks, and the abundances of polar lipids were highest in the homo–past sheep milk and lowest in the homo–heat sheep milk (Figure 7-2B). As discussed above, large proportions of the homogenized MFGs would have been incorporated within the protein matrix, with only a proportion of the small native MFGs remaining in the liquid phase at an early stage of gastric digestion, which could contribute to the lower abundances of TGs but the higher abundances of polar lipids in the G20 of the homogenized sheep milks than the raw sheep milk. The homo–heat sheep milk might have

most of the small native MFGs incorporated into the protein matrix because of the interactions between denatured whey protein/casein-coated MFGs and the protein matrix. However, for the G180, the raw and homogenized (homo–past and homo–heat) sheep milks were separated but the homo–past and homo–heat sheep milks appeared to overlap with each other (Figure 7-2A). The G180 showed lower abundances of 43 TGs and higher abundances of seven TGs in raw sheep milk than in homogenized sheep milk (Figure 7-2B), which could be attributed to the release of more homogenized MFGs from the protein matrix at a late stage of gastric digestion, compared with the raw sheep milk. No differences in the polar lipids and DGs of the G180 between the raw and homogenized sheep milks were found (Figure 7-2B). This was a consequence of gradually emptying of small MFGs in both the raw and homogenized sheep milks during gastric digestion, leading to the low abundance of polar lipids in the G180 for both the raw and homogenized sheep milks.

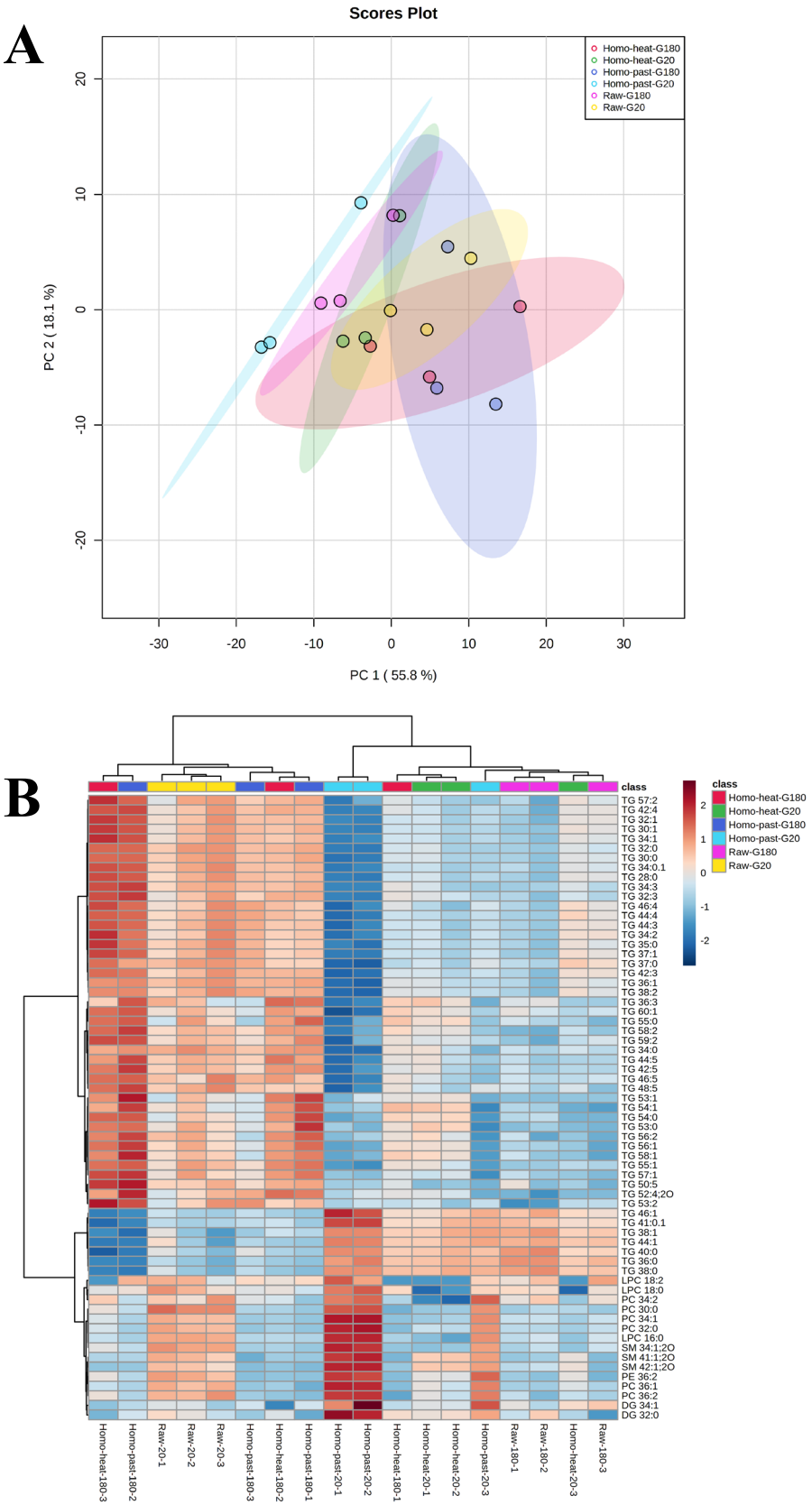

Figure 7-2. Lipidome differences of the gastric digesta emptied at 20 and 180 min among raw, homogenized and pasteurized (homo–past), and homogenized and heated (homo–heat)

sheep milks. (A) Principal component analysis and (B) heatmap (B) of all lipids in the gastric digesta. The heatmap colors reflect the abundance of milk lipids (mean-centered and divided by the standard deviation of each variable).

7.4.1.3. Comparison of lipidome among intestinal digesta

Lipidomics analysis detected 150 lipid species from six different lipid classes, i.e., 131 TGs, six PCs, five DGs, four LPCs, three SMs, and one PE. The abundance of these lipids was very low as most of the lipid had been hydrolyzed by the lipase after 2 h of small intestinal digestion. The differences in the lipidomic compositions of the intestinal digesta after 120 min of digestion of the G20 (G20-Int) and the G180 (G180-Int) among the differently processed sheep milks are shown in Figure 7-3. The PCA shows that the G20-Int of the raw sheep milk and the other digesta appeared to be separated, and that the other groups tended to overlap with each other. This indicated that the lipid composition of the G20-Int of the raw sheep milk was different from those of the other small intestinal digesta samples. The heatmap shows the 11 significantly ($P < 0.05$ from an ANOVA of inter-types comparison) different lipids in the small intestinal digesta of the differently processed sheep milks. The heatmap clearly shows that the G20-Int of the raw sheep milk was more abundant in nine lipids (seven TGs and two DGs) but less abundant in two lipids (one SM and one LPC) than the other small intestinal digesta. By comparison with the other digesta samples, the G20 of the raw sheep milk had the highest fat content, less surface area of the MFGs available for the enzyme to contact, and a relatively intact MFGM because of the low pepsin activity at an early stage of gastric digestion (Z. Pan et al., 2021). In contrast, the surface proteins of the G180 in all types of sheep milk were hydrolyzed by gastric pepsin to a greater extent because of the increased pepsin activity at low pH and a longer digestion time. Additionally, the MFGs of homogenized sheep milk had a larger surface area for

enzymes and bile to interact with compared with those of raw sheep milk, possibly leading to a faster lipolysis rate of the MFGs in both the G20 and the G180. Therefore, the G20 of the homogenized sheep milks and the G180 of all types of sheep milk showed differences in the abundances of TGs compared with the G20 of the raw sheep milk in the small intestinal phase.

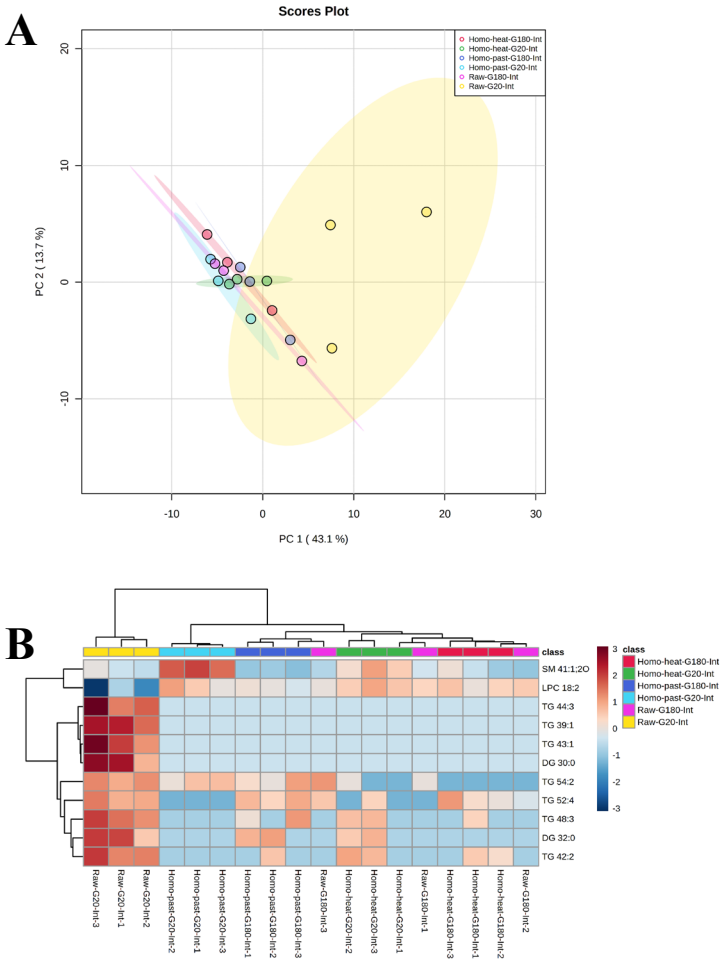

Figure 7-3. Lipidome differences of the intestinal digesta after 120 min of digestion of the gastric digesta emptied at 20 and 180 min, among the raw, homogenized and pasteurized (homo–past), and homogenized and heated (homo–heat) sheep milks. (A) Principal component analysis and (B) heatmap of lipids in the intestinal digesta. The heatmap colors reflect the abundance of milk lipids (mean-centered and divided by the standard deviation of each variable).

7.4.2. Comparison of lipolysis rates during small intestinal digestion

The amounts of FFAs released after small intestinal digestion of the G20 and the G180 of the raw, pasteurized, homo–past, and homo–heat sheep milks are presented in Figures 7-4A and 7-4B. For all digesta samples, the amount of FFAs released increased markedly at the beginning of digestion, followed by a progressive slowing down, leading to a pseudo-plateau after 10 min. For the G20-Int, the amount of total FFAs released was highest in the raw sheep milk after 120 min of intestinal digestion, followed by the pasteurized, homo–heat, and homo–past sheep milks (Figure 7-4A). For the G180-Int, the amount of total FFAs released followed the order: homo–past > homo–heat > pasteurized > raw after 120 min of intestinal digestion. These results are in line with the fat contents of gastric digesta reported in our previous study (Z. Pan et al., 2021), which showed the same order in the fat contents of the G20 and the G180 of differently treated sheep milks.

This result showed that the release of FFAs was nearly complete after 10 min of intestinal digestion in all samples, regardless of the fat content or the structure of the MFGs in the gastric digesta. However, the rate of FFA release during intestinal digestion showed differences among the differently processed sheep milks. The rate of FFA release is shown in Figure 7-4C. The FFAs in raw sheep milk were released at a significantly ($P < 0.001$) slower rate in the G20 than in the G180. One possible explanation is that the fat content of the G180 was lower than that of the G20, resulting in a higher ratio of enzyme to fat in the G180 than in the G20 and thus a faster release rate of fatty acids in the G180. Another possibility is that the MFGs of the G180 in the raw sheep milk contained less MFGM protein and thus were more vulnerable to pancreatic lipase than those of the G20. Previous studies showed that the degree of hydrolysis of MFGM proteins by pepsin increased with increasing digestion time because of the increased pepsin activity (Wang et al., 2019; Ye et al., 2011). It is therefore likely that the MFGM proteins (such as butyrophilin, PAS 6/7, and

mucins) were digested to a greater extent at 180 min than at 20 min of gastric digestion. As a consequence, MFGs with less MFGM in the G180 showed a faster fatty acid digestion rate than those in the G20. In contrast, the G20 of the homo–past and homo–heat milks showed significantly ($P < 0.01$) higher rates of FFA release than the G180. Our previous study showed that most of the MFGs of homo–past and homo–heat sheep milks were released in their intact status to the small intestine at 20 min of gastric digestion, whereas they flocculated via protein–peptide or peptide–peptide interactions at 180 min of gastric digestion (Z. Pan et al., 2021). The protein/peptide coating on the surfaces of the MFGs in homogenized sheep milk might reduce the surface area of the MFGs with which pancreatic lipase can interact. This could slow down the rate of hydrolysis of the MFGs by pancreatic lipase during small intestinal digestion, leading to a lower release rate of fatty acids in the G180 than in the G20.

For the G20, the homo–heat milk showed the highest digestion rate of the MFGs, followed by the homo–past, pasteurized, and raw milks (Figure 7-4C). The release rate of FFAs was significantly ($P < 0.01$) higher in the homogenized milks than in the unhomogenized milks (Figure 7-4C). These findings are consistent with previous findings for cow milk, which showed that homogenized and heat-processed cow milk had a higher digestion rate of MFGs at the initial stage compared with raw milk (Liang et al., 2017; Ye et al., 2010; Zhao et al., 2019). The MFGs in homogenized milk have a higher surface area than in raw milk, and more milk proteins (mainly caseins) are adsorbed to the surface of the MFGs after the homogenization of milk. This could increase the affinity of MFGs to pepsin and pancreatic lipase (Zhao et al., 2019). Additionally, it has been reported that caseins are less resistant to hydrolysis by pepsin than some MFGM proteins (especially glycoproteins) (Le et al., 2012) and that MFGM phospholipids could inhibit lipid hydrolysis by pancreatin (Ye et al., 2010). The MFGs of homogenized milk contained less MFGM but

more caseins on the surface than those of unhomogenized milk, which would accelerate the release of FFAs in homogenized milk. These different rates of FFA release might also be affected by the digestive content of the gastric digesta, as the ratio of enzymes to digestive substrates was different among the samples. Further studies may be required to investigate the effect of the concentration of digestive substrates on the lipolysis rate during the small intestinal digestion of differently processed milks.

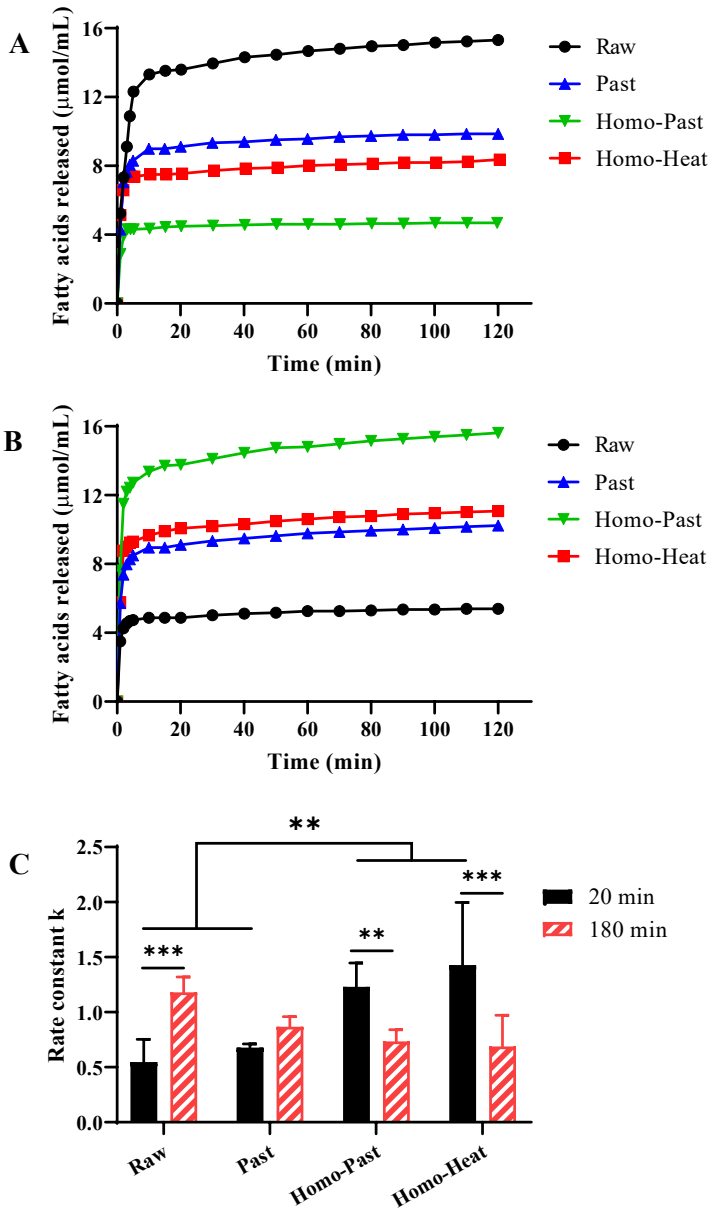

*Figure 7-4. Total fatty acids released from the gastric digesta emptied at (A) 20 and (B) 180 min from differently processed sheep milks after 120 min of in vitro intestinal digestion: ●, raw milk; ▲, pasteurized (past) milk; ▼, homogenized and pasteurized (homo–past) milk; ■, homogenized and heated (homo–heat) milk. (C) The rate constant k of fatty acids released from the gastric digesta emptied at 20 (black without pattern) and 180 (red with lines) min of differently processed sheep milks after 120 min of in vitro intestinal digestion. Significant difference levels: **, $P < 0.01$; ***, $P < 0.001$.*

7.4.3. Lipolysis during small intestinal digestion

The degree of lipolysis during digestion was expressed as the percentage of released FFAs (in mg) versus the total fatty acids present in the digesta after 2 h of small intestinal digestion. The percentages of individual fatty acids and total FFAs released from the G20 and the G180 of all the sheep milks after 2 h of intestinal digestion are shown in Figure 7-5 and Table 7-1, respectively. After 2 h of intestinal digestion, all sheep milk samples showed a similar pattern of FFA release. For instance, a large proportion of the lipids in the G20 of the raw sheep milk was digested, with a range from ~89 to 98% of the FFAs being released (Figure 7-5). The percentage of released fatty acids reached over ~85% for all types of fatty acids in both the G20 and the G180 and little differences in the individual fatty acids between the G20 and the G180 were found regardless of the processing treatment (Figure 7-5). This indicated that the release of fatty acids was almost complete within 20 min for all types of sheep milk, which is in line with the result shown in Figure 7-4. For the G180 of the raw sheep milk, a similar lipolysis degree was observed, with 92.5% of the total FFAs being released, compared with ~94.4% for the G20 (Table 7-1). However, the amount of total fatty acids released was different among the differently processed sheep milks (Table 7-1), which was associated with the fat content of the gastric digesta; our previous study reported that the fat content of digesta followed the order: raw > pasteurized > homo–heat > homo–past in the G20 and homo–past > homo–heat > pasteurized > raw in the G180 (Z. Pan et al., 2021).

The present study showed little difference in the lipolysis degree among the differently processed sheep milks (Table 7-1). This is different from previous results for cow milk reported by Zhao et al. (2019) who found that homogenization and heat processing of cow milk resulted in a higher degree of lipolysis after 2 h of simulated small intestinal digestion. The differences between this study and the previous study may be

attributed to the increased concentration of lipase activity adapted from the INFOGEST protocol (Brodkorb et al., 2019). The INFOGEST protocol recommended adding porcine pancreatin suspension into the digestion mixture to achieve a trypsin activity of 100 U/mL, which was converted to a concentration of 20 mg/mL for porcine pancreatin in this study. The concentration of pancreatin was much higher than that reported previously by Zhao et al. (2019) who used a porcine pancreatin concentration of 1.6 mg/mL. The higher enzyme concentration may have narrowed the gap in the degree of lipolysis among the differently processed sheep milks. This needs to be confirmed in future work by testing the impact of different concentrations of enzymes on the lipolysis degree of differently processed milks. Additionally, the ratio of enzyme to substrate could be different between this study and previous studies, which could also contribute to the differences in the lipolysis degree.

The lipolysis degree (90–97%) of the differently processed sheep milks presented here is much higher than the expected theoretical maximum value (~67%) (Corstens et al., 2018). The specific chemical compositions of sheep MFGs may contribute to their high lipolysis degree during digestion. Previous studies showed that sheep milk had higher concentrations of short-chain and medium-chain fatty acids than cow, goat, and human milks (Balthazar et al., 2017). Therefore, these fatty acids can quickly disperse into the aqueous phase after hydrolysis by lipase, reducing the inhibitory effect of interface saturation on the surface of the MFGs and thus promoting the digestion of the MFGs. Moreover, porcine pancreatin could contain bile-salt-stimulated lipase and pancreatic-lipase-related protein-2 (Salhi et al., 2020), which can nonspecifically hydrolyze various substrates, such as TGs, DGs, MGs, cholesteryl esters, and the esters of fat-soluble vitamins, and thus could drive the hydrolysis towards completion (Kim Ha & Lindsay, 1993; Li et al., 2007; Salhi et al., 2021). For instance, Salhi et al. (2021) showed that gastric lipase and pancreatic lipase hydrolyzed around two-thirds of the total ester bonds in milk, producing

MGs and FFAs during digestion, and that adding bile-salt-stimulated lipase resulted in the hydrolysis of MGs. The concerted action of these three lipases could result in the complete digestion of milk TGs, generating free glycerol and FFAs as the end products and not *sn*-2 MGs and FFAs. Therefore, the higher concentration of porcine pancreatin used in the current study probably had higher concentrations of bile-salt-stimulated lipase and pancreatic-lipase-related protein-2, which would contribute to the greater extent of lipolysis of sheep MFGs.

