Structural changes in milk of different species during digestion

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To my family and my husband, Upal

for all your love and support

Abstract

Cow milk is the most abundant type of mammalian milk produced in the world. It has been widely explored industrially as well as academically. However, non-cow milk (e.g. water buffalo, goat, and sheep milk) consumption is significant and forms an important nutritional source for people in many countries. The interest in non-cow milks has increased because of several anecdotal experiences reported about the nutritional and digestive benefits of these milks. However, there is very little scientifically validated information available.

The overall objective of this PhD study was to investigate how some of the non-cow milks (such as goat and sheep milk) are structurally different (or similar) to cow milk, especially in their coagulation behaviour under the gastric environment. The potential implications of structural changes on the delivery of nutrients under dynamic gastric digestion conditions were also explored. Dynamic *in vitro* and *in vivo* gastric digestion models were employed for this study.

It was found that milk from different species vary in their natural macronutrient composition, structure, and acid-gelation behaviour. The fundamental mechanism of coagulation of proteins under the dynamic *in vitro* gastric digestion conditions was found to be similar for different species milk. The *in vivo* gastric digestion studies revealed comparable results, although goat and sheep milk curds had relatively lower rates of strengthening and relatively more open microstructure. Both the dynamic *in vitro* and *in vivo* studies revealed that the release of fat globules from the coagulated curd was directly proportional to the breakdown

(or hydrolysis) of the protein in the curd during gastric digestion. The studies clearly showed that the curd formation and its disintegration in the stomach is a key factor influencing the rate of delivery of macronutrients to the small intestine.

The results from this thesis contribute to the knowledge of how composition along with structure impact the release of nutrients at various stages of gastric digestion of different mammalian milks. The information gained from this study might have important consequences for developing dairy products with improved structures for controlled delivery or release of nutrients to meet the special dietary needs of consumers.

Contents

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List of tables

List of figures

List of peer-reviewed publications, posters, and presentations

Publications

1.**Roy, D.**, Ye, A., Moughan, P. J., and Singh, H. (2021). Impact of gastric coagulation on the kinetics of release of fat globules from milk of different species. *Food and Function, 12, 1783-1802.* https://doi.org/10.1039/D0FO02870C

2. **Roy, D.**, Ye, A., Moughan, P. J., and Singh, H. (2021). Structural changes in cow, goat and sheep skim milk during dynamic *in vitro* gastric digestion. *Journal of Dairy Science, 104(2), 1394-1411*. https://doi.org/10.3168/jds.2020-18779

3. **Roy, D.**, Ye, A., Moughan, P. J., & Singh, H. (2020). Composition, structure, and digestive dynamics of milk from different species—A Review. *Frontiers in Nutrition (Food Chemistry), 7*(195). https://doi.org/10.3389/fnut.2020.577759

4. **Roy, D**., Ye, A., Moughan, P. J., & Singh, H. (2020). Gelation of milks of different species (dairy cattle, goat, sheep, red deer, and water buffalo) using glucono-δ-lactone and pepsin. *Journal of Dairy Science, 103(7), 5844-5862.* https://doi.org/10.3168/jds.2019-17571

5. Ye, A., **Roy, D.**, & Singh, H. (2020). Structural changes to milk protein products during gastrointestinal digestion. In M. Boland & H. Singh (Eds.), *Milk Proteins (Third Edition)* (pp. 671-700): Academic Press. https://doi.org/10.1016/B978-0-12-815251-5.00019-0

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Posters

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2. Gelation properties of acid gels of milk from different species. 2nd LMWZ University Consortium and International Food Industry Forum, 17–18th Nov 2017, Hangzhou, China.

3. Rheological and structural properties of bovine and non-bovine skim milk acid gels. 6th Delivery of Functionality in Complex Food Systems Conference, 5–8th Nov 2017, Auckland, New Zealand.