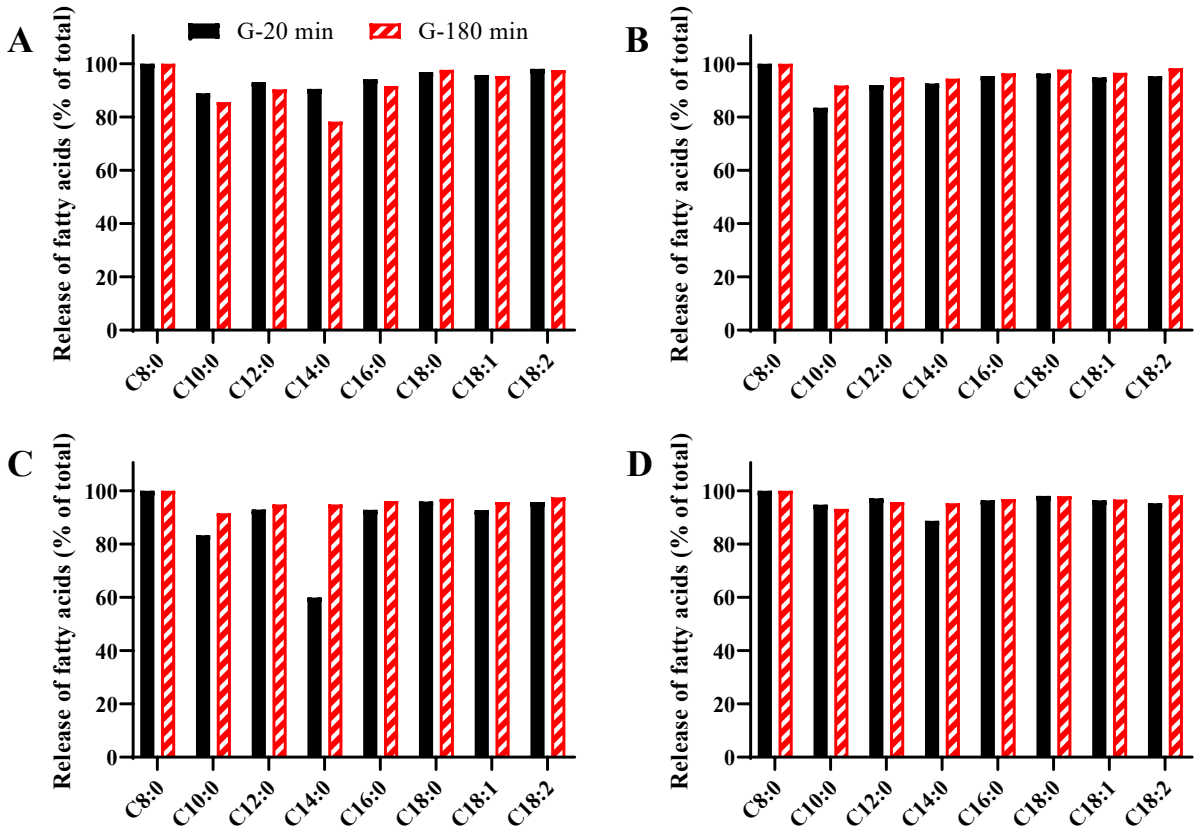

Figure 7-5. Individual fatty acids released after 2 h of small intestinal digestion of the gastric digesta emptied at 20 min (G20, black without pattern) and 180 min (G180, red with right-slanting lines) of raw (A), pasteurized (B), homogenized and pasteurized (C) and homogenized and heated (D) sheep milks.

Table 7-1. Individual fatty acids released after 2 h of small intestinal digestion of the gastric digesta emptied at 20 min (G20, black without pattern) and 180 min (G180, red with right-slanting lines) of raw (A), pasteurized (B), homogenized and pasteurized (C) and homogenized and heated (D) sheep milks.

sample	raw	pasteurized	homo–past	homo–heat
total fatty acids (mg)				
G20	18.461	11.961	10.002	14.142
G180	7.064	18.572	27.074	23.405
FFAs (mg)				
G20	17.424	11.301	9.073	13.523
G180	6.536	17.893	25.938	22.625
total FFAs released (% of total fatty acids)				
G20	94.4	94.5	90.7	95.6
G180	92.5	96.3	95.8	96.7

7.5. Conclusions

This study demonstrated the effect of heat treatment and homogenization on the lipid digestion of sheep milk during *in vitro* gastrointestinal digestion. The lipidome results showed that heat treatment (pasteurization at 75 °C/15 s and heating at 95 °C/5 min) of sheep milk resulted in a different abundance of TGs probably because the heat treatment of milk could induce the hydrolysis of TGs. The lipid composition showed differences in the gastric digesta among the differently treated sheep milks, indicating that heat treatment and homogenization have an impact on the release of fat during gastric digestion. For the intestinal phase, lipolysis of the milk fat was nearly complete in the first 10 min for all types of sheep milk, and the lipolysis rate was affected by the processing treatment. Homogenization of sheep milk significantly increased the lipolysis rate compared with unhomogenized sheep milk but did not change the lipolysis degree during digestion.

Our findings showed that these differences are directly related to the differences in sizes and surface structures of the MFGs and the formation of gastric curds, which affect the interactions between MFGs and enzymes and thus the lipid composition. However, no significant differences in the lipid composition of the small intestinal digesta and the lipolysis degree among the differently processed sheep milk were found. This study contributes to an understanding of how heat treatment and homogenization affect lipid digestion behavior under simulated gastrointestinal conditions.

Chapter 8. Overall discussion and future work

8.1. Overall discussion

The development of the sheep dairy industry has drawn great attention because of a growing consumer interest in sheep milk and its proteins. However, sheep milk products in a liquid form are not widely available around the world. One of the important reasons is the low heat stability of sheep milk which leads to the coagulation of proteins during high heat treatment and thus the difficulties in producing long shelf-life sheep milk. Critical parameters controlling protein aggregation during the heat treatment of sheep milk, especially under UHT processing are unclear. Additionally, heat-induced structural changes in sheep milk that eventually result in coagulation have not yet been determined. Although protein interactions in cow milk under various heating conditions have been widely studied, this knowledge cannot be directly extrapolated to sheep milk because of compositional and structural differences between sheep milk and cow milk.

In vitro studies revealed the complex digestion process of milk from different species. The composition and structure of milk protein and fat play an important role in delivering nutrients during gastrointestinal digestion, especially in the gastric phase. In addition, commercial processing treatments (such as thermal processing and homogenization) alter the structure and composition of milk protein and fat, leading to the different digestion behavior of milk. However, the effect of processing treatment on the digestion process of sheep milk within the gastrointestinal phase is still unknown.

8.1.1. Heat-induced protein interactions in SSM

This study confirmed that heat treatment (75–90 °C for 0.5–30 min) of SSM increased the casein micelle size to greater extent compared with previous results on cow skim milk. Several interactions, such as the association of denatured whey proteins with

casein micelles and aggregation of casein micelles, contributed to the marked increase in casein micelle size. The extent of association of denatured whey proteins with casein micelles occurred was greater in SSM than in cow milk reported previously, which partially contribute to the increase in casein micelle size. This was probably due to the higher proportion of α_{S2}-casein in sheep milk as the α_{S2}-casein could also interact with β-LG via the thiol-disulfide exchange, thereby providing more active sites on the casein micelles for denatured whey proteins to interact with (Farrell et al., 2009). On the other hand, the aggregation behavior among casein micelles at heating temperatures of 80–90 °C was observed in SSM, and this phenomenon has not been found in cow milk. The casein micelle aggregation in cow milk is commonly observed on extended heating at high temperatures (120–140 °C).

The heat stability of SSM at 140 °C and the protein interactions occurring in SSM at pHs 6.8–7.0 at different heating times ranging from 120 to 500 s were also explored. The HCT–pH profile showed that SSM had maximum HCT at pH 6.9 and became increasingly unstable at higher and lower pH values. The results also confirmed that sheep milk has lower heat stability than cow milk, in comparison with the HCT–pH profile reported previously for cow milk. Previous studies have shown that some heat-induced changes in milk, such as the decrease in pH, deposition of calcium phosphate onto micelles, association of whey proteins with casein micelles and dissociation of caseins (in particular κ-casein), could promote instability of milk (Singh, 2004). Sheep milk used in this study showed a lower natural pH and higher ionic calcium concentrations than cow milk, indicating that the electrostatic repulsion among casein micelles could be lower in sheep milk than in cow milk because of the lower negative charge of casein micelles in sheep milk. High ionic calcium concentrations could also result in more calcium phosphate depositing on the casein micelles during heating. Additionally, the lower concentration of κ-casein, the lower

proportion of κ-casein on the casein micelles and the higher level of dissociation of κ-casein were observed in SSM than in cow skim milk. Whey protein associated with the micelles may help connect casein micelles via cross-linking of whey proteins bound onto different micelles (Singh, 2004). The high concentrations of protein and α_{S2}-casein in sheep milk probably allow more frequent contact among whey protein-coated casein micelles, leading to the aggregation of casein micelles via thiol-disulfide interchange reactions. All these differences make the casein micelles of sheep milk more unstable, potentially resulting in the aggregation of casein micelles at a low-temperature range of 80–90 °C and 140 °C.

Numerous hypotheses (such as high casein content and unstable casein micelles induced by the formation of κ-casein/whey protein complexes) have been made for the heat-induced aggregation of sheep milk proteins. However, other chemical and physical reactions involved in protein aggregation have not yet been discussed in the literature. As proposed and discussed in Chapter 4 and 5, the low heat stability of SSM at low pH (< 6.9) could be due to salt-induced coagulation because of its high ionic calcium concentration, the low surface charge of casein micelles, a reduced electrostatic repulsion and a collapse of the hairy layer induced by charge neutralization. It has been proposed that the important determinant of heat stability might be the pH and ionic calcium values; low pH or high ionic calcium concentration contribute to the low heat stability of milk and vice versa. SSM showed a higher concentration of ionic calcium than cow milk at the same pH values as reported previously. At a lower pH (< 6.9), the surface charge of casein micelles and electrostatic repulsion among casein micelles of SSM could be low because of charge neutralization. Additionally, the hairy layer of κ-casein may collapse during heating as increasing temperature further decreases the pH and surface charge, and increases ionic calcium concentration, which allows casein micelles to get closer and aggregate together. Similar to cow milk, whey proteins of SSM preferentially associated with casein micelles

at more acidic pHs. The relatively high proportions of whey proteins associated with casein micelles at low pH may promote aggregation of casein micelles via cross-linking of whey proteins bound onto different micelles.

In unheated SSM, the dissociation of κ-casein increased significantly with increasing pH. However, little changes in the level of κ-casein of casein micelles were reported in previous studies on cow milk. It has been reported that the electrostatic repulsions are enhanced with increasing pH because of the lowered calcium activity and increased surface charge. It is possible that the electrostatic repulsions among caseins of SSM casein micelle were greater than that of cow milk casein micelle probably because of their different casein composition; the increased electrostatic repulsions between κ-casein and other micelle components resulted in the partial dissociation of κ-casein at higher pH values. At pH > 6.9, increasing amounts of caseins (especially κ-casein) dissociated from casein micelles after heating at 140 °C. A greater proportion of the dissociated κ-casein complexed with whey proteins and was presented in the serum phase at higher pH. The formation of κ-casein/whey protein complexes may sterically hinder the contact of κ-casein with casein micelles, thereby inhibiting the reassociation of κ-casein with casein micelles. This could create more unstable hydrophobic areas on micelles that allow micelles to get closer and promote the aggregation of κ-casein-depleted micelles. In both cases (pH < 6.9 and pH > 6.9), the heat-induced structural rearrangement of casein micelles is largely dependent on the serum environment (such as pH and calcium) that influences electrostatic interactions of the caseins within and among the micelles.

The occurrence of maximum heat stability at pH 6.9 was probably due to the critical level of ionic calcium concentration, surface κ-casein on micelles and electrostatic repulsion that keep the casein micelle relatively stable. The results discussed in Chapter 5 suggested that there was a sharp boundary between unstable SSMs that produced high

amounts of sediment and stable SSM that produced very low amounts of sediment. A small adjustment in the pH values at the boundary region could convert SSMs from those that produce low amounts of sediment to those that produce high amounts of sediment or vice versa. It is likely that the attractive and repulsive forces of SSM casein micelles at pH 6.9 reach a critical level that prevents the dissociation of caseins from casein micelles and aggregation of casein micelles. The pH adjustment, heat-induced deposition of calcium phosphate onto the micelles and association of whey proteins with casein micelles can alter the surface/internal charge, distribution of calcium or steric repulsion for micelles. When the attractive and repulsive forces exceed the critical level (possibly at pH < 6.9 or > 6.9), protein aggregation would occur to a greater extent.

8.1.2. Protein digestion in differently processed sheep milk

The effect of heat treatment and homogenization on the dynamic *in vitro* gastric digestion of sheep milk was explored in the present study. All sheep milk samples formed structured curds in the stomach. The curds formed from raw sheep milk were intact. Pasteurization of sheep milk reduced the structural integrity of the curds, resulting in fragmented curds at the end of digestion. In comparison with the curd structure of pasteurized cow milk, the effect of pasteurization on curd formation appeared to be more pronounced for the sheep milk. This is likely related to the low heat stability of sheep milk, which results in a greater extent of whey protein denaturation and association of denatured whey proteins with casein micelles than cow milk (as shown in Chapter 3). More associated whey proteins in sheep milk are involved in the formation of curds than cow milk and hinder the casein–casein interaction; denatured whey proteins are more susceptible to proteolysis by pepsin. Therefore, pasteurized sheep milk with high levels of whey protein denaturation had a greater extent of curd breakdown during gastric digestion compared with pasteurized cow milk.

The curds formed from the homogenized (homo–past and homo–heat) sheep milks had a much looser and fractured structure than those formed from the unhomogenized (raw and pasteurized) sheep milks because of the inclusion of smaller fat globules into the curd. By comparison with the curds of homo–past cow milk reported in the literature, which showed grainy and continuous structures, homo–past sheep milk contained considerably small curd grains and no continuous structural curds. This indicated that the combination of pasteurization and homogenization has a greater impact on the macrostructures of sheep milk curds than that of cow milk curds. One possible explanation is the differences in the extent of whey protein denaturation between sheep milk and cow milk. Homogenized MFGs are covered by caseins and whey proteins and thus could act as pseudo-protein particles to participate in the formation of protein matrix during gastric digestion. Pasteurization of homogenized milk may result in a greater amount of whey proteins associated with either casein micelles or MFGs in sheep milk than in cow milk because of the lower heat stability of sheep milk, which is likely to affect the interactions between homogenized MFGs and other proteins to a greater extent than that in cow milk. Therefore, the curd structure could be different between homo–past sheep and homo–past cow milk. Another possible explanation is the different protein composition between sheep milk and cow milk. As different types of casein have different physicochemical properties (such as hydrophobicity, calcium-binding ability and water-holding capacity), the different protein compositions may affect the protein network formation and result in a different structure of curds during gastric digestion.

The homogenization of sheep milk followed by intense heating at 90 °C for 5 min resulted in crumblier curds compared with homogenization coupled with pasteurization because of the incorporation of more whey proteins into the curd. The curd structure of homo–heat milk did not show obvious differences between sheep milk and cow milk. This

could be attributed to the similar extent of whey protein denaturation in sheep milk and cow milk. Based on the kinetics of whey protein denaturation reported in Chapter 3 for sheep milk and in previous studies for cow milk (Oldfield, 1996), heating milk at 90 °C for 5 min could denature virtually all whey proteins in both sheep milk and cow milk. Therefore, it can be hypothesized that the fully denatured whey proteins in both cow and sheep milk could narrow down the differences in curd structure.

The differently structured curds resulted in different rates of the breakdown of the curds and protein hydrolysis by pepsin and thus affected the gastric emptying rate. The relative rates of fat and protein released from the curds to the emptied digesta were markedly affected by homogenization but not thermal processing. Curds of homogenized sheep milk showed a faster release of protein, fat, and calcium than that of unhomogenized sheep milk, resulting in higher fat and protein contents in the emptied digesta of the homogenized sheep milks. This is attributed to the looser and crumbly structure of curds, which allows the gastric fluid and pepsin to penetrate into the curds and gives a greater surface area for pepsin to interact with proteins. Flocculation of the fat globules was observed in the digesta of the homogenized sheep milks, and most of the fat globules were incorporated into the protein/peptide particles.

8.1.3. Lipid digestion in differently processed sheep milk

Processing treatments also had an impact on the lipid digestion behavior. In raw sheep milk, parts of MFGs were entrapped in the curds because of the intact structure of MFGs and the remaining MFGs were present in the liquid phase at the beginning of gastric digestion. Those entrapped MFGs tended to coalesce and form larger MFGs within the curds with increasing digestion time as the MFGM proteins were hydrolyzed by pepsin and make the MFG unstable. Those larger MFGs could be released gradually when the

surrounded protein layer was digested. However, the release of MFGs from the curd of raw sheep milk was slow. The curd formed from raw sheep milk had an integrated and compact structure, impeding the release of MFGs from the curd. Additionally, this integrated curd structure could also hinder the penetration of pepsin and SGF into the curds, which slows down the proteolysis rate and breakdown of curds and thus further limits the release of the entrapped MFGs from the curds.

For homogenized sheep milk, the gastric digestion behavior of MFGs was different with raw sheep milk. Homogenization of sheep milk resulted in a reduced MFG size and increased surface areas that are covered by proteins (mainly caseins). This resulted in the incorporation of homogenized MFGs in the curd as the homogenized MFGs could act as pseudo protein particles to participate in the formation of protein network through casein-casein or casein-whey protein interactions. The amount of homogenized MFGs released from curds were increased (Chapter 6) as the digestion progressed. This is attributed to the looser and fragmented structure of curds, which provides more surface area for pepsin to digest the proteins that surround MFGs at a faster rate and results in the increased amount of homogenized MFGs released to the liquid phase of the stomach.

Different lipid digestion behaviors were also observed between homo–past and homo–heat sheep milk. As discussed in Chapter 7, there could be some native small MFGs that are less likely to be affected by homogenization. For homo–past sheep milk, those native small MFGs remained in the liquid phase as they had a relatively intact surface structure. However, intense heat treatment of homogenized sheep milk could lead to the association of denatured whey proteins with MFGs, thereby resulting in the incorporation of small MFGs into the curds through casein–whey protein or whey protein–whey protein interactions. As a result, more native small MFGs would be emptied out from the stomach at an early stage of gastric digestion in homo–past sheep milk than in homo–heat sheep

milk. Overall, those differences in lipid digestion in gastric phase among differently processed sheep milk are dependent on the surface structure of MFGs. Those different MFGs could result in the different structures of gastric curds, interactions between substrates and enzymes in the stomach and consequently the composition of MFGs emptied at different time points.

There appeared to be minor differences in lipid digestion in the simulated small intestinal digestion among differently processed sheep milk. Homogenized sheep milk showed a higher lipolysis rate than unhomogenized (raw and pasteurized) sheep milk (Chapter 7). This could be associated with the differences in the surface area and interfacial composition of MFGs emptied from the stomach between unhomogenized and homogenized sheep milk. However, the lipolysis degree after 2 h of small intestinal digestion were similar among differently processed sheep milk, which could be linked to the high concentration of pancreatin adapted from INFOGEST protocol or the high ratio of enzyme to substrate. Therefore, it can be concluded that the concentrations of pancreatin and bile salt derived from INFOGEST protocol are high enough to digest all sheep milk fat regardless of processing treatments although the lipolysis rate at the early stage of small intestinal digestion differs between differently processed sheep milks.

8.2. Recommendations for future work

8.2.1. Effect of whey protein in bridging casein micelles during heating SSM

Heat treatment (80–90 °C) of SSM resulted in the aggregation of casein micelles, which may be partially due to the whey proteins bound onto different micelles. However, the bridging role of whey proteins on the casein micelles has not been elucidated. The bridging effect of whey proteins among casein micelles could be validated by comparing the protein interactions of SSM in the presence or absence of whey proteins during heating.

8.2.2. Fortification of SSM with different food stabilizers: Impact of physicochemical changes on stability to processing

As adjusting the pH of milk may not be permissible in the industry, the addition of food stabilizers to the sheep milk may be necessary to produce UHT-treated sheep milk products with low sedimentation levels. Although there are some UHT sheep milk products with the addition of food stabilizers available in the market, how these different types of stabilizers affect sheep milk stability is still unclear. The effects of various food stabilizers, such as sodium phosphate and sodium citrate, on protein interactions and SSM stability could be investigated further.

8.2.3. Digestion behavior of infant formulae made from sheep milk

A growing number of infant formulae made from sheep milk have been developed, but their digestion behavior under infant gastrointestinal conditions is unknown. Investigations on the infant digestion behavior of various infant formulae using an infant gastric simulator should be carried out.

8.2.4. Effect of whey protein denaturation on curd formation of sheep milk

This study showed that pasteurization of milk results in different structures of curds formed from sheep milk and cow milk, which is probably caused by the different extent of whey protein denaturation and association of denatured whey proteins with casein micelles. It could be concluded that the extent of denaturation of whey proteins can affect the formation of curds and thus controlling the release of nutrients from the stomach to the small intestine. Various heating conditions (temperature and time) could be used to denature whey proteins to a different extent and investigate how these changes affect their digestion behavior within the gastrointestinal tract.

8.2.5. Gastric lipid digestion and its effect on intestinal digestion

The effect of gastric lipase on the digestion of differently processed sheep milk using a dynamic HGS is still unknown. Curds with different structures could have different rates and extents of lipolysis during gastric digestion, and this may also influence lipid digestion in the small intestine.

8.2.6. Effect of MFG size on lipid digestion in the gastrointestinal tract

This study showed that different-sized MFGs have different compositions of lipid and protein. These MFGs with different sizes may be involved in the formation of milk curds differently, potentially affecting the digestion behavior of milk protein and lipid. Ultrafiltration equipment may be used to separate MFGs with different sizes and reconstitute SSM with different-sized MFGs. Thus, the obtained reconstitute sheep milk can be used to investigate their digestion behavior.

8.2.7. Effect of non-protein components on the heat stability of sheep milk

The presence of non-protein components, including cations and anions, can influence the serum environment, thereby causing variations in chemical and physical interactions, such as electrostatic interactions, among proteins. Due to the distinct protein compositions found in sheep milk compared to cow milk, these alterations in the serum environment can have differential effects on protein interactions in sheep milk. Investigating these dynamics would provide a deeper understanding of how non-protein components influence the heat stability of sheep milk.

8.2.8. Heat stability of sheep milk from different breeds

Sheep breeds exhibit significant variations in their milk protein composition, leading to potential differences in protein interactions and dissociation patterns during heating. Therefore, it is important to note that there may be limitations to the applicability

of this PhD research, and further investigation is required to explore the heat stability of sheep milk across different breeds.

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Appendix A: Literature review

Introduction

Milk is an important daily food for human consumption, especially for infants, it is the primary source of nutrition. The main source of milk for producing infant formula (IF) is ruminant milk (i.e., cow milk) (EFSA Panel on Dietetic Products & Allergies, 2014). Worldwide, the most commonly consumed milk is derived from cattle, around 81.6% of the world milk production is derived from cow milk (675 million tons) in 2017, followed by milks from other species such as buffalo (14.5%), goat (2.2%), sheep (1.3%), and camel (0.3%) (Food & Nations, 2017). However, the composition and function of milk proteins from different ruminant animals vary significantly due to various factors (i.e., diet, environmental factors, stage of lactation, age and fodder) (Claeys et al., 2014). Cow milk allergy also frequently occurs in infants (Park, 1994). Therefore, sheep milk is promoted as a good alternative to cow milk for human due to its lower allergy, higher major nutritional contents (Park et al., 2017).

Sheep is traditionally used as the source of meat and wool. However, today sheep's milk is popular for consuming as various cheeses and yoghurt products because it contains a higher level of total solids and major nutrients than cow milk. The sheep milk constituents offer much more energy and the necessary nutrients for human growth and health. For example, sheep milk can be a source of new bioactive peptides that can be used as health ingredients and for the development of novel functional foods. Sheep milk also contains antibodies that protect the lambs against infection. Furthermore, sheep milk is also a superior source than cow milk in supplying all 10 essential amino acids, all vitamins (except folate) and most minerals are higher in sheep milk than in cow milk (Park et al., 2017).

The sheep dairy industry has developed rapidly in recent years all over the world, but the fresh sheep milk in liquid form is still not available in most markets. Liquid sheep milk can only be found at some small farms or rural areas, such as the mid-east Asian or Mediterranean basin. One of the main reasons is that sheep milk is highly sensitive to high temperatures (Raynal & Remeuf, 1998). For instance, the protein of sheep milk can be easily coagulated under ultra-high temperature (UHT) processing conditions, which makes it unable to be produced as a long shelf life liquid product (Martinez Alonso et al., 2009). Currently, structural changes of proteins and lipids in cow and goat milk during the processing treatment have been investigated and compared in-depth, however, those differences on sheep milk were only superficially discussed (Kalyankar et al., 2016).

It is widely known that the composition of sheep milk is very different from cow milk, and the variance in composition could play an important role in the digestion behavior, nutrient release and absorption, and their influence on gut health (Ye, Cui, et al., 2019). In addition, it has been reported that people who are unable to digest cow or goat milk had no trouble to digest sheep milk due to its different protein composition (Wendorff & Kalit, 2017). The digestion behavior and absorption of nutrients from milks with different compositions from different species have been studied using *in vitro* digestion models (Dalziel et al., 2018; Gallier et al., 2012b; Maqsood et al., 2019), but the effect of processing treatments on the digestion behavior of sheep milk is still unknown.

Recently, a growing interest in sheep dairying in New Zealand, northern Europe, Australia, India, and the United States for infant nutrition creates new opportunities for developing a high value for sheep milk (Claeys et al., 2014; Kalyankar et al., 2016). To develop a competitive sheep milk industry, the information on the effect of processing treatment on sheep milk and how it influences the digestion behavior should be established. Building the knowledge of processing treatment on digestion behavior in sheep milk could

provide important information for developing new milk products and position sheep milk as a better food source for infants (Recio et al., 2009).

Sheep milk components

Sheep milk is an ideal alternative to cow milk products due to its higher level of protein, fat, and calcium by casein unit, which gives sheep milk an advantage of specific taste, texture, and healthy image (Anifantakis, 1986). But chemical compositions of sheep milk change over time and are different from individual animals based on several factors, such as the stage of lactation, environmental factors (season), age, and fodder (Claeys et al., 2014).

Proteins

Casein

The casein fraction shows approximately 80% of the total sheep milk protein, mainly consisting of four fractions: β-casein (~61.6%), α_{S2}-casein (~22.8%), α_{S1}-casein (~6.7%), and κ-casein (~8.9%) (Selvaggi et al., 2014). Besides, there is a γ-casein fraction that can be obtained after hydrolyzing β-casein by the enzyme plasmin. The proportion of casein fractions in sheep milk shows much higher α_{S2}-casein and β-casein content but lower α_{S1}-casein than cow milk (32.7% β-casein, 10.3% α_{S2}-casein, and 39.7% α_{S1}-casein).

Around 60% of the total casein in sheep milk is β-casein that is presented as the multiply phosphorylated forms, β_1- and β_2-casein, showing the similar amino acid composition with cow milk β-casein (Selvaggi et al., 2014). β-casein has been reported as the least calcium-sensitive casein, thus precipitates at 37 °C in the range of 8–15 mM calcium concentration (Farrell et al., 1988). Besides, the casein micelle stability, the availability and distribution of calcium can be affected by the presence of multiply phosphorylated β-casein (Amigo et al., 2000). Unlike cow milk, which can easily cause β-

casein dissociation from the casein micelles, solubilization of calcium phosphate and decrease in micelle size under 4 °C condition (Raynal & Remeuf, 2000), the β-casein in sheep milk does not induce dissociation at the casein micelle surface or cause it to diffuse into the interior at the same cold storage-conditions. Consequently, the rennet coagulation rates and gel firmness of sheep milk are not affected by the cold storage conditions (Wendorff & Kalit, 2017).

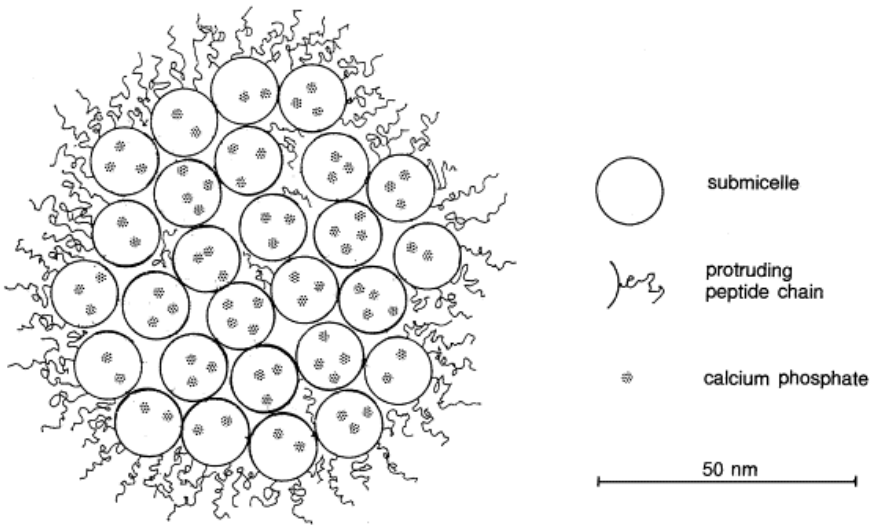
α_S-casein is the largest proportion of caseins in milk and plays a vital role in casein micelle assembly. It contains α_{S1}-casein and α_{S2}-casein. The α_{S1}-casein is the dominant protein (~40%) in cow milk proteins, but it only represents around 6% in goat and 7% in sheep milk proteins. The difference may influence the processing properties of sheep milk. According to Devold et al. (2010) research, the casein micelle size of goat milk is negatively correlated to the content of α_{S1}-casein. Besides, α_{S1}-casein can significantly affect the gel strength after adding the rennet to goat milk (Devold et al., 2010). Since sheep milk has a similar level of α_{S1}-casein with goat milk, it indicates that the content of α_{S1}-casein in sheep milk may also affect the technological properties of casein micelle. The content of α_{S2}-casein varies in different breeds of sheep, with a range of 13–30% (Balthazar et al., 2017; B Ingham et al., 2018), but cow milk only contains around 10% of α_{S2}-casein. It has been known that α_{S2}-casein is the most calcium-sensitive casein, leading to sedimentation of α_{S2}-casein at calcium concentration less than 2 mM (Aoki et al., 1985). Consequently, the structure and properties of casein micelle can be easily affected by the α_{S2}-casein content that can curtail the secretion of the other casein proteins and calcium-phosphate (Kolb et al., 2011).

Unlike α_{S2}-casein, α_{S1}-casein, and β-casein, which are calcium-sensitive and correspond to the aggregation of nanometer-sized clusters, κ-casein is known as the calcium-insensitive protein in milk, which limits the growth of casein micelle size

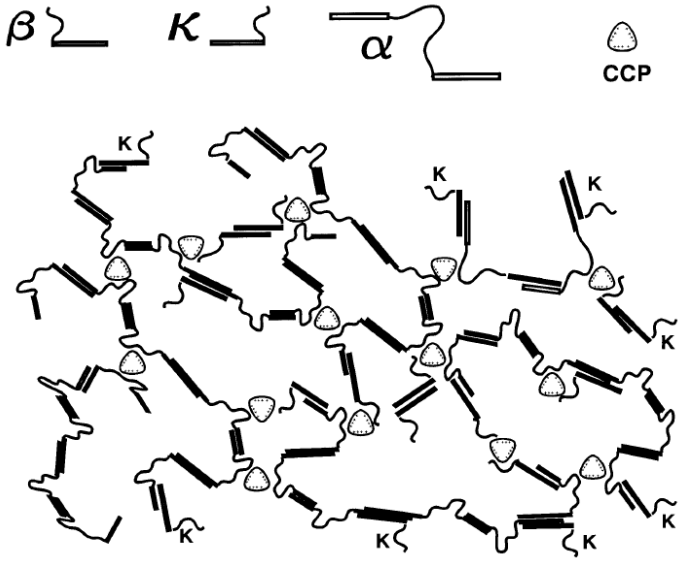
(Huppertz et al., 2018). κ-casein remains soluble state at the calcium concentration less than 15 mM as it usually contains only one phosphoserine residue and does not bind calcium strongly compared to the other three caseins. Besides, κ-casein also shows strong self-association ability in the solution that is caused by interaction among the hydrophobic C-terminal region and then forms micelles. Moreover, κ-casein can form amyloid-like fibrils when stirring at 25 °C. But the aggregating behavior of κ-casein can be inhibited by β-casein (Ossowski et al., 2012). These findings indicate that the aggregation behavior of κ-casein has influences on the structure and stability of casein micelles. However, the difference of κ-casein between the cow and sheep milk is still unclear.

Casein micelle structure

The caseins normally exist in milk in the form of large spherical-shaped colloidal particles (~50–600 nm in diameter, mean ≈160 nm) which is known as casein micelles (Dalgleish & Corredig, 2012; Fox & Brodkorb, 2008). Different casein micelle models in cow milk have been proposed in many studies, which can be a reference for casein micelle structure of sheep milk because casein micelle structure is similar in cow and sheep milk (Park et al., 2017). In 1982, the sub-micelle model (Appendix A Figure 1) was proposed (Schmidt, 1982), in which sub-micelles are bonded by colloidal calcium phosphate. In addition, it is believed that most or all of the κ-casein bind on the surface of casein micelle and forms a highly hydrated 'hairy layer' that provides steric stabilization, which can limit the growth of casein micelle size caused by aggregation among the other three caseins (Horne, 2006). On the other hand, in the dual-binding model (Appendix A Figure 2) of casein micelle structure (Horne, 1998), the individual casein molecules (α_{S1}-casein, α_{S2}-casein and β-casein) crosslink through hydrophobic regions and hydrophilic regions and bridging across calcium phosphate nanoclusters, whereas κ-casein is located at the surface of casein micelles and act as a terminator.



Appendix A Figure 1. Submicelle model of casein micelle, from Walstra (1999).



Appendix A Figure 2. Dual bonding model of the structure of casein micelle, from Horne (1998).

These differences in casein composition lead to different casein micelle characteristics, such as size, hydration, and mineralization (Balthazar et al., 2017). The casein micelles of sheep milk show higher mineralization degrees and are less hydrated and heat-stable compared with cow milk (Raynal-Ljutovac et al., 2007). The calcium can cause

rennet coagulation, in which the cross-linking and aggregation of the para-κ-casein would occur (Guinee & O'Brien, 2010). Meanwhile, the sheep milk casein micelle is higher in calcium content than cow milk, which gives a significant technological advantage for using sheep milk to produce cheese products without adding extra $CaCl_2$ additive (Park, 2007). Besides, sheep milk requires less rennet or chymosin to produce a fine curd compared to cow milk (Wendorff & Kalit, 2017). Furthermore, the protein surface hydrophobicity values of the sheep milk samples were also lower than those of the cow milk. These results indicate that the casein structure of the sheep milk is more compact and contains less hydrophobic sites on the micelle surface (Yuksel et al., 2012).

Whey protein

Whey proteins are the second basic proteins in sheep milk, mainly including α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), but immunoglobulins, serum albumin, lactoferrin, proteose-peptone and other minor proteins also exist at a lower concentration in sheep milk (Appendix A Table 1) (Ramos & Juarez, 2011). Whey proteins are globular proteins: they have a uniform distribution of hydrophobic/hydrophilic amino acids and compactly folded peptide chains (Mulvihill & Donovan, 1987). Whey proteins are the valuable by-product of cheese manufacturing since it contains bioactive proteins, such as lactoferrin and lactoperoxidase, with high level and quality protein content (Navarro et al., 2015). Also, whey protein concentrates of sheep milk show better foam overrun, foam stability, and gel strength than that of cow and goat milk (Ullrey et al., 2011).

Appendix A Table 1. Whey proteins distribution in sheep, goat, and cow whey (% of total whey protein).

Whey protein	Sheep	Goat	Cow
β-Lg (%)	74.0	58.6	64.9
α-La (%)	14.8	27	15.6
Serum albumin (%)	4.1	4.0	6.5
Immunoglobulin (%)	7.3	9.7	13

Source: Data from (Casper et al., 1998).

β-Lactoglobulin

β-Lg, the most prevalent protein in whey, comprises around 74% of the major whey proteins in sheep milk (Ramos & Juarez, 2011). It has a native internal structure and consists of different noncovalent bonds that exist within or between proteins, which mainly dominants the properties of whey proteins (Relkin & Mulvihill, 1996). Three genetic variants of β-Lg in sheep milk have been investigated: β-Lg A, β-Lg B, and β-Lg C. β-Lg A and β-Lg B can be detected in almost all of the sheep breeds, whereas β-Lg C is rarely seen and confined in some specific breeds (Ramos & Juarez, 2011). The relative amounts of β-Lg in sheep milk rises in midseason and then gradually decreases toward the end of the lactation.

α-Lactalbumin

α-La is the second most prevalent whey protein in sheep whey, consisting of approximately 14.8% of total sheep milk whey proteins. α-La in sheep milk is closely homologous to that in cow milk (Ramos & Juarez, 2011). It contains 4 disulphide bonds and a high percentage of essential amino acids but no –SH and phosphate groups. α-La of sheep whey is also described as a metalloprotein containing one calcium atom per molecule, and the biological function of α-La is involved in the synthesis of lactose. There are two

genetic variants (α-La A and B) in sheep milk, although α-La B is very uncommon in most of the sheep breeds (Ramos & Juarez, 2011). Relative amounts of α-La in sheep milk decrease throughout the season.

Lipids

Lipids are another essential component of sheep milk, providing unique physical and sensory characteristics as well as nutritional properties. Lipids in sheep milk are presented in the form of spherical droplets, and the average size of fat globules in sheep milk is the smallest (less than 3.5 μm) followed by goat and cow milk (Kalyankar et al., 2016), which is a big advantage for digestibility compared to goat and cow milk (Park, 1994).

The structure and composition of the milk fat globule membrane (MFGM) in sheep milk are similar to cow and goat MFGM, and around 1% of total fat volume in milk exists in the form of the fat globule membrane (Kalyankar et al., 2016). The MFGM is a complex layer surrounding the surface of milk fat globules. The membrane, about 10 to 20 nm thick, mainly containing proteins, phospholipids, glycoproteins, cholesterol, cerebrosides and other minor components. The MFGM plays a vital role in preventing the flocculation and coalescence of fat globules in milk and protecting the milk fat against enzymatic hydrolysis. However, the physical properties and composition of MFGM can be significantly influenced by processing treatments, such as heat and homogenization (Lee & Sherbon, 2002).

Minerals

Minerals in sheep milk have not been investigated as much as that in cow milk. Sheep milk contains approximately 0.9% of total minerals in comparison with 0.7% in cow milk. Sheep milk is higher in calcium, phosphorous, magnesium, iron, and zinc levels than

cow and goat milk sources (Appendix A Table 2). The average amount of calcium in sheep milk is about 193.5 mg/100 g, which shows around 36% more calcium than cow milk and 31% more than goat milk. However, the content of minerals in sheep milk is not in a constant state and can be affected by different factors like breed, diet effects, the stage of lactation, nutritional status of the individual animal, genetic variants and environmental conditions (i.e., temperature). According to the observed mineral profile of sheep, cow and goat milk, it is believed that sheep milk is a better source of dietary minerals than cow and goat milk (Park, 2009; Park et al., 2007; Raynal-Ljutovac et al., 2008)

Appendix A Table 2. Range and mean values of mineral contents in sheep, cow, and goat milk.

	mg/100g		
Mineral	Sheep	Cow	Goat
Sulphur	29	32	28
Phosphorus	124–158	119	121
Sodium	44	58	41
Potassium	136	152	181
Calcium	195–200	122	134
Magnesium	18	12	16
Chlorine	160	100	150
Manganese	0.053–0.09	0.02	0.032
Iron	0.08	0.08	0.07
Copper	0.04	0.06	0.05
Zinc	0.52–0.75	0.53	0.56
Iodine	0.02	0.02	0.02
Selenium	1.0–3.1	0.96	1.33

Source: Data from (Park et al., 2017; Park & Haenlein, 2013; Park et al., 2007).

Processing of sheep milk

Pasteurization

The moderate heat treatment on milk does not induce significant aggregation or disruption of the protein structures (Dalgleish & Corredig, 2012). Pasteurization is defined as a way to heat every particle of milk in properly operated equipment at 62.8 °C for 30 minutes (vat pasteurization) or 71.7 °C for 15 seconds (HTST pasteurization), or to holds the milk in the equipment continuously at or above the HTST temperature for the specific time (US Food and Drug Administration). The HTST pasteurization is the most commonly used technology in the dairy industry, while the vat pasteurization is not used in commercial drinking milk production as it is a batch procedure. According to the US FDA requirement, if the dairy material contains 10% fat or more, and/or greater than 15% total solids content and/or containing added sweeteners, the specified temperature should be increased by 2.8 °C (New Zealand Food Safety Authority, 2009). Higher temperatures (e.g., up to 90 °C) could increase shelf-life and keep the quality of pasteurized milk (Park & Haenlein, 2013).

UHT

UHT (135–150 °C for few seconds) is a sterilization treatment aiming to sterilize various microbial groups in milk and provide a longer shelf-life product at ambient temperature. Unlike pasteurization, which cannot kill all microorganisms in milk, UHT treatment can largely reduce the number of viable pathogens without changing the nutritional value significantly, and therefore minimize the risk of infection and extend the shelf life of milk (Raynal-Ljutovac et al., 2007). However, the UHT method has significant disadvantages for sheep milk. UHT sheep milk is not stable during storage. The main phenomena like high sedimentation once UHT sheep milk is packaged can occur, which is mainly attributed to the formation of protein aggregates and precipitates during heat

treatment (Martinez Alonso et al., 2009). In fact, no products from sheep milk subjected to UHT treatment are known in the present technological level which can preserve UHT sheep milk with satisfied organoleptic qualities more than 90 days at room temperature.