Oral presentations at International conferences

1. *Invited talk*: Composition, structure and digestive dynamics of milk from different Species. 16th International Symposium on Milk Genomics and Human Health, 12–14th Nov 2019, Aarhus, Denmark.

2. Structural dynamics of cow, goat and sheep milk during *in vitro* and *in vivo* gastric digestion. Food Structures, Digestion, and Health (FSDH) International Conference, 30th Sept – 3rd Oct 2019, Rotorua, New Zealand.

3. Insights into the structural dynamics of bovine and non-bovine milks during digestion in piglets. 3rd 'Feeding the Future' Infant Nutrition Council Conference, 4–5th Apr 2019, Melbourne, Australia.

Key oral presentations locally in New Zealand

1. Milks, Curdling and Digestion. Three Minute Thesis (3MT) Heat and Final, 14th Aug 2019, Massey University, Palmerston North, New Zealand.

2. Structural changes in bovine and non-bovine milks during digestion. Industry Ownership Group Workshop, New Zealand Milk Means More (NZ3M), 21st May 2019, Palmerston North, New Zealand.

3. Composition, structure, and curd forming properties of bovine and non-bovine milks. Structure Design for New Food Applications Workshop, 14th Nov 2018, Palmerston North, New Zealand.

4. Exploring the potential of bovine and non-bovine milks – beyond composition. Inaugural Dairy Industry Workshop, 29–30th Aug 2018, Palmerston North, New Zealand.

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Additional publications (as part of internship in China)

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Chapter 1: Introduction

Milk is the first source of food for meeting the complete nutritional and energy requirements of the new-born or young of any mammalian species during the early years of their growth and development. Humans have culturally evolved over the years to consume milk from other species (such as cow, buffalo, goat, and sheep) during all the stages of life and thus, milk has become an integral part of a healthy and nutritionally balanced diet for humans (Pereira, 2014). The traditional dairy industry, which is based mainly on cow milk, is becoming increasingly diversified with non-cow milks such as goat and sheep milk; this is due to consumer perception that these milks have better digestion properties than cow milk (Haenlein & Wendorff, 2008; Park & Haenlein, 2006b; Park *et al.*, 2007). However, only little scientific information or research is available on the digestion behaviour of non-cow milks.

Milk (irrespective of any mammalian species) is 'nature's most complex oil-in-water emulsion system' (Singh & Gallier, 2017), partly because of its unique production process in the mammary glands, partly because of the special structure of its naturally-assembled nutrients (fat globules and casein proteins), and partly because of its distinctive coagulation behaviour in the stomach, compared to the artificially engineered emulsions. Restructuring during the gastric digestion process is the main driver in influencing the delivery, absorption, and metabolic responses of nutrients from complex structured foods (such as milk). However, despite the years of milk consumption, there is still limited fundamental understanding of the underlying physiochemical and structural changes in the milk matrix during the dynamic

gastric digestion process. Only a few recent studies have focused on understanding the structural changes that occur in the milk matrix during the dynamic gastric digestion (Ye *et al.*, 2016a, 2016b, 2017; Ye *et al.*, 2019b). However, all of these previous studies were focused on cow milk and an in-depth investigation of the influence of dynamic gastric processes on milk digestion is required. The unique make-up of milk (in terms of composition and structure) can vary depending on species. Such differences are considered to provide a digestive advantage to some non-cow milk such as goat milk. Most of the previous studies on non-cow milks were carried out using static *in vitro* digestion models. To understand the physicochemical modification as well as digestive dynamics of a complex structured fluid such as milk, the use of dynamic *in vitro* gastric digestion models is essential. Moreover, there is a lack of *in vivo* studies to investigate the structural changes in proteins and fats during gastric digestion of milk as well as to validate the observations noted using dynamic *in vitro* gastric digestion systems. Hence, this thesis describes comprehensive studies aimed at gaining new fundamental insights about the behaviour of different mammalian milks under both *in vitro* and *in vivo* conditions of dynamic gastric digestion.