Heat stability of sheep milk

The heat stability of sheep milk is lower than cow milk and has not been extensively studied so far (Park et al., 2007; Raynal-Ljutovac et al., 2007). The functional and nutritional properties of sheep milk can be affected by the heat treatment (i.e., temperature), milk composition, or pH, which in turn results in numerous chemical and physical reactions (Dumitraşcu et al., 2012). For instance, the casein micelle size would be increased from 25% to 75% in sheep milk under 75–90°C for 30 s to 10 min but remained unchanged in cow milk (Raynal & Remeuf, 1998). The lower heat stability of sheep milk than cow milk could be due to the different proportion and genetic polymorphs of contained proteins, although sheep milk has the same protein components as observed in cow milk. Furthermore, the higher ionic calcium, higher micellar mineralization, and lower colloidal stability could lead to the low heat stability of sheep milk (Raynal-Ljutovac et al., 2007). The maximum heat stability of sheep milk is showed at about pH 7.0 that is higher than cow milk (pH 6.7) (Fox & Hoynes, 1976; Muir & O'Dea, 1992), thus, raw sheep milk are more heat-sensitive than cow milk due to its lower natural pH (6.39–6.5) than cow milk (pH 6.65–6.71).

Thermal denaturation of whey protein

In comparison with cow milk, the higher level of whey protein denaturation for sheep milk can be observed when heating at 80–90 °C, but not at lower temperatures (Law, 1995; Raynal & Remeuf, 1998). At the pasteurization condition (65 °C, 30 min), about 15% of the whey proteins in sheep milk were denatured, while only 2.3% of whey proteins in cow milk are denatured under the same conditions (Molik et al., 2012). The higher ratio β-

Lg/α-La and higher whey protein content in sheep milk might be the reason causing the faster kinetic of whey protein denaturation in sheep milk than cow milk (Law & Leaver, 1997). In addition, α-La has higher heat stability than β-Lg in bovine and non-bovine milk (Dumitraşcu et al., 2013), but the α-La in sheep milk is less resistant to thermal denaturation than β-Lg in cow and goat milk (Pintado & Malcata, 1996). Moreover, two genetic variants of β-Lg also show differences in heat stability. For instance, at a heating temperature higher than 85 °C, the β-Lg A shows a higher level of denaturation than β-Lg B (Rampilli et al., 1992). Calavia and Burgos (1998) also observed a higher denaturation temperature (74.3 °C) for the β-Lg A of sheep milk at pH 6.6 compared with β-Lg B (70.0 °C) in the heat temperature range of 30 to 100 °C. Furthermore, the reactivity of denatured β-Lg will be increased after the denaturation of most whey proteins due to the exposure of the SH- group (Molik et al., 2012).

Interactions between whey proteins and caseins/casein micelles

Major whey proteins (β-Lg, α-La, lactoferrin, and serum albumin) can interact with caseins and form aggregates after heat denaturation (Wang et al., 2018). For instance, most of the denatured whey proteins (especially β-Lg) can covalently bound to κ-casein, and the complex of β-Lg/κ-casein can create unstable hydrophobic areas and allow the casein micelles to get closer and associate (Van Hooydonk et al., 1987), leading to the increase in casein micelle size (Anema & Li, 2003a). In previous studies, the heated-induced complexes could be dissociated by reducing agents, and the formation of the complexes could also be prevented by thiol-blocking agents (Sawyer et al., 1963), indicating the involvement of the free thiol group of β-Lg in the interactions. Besides, the intermolecular disulphide bonds were involved in the formation of denatured β-Lg/κ-casein complexes (Grindrod & Nickerson, 1967; Sawyer, 1969). A subsequent study also corroborated that the free thiol group of β-Lg was exposed after heat treatment, leading to a series of thiol-

disulphide exchange reactions between β-Lg and other denatured β-Lg or κ-casein (Cho et al., 2003). Moreover, the present of β-Lg during heating help interact α-La with κ-casein, because the α-La could not directly interact with κ-casein (Baer et al., 1976; Elfagm & Wheelock, 1978). Apart from the disulphide bonds, noncovalent bonding is also involved in the interaction between denatured whey proteins and κ-casein, especially in the early stage of heating and at the lower heating temperatures (Anema, 2000). However, most of these studies were conducted in the model system using purified proteins. The protein composition is more complex in milk, containing numerous types of protein that could potentially interact through thiol-disulphide exchange or noncovalent bonding with other proteins upon heat treatment, such as α_{S2}-casein, α-La and bovine serum albumin (Anema, 2014; Oldfield, Singh, & Taylor, 1998). α_{S2}-casein could also participate in the interactions through thiol-disulphide exchange reactions with denatured thiol-bearing whey proteins (especially β-Lg) due to its disulphide bonds (Anema, 2014). The interactions between α_{S2}-casein and denatured whey proteins in pasteurized milk can be limited by the lower accessibility of α_{S2}-casein because of the location of α_{S2}-casein in the interior of casein micelles, although some interactions occurred under UHT conditions (Patel et al., 2006). Interestingly, the disulphide bond of α_{S2}-casein could become more accessible to thiol groups of denatured whey proteins after pressure treatment as the micelle structure can be disrupted by pressure (Patel et al., 2006).

The interaction between denatured whey protein and casein micelles can be easily influenced by several factors, such as heating temperature, holding time, heating-up time, the concentration of milk and individual protein, pH, and the level of milk salts (Anema, 2018b; Anema & Li, 2003a, 2003b; Dumpler & Kulozik, 2015; J.E.O'Connell & P.F.Fox, 2011; Sutariya et al., 2017; Vasbinder & De Kruif, 2003). For example, the milk needs more heating up time when subjected to laboratory thermal water bath, in which

approximately 80% of the denatured β-Lg associated with casein micelles (Corredig & Dalgleish, 1996; Smits & Brouwershaven, 1980). In contrast, the milk needs much less heating up time when subjected to commercial heating systems, in which only around 55% of the denatured β-Lg and α-La associated with casein micelles. The rest of the denatured whey proteins remained in the serum phase of milk as disulphide-bonded and hydrophobically associated aggregates (Oldfield, Singh, & Taylor, 1998; Singh & Creamer, 1991a). Besides, it has been described that the negative charge of micelles in cow milk increased with increasing the level of association of whey protein with casein micelles at 80–100 °C for 20 min, which can help prevent the aggregation of sheep milk (Singh & Fox, 1987b). The same hypothesis of β-Lg stabilization for sheep milk in the pH range of 6.4–6.8 is also given by Fox and Hoynes (1976).

The degree of interaction between denatured whey proteins and casein micelles can dramatically be affected by the pH of milk at heating. For instance, at a high heating temperature of 140 °C, the heat stability of cow milk increased with increasing pH, reaching the maximum at approximately pH 6.7, and then decreased to a minimum at around 6.9, followed by growing again with further rising pH (Rose, 1961). When milk was heated at high temperature (90–140 °C) for 30 min at pH below 6.7, the microscopy image showed that the surface of casein micelles was complexed by denatured whey proteins in a filamentous form, whereas the denatured whey proteins were found in the serum phase when heated at higher pH (above 6.7) (Creamer et al., 1978; Creamer & Matheson, 1980; Kudo, 1980). It has been evidenced that the denatured whey proteins could co-sediment with casein micelles at low pH (around 6.5), while most of the denatured whey proteins with high level of κ-casein were dissociated from casein micelles at high pH (above 6.8), in which whey proteins (particularly β-Lg) play an important role in the heat-induced pH-dependent dissociation of κ-casein (Kudo, 1980; Singh & Fox, 1985; Singh & Fox, 1987a,

1987b). The subsequent studies had confirmed that when milk was heated at pH below 6.7, about 80–90% of denatured whey proteins associated with casein micelles, but this level of association decreased to 20% when pH greater than 6.8 (Anema & Klostermeyer, 1997; Oldfield et al., 2000). Besides this, the heat-induced dissociation of κ-casein is pH-dependent ranging from 6.5–7.1, with linearly increasing level of κ-casein in the serum phase when the pH increased throughout the range, and the level of κ-casein in the serum phase is correlated with the level of denatured whey proteins in the serum phase as the reaction preferentially occurs in the serum phase between denatured whey proteins and κ-casein and/or whey protein-κ-casein complexes (Anema, 2007, 2008).

The degree of denaturation of whey proteins and the level of association of denatured whey proteins with casein micelles significantly affect the changes in casein micelle size. For example, whey protein denaturation (0–80%) in sheep milk increased with increasing heating temperature (75–90 °C) and holding time (0–10 min), which leads to the increase in the casein micelle size of sheep milk from 0% (75°C for 1–10 minutes) to 75% (85 °C for 1min and 90 °C for 30 s) in comparison with raw sheep milk (Raynal-Ljutovac et al., 2007). However, the casein micelle size only changes a little during heat treatment if the whey proteins are removed from the skim milk (Anema & Li, 2003a). It has been reported that adding β-Lg to the whey protein-depleted skim cow milk considerably increased the casein micelle size at the same heating condition (Anema & Li, 2003a). Thus, one reason for the changes in casein micelle size of skim milk caused by heat treatment is the association of denatured whey proteins with casein micelles.

The interaction between denatured β-Lg and κ-casein on the surface of casein micelles during heating has been confirmed, which provides steric repulsion and negative charge against aggregation among casein micelles. As a result, the interaction effect of the association of β-Lg A&B with κ-casein can help stabilize the sheep milk when subjected to

high temperature, which is even more profound if the amount of κ-casein increases (Calavia & Burgos, 1998). However, the low level of κ-casein seems to be one of the reasons why sheep milk shows lower heat stability than cow milk (Fox & Hoynes, 1976). Besides, heat treatment can also cause calcium phosphate precipitation, increasing the proportion of large micelles (Jeurnink & De Kruif, 1993). In 1976, Fox and Hoynes (1976) described that removing colloidal calcium phosphate from sheep milk only has little influence on its heat stability. Moreover, sheep milk casein micelles are more mineralized but less hydrated than cow milk casein micelles (Remeuf et al., 1989), and could produce a higher level of micelle aggregation than that in cow milk due to its lower colloidal stability (Van Hooydonk et al., 1987). The interactions and aggregation of casein micelles can be easily caused by the high casein content in sheep milk. Nevertheless, casein dissociation (especially κ-casein) occurs when milk is subjected to severe heating treatment, which leads to an increase not only in the casein micelle size but also in the number of protein particles that are smaller than casein micelle (Singh & Latham, 1993). These changes probably occur at a different level in different types of milk.

With increasing heating intensities, significant changes in milk casein micelles can be observed, especially in small ruminant milk. It has been investigated that severe heat treatment, particularly 90 °C or above, can cause dissociation of κ-casein and/or the κ-casein/β-Lg complex from the micelles (Singh & Creamer, 1991b; Singh & Fox, 1987a; Van Hooydonk et al., 1987). It has been confirmed that the formation of κ-casein/β-Lg complexes performs less efficiency in stabilizing α_S-casein and β-casein in the presence of calcium ions than uncomplexed κ-casein (Zittle et al., 1962), suggesting that the interaction may prevent κ-casein from stabilizing other caseins after heat treatment, and they could reassociate with casein micelles or form larger aggregates on subsequent cooling. Consequently, the removal of κ-casein or κ-casein/β-Lg complexes can induce aggregation

of casein micelles (Huppertz et al., 2018) and form larger particles, which may cause sedimentation of protein particles (Dalgleish, 1992). According to Gaur et al. (2018), sediments from UHT treated milk mostly consists of κ-casein-depleted casein micelles, with only low levels of denatured whey proteins (β-Lg and α-La). For instance, when milk was heated at 90 °C for 30 min at natural pH, about 30–40% of κ-casein dissociated from casein micelle (Anema, 2007), and a higher level of κ-casein (up to 60%) dissociated when milk was treated with commercial direct or indirect UHT processing conditions (140 °C for 3 s) at natural pH (Anema, 2017). Therefore, UHT-treated milk could dissociate κ-casein from casein micelles (Anema, 2017). Because the remarkable stability of casein micelles relies on the κ-casein at the surface of the casein micelle, the casein micelle that lost κ-casein is less stable and easily produce more aggregates via calcium-induced bridging when the pH of milk is lower and/or the ionic calcium level is higher than the critical levels, which thus increases the rate of sedimentation through forming larger particles (Anema, 2018a). However, limited studies presented indicate the heat stability of sheep milk currently, although the mechanism of coagulation of cow milk proteins under heat treatment has been widely investigated.

Homogenization

Homogenization is a typical way to reduce the average size of fat globules in milk to 1 μm or less and has been broadly used in the production of fluid milk, yoghurt-type products, ice cream, and cheeses, which allows whole milk or half-fat milk to be effectively treated and increase the physical shelf-life through preventing fat separation (Britz & Robinson, 2008). Sheep milk naturally has a lower average fat globule size (3.99 μm) than cow milk (4.42 μm) (Mehaia, 1995), which makes sheep milk not form a cream line in fluid milk (Rauschenberger, 2001). Homogenization of milk can also lead to the color whiten, more full-bodied flavor, and better mouth-feel (Bylund, 1995), but the increasing

homogenization pressure reduces the total amount of fat extracted from the milk. However, high-pressure homogenization (~350 MPa) does not cause apparent differences in the fatty acid composition of raw milk (Park et al., 2017). Moreover, micellar casein in milk can be incorporated into the new and smaller fat globule membrane after homogenization so that the new fat globules will react differently from the native fat globules (Wendorff & Kalit, 2017).

In the production of set-type yoghurt, homogenization can increase viscosity and reduce the separation of serum and fat, whereas it has no consistent effect on the stirred-type yoghurt (Muir & Tamime, 1993). Furthermore, homogenization helps reduce the firmness of the yoghurt gel from sheep milk for producing yoghurt drinks (Kurmann, 1986). Homogenization is typically not applied in the manufacture of semi-soft and hard cheeses as it tends to increase gel firmness and retard whey syneresis in the renneted milk (Storry et al., 1983), but it can help improve the body and texture of reduced-fat cheese (Lomholt & Qvist, 1999; Metzger & Mistry, 1994).

Digestive system

This section covers the digestive system, including the mouth, stomach, and small intestine, and reviews the *in vitro* gastrointestinal (GI) digestion behavior of milk components based on current literature.

The mouth

The food is digested in the following three phases: mouth, stomach, and intestine. In the mouth, food is firstly bitten by the teeth followed by chewing, mastication, transportation, bolus formation, and swallowing (Chen, 2009). The physicochemical conditions of the mouth are shown in Appendix A Table 3. The food is broken into an appropriate size and then transported through the esophagus to the stomach. During this

progress, the pH and the enzyme content of saliva are essential factors that influence the digestion of milk proteins and fat in the subsequent gastric phase (Liu et al., 2019). Lingual lipase secreted from the tongue is the first enzyme to hydrolyze milk triglycerides (TAGs). However, the level of lipase is low in the newborn and only increases after weaning (Jensen et al., 1982). Thus, ingesting food and water is the main function of the mouth in the infant digestive system, which helps prepare the ingested food for chemical digestion and absorption in the gastric phase. Milk is an ideal food for infants since it can be automatically swallowed and transported into the stomach by the infant.

Appendix A Table 3. Physicochemical conditions of human digestion phases.

Human digestion phase	Functions	Conditions (adult)
Mouth	Chewing for breaking down food and mixing with saliva	pH: 5–7 Enzymes: amylase, lingual lipase Transition time: 5 s–2 min Saliva flow rate: 0.042–1.83 mL/min (unstimulated), 0.77–4.15 mL/min (stimulated) Biting force: 110–370 N Others: electrolytes, mucus, proteins
Gastric	Mechanical mixing and biochemical hydrolysis of ingested bolus	pH: 1–3 Enzymes: pepsin, gastric lipase Transition time: 15 min–4 h Gastric juice secretion: 1–3 L/day Frequency of contractions: 3 cycles/min Others: mucous, ions
Small intestine	Enzymatic catalysis of macromolecules to micromolecules and absorption of nutrients	pH: 6–7.5 Enzymes: pancreatic lipase, protease, amylase Transition time: 1–5 h Pancreatic juice secretion: ~ 1.5 L/day Shape: 2.5–3 cm in diameter and 6.9–7.1 m in length Others: bile, sodium bicarbonate, Ca^{2+}, Mg^{2+}

Source: Data from (Liu et al., 2019).

The stomach

The stomach, a roughly J-shaped enlargement of GI tract (GIT), is composed of the cardia, fundus, body (corpus), pyloric antrum, and pylorus according to anatomy (Appendix A Figure 3). The stomach connects the esophagus to the duodenum (the first portion of the small intestine). One of the functions of the stomach is to mix and storage food before release into the small intestine phase. Several minutes after the food bolus is ingested, peristaltic movements pass over the stomach every 15 to 25 seconds, in which few peristaltic waves appear at the fundus, and most waves occur at the body of the stomach and intensify when they reach the antrum (Tortora, 2018). The peristaltic wave that transfers food from the body of the stomach down into the antrum is known as propulsion. The pyloric sphincter normally keeps in the state of almost close, which helps prevent large particles from going into the duodenum. The gastric emptying of food only occurs when the food particles have been broken down to relatively fine particles (< 2 mm in size) (Low, 1990). Because of this, the food particles that do not fit through the narrow pyloric sphincter are push back into the body of the stomach (known as retropulsion), mixed with gastric juice and milled again. The several repeated repulsion results in forming a soupy liquid which is called chyme (Tortora, 2018). Besides, the process of gastric emptying is slow, in which the pyloric sphincter only allows approximately 3 ml of chyme to go through at a time. Therefore, some parts of food could remain in the fundus for dozens of minutes without mixing with gastric juice and be continuously digested by salivary amylase (Tortora, 2018). However, the chyme can be soonly mixed with gastric juice, leading to the inactivation of salivary amylase and the activation of lingual lipase generated by the tongue (Tortora & Derrickson, 2008).

Appendix A Figure 3. Anterior view of regions of the stomach (Tortora, 2018).

Approximately 1-3 liters of gastric juice is secreted by the fundus, which contains hydrochloric acid (HCl), potassium ions (K^+), bicarbonate ions (HCO_3^-) mucus, and enzymes. The mechanical force for mixing, disrupting and transporting the ingested food bolus is generated by the antrum (McClements & Li, 2010; Tortora, 2018). The hydrogen ions (H^+) and chloride ions (Cl^-) are separately secreted into the stomach lumen by parietal cells, but the function of both ions acts as HCl (Grabowski & Tortora, 2000). The secretion of HCl keeps the pH in the stomach at 1–3 under fasting conditions and the pH could increase to 5–6 once the food bolus enters, followed by slowly decrease to about 2 in the next hour (Liu et al., 2019). The acid produced in the stomach provides an efficient penetration barrier for the microorganisms in the ingested food and activates and promotes the pepsin to digest protein. Besides, only about 10–30% of the ingested lipid can be broken down by the gastric lipase (Favé et al., 2004).

Pepsins

Pepsin, secreted by the mucous cell, is the only proteolytic enzyme in the stomach. Pepsin can break down the protein chain with many amino acids into smaller peptide fragments through severing certain peptide bonds between amino acids (Tortora, 2018). Pepsin can be fully activated under the very acid condition of the stomach (pH 2.0), but it also can be inactivated at a higher pH above 6.5. The peak pepsin output was ~68100 U/h (Anson unit) in young (18–34 years old) and middle-aged (35–64 years old) group (Feldman et al., 1996). Ulleberg et al. (2011) reported that the pepsin activity of human gastric juices from 18 individuals (12 females and 6 males) ranged from 7 to 70 U/mL, with an average pepsin activity of 37 ± 21 U/mL, in addition, the pH, protein concentration and pepsin activity of the human pooled gastric juice from the same group is 1.7, 1.2 mg/ml and 26.7 U/ml, respectively. Besides, the pepsin activity among different age groups (infants, children, and adults) did not show statistically noticeable differences (Dipalma et al., 1991).

The small intestine

The small intestine phase is divided into three regions including duodenum, jejunum and ileum (Appendix A Figure 4). The small intestine is the region where the digestion is completed and the most absorption occurs. The environment of the intestinal tract is complex, which contains bile, pancreatic juice, co-enzymes, various salts, phospholipids, yeasts and various bacteria (Singh & Ye, 2013). The principal enzymes of the digestive enzymes are shown in Appendix A Table 4.

Appendix A Figure 4. Anterior view of the small intestine.

Appendix A Table 4. Principle enzymes in the digestive system.

Enzyme	Substrates	Products
Saliva		
Salivary amylase	Starches (polysaccharides)	Maltose (disaccharide), maltotriose (trisaccharide), and α-dextrins
Lingual lipase	Triglycerides (fats and oils) and other lipids	Fatty acids and diglycerides
Gastric juice		
Pepsin	Proteins	Peptides
Gastric lipase	Triglycerides (fats and oils)	Fatty acids and monoglycerides
Pancreatic juice		
Pancreatic amylase	Starches (polysaccharides)	Maltose (disaccharide), maltotriose (trisaccharide), and α-dextrins
Trypsin	Proteins	Peptides
Chymotrypsin	Proteins	Peptides
Elastase	Proteins	Peptides
Carboxypeptidase	Amino acid at carboxyl end of peptides	Amino acids and peptides
Pancreatic lipase	Triglycerides (fats and oils) that have been emulsified by bile salts	Fatty acids and monoglycerides
Nucleases		
Ribonuclease	Ribonucleic acid	Nucleotides
Deoxyribonuclease	Deoxyribonucleic acid	Nucleotides
Small intestinal juice		
α-Dextrinase	α-Dextrins	Glucose
Maltase	Maltose	Glucose
Sucrase	Sucrose	Glucose and fructose
Lactase	Lactose	Glucose and galactose
Enterokinase	Trypsinogen	Trypsin
Peptidases		
Aminopeptidase	Amino acid at amino end of peptides	Amino acids and peptides
Dipeptidase	Dipeptides	Amino acids
Nucleosidases and phosphatases	Nucleotides	Nitrogenous bases, pentoses, and phosphates

Source: Data modified from (Tortora, 2018).

When the gastric chyme passes into the small intestine, the gastric acid in the chyme can be rapidly neutralized by the alkaline mucus (pH 7.6) secreted by the submucosa of the duodenum (Tortora, 2018). The high concentration of bicarbonate ions (HCO_3^-) contributes to the alkaline pH of intestinal juice. The neutral pH of chyme can maximize the pancreatic enzymes activity (Golding & Wooster, 2010). In the small intestine phase, around 70-90% of lipids are hydrolyzed into free fatty acids (short-chain or long-chain fatty acids) and diacylglycerols by lipases, although some lipid digestion (~10–30%) occurs in the stomach (Singh & Gallier, 2014). There are three types of lipases in the whole digestive system: lingual lipase, gastric lipase, and pancreatic lipase. The digestion of lipids is mainly catalyzed by pancreatic lipase, which can function efficiently at pH 7 (Singh & Ye, 2013), although the optimum pH is between 8–9 (Patton & Carey, 1981). But the enzymatic reaction of pancreatic lipase with lipids requires the involvement of bile salts, calcium and co-lipase (Hur, Joo, et al., 2011). The binding degree of pancreatic lipase with lipids is mainly affected by the competitive adsorption with bile salts, the interfacial electrical charge, chyme or other surfactants (McClements et al., 2008). The co-lipase, a kind of co-enzymes containing hydrophilic and hydrophobic groups, can interact with pancreatic lipase and form an enzyme complex that can adsorb at the oil-water interface and then access to the lipid substrate more quickly (Bauer et al., 2005).

When the large fat globules enter the small intestine, it firstly is broken down into several small fat globules (a process known as emulsification) and then digested. Bile salts secreted by bile contains the sodium salts and potassium salts of bile acids, which has high surface activity and plays an important role in both digestion and absorption of lipids (Grabowski & Tortora, 2000). Bile salts are a native biosurfactant, containing a hydrophobic region and a hydrophilic region (Euston et al., 2013). The nature of bile salts makes them emulsify a large fat globule: the hydrophobic regions of bile salts adsorb

readily at the oil-water surface of the fat globule, while the hydrophilic regions of bile salts interact with the intestinal chyme (McClements et al., 2008). Therefore, the large fat globules can be easily deformed into small lipid globules, facilitating the digestion of lipid by providing a large surface area that gives accessibility of the pancreatic lipase complex to the bile salts coated fat globules (Sarkar et al., 2016). The addition of bile salts could promote the lipid digestion and improve the digestibility of both adsorbed and unadsorbed proteins in the emulsion (Gass et al., 2007).

In vitro human digestion models

Many different *in vitro* digestion models have been widely used to investigate the digestibility, structural changes, risk assessment of ingested food and release of food components under simulated GI conditions due to its significant advantages in saving time and cost, and a certain level of reproducibility compared to *in vivo* digestion studies (Hur, Lim, et al., 2011; Kong & Singh, 2010). However, the result from different *in vitro* digestion models often shows differences as they are designed in different ways. Several factors can significantly affect the result of *in vitro* digestion, such as sample characteristics, enzyme activities, ionic composition, digestion time, type of system and applied mechanical stresses (Hur, Lim, et al., 2011). The chemical composition in the digestion models is one of the important differences, including the type and concentration of digestive enzymes (i.e., pepsin, pancreatin, trypsin, chymotrypsin, lipase, peptidase, and α-amylase), bile salts, mucin, salts, buffer, the surface-active components, etc. (Almaas et al., 2006; Hur et al., 2009; Hur, Lim, et al., 2011; Lucas-González et al., 2018). Another important variation between digestion models is the mechanical stresses and the fluid flow rates used in the different digestive stages (Brandon et al., 2006; McClements et al., 2009). Although various enzymes and digestion equipment are used for *in vitro* digestion, the digestion temperature always remains at 37 °C in most of *in vitro* digestion research.

Several *in vitro* GI models have been designed to simulate the food digestion, ranging from the single static system to multi-compartmental and dynamic systems. The static models are the most popular used and have been successfully used to imitate the gastric and small intestinal digestion in three sequential phases (oral, gastric and small intestinal) (Lucas-González et al., 2018). In different digestive phases, the ingested food is mixed with simulated artificial saliva, gastric and small intestinal fluids, respectively, and incubated for a certain time and at a specific temperature and pH. However, these digestion models do not have consensus on digestive conditions, making these studies among different research teams incomparable. To mitigate the impact of these differences, the COST INFOGEST network presented a standardized protocol based on an international consensus (Brodkorb et al., 2019). The INFOGEST protocol aims to simulate the physiological conditions of the upper GI tract (oral, gastric and small intestinal phases) and help produce more comparable data of *in vitro* digestion in the future, which is increasingly useful in predicting the result of *in vivo* digestion in some cases (Bohn et al., 2018).

Obviously, these static models oversimplify the mixing pattern and cannot properly mimic fluid mechanics and mechanical forces of the human gastric digestion. Therefore, the *in vitro* GI static model do not produce all the dynamic digestion process under *in vivo* conditions, where the biochemical environment keeps changing, and the physical condition such as the continuous peristaltic movement of stomach walls has a significant influence on the breakdown of ingested food and the release of nutrients (Golding & Wooster, 2010). At this point, the dynamic digestion model can help address the defect of the static digestion model. The most important factors for designing a dynamic digestion model are geometry, biochemistry, and physical forces (Lucas-González et al., 2018) that have been differently utilised in the dynamic digestion models developed by (Kong & Singh, 2010; Vardakou et al., 2011). Besides, the geometry of the fundus and antrum of the stomach and/or the

duodenum have been also designed in some more advanced dynamic digestion models (Thuenemann, 2015). These designs help simulate the physical forces exerted on the digesta during transit through the GIT, which in turn help mimic the inhomogeneous nature of the digesta and localize the biochemical environments as *in vivo* (Thuenemann, 2015). The general comparison between static and dynamic digestion models is shown in Appendix A Table 5.

Appendix A Table 5. General comparison between static and dynamic digestion models.

Static	Dynamic
Type of study Particularly useful for gastric and/or intestinal digestion steps	Applicable in total digestion studies
Type of food Predominantly used for simple food and isolated or purified food components	Complex food
Major food applications Macronutrients • Protein • Lipid • Carbohydrate Release of micronutrients bio-accessibility of minerals from simple food matrics	• Protein digestion • Lipid separation • Peptide production Bio-accessibility of macronutrients, minerals, fat- and water-soluble vitamins, and bioactive compounds from complex food matrics
Advantages Reproduce biochemical processes and control conditions Rapid and simple Cost-effective	Reproduce the complex physical forces as observed *in vivo* Simulate the inhomogeneous nature of digesta and localized biochemical environments
Limitations No simulation of the biomechanical aspects of food digestion (stomach contraction, peristaltic movements, gastric emptying or continuous pH changing and secretion flow rates)	Unable to automate the secretory and emptying patterns Not transparent: Visual observations not possible during antral processing No validation with *in vivo* data

Digestion of milk

Digestion of milk protein

Milk as an important source of protein for human has been widely studied in both *in vitro* and *in vivo* digestion. The milk composition and processing treatment have significant impacts on digestion behavior (Boland & Singh, 2020). As mentioned above, milk mainly contains two types of proteins: caseins and whey proteins, and they show different digestion behavior depends on their structures. Caseins have a loose, highly flexible, and disordered conformation, leading to more natural exposure to hydrolysis by

pepsin during gastric digestion, whereas whey proteins exhibit a globular and well-defined three-dimensional (3D) structure, resulting in extremely resistance to proteolysis. However, caseins are known as 'slow proteins' in releasing amino acids in the plasma though caseins are more sensitive to hydrolysis by pepsin. Unlike caseins, whey proteins rapidly increase the amino acids contents in the plasma (Boirie et al., 1997). The difference can be attributed to the formation of coagulum of caseins in the stomach by the joint effects of acid secretion and digestive enzymes. The coagulum can stay in the stomach for a longer time than whey proteins, which remains soluble and is rapidly released into the small intestine. Recent studies have confirmed that unheated skim milk formed a firm, dense and cheese-like clot with porous structures during *in vitro* gastric digestion, and this structure became tighter and less permeable with increasing digestion time (Wang et al., 2018). The hydrolysis of κ-casein by pepsin can contribute to the formation of the clot as the clot formed at pH higher than 6.2, which has been supported by the observation that the level of κ-casein decreased rapidly at first 20 min of gastric digestion, and virtually all of κ-casein disappeared after 40 min according to the SDS-PAGE profile of both clot and digesta, whereas the αS-caseins and β-casein remained roughly unchanged with this digestion time (Ye et al., 2016b). The result also showed that the formation of the clot does not require the involvement of whey proteins, and whey proteins could be emptied out from the stomach during digestion (Ye et al., 2016b). Whey proteins, particularly β-Lg, are not affected by proteolysis in gastric digestion, protected by the phosphatidylcholine from the gastric mucosa on β-Lg to hydrolysis by trypsin and chymotrypsin (Mandalari et al., 2009). The same protective result of phospholipids from the gastric mucosa was also observed in the hydrolysis of α-La by pancreatic enzymes (Moreno et al., 2005). However, the β-Lg located at the interface of lipid droplets was more sensitive to pepsinolysis compared to that in the solution due to its conformation changes, whereas the α-La located at the oil-water interface showed better

resistance to digestion than that in the solution (Macierzanka et al., 2009). Recent *in vivo* study based on breastfeeding preterm neonates suggested that α-La has the highest resistance to gastric digestion in human milk proteins (De Oliveira et al., 2017). Unlike β-Lg and α-La, lactoferrin could be easily degraded during *in vitro* gastric digestion as proteolytic processing act on the cleavage sites mostly located on the surface and mainly on the non-glycosylated half of lactoferrin (Furlund et al., 2013).

Digestion of milk fat

Whole milk can also form the structured clot during gastric digestion, in which the fat globules were also involved in the formation of the clot (Ye et al., 2016b), evenly distributed in the clot matrix, and released when the matrix of the clot was broken down (Ye et al., 2016b). The close-knit structured clot became denser when the gastric digestion time increased, and the fat globules within the clot prone to aggregate and coalesce. The release of fat from the stomach to the small intestine can be affected by the structure and break down of the clot during gastric digestion. It has been reported that fat was proportionally released when the clot from three milk samples (raw, homogenized and homogenized-heated milk) was broken down during gastric digestion. In all three milk samples, a linear correlation between the release of fat globules from the clot and disintegration of the clot during gastric digestion was observed, and the slope of the regression line is close to 1 (Ye et al., 2017). It is worth noting that, in raw milk, fat globules could only be involved in the clot via entrapment because no association between milk fat globule membrane (MFGM) and proteins could be found in the matrix of the clot (Ye et al., 2020). In contrast, when the milk is homogenized, the lipids droplets become smaller and are stabilized by caseins covering the oil-water interface, consequently participating in the formation of the clot. In homogenized-heated milk, the surface of fat globules could interact with both caseins and whey proteins and therefore become a part of the clot matrix.

Confocal microscopy has shown that fresh whole milk and homogenized milk had similar close-knit structured clot at the initial stage of formation of clot matrix, whereas the structure of clot in homogenized-heated milk was much more open (Ye et al., 2017). At the early stage of digestion (20 min), fat globules seem to be clumped and the size of it in the aqueous phase appears to be larger than in undigested homogenized milk, indicating that the coalescence of fat globules occurred when the MFGM protein surrounding the fat globules was hydrolyzed by pepsin (Ye et al., 2017). Consequently, the difference in the structure of differently treated milk can influence the delivery of the fat globules to the small intestine.

Impact of processing on digestion of milk

Recent research on digestion has displayed a growing interest in understanding how the processing treatment influences the food structure and matrix design and how it affects the rates of nutrient digestion and bioavailability (Singh & Gallier, 2014). Milk processing (i.e., heat treatment and homogenization) has considerable impacts on the structure of protein, the milk fat globule, and MFGM, such as denaturation of milk protein and fat globule membrane protein, disruption of the milk fat globules into smaller droplets, which in turn leads to different digestion behavior and bioavailability of the milk fat (Gallier et al., 2012a; Gallier et al., 2013).

Heat treatment is one of the most commonly used processes in dairy manufacturing. As mentioned above, the structure of milk compositions (such as whey protein and caseins) can be differently affected by heat treatment, leading to structural changes in these components at different levels. For whey proteins, which has highly folded and compact conformation and shows resistance against digestion, heat treatment could unfold the globular structure, making whey proteins more sensitive to hydrolysis by digestive enzymes

(Barbé et al., 2013; Inglingstad et al., 2010; Peram et al., 2013; Zeece et al., 2008). For example, when skim milk was heated at 90 °C for 20 min, the denatured whey proteins and caseins could form a loose, fragmented, and porous network-structured clot, while unheated milk which formed a firm, dense-structured clot, creating more contact areas of clots for SGF containing pepsin and therefore resulting in faster hydrolysis of caseins by pepsin in heated milk than in unheated milk (Ye et al., 2016b). Besides, both β-Lg and α-La were gradually hydrolyzed into peptides along with caseins during gastric digestion. It has also been reported that in-can sterilized milk, followed by UHT (140 °C for 4 s) milk, showed more rapid degradation of proteins than untreated and pasteurized (72 or 85°C for 15 s)-homogenized milk during both *in vitro* and *in vivo* gastric digestion (Wada & Lönnerdal, 2014; Ye, Liu, et al., 2019), which is also attributed to the formation of looser structured clots with more and larger voids after sterilization and UHT treatment (Ye, Liu, et al., 2019). The similar result was also reported by (Mulet-Cabero et al., 2019), in which the influence of two types of heat treatment (pasteurization 72 °C for 15 s; UHT treatment 140 °C for 3s) on the digestion behavior of milk was investigated using a semi-dynamic adult *in vitro* model. These results indicate that whey proteins can be involved in the formation of clots during the gastric digestion of heated milk, which might be due to two main reasons: association of denatured whey proteins with casein micelles during heat treatment (Anema & Li, 2003a, 2003b) and the association of whey proteins/κ-casein complexes with casein micelles (Donato et al., 2007). Unlike unheated milk, in which both intact β-Lg and α-La could be observed in the digesta throughout the period of gastric digestion, the emptied digesta from heated milk did not contain intact caseins and whey proteins, indicating that denatured whey proteins in heated milk were hydrolyzed rapidly.

Homogenization of milk could increase the stability of the milk fat through reducing the lipids droplets size. As described above, the smaller fat globules in homogenized milk

are stabilized by the adsorption of milk proteins onto the surface of the fat globules. In homogenized and heated milk, denatured whey proteins could interact with the caseins adsorbed on fat globules and MFGM proteins through disulphide bonds (Michalski & Januel, 2006), leading to changes in the interactions of proteins during gastric digestion and therefore forming clots with more fragmented and crumbled structures during *in vitro* gastric digestion compared to raw and homogenized milk (Mulet-Cabero et al., 2019). Ye et al. (2017) reported that homogenized milk underwent a rapid decrease in the intensity of protein bands at earlier digestion stage, indicating higher hydrolysis of proteins in homogenized milk than in untreated milk, which was in agreement with the faster breakdown of the clot. Moreover, the band of β-Lg and α-La was very faint in the clot from both untreated and homogenized milk, suggesting that whey proteins did not remarkably participate in the formation of clots and remained in the aqueous phase (Ye et al., 2017).

Digestion of non-bovine milk

The most important non-bovine milk is breast milk, which can satisfy the nutritional requirements of the infant (Happe & Gambelli, 2015). Breastfeeding is not always available for all babies (Organization, 2001) and then infant formula mostly based on bovine milk can be the important alternative for breast milk due to the large production volumes of bovine milk and the proven functionalities of its components (Park et al., 2017). In recent years, there is a growing interest in consuming non-bovine milk due to its high-value energy and potential to develop nutritional and functional milk products. Therefore, it is necessary to investigate more information on the digestion behavior of non-bovine milk.

Milk from different species varies in composition, physicochemical properties and structure, leading to different digestion behaviors during digestion and influencing the kinetics of digestion and bioavailability of nutrients. For instance, goat milk has lower α_{S1}-

casein and higher β-casein contents compared to cow milk, and the infant formulae made from goat milk formed smaller flocs of aggregated protein and oil droplets during gastric digestion, resulting in faster protein digestion in goat milk infant formulae than in cow milk infant formulae (Claeys et al., 2014; Ye, Liu, et al., 2019). Besides, the casein from goat milk is more likely to be efficiently digested than that from cow milk, and peptide profiles of goat milk show both similarities and differences compared to cow milk after *in vitro* digestion (Hodgkinson et al., 2018; Hodgkinson et al., 2019).

Lönnerdal et al. (1984) investigated the effect of individual components within the cow milk-based formula on the bioavailability of zinc. They observed that lowering the ratio of casein:whey protein of cow milk to mimic human milk helped increase the absorption of zinc compared to non-adjusted cow milk. Lönnerdal and Glazier (1988) also reported that a larger proportion of insoluble zinc formed in cow milk than in human milk under gastric conditions, and a higher proportion of zinc bound to proteins in cow milk compared to human milk was also found. Subsequent study has confirmed that the bioavailability of zinc was adversely influenced by casein content, the higher casein:whey protein ratio of the infant formula, the lower bioavailability of zinc obtained (Drago & Valencia, 2004). In cow milk, caseins are the principle fraction of proteins that binds zinc, while whey proteins and fat bind only a small amount of zinc, resulting in entrapment of zinc in the clot during gastric digestion and hence incompletely digestion of zinc in the small intestine due to the unavailable absorption of a remarkable proportion of zinc (Sandström et al., 1983; Singh et al., 1989). In contrast, human milk has a significantly lower ratio of casein:whey protein than cow milk, which in turn results in higher bioavailability of zinc from human milk compared to cow milk within the GIT.

Jasińska (1995) conducted a study to examine the hydrolysis of casein micelles in raw milk from 4 species (human, goat, mare, and two breeds of cow). Results showed that

the degree of hydrolysis caseins by pepsin were 80%, 65%, 45%, 42%, and 23% for human, goat, mare, black and white cow, and red polish cow milk, respectively, and the degree of hydrolysis of caseins by trypsin were 100%, 96%, 92%, 90%, and 76% for human, goat, mare, black and white cow, and red polish cow milk, respectively. The differences in the casein hydrolysis among different species could be attributed to the different physicochemical properties of caseins. For instance, the percentage of β-casein accounts for up to 60% and 66.7% in goat and human milk, respectively, while only about 45% and 35.7% for the mare and cow milk, respectively (Potocnik et al., 2011). Besides, the difference in the degree of hydrolysis of casein between the black and white cow and red polish cow milk was caused by the size and geometries of the micellar aggregates in the milk.

Almaas et al. (2006) compared the *in vitro* digestion of bovine milk and two types of caprine milk (high and low contents of α_{S1}-casein) using human gastric and duodenal enzymes. They found that caseins and whey proteins (particularly β-Lg) in caprine milk was hydrolysed faster than bovine milk, but the two types of caprine milk with high and low α_{S1}-casein did not show obvious differences. A similar study was conducted by Opatha Vithana et al. (2012) to compare the *in vitro* digestion of protein from red deer and cow milk. They found that the protein of deer milk was degraded by commercial proteolytic enzymes more rapidly and produced more peptides than cow milk. In addition, the higher digestibility of caseins, α-La, and immunoglobulin in deer milk was also observed, whereas the digestibility of β-Lg from both species was low.

Dalziel et al. (2018) compared the effect of sheep, cow skim milk before and after fermentation on the rate of GI transit using X-ray imaging technique. The GI transit was tracked at three-time points for identifying the location of metallic beads that exit from the stomach (4h), small intestine transit (9h), and large intestine transit (12h). They reported

that the GI transit was more rapid for sheep milk than cow milk of both unfermented and fermented types, and a similar pattern was also observed for the fermented milk from both species. The faster GI transit for sheep milk in comparison with cow milk demonstrated the prominent differences between species, regardless of whether the milk was fermented or not.

Tagliazucchi et al. (2018) conducted a study to compare the digestibility, biological activities, and peptidomic profiles from skimmed cow, goat, sheep, and camel milk. They observed that, after *in vitro* digestion, the protein digestibility increased in the order of cow < camel ≈ sheep < goat, while the angiotensin-converting enzyme (ACE)-inhibitory activity was in the order of sheep > goat > camel > cow and the dipeptidyl peptidase-IV (DPP-IV)-inhibitory activity was shown in the order cow > goat > sheep > camel. Besides, goat and sheep milk had the highest similarity in peptides sequence according to the peptidomic analysis after *in vitro* digestion.

Besides the protein digestion, the lipid digestion is also important due to the difference in lipidomic profiles and structures of milk fat from different species. The difference in milk fat globule size and structure could also influence the digestion of fat differently. The capture and subsequent release of the fat globules from the clot formed in the stomach could be different depends on the composition and structures of proteins, and the composition and structural properties of milk protein. Fats are also varied in different species, therefore, leading to differences in the digestion of milk fat from different species.

Alférez et al. (2001) investigated the effects of milk fat from cow and goat on the digestive utilization and biochemical parameters related to the metabolism of lipids using rats. Three types of diets, including olive oil (as standard diet), fat obtained from lyophilized goat milk and lyophilized cow milk, respectively, were fed to the rats. Results showed that

the digestibility of fat showed in the order: olive oil > goat milk-based milk > cow milk-based diet. The authors demonstrated that the higher digestibility coefficient of goat milk fat could be attributed to the higher medium-chain triglyceride content (36%) in goat milk fat than that in cow milk fat (21%). In addition, the smaller fat globule size in goat milk contributed to the faster digestion of lipids, with providing more contact surface to the lipase during digestion. The consumption of goat milk was also considered to reduce the synthesis of endogenous cholesterol and intestinal absorption.

Meena et al. (2014) compared the digestion of milk fat from four species (goat, cow, buffalos, and camel) using an *in vitro* intestinal digestion model. They found that the buffalo showed the biggest fat globule size, followed by cow, goat, and camel. However, camel milk fat had the highest digestibility, followed by goat, cow, and buffalo, suggesting that the fat digestibility was negatively correlated with the size of the fat globules. Besides, the level of free fatty acids released from the milk samples decreased in the order goat ≈ camel > cow > buffalo, indicating the better digestion of fat in goat and camel milk because of their smaller size of fat globules.

Research gaps related to the effect of processing on sheep milk

As discussed above, the heat treatment of sheep milk can cause pronounced physicochemical changes, such as the coagulation of proteins, leading to sedimentation and hence poor quality and shelf-life. UHT milk is known to form a sediment layer during storage, and this layer usually is thin but robust and cannot be easily dislodged. Besides, the casein micelles precipitate at the bottom of the pack when stored for several months, but this phenomenon is reversible by inversion or shaking. Sedimentation is considered as a defect when it becomes excessive and forms a thick layer of deposition after manufacture. The formation of the thick sediment layer can be found in the manufacture of UHT sheep

milk, limiting the production of UHT sheep milk (Martinez Alonso et al., 2009). The lower heat stability of sheep milk than cow milk, due to various factors (i.e., milk composition, pH, protein structures), has been reported. But the mechanism of formation of sediment in UHT sheep milk has not yet been well investigated. Therefore, the structural changes and interactions between protein components in sheep milk during heat treatment will be studied in this project.

Research gaps related to digestion of sheep milk

To date the *in vitro* digestion research has mainly focused on cow and goat milk, the information about the digestion behavior of sheep milk is unclear. In addition, the influence of processing treatments on the digestion behavior of sheep milk has not yet been reported. Therefore, it is important to investigate the digestion behavior of sheep milk and provide more valuable information in manipulating the digestion rates of nutrients in milk products and contributing to the development of sheep dairy industry.

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Appendix B: Statement of Contribution – Doctorate with Publications/Manuscripts

DRC 16

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Zheng Pan
Name/title of Primary Supervisor:	Professor Aiqian Ye
In which chapter is the manuscript /published work:	Appendix A

Please select one of the following three options:

○ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

◉ The manuscript is currently under review for publication – please indicate:

- The name of the journal:

Food Chemistry

- The percentage of the manuscript/published work that was contributed by the candidate: 90.00
- Describe the contribution that the candidate has made to the manuscript/published work:

The candidate contributes to the conceptualization, designed and conducted the experiment, analyzed all the experimental data with assistance from Professor Aiqian Ye in developing an appropriate method and Dr Karl Fraser in analyzing the lipidomic data. The candidate wrote the original draft of the manuscript, with changes and corrections completed with input from all supervisors.

○ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Zheng Pan Digitally signed by Zheng Pan Date: 2023.02.21 17:01:12 +13'00'
Date:	21-Feb-2023
Primary Supervisor's Signature:	Aiqian Ye Digitally signed by Aiqian Ye DN: cn=Aiqian Ye, c=NZ, o=Massey University, ou=SF&AT, email=a.m.ye@massey.ac.nz Date: 2023.02.22 14:09:28 +13'00'
Date:	22-Feb-2023

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

GRS Version 5 – 13 December 2019
DRC 19/09/10

Appendix B: Comparative lipidomics analysis of different-sized fat globules in sheep and cow milks

The contents of this article have been submitted to Food Chemistry.

Abstract

The effect of milk fat globule (MFG) size and species (sheep versus cow) on the lipid and protein compositions of sheep and cow milks was studied. The MFGs in raw cow and sheep milks were separated into six significantly different-sized (1–5 μm) groups by a gravity-based separation method, and their fatty acids, their lipidomes and the protein compositions of their MFG membranes were determined. The proportions of polar lipids and glycoproteins increased with decreasing MFG size in both sheep milk and cow milk, whereas the fatty acid composition showed few differences among the MFG groups. The average size of each MFG group was comparable between sheep milk and cow milk. Sheep milk contained higher proportions of short-chain fatty acids, medium-chain fatty acids and sphingomyelin than cow milk in all MFG groups. The proportion of glycoproteins was higher in cow MFG membrane than in sheep MFG membrane. The results suggested that the lipid and protein compositions were markedly species and size dependent.

Keywords: Fatty acid, Lipidome, Polar lipids, Milk fat globule membrane, Phospholipids, Glycoprotein

Introduction

Milk fat globules (MFGs) are secreted in a diverse range of sizes (0.2–15 μm) depending on the animal species (Argov et al., 2008), the lactation stage (Mesilati-Stahy & Argov-Argaman, 2014), the season (Briard et al., 2003; Li, Delger, et al., 2022) and animal nutrition (Lopez et al., 2008). There can be significant differences in the compositions of

the core lipids and the milk fat globule membrane (MFGM) between small and large MFGs (Briard et al., 2003; Lopez et al., 2011; Mesilati-Stahy et al., 2011; Michalski et al., 2003). The differences in the lipid compositions of different-sized MFGs in cow milk have been well researched. For instance, small cow MFGs contained more medium-chain and polyunsaturated fatty acids than large cow MFGs (Lopez et al., 2011; Mesilati-Stahy et al., 2011). Some studies have also reported the relationship between the size of the native MFGs and the protein composition of the MFGM. Lu et al. (2016) analyzed the MFGM protein compositions of two different-sized cow MFG fractions (obtained using a centrifugation method) and showed that lactadherin, lactoferrin/lactotransferrin, fatty-acid-binding protein, cluster of differentiation 14 and mucins 1/4/15 were enriched in larger MFGs (7.6 ± 0.9 μm) compared with smaller MFGs (3.3 ± 1.2 μm). However, these differences between different-sized MFGs have not been studied in sheep milk.

Cow, buffalo, goat and sheep milks are among the most consumed animal milks in the world. Some studies have compared the overall lipid compositions of sheep milk and cow milk. For instance, Pietrzak-Fiećko and Kamelska-Sadowska (2020) compared the lipid contents of milks from different mammalian species and showed that sheep milk had the highest fat content, whereas cow milk had the highest cholesterol concentration. Teng et al. (2020) determined the fatty acid compositions of sheep milk and cow milk and found that sheep milk contained a higher percentage of unsaturated fatty acids but a lower percentage of saturated fatty acids than cow milk.

Although the overall differences in the milk fat between different species have been studied, the dominant view of milk fat overlooks the influence of MFG size on the compositional differences between species. A clear understanding of the differences in the compositions of the milks from different mammalian species and the compositions of different-sized MFGs could encourage the consumption of different milk sources, not just

for nutritional reasons, but also as a targeted supplement to improve human development and health (Thum et al., 2023). Therefore, determining the lipid and protein compositions of different-sized MFGs could provide more information for the development of new products with different technological properties and an understanding of their potential impact on digestion and postprandial metabolism. Previous studies on the composition of MFGs and the MFGM as a function of different MFG sizes have focused exclusively on cow milk (Lu et al., 2016; Mesilati-Stahy et al., 2011); the compositional variations in MFG size fractions between sheep milk and cow milk have yet to be elucidated.

This study aimed to understand the size-dependent lipid and protein compositions of the MFGs in sheep milk, and the compositional variations between sheep milk and cow milk. Different-sized MFGs of sheep milk and cow milk were separated according to a gravity-based separation method, resulting in six MFG size groups. The protein and lipid compositions of each MFG group in sheep milk and cow milk were analyzed and compared.

Materials and methods

Milk collection

Bulk raw cow milk and sheep milk in mid lactation were collected from Massey University No. 4 dairy farm (Palmerston North, New Zealand) and Fernglen Farm Limited (Masterton, New Zealand) respectively at 4 °C and sent to the laboratory using chill boxes. The compositions of the milks were analyzed using a MilkoScan FT1 (Foss Electric, Hillerød, Denmark) and are shown in Appendix B Table 1. The milk collection was replicated three times on different days.

Appendix B: Comparative lipidomics analysis of different-sized fat globules in sheep and cow milks

Appendix B Table 1. Fatty acid compositions (g/100 g fat) of different-sized milk fat globule groups from sheep milk and cow milk[a].

Fatty acid	Species	Whole milk	Cream	F1	F2	F3	F4	F5	*P* value (groups)	*P* value (species)
C4:0	Sheep	2.57 ± 0.16	2.28 ± 0.05	2.45 ± 0.06	2.48 ± 0.02	2.41 ± 0.01	2.48 ± 0.04	2.29 ± 0.06	0.0018	ns
	Cow	2.55 ± 0.21	2.56 ± 0.14	2.64 ± 0.13	2.59 ± 0.09	2.59 ± 0.08	2.20 ± 0.40	2.11 ± 0.12	0.0248	
C6:0	Sheep	2.74 ± 0.12	2.58 ± 0.19	2.67 ± 0.16	2.66 ± 0.13	2.65 ± 0.13	2.65 ± 0.14	2.45 ± 0.14	ns	< 0.0001
	Cow	2.14 ± 0.12	2.18 ± 0.04	2.19 ± 0.03	2.19 ± 0.04	2.17 ± 0.04	2.01 ± 0.14	1.77 ± 0.10	0.0002	
C8:0	Sheep	2.84 ± 0.24	2.73 ± 0.29	2.77 ± 0.22	2.74 ± 0.20	2.78 ± 0.18	2.83 ± 0.17	2.64 ± 0.23	ns	< 0.0001
	Cow	1.37 ± 0.04	1.40 ± 0.04	1.39 ± 0.01	1.40 ± 0.02	1.42 ± 0.07	1.33 ± 0.01	1.19 ± 0.06	< 0.0001	
C10:0	Sheep	8.90 ± 1.08	8.92 ± 1.44	8.75 ± 0.99	8.70 ± 0.95	8.85 ± 0.89	9.27 ± 0.81	9.10 ± 1.02	ns	< 0.0001
	Cow	3.38 ± 0.19	3.33 ± 0.06	3.26 ± 0.12	3.31 ± 0.12	3.31 ± 0.14	3.30 ± 0.09	3.17 ± 0.12	ns	
C10:1	Sheep	0.28 ± 0.02	0.35 ± 0.18	0.28 ± 0.02	0.29 ± 0.02	0.30 ± 0.02	0.34 ± 0.02	0.37 ± 0.02	ns	ns
	Cow	0.27 ± 0.01	0.32 ± 0.07	0.29 ± 0.02	0.29 ± 0.02	0.30 ± 0.02	0.32 ± 0.04	0.26 ± 0.01	ns	
C12:0	Sheep	5.01 ± 0.77	4.88 ± 0.77	4.84 ± 0.66	4.83 ± 0.64	5.07 ± 0.56	5.38 ± 0.57	5.40 ± 0.74	ns	< 0.0001
	Cow	3.61 ± 0.16	3.66 ± 0.27	3.63 ± 0.18	3.68 ± 0.16	3.83 ± 0.41	3.66 ± 0.14	3.43 ± 0.07	ns	
C14:0	Sheep	11.13 ± 0.88	10.94 ± 0.75	10.88 ± 0.62	10.86 ± 0.68	11.06 ± 0.55	11.48 ± 0.51	11.40 ± 0.77	ns	0.0221
	Cow	11.52 ± 0.37	11.50 ± 0.52	11.61 ± 0.44	11.75 ± 0.35	11.73 ± 0.42	11.66 ± 0.29	10.89 ± 0.19	ns	
C14:1	Sheep	0.21 ± 0.05	0.22 ± 0.03	0.20 ± 0.02	0.25 ± 0.07	0.28 ± 0.06	0.25 ± 0.01	0.28 ± 0.01	ns	< 0.0001
	Cow	0.80 ± 0.05	0.79 ± 0.06	0.82 ± 0.06	0.84 ± 0.05	0.86 ± 0.05	0.87 ± 0.05	0.81 ± 0.05	ns	
C15:0	Sheep	1.31 ± 0.09	1.28 ± 0.05	1.28 ± 0.04	1.28 ± 0.05	1.28 ± 0.06	1.28 ± 0.04	1.23 ± 0.08	ns	< 0.0001
	Cow	1.09 ± 0.06	1.11 ± 0.10	1.09 ± 0.10	1.12 ± 0.08	1.13 ± 0.09	1.14 ± 0.12	1.05 ± 0.05	ns	
C16:0	Sheep	23.72 ± 1.13	23.45 ± 0.79	23.38 ± 0.59	23.18 ± 0.80	23.49 ± 0.61	24.19 ± 0.54	24.23 ± 0.91	ns	< 0.0001
	Cow	27.60 ± 1.24	27.80 ± 1.54	27.17 ± 1.39	27.49 ± 1.31	27.41 ± 1.40	27.60 ± 1.20	25.86 ± 0.44	ns	
C16:1	Sheep	1.27 ± 0.16	1.41 ± 0.01	1.42 ± 0.04	1.57 ± 0.18	1.56 ± 0.22	1.34 ± 0.11	1.45 ± 0.25	ns	< 0.0001
	Cow	1.68 ± 0.23	1.60 ± 0.10	1.79 ± 0.07	1.79 ± 0.08	1.83 ± 0.13	1.82 ± 0.10	1.55 ± 0.05	ns	
C17:0	Sheep	0.65 ± 0.03	0.71 ± 0.10	0.65 ± 0.05	0.63 ± 0.04	0.62 ± 0.05	0.61 ± 0.05	0.57 ± 0.05	ns	< 0.0001
	Cow	0.84 ± 0.04	0.85 ± 0.03	0.84 ± 0.05	0.84 ± 0.05	0.81 ± 0.05	0.80 ± 0.05	0.79 ± 0.01	ns	
C18:0	Sheep	7.87 ± 1.51	8.13 ± 1.59	7.64 ± 1.55	7.30 ± 1.47	6.95 ± 1.44	6.30 ± 1.30	5.57 ± 1.14	ns	< 0.0001
	Cow	10.71 ± 0.83	10.95 ± 0.58	10.50 ± 0.70	10.30 ± 0.68	9.87 ± 0.70	9.57 ± 0.77	9.58 ± 0.24	ns	
C18:1	Sheep	20.37 ± 1.98	20.69 ± 2.03	20.61 ± 1.94	20.20 ± 1.91	19.78 ± 1.86	19.16 ± 1.76	17.48 ± 1.60	ns	< 0.0001
	Cow	23.29 ± 2.18	23.73 ± 1.78	23.62 ± 1.80	23.38 ± 2.03	22.89 ± 2.02	22.60 ± 2.16	22.26 ± 0.81	ns	
C18:2	Sheep	2.49 ± 0.24	2.44 ± 0.29	2.53 ± 0.31	2.59 ± 0.37	2.49 ± 0.35	2.46 ± 0.33	2.24 ± 0.34	ns	< 0.0001
	Cow	1.51 ± 0.15	1.66 ± 0.22	1.62 ± 0.09	1.53 ± 0.09	1.61 ± 0.18	1.56 ± 0.12	1.67 ± 0.22	ns	
CLA	Sheep	1.77 ± 0.32	1.79 ± 0.32	1.73 ± 0.27	1.75 ± 0.24	1.75 ± 0.23	1.74 ± 0.28	1.78 ± 0.27	ns	< 0.0001

Appendix B: Comparative lipidomics analysis of different-sized fat globules in sheep and cow milks

	Cow	1.03 ± 0.09	1.03 ± 0.04	1.08 ± 0.06	1.08 ± 0.07	1.09 ± 0.09	1.11 ± 0.11	1.29 ± 0.16	ns	
C18:3	Sheep	1.81 ± 0.14	1.90 ± 0.15	1.87 ± 0.05	2.05 ± 0.19	1.98 ± 0.23	1.71 ± 0.15	1.51 ± 0.17	0.0232	< 0.0001
	Cow	0.79 ± 0.06	0.82 ± 0.08	0.86 ± 0.11	0.77 ± 0.03	0.82 ± 0.11	0.72 ± 0.11	0.80 ± 0.08	ns	
Others	Sheep	5.05 ± 0.98	5.30 ± 0.70	6.04 ± 0.21	6.64 ± 0.49	6.69 ± 0.26	6.53 ± 0.59	10.01 ± 2.05	0.0004	ns
	Cow	5.82 ± 1.34	4.71 ± 0.36	5.58 ± 0.44	5.65 ± 0.88	6.31 ± 0.71	7.74 ± 1.71	11.51 ± 2.29	0.0003	

[a]*Means ± standard deviations.*

Abbreviations: CLA, conjugated linoleic acid; ns, no significant difference.

P value (groups) shows differences among the different milk fat globule groups. P value (species) shows differences between sheep milk and cow milk. The group and species variations were analyzed using one-way analysis of variance.

MFG separation

Different-sized MFGs were separated using a gravity-based separation method described by Ma and Barbano (2000) with slight modifications. A 60 mL milk sample was separated into a syringe (60 mL capacity) without the needle instead of a cylindrical plastic column. The syringe with the milk sample was held vertically (tip side down) at 17 °C for 20 h in a climate chamber ICH110eco (Memmert GmbH + Co. KG, Schwabach, Germany). The skim milk was divided into five fractions and was drained from the bottom of the syringe by gently pushing the plunger. The different MFG fractions from top to bottom were defined as F1 to F5. Fraction F5 was 5 mL, and the other fractions (F1–F4) were 10 mL each. The cream layer at the top of the milk was also collected.

Determination of MFG size

The MFG sizes of the whole milk, the cream and the five fractions of skim milk were determined using a Malvern MasterSizer 2000 (Malvern Instruments Ltd., Malvern, UK). All milk samples were diluted in a solution containing 2% sodium dodecyl sulphate

(SDS) and 20 mM EDTA, pH 6.7, to dissociate the casein micelles. Each sample was measured in triplicate.

Fatty acid analysis

The total lipids were extracted from the milk samples using the B&D method (Bligh & Dyer, 1959). Briefly, the lipids were extracted by mixing 5 mL of milk sample with 5 mL of chloroform:methanol (1:2, v/v) and the organic phases at the bottom were collected. The extraction procedure was repeated twice, and the extracted organic phases were pooled. After evaporation of the solvent in an evaporator (Thermo Fisher Scientific, IL, USA), 2 mL of methanol was added to dissolve the lipids. The total amounts of individual fatty acids were analyzed using a protocol developed by Zhu et al. (2013). Briefly, 200 μL of the extract was transferred to a 10 mL screw-cap glass tube. After the solvent had been evaporated using the evaporator, 0.5 mL of nonadecanoic acid (C19:0; CAS No. 646-30-0; molecular weight = 298.5) in heptane (1 mg/mL) as internal standard, 0.7 mL of 10 *M* NaOH and 5 mL of methanol were added. The well-sealed tubes were incubated in a water bath at 55 °C for 1.5 h. The tubes were then allowed to cool to room temperature and 0.58 mL of 12 *M* H_2SO_4 was added. The tubes were well mixed by manually shaking and were incubated again in the water bath at 55 °C for 1.5 h. They were then cooled to room temperature and centrifuged at 3500 × *g* for 10 min at 20 °C. The heptane layer was transferred to a 350 μL glass insert fitted in an autosampler vial and stored at –18 °C before gas chromatographic analysis.

The fatty acid composition was determined using an Agilent 7890 system equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA). The oven temperature was initially held at 180 °C for 5 min, then programmed to 210 °C at a rate of 1 °C/min and held at 210 °C for 25 min. The temperatures of both the flame ionization

detector and the injector were set at 270 °C. Peak identification was based on the relative retention times of the internal standard. The amount of each fatty acid was calculated by comparing its peak area with that of the internal standard, and the total amount of fatty acids was obtained by summing the calculated amount of each fatty acid. The fatty acids were recorded as the percentage of total fatty acids (w/w) within each sample.

Lipidomics of different-sized MFGs

The milk was thawed and thoroughly shaken by hand to mix (60 s) prior to aliquoting. The extraction method was a biphasic liquid–liquid extraction, as used in the laboratory for untargeted metabolomics. The lower organic phase containing lipids was measured by liquid chromatography coupled to mass spectrometry (LC–MS). Briefly, 300 μL of milk was mixed with 800 μL of prechilled (–20 °C) chloroform:methanol (50:50, v/v), agitated for 30 s and placed in a –20 °C freezer for 60 min to allow protein precipitation; this was followed by the addition of 400 μL of water, vortex-mixing for 30 s and centrifugation at 11,000 rev/min and 4 °C for 10 min in an Eppendorf Centrifuge 5427 R (Eppendorf AG, Hamburg, Germany). A 200 μL aliquot of the lower organic layer was removed and evaporated to dryness under a stream of nitrogen. Pooled lipid quality control (QC) samples for sheep milk or cow milk were prepared by combining 50 μL of the lower organic phase from each sample of sheep milk or cow milk in a new tube. The pooled QC samples were well mixed and dried under a stream of nitrogen. These dried samples were stored at –80 °C until analysis.

The lipid extracts were analyzed using a Shimadzu Nexera-x2 Ultra Performance Liquid Chromatography® system coupled to a Shimadzu LC–MS-9030 mass spectrometer. A 2 mL sample was injected on to a Waters CSH-C18 column (2.1 mm × 100 mm, 1.7 μm particle size) and the column oven was set to 60 °C. The chromatographic conditions were

as follows: total run time, 15 min; flow rate, 400 μL/min; solvent A, 10 m*M* ammonium formate and a mixture of water, acetonitrile and isopropanol in a ratio of 5:3:2 (v/v/v); solvent B, 10 m*M* ammonium formate and a mixture of water, acetonitrile and isopropanol in a ratio of 1:9:90 (v/v/v). The solvent gradient program was as follows: 10–45% solvent B (0–2.7 min), 45–53% solvent B (2.7–2.8 min), 53–65% solvent B (2.8–9.0 min), 65–89% solvent B (9.0–9.1 min), 89–92% solvent B (9.1–11.0 min) and 92–100% solvent B (11.0–11.1 min), and held for 0.8 min (11.1–11.9 min) before returning to the starting conditions of 10% solvent B in 0.1 min (11.9–12.0 min); before injection of the next sample, the column was re-equilibrated under the starting conditions for 15 min (Abshirini et al., 2021). Mass spectrometry analysis was performed in positive ion mode. The following mass spectrometer conditions were used: gas temperature, 150 °C; nebulizing gas flow rate, 2.0 L/min; heater gas flow rate, 10 L/min; interface temperature, 300 °C; drying gas flow rate, 10 L/min; desolvation line temperature, 250 °C; heater block temperature, 400 °C; source voltage, +4.0 kV; sheath gas flow rate, 10 L/min. Spectra were obtained over the range 250–1250 *m/z* and the data-independent acquisition data were collected in 20 *m/z* windows from 300 to 1100 *m/z*. High-purity nitrogen was used for the drying and collision gases.

Isolation of MFGM material

The MFGM material of the sheep milk and the cow milk was isolated from milk samples containing different-sized MFGs using a centrifugation method described by Ye et al. (2002) with slight modifications. Phosphate-buffered saline (PBS; containing 0.137 *M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4 and 1.8 m*M* KH_2PO_4, pH 6.8) was used as the washing solution in this study instead of simulated milk ultrafiltrate. Briefly, the milk samples containing different-sized MFGs were centrifuged at 15,000 × *g* for 20 min at 20 °C using a temperature-controlled ultracentrifuge Avanti JXN-26 (Beckman Coulter, Brea, CA, USA) and the top layer (cream) was collected using a spatula. The cream was washed with

PBS to remove proteins that did not associate with the MFGM. The cream was suspended in 10 volumes of PBS and allowed to stand for 1 h at room temperature. The top layer of this mixture was collected after it had been centrifuged at 15,000 × *g* for 20 min at 20 °C. The washing step in PBS was done twice. The washed cream was stored at 4 °C before further analysis.

MFGM protein composition analysis

The protein composition of the washed cream was determined by SDS-polyacrylamide gel electrophoresis (PAGE). The washed cream was mixed with a sample buffer, containing 0.5 *M* Tris-HCl, 10% glycerol, 10% (w/v) SDS (20 mL), 0.01% bromophenol blue and 100 m*M* dithiothreitol, to reach a protein concentration of 1 mg/mL. The mixture was vortexed and heated at 70 °C for 10 min in a temperature-controlled water bath.

Before loading on to the gel, the mixture was centrifuged at 11,000 × *g* for 5 min to remove the fat. A 10 μL aliquot of the mixture was loaded into each well of a 4–15% polyacrylamide gel. The same amount of molecular weight standard protein solution (Bio-Rad Laboratories, Hercules, CA, USA) was also added to the well for identifying the MFGM proteins. The protein bands were fixed by 5% glutaraldehyde solution (Sigma-Aldrich, Poole, UK) and stained with Coomassie brilliant blue G-250 (0.03 g in 100 mL of 10% acetic acid solution) for 30 min. Visualization of the gels was performed using a Molecular Dynamics Model PD-SI computing laser densitometer (Molecular Dynamics Inc., Sunnyvale, CA, USA), and the result was analyzed using Image Lab software version 6.1 (Bio-Rad Laboratories).

Data processing

Data processing of the untargeted LC–MS lipidomics data was performed using the untargeted data processing software package MS-DIAL (v. 4.90; http://prime.psc.riken.jp/compms/msdial/main.html), which contains the LipidBlast database internally (v. 2022, https://fiehnlab.ucdavis.edu/projects/LipidBlast) (Tsugawa et al., 2015). The data-independent acquisition spectra were used to identify the aligned peaks. The lipidomic features were searched against the built-in lipid library in-silico-generated lipid fragmentation spectra. The locally weighted scatterplot smoother (LOWESS) regression analysis and the pooled QC samples were used to correct run-order and normalize the resultant peak intensity Appendix B Table. Features within the pooled QC samples with an average QC-to-blank sample ratio of less than 5 and a coefficient of variation of 30% were removed. In total, the full datasets for 21 sheep milk samples and 21 cow milk samples were included in the lipidomic analysis.

Statistical analysis

The peak intensity of each individual fatty acid was converted to a relative proportion of the total lipids. A one-way analysis of variance (ANOVA) test followed by multiple comparisons was used to verify differences in the abundances of the fatty acids in sheep milk and cow milk. These analyzes were carried out using GraphPad Prism v. 8.4.0 software (GraphPad Software). The lipidomics data were transformed by generalized log-transformation and auto-scaling to correct for heteroscedasticity, to reduce the skewness of the data and to mask effects. Principal component analysis (PCA) identified differential lipid metabolites between different-sized MFG groups and between sheep milk and cow milk. The heatmap was clustered by Euclidean distance and Ward's minimum variance method. The PCA and the heatmap analysis were produced using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

Results and discussion

MFG size

The particle size distributions of the whole milk, the cream and fractions F1–F5 obtained from cow milk and sheep milk are shown in Appendix B Figures 1A and 1B, respectively. The cream of both cow milk and sheep milk showed a peak at size larger than 17 μm, whereas only a single peak (< 17 μm) was observed in the other milk samples. The average fat globule size (D43) of the whole milk, the cream and fractions F1–F5 is shown in Appendix B Figure 1C. For both sheep milk and cow milk, the fat globule size was highest in the cream, followed by the whole milk and fractions F1, F2, F3, F4 and F5. Statistical analysis showed that there were significant ($P < 0.05$) differences in the MFG size between each of the size groups, and that there were no significant ($P > 0.05$) differences in the fat globule size in each corresponding fraction between sheep milk and cow milk. This finding for the average MFG sizes of sheep whole milk and cow whole milk does not agree with previously reported findings that sheep milk has a smaller fat globule size than cow milk (Crowley et al., 2017; Roy et al., 2021a). It has been proposed that maternal physiology, breed, herd nested within a herd, season and diet can result in MFG size variations within species (Thum et al., 2023). Therefore, the difference in the MFG sizes of sheep whole milk and cow whole milk between this and previous studies may be due to the different milk sources used.

Fatty acid profile of different-sized MFGs

The fatty acid compositions of the different-sized MFG groups were determined by gas chromatography. Appendix B Table 1 presents an overview of the major fatty acid composition; unknown minor fatty acids were grouped and are presented as “Others”. Six fatty acids (C10:0, C12:0, C14:0, C16:0, C18:0 and C18:1) in the whole milk, cream and

fractions F1–F4 accounted for over ~ 76% and ~ 78% of the total fatty acids for sheep milk and cow milk respectively; these fatty acids were lower in fraction F5 of the sheep milk (~ 73%) and the cow milk (~ 75%). Fraction F5 of sheep milk had the lowest proportions of C4:0 and C18:3 and the highest proportion of C16:0 compared with the other MFG groups. However, cow milk showed a different pattern; fraction F5 had the lowest proportions of C4:0, C6:0 and C8:0 and the other major fatty acids did not show significant changes among the MFG groups. Both sheep milk and cow milk showed an increasing trend for the "Others" unknown fatty acids as the MFG size decreased. This indicated that the MFGs in the sheep milk and the cow milk had similar fatty acid composition patterns in the different fractions and that the MFG size had little impact on the composition of the major fatty acids.

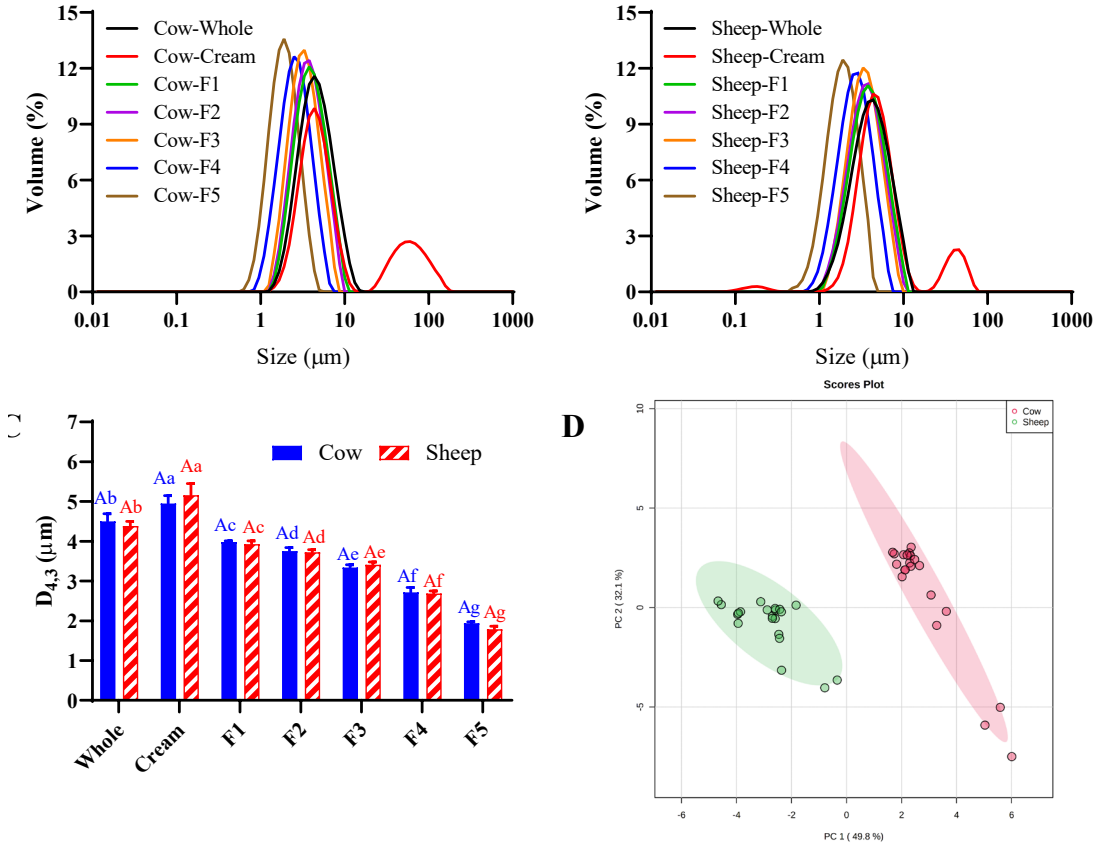
Statistical analysis of the fatty acid composition showed that the proportions of C4:0 and C18:3 in the sheep MFGs were significantly ($P < 0.05$) affected by the MFG size but that the proportions of the other fatty acids did not show a significant difference among the MFG groups; the cow milk fatty acids showed significant differences in the proportions of C4:0, C6:0 and C8:0 between fraction F5 and the other MFG groups whereas the other fatty acids did not show significant differences among the MFG groups (Appendix B Table 1). This indicated that the composition of the major fatty acids was not influenced by the MFG size in both sheep milk and cow milk. This is different from previous findings reported by Mesilati-Stahy et al. (2011) and Lu et al. (2016), who showed that smaller MFGs contained more unsaturated fatty acids (such as C18:1, C18:2 and C18:3) than larger MFGs but no significant differences were reported for C4:0, C6:0 and C8:0. The reason for the differences between the results presented here and in previous studies is unclear. The variation may be due to differences in breed, diet, feeding, season and stage of lactation, as these factors have been proven to affect the fatty acid composition of milk (Mohsin et al., 2019).

It should be noted that the markedly increased "Others" unknown fatty acids in the smallest MFG fraction (Appendix B Table 1) have not previously been reported. The results for these unknown fatty acids must be interpreted with caution because the raw milk samples may have contained some indigenous bacteria that could contain some odd- and branched-chain fatty acids (such as iso C14:0, iso C15:0, anteiso C15:0, iso C16:0, iso C17:0 and anteiso C17:0) in the bacterial membrane lipids (Vlaeminck et al., 2006). These indigenous bacteria in milk may have precipitated to the bottom during separation, leading to an increased proportion of unknown fatty acids. However, the bacterial content was not clarified in the study. The bacterial content in each fraction should be further studied to verify if the existence of bacteria affected the fatty acid composition.

Sheep milk contained overall higher proportions of C6:0, C8:0, C10:0, C12:0, C14:0, C15:0, C18:2, conjugated linoleic acid and C18:3 and lower proportions of C14:1, C16:0, C16:1, C17:0, C18:0 and C18:1 than cow milk, but no significant ($P > 0.05$) differences were found for C4:0, C10:1 and the "Others" unknown fatty acids (Appendix B Table 1). This is in agreement with previously reported results that the fatty acid content of sheep milk has comparable C4:0, more medium-chain fatty acids (MCFAs, including carbon numbers between 6 and 15) but fewer long-chain fatty acids (LCFAs, including carbon numbers larger than 16) than cow milk (Balthazar et al., 2017).

To investigate the difference in the distribution of fatty acids between sheep milk and cow milk, PCA was performed and the results are shown in Appendix B Figure 1D. The fatty acid compositions of sheep milk and cow milk were completely separated. The clustering highlights that the lipid compositions of the different-sized MFG groups were more similar to each other within the milks from the same species than to the milks from different species. According to the fatty acid composition shown in Appendix B Table 1, sheep milk contained more MCFAs than cow milk in all the MFG groups, in accordance

with previous studies (Ramos & Juarez, 2011; Teng et al., 2020). These differences in the MCFAs may have contributed to the complete separation between sheep milk and cow milk in the PCA plot (Appendix B Figure 1D).



Appendix B Figure 1. Particle size distributions of the whole milk, cream and fractions F1–F5 obtained from (A) cow milk and (B) sheep milk. (C) Fat globule sizes of the whole milk, cream and fractions F1–F5 obtained from cow milk (blue bars with no pattern) and sheep milk (red bars with lines). Different capital letters represent significant ($P < 0.05$) differences between cow milk and sheep milk. Different lower-case letters represent significant ($P < 0.05$) differences among the fat globule groups. (D) Principal component analysis of the differences in the fatty acid compositions of sheep milk and cow milk.

Lipid composition of different-sized MFGs

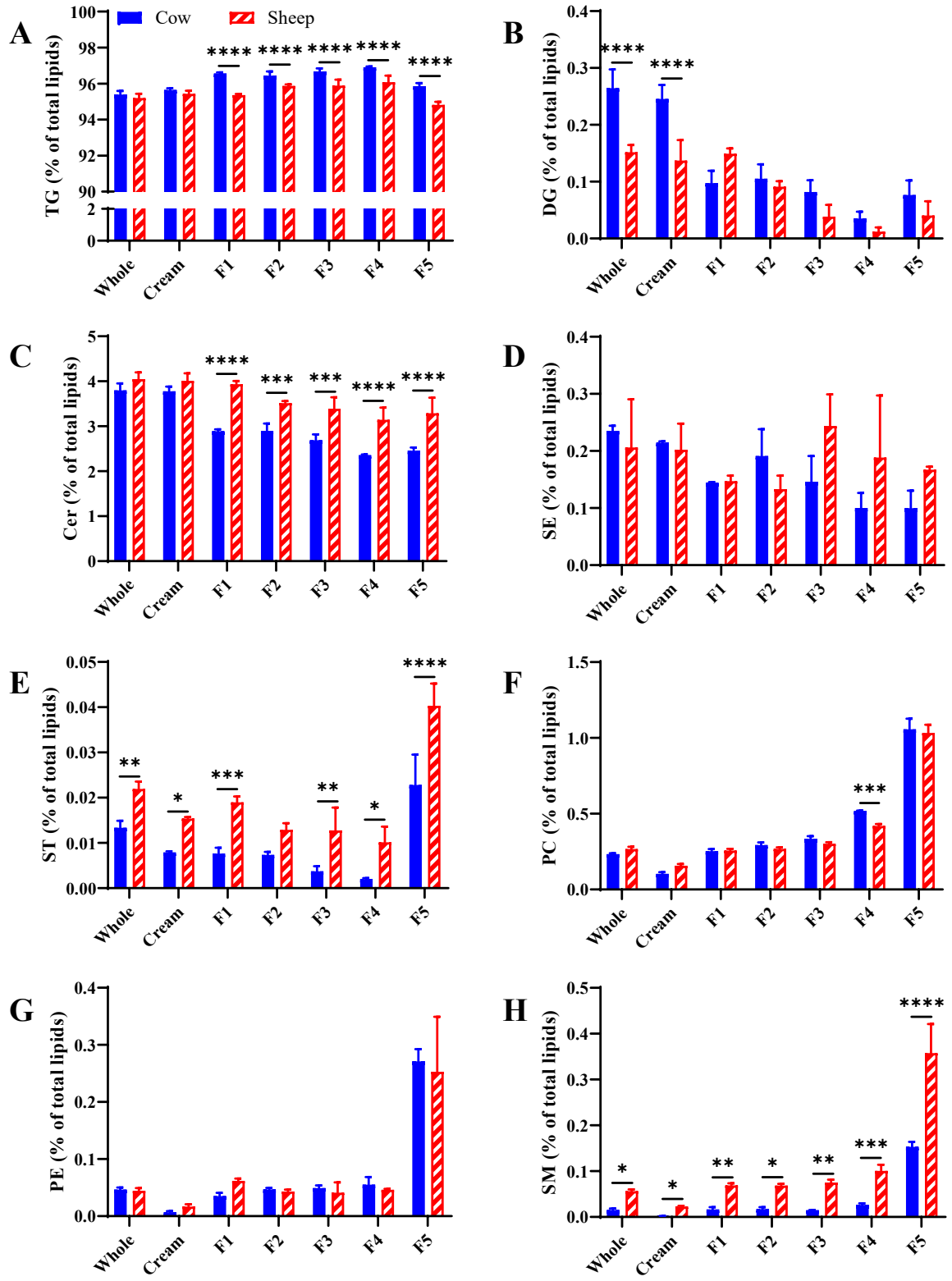
The total lipid composition for each major lipid class of sheep milk and cow milk was calculated by summing the peak areas and creating a sum normalized concentration; the results are shown in Appendix B Figure 2. Lipidomics analysis detected 231 lipid species from six different lipid classes that included triglyceride (TG), ceramide, diglyceride (DG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), sterol ester and sterol. TGs (167) were the predominant lipids, followed by ceramide (34), DGs (11), PC (9), PE (5) and SM (3) for both sheep milk and cow milk. The relative proportions of DG and ceramide decreased with decreasing MFG size, whereas TG, PC, PE and SM showed an increasing trend as the MFG size decreased. This suggested that small MFGs contain more TG and polar lipids (including PC, PE and SM) but less DG and ceramide than large MFGs.

Sheep milk had significantly ($P < 0.05$) lower proportions of TG but significantly ($P < 0.01$) higher proportions of ceramide than cow milk in fractions F1–F5 (Appendix B Figures 2A and 2C). Sheep milk contained a significantly ($P < 0.01$) lower proportion of DGs than cow milk in the whole milk and the cream, whereas no differences were found for fractions F1–F5 (Appendix B Figure 2B). The proportion of sterol was significantly higher in sheep milk than in cow milk (Appendix B Figure 2E), whereas there was no significant ($P > 0.05$) difference in sterol ester between sheep milk and cow milk (Appendix B Figure 2D). For polar lipids, there were no significant ($P > 0.05$) differences in the PC and PE contents between sheep milk and cow milk (Appendix B Figures 2F and 2G). However, sheep milk had a significantly higher ($P < 0.01$) proportion of SM than cow milk in all milk fractions (Appendix B Figure 2H).

The polar lipid composition for both sheep milk and cow milk revealed that the concentrations of PC, PE and SM increased with decreasing MFG size (Appendix B Figures 2F–2H), indicating that the smaller MFGs were coated with more polar lipids. The results are consistent with that reported by Lopez et al. (2011), who compared the concentrations of polar lipids in whole (~ 4.2 μm), small (~ 1.6 μm) and large (~ 6.5 μm) MFGs and found that small MFGs contained the highest concentration of polar lipids (8.91 mg/g fat), followed by whole MFGs (6.25 mg/g fat) and large MFGs (2.72 mg/g fat); however, the results of the current study differ from the previous findings for cow milk reported by Mesilati-Stahy et al. (2011), who showed few differences in the levels of PE and PC in different-sized MFG groups, and that the level of SM increased as the MFG size decreased. Variations in polar lipids between studies can possibly be attributed to the different analytical methods or milk sources used, such as milk from different breeds, stage of lactation, seasonal variations and conditions of feeding (Ménard et al., 2010; Tai et al., 2022). With respect to the polar lipid species, the results are in accordance with previous literature for milk; that is, PE, PC and SM are the major polar lipids characterized for milk samples (Et-Thakafy et al., 2017; Lu et al., 2016; Ménard et al., 2010; Mesilati-Stahy et al., 2011).

When the compositions of the polar lipids were compared, there were significant ($P < 0.001$) differences in their total concentrations (including PC, PE and SM) between sheep milk and cow milk in the whole milk (0.285 versus 0.351%), the cream (0.108 versus 0.187%) and fraction F1 (0.291 versus 0.367%), but not in the MFG fractions with smaller size (F2–F5) ($P > 0.05$). The concentration of SM in all MFG groups was significantly ($P < 0.05$) higher in sheep milk than in cow milk (Appendix B Figure 2H), whereas no significant ($P > 0.05$) differences were observed for PC and PE in most of the MFG groups (Appendix B Figures 2F and 2G). This is in line with a previous study, which showed that

sheep milk contained a significantly ($P < 0.001$) higher proportion of SM but comparable proportions of PC and PE when compared with cow milk (Et-Thakafy et al., 2017). The SM content has been associated with different MFGM features such as structures and melting temperatures, which probably influence the interfacial properties of the MFGM and thus the functional properties and the digestion behavior of the fat globules (Et-Thakafy et al., 2017; Tai et al., 2022).



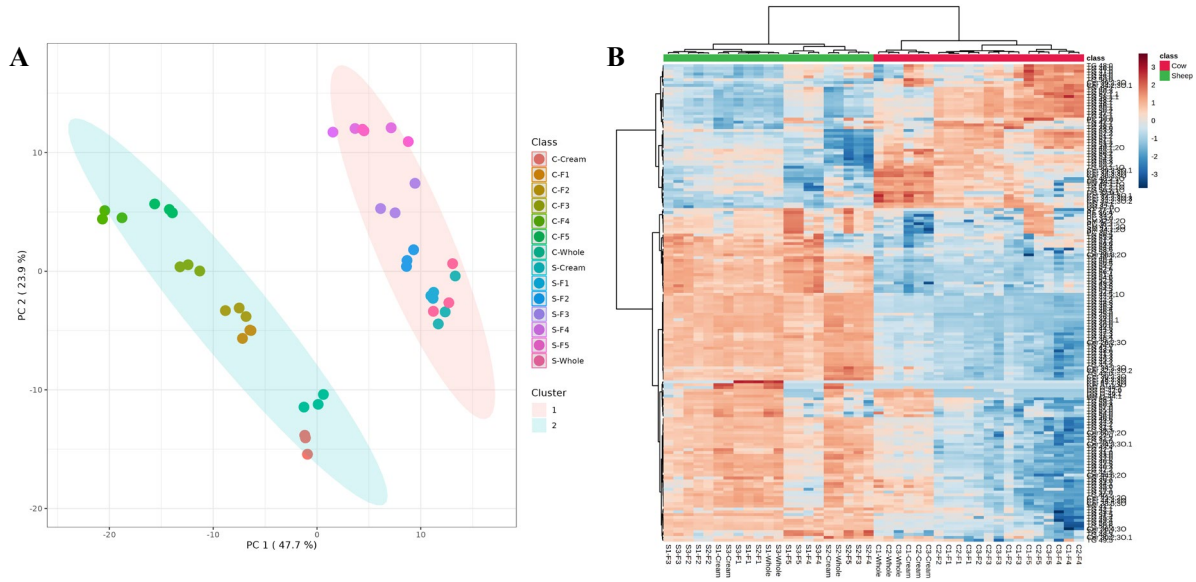
Appendix B Figure 2. (A) Triglyceride (TG), (B) diglyceride (DG), (C) ceramide (Cer), (D) sterol ester (SE), (E) sterol (ST), (F) phosphatidylcholine (PC), (G)

phosphatidylethanolamine (PE) and (H) sphingomyelin (SM) contents in different-sized milk fat globules of cow milk (blue bars with no pattern) and sheep milk (red bars with lines). Level of significance: ∗, 0.01 < P < 0.05; ∗∗, 0.001 < P < 0.01; ∗∗∗, P < 0.001; ∗∗∗∗, P < 0.0001.

Several studies have reported the phospholipid composition of different-sized MFGs in cow milk. Mesilati-Stahy et al. (2011) showed that large MFGs contain more PE and PC than small MFGs. In contrast, Lu et al. (2016) compared the lipid compositions of two different-sized MFG groups (7.6 ± 0.9 versus 3.3 ± 1.2 μm) in cow milk and showed that small MFGs had higher concentrations of PE and PC than large MFGs, which is in agreement with the findings in the current study. The reason for the differences between these studies is unclear. It has been suggested that the lipid composition of milk could be affected by physiological characteristics, such as weight, somatic cell count, pregnancy, day in milk, parity and milk production traits, including milk yield, fat yield, protein content, fat content and the ratio of fat to protein, on the individual animal level (Ceciliani et al., 2021). Therefore, these differences in the lipid composition among different studies could be due to the different milk sources used.

The difference in the lipidomes between sheep milk and cow milk is shown in Appendix B Figure 3. The PCA of the lipidomics data provided evidence of the considerable differences between the lipidomes of sheep milk MFGs and cow milk MFGs (Appendix B Figure 3A). The PCA highlights that 47.7% of the variance within the samples was in principal component 1, which clearly separated the species. A further 23.9% of the variance is explained in principal component 2, which separated the MFG groups with different sizes. The heatmap showed 169 significantly different lipids (FDR *P*-value < 0.05 from a t-test of interspecies comparison) between sheep milk and cow milk (Appendix B

Figure 3B). The sheep milk and the cow milk were clearly grouped into two different clusters. The abundances of 51 lipids were higher in cow milk than in sheep milk, in which TG (~ 74%) was the largest group followed by ceramide (~ 16%) and DG (~ 8%). Sheep milk had higher abundances of the remaining 118 lipids than cow milk, with TG, ceramide, polar lipids (including PC, PE and SM) and DG accounting for ~ 75, 14, 7 and 3% respectively.



Appendix B Figure 3. Differences in lipidomes of different-sized milk fat globule groups in sheep milk (S) and cow milk (C). (A) Principal component analysis and (B) heatmap of lipids in sheep milk and cow milk samples. The heatmap colors reflect the abundance of milk lipids (mean-centered and divided by the standard deviation of each variable).

Lipidomes of different-sized MFGs in sheep milk

The differences in the lipidomes among the different fractions of sheep milk are shown in Appendix B Figure 4A. The PCA plot shows that the whole milk and cream samples overlapped markedly, and that the other fractions were well separated from each other (Appendix B Figure 4A1). The 134 different lipids (*P*-value < 0.05 from an ANOVA

of inter-fraction comparison) are shown in the heatmap (Appendix B Figure 4A2). Overall, 83 lipids were higher in the whole sheep milk, the cream and fractions F1 and F2, in which TG (~ 69%) was the predominant lipid, followed by ceramide (~ 22%) and DG (~ 9%). In contrast, the other 51 lipids were higher in the other smaller MFG fractions (F3–F5), in which TGs (~ 61%) and polar lipids (~ 31%, including PC, PE and SM) were the predominant groups.

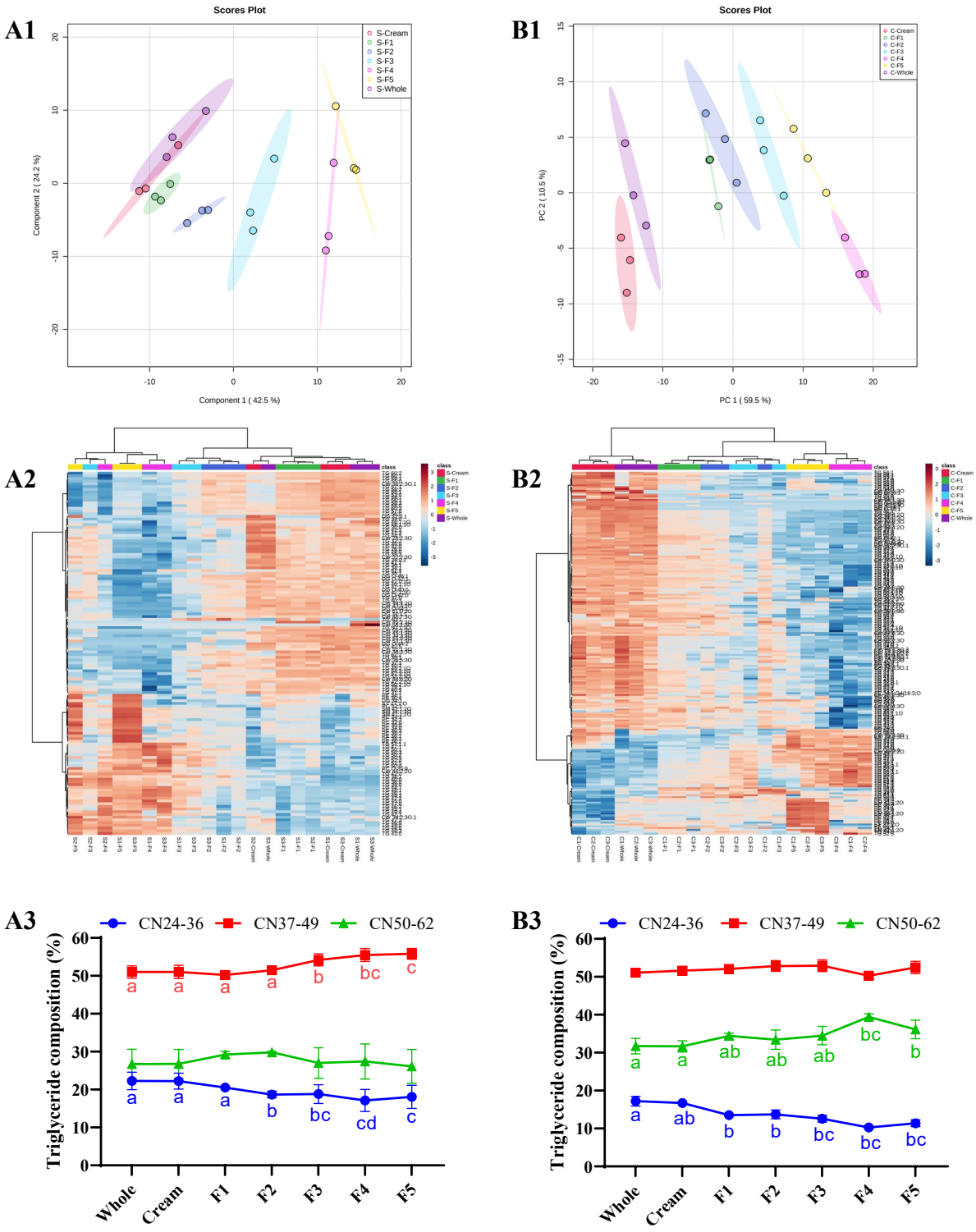
Appendix B Figure 4A3 shows the fractional variation in the TG composition. The TGs were divided into low molecular weight (LMW; CN24–CN36, CN is the total carbon number of the three fatty acids in the TG), medium molecular weight (MMW; CN37–CN49) and high molecular weight (HMW; CN50–CN62) TGs, as reported by Pacheco-Pappenheim et al. (2021). The LMW TGs decreased with decreasing MFG size; the MMW TGs increased as the MFG size decreased; the HMW TGs remained roughly unchanged with the MFG size. Statistical analysis showed that the small MFGs (fractions F3–F5) in sheep milk contained significantly ($P < 0.05$) lower proportions of LMW TGs but significantly ($P < 0.05$) higher proportions of MMW TGs than the large MFGs (whole milk, cream and fractions F1 and F2). These differences in the abundance of TGs and polar lipids drove the different-sized MFG groups to separate from each other.

Lipidomes of different-sized MFGs in cow milk

The lipidome results of the different-sized MFG groups in cow milk are shown in Appendix B Figure 4B. The PCA plot shows that each fraction was distributed separately from the left side (larger MFGs) to the right side (smaller MFGs) in cow milk; similar to sheep milk (Appendix B Figure 4A1), the cream of cow milk was close to the whole milk (Appendix B Figure 4B1). The heatmap shows the 199 different lipids (P-value < 0.05 from an ANOVA of inter-fraction comparison) in all milk fractions (Appendix B Figure 4B2); 141 lipids were identified as less abundant lipids in small-sized MFG groups (including

fractions F4 and F5), in which TG, ceramide and DG accounted for ~ 75, 18 and 7% respectively. The other 58 lipids (~ 64% TGs and ~ 29% polar lipids) were the most abundant classes in fractions F4 and F5, compared with whole milk, cream and fractions F1–F3. The lipidome results for sheep milk (Appendix B Figure 4A) and cow milk (Appendix B Figure 4B) suggested that smaller MFGs contain more polar lipids.

Appendix B Figure 4B3 shows the fractional variation in TG composition. The LMW TGs decreased with decreasing MFG size; the MMW TGs remained roughly unchanged with decreasing MFG size; the HMW TGs increased as the MFG size decreased. Statistical analysis showed that the smaller MFG groups (fractions F4 and F5) contained significantly ($P < 0.05$) more HMW TGs but significantly ($P < 0.05$) fewer LMW TGs than the larger MFG groups (whole milk, cream and fractions F1 F2, and F3). Similar to the findings for sheep milk (Appendix B Figure 4A), these differences in the TGs and polar lipids between the MFG groups drove the small MFGs to be different from the large MFGs. In comparison with sheep milk, cow milk contained significantly ($P < 0.05$) lower proportions of LMW TGs in all MFG groups, which is in line with the fatty acid composition result that cow milk contained fewer MCFAs than sheep milk (Appendix B Table 1).



Appendix B Figure 4. Differences in lipidomes of different-sized milk fat globule groups in (A) sheep milk and (B) cow milk. (A1 and B1) Principal component analysis, (A2 and B2) heatmap and (A3 and B3) fractional variation of milk fat triglycerides. The heatmap colors reflect the abundance of milk lipids (mean-centered and divided by the standard deviation

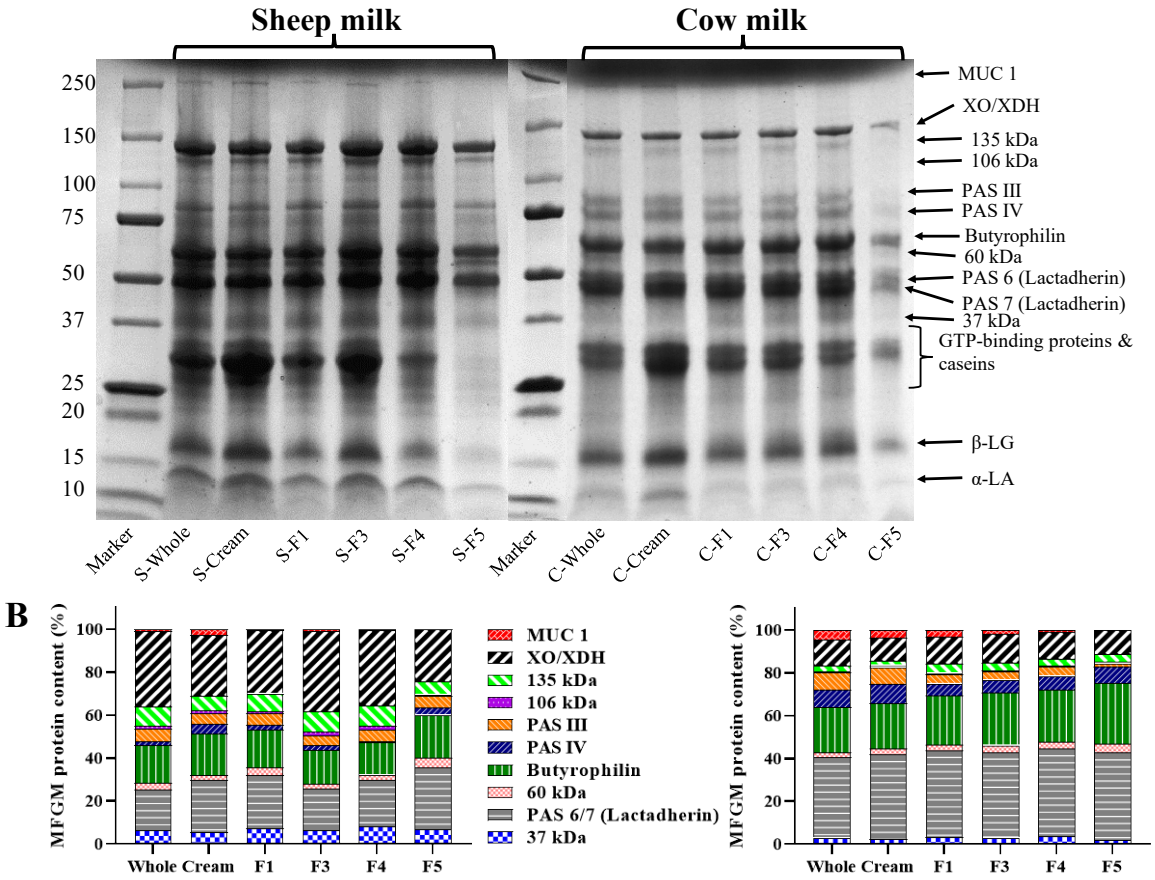
of each variable). Low molecular weight [carbon numbers (CN) 24–36], medium molecular weight (CN37–CN49) and high molecular weight (CN50–CN62) triglyceride groups.

Protein composition of MFGM

Appendix B Figure 5A shows the SDS-PAGE profile of the MFGM proteins in the different-sized MFG groups. Sheep milk and cow milk had similar MFGM protein compositions. The only difference was a single band at periodic acid Schiff (PAS) 6 and PAS 7 (PAS 6/7 are also known as lactadherin) positions in sheep milk, whereas cow milk had two bands. This is in agreement with previous results reported by Cebo and Martin (2012), who compared the MFGM proteins in cow milk and non-cow milk using SDS-PAGE and mass spectrometry analysis and identified two bands for cow milk lactadherin but a single band for sheep milk lactadherin.

The detailed MFGM protein (molecular weight ≥ 37 kDa as a whole) composition of sheep milk and cow milk is shown in Appendix B Figures 5B and 5C respectively. Xanthine dehydrogenase/oxidase, PAS 6/7 (lactadherin) and butyrophilin were the major MFGM proteins in both sheep milk and cow milk. The results are generally in agreement with previous reports of Thum et al. (2023), who stated that butyrophilin, xanthine dehydrogenase/oxidase, lactadherin and adipophilin are enriched in cow MFGM and sheep MFGM. However, the compositions of the individual MFGM proteins were different between sheep milk and cow milk. Sheep MFGM contained higher proportions of xanthine dehydrogenase/oxidase and bands at 135, 106 and 37 kDa but lower proportions of mucin 1 (MUC 1), PAS IV (also known as cluster of differentiation 36), butyrophilin and PAS 6/7 than cow MFGM. This indicated that sheep milk contained more non-glycosylated proteins (including xanthine dehydrogenase/oxidase and adipophilin) but fewer glycoproteins

(including MUC 1, PAS III and PAS IV) than cow milk. For the different-sized MFGs, both sheep milk and cow milk showed similar patterns, in which smaller MFGs contained lower proportions of MUC 1, PAS III and PAS IV but higher proportions of proteins at 135, 106 and 60 kDa, butyrophilin and PAS 6/7 than larger MFGs. This suggested that small MFGs have more glycoproteins than larger MFGs. The differences in the MFGM protein composition among the MFG groups can be attributed to the different affinities of the MFGM for proteins as the size-dependent fatty acid and polar lipid composition could lead to different surface polarities among the MFG fractions (Lu et al., 2016).



Appendix B Figure 5. (A) SDS-PAGE profile of milk fat globule membrane (MFGM) isolated from different- sized fat globules of sheep milk (S) and cow milk (C). The protein compositions of (B) sheep MFGM and (C) cow MFGM. MUC 1, mucin 1; XO/XDH, xanthine oxidase/xanthine dehydrogenase; β-LG, β-lactoglobulin; α-LA, α-lactalbumin.

Conclusions

This study showed that gravity-based separation effectively separated milk into six significantly different-sized MFG groups. The lipid and protein compositions of the different-sized MFG groups in sheep milk and cow milk were compared. The MFG size had little impact on the composition of the fatty acids in both sheep milk and cow milk, whereas smaller MFGs had higher proportions of polar lipids (including PC, PE and SM) but lower proportions of LMW TGs than larger MFGs in both milks. The TG composition showed that the MMW TGs of sheep milk and the HMW TGs of cow milk increased with decreasing MFG size. More glycoproteins were observed in small MFGs than in large MFGs.

The lipid and protein compositions were significantly different between sheep milk and cow milk. The MFGs of sheep milk had higher proportions of short-chain fatty acids, MCFAs, SM and LMW TGs than those of cow milk in all size groups. Sheep milk contained more non-glycosylated proteins but fewer glycoproteins in the MFGM than cow milk. These differences might potentially affect the functional properties and digestion behaviors of the MFGs of sheep milk and cow milk.