The key objectives and the corresponding questions of this research were:

Objective 1: To understand the composition, structure and gelation properties of milk from different species (cow, goat, sheep, deer and buffalo) using an acid precursor (glucono-δ-lactone) and/or enzyme (pepsin).

1. How do the milk proteins from different species vary in gelation properties relevant to digestive conditions?

Objective 2: To investigate the structural changes in skim milk from different species (cow, goat and sheep) using an *in vitro* dynamic gastric digestion model.

2. Do the milk from various species vary in coagulation behaviour during gastric digestion? What are the factors influencing the gastric digestion behaviour of milk?

Objective 3: To determine the impact of restructuring and destructuring of whole milk from different species (cow, goat and sheep) on the state and release of milk fat globules during gastric digestion.

3. How do milk fat globules behave during coagulation of milk during gastric digestion? What is the impact of milk coagulation on fat digestion? Are there any differences in the digestion of milk fat globules from different species?

Objective 4: To explore the gastric digestion behaviour of whole milk from different species (cow, goat and sheep) and its impact on overall digestion using a physiologically relevant animal model (piglet).

4. Are the changes observed during *in vitro* gastric digestion of milk from different species are valid under *in vivo* conditions? What happens to the milk proteins and fat during *in vivo* gastric digestion?

Chapter 2: Literature review[1]

The aim of this chapter is to review the published work on the composition, structure, and digestive dynamics of milk from different mammalian species. General properties of some non-cow milks, in comparison with human and cow milk, particularly focusing on their protein profile, fat composition, hypoallergenic potential, and digestibility, are summarized. Furthermore, the implications of coagulation behaviour of different milks in stomach on nutrient release and controlled delivery are also covered.

2.1 Introduction

Milk has evolved to meet the nutritional and physiological requirements of the neonate. Milk is thus regarded as a high-quality food, nutritionally. Humans are known to have consumed cow (*Bos taurus*) and non-cow (such as goat and sheep) milks as part of their diet since prehistoric times (Dunne *et al.*, 2012; Evershed *et al.*, 2008). As a convenient source of nutrition, cow milk is the most-consumed milk worldwide because of its widespread availability and large production volumes. Non-cow milks are of nutritional importance to people in developing countries as well as in geographical areas in which the climate is unsuitable for the survival of dairy cow (Faye & Konuspayeva, 2012; Park & Haenlein, 2006a). For example, buffalo milk in Asia, sheep milk in Europe and the Mediterranean basin

[1]Part of the contents presented in this chapter has been published as a peer-reviewed paper and chapter: Roy, D., Ye, A., Moughan, P. J., & Singh, H. (2020). Composition, structure, and digestive dynamics of milk from different species—A Review. *Frontiers in Nutrition (Food Chemistry)*, *7*(195), and Ye, A., Roy, D., & Singh, H. (2020). Structural changes to milk protein products during gastrointestinal digestion. In M. Boland & H. Singh (Eds.), *Milk Proteins (Third Edition)* (pp. 671-700): Academic Press.

(including the Middle East), camel milk ("the white gold of the desert") in Africa, goat milk ("the cow of the poor") in Africa and southern Asia, horse milk in the steppe areas of central Asia, yak milk on the Tibetan plateau, reindeer milk in northern Scandinavia, musk ox milk in the Arctic, and mithun milk in the hilly regions of the Indian subcontinent (Faye & Konuspayeva, 2012; Verduci *et al.*, 2019).

Of the total world milk production, the proportion of total non-cow milk production has increased from ~9% in 1961 to 19% in 2018 (Figure 2.1). Of the total global non-cow milk production, buffalo milk has nearly tripled, camel milk has nearly doubled, and goat milk has slightly increased during this period. No world statistics on the amounts of milk produced from other dairy species, such as yak, horse, donkey, deer, musk ox, and llama, are available. Much of the non-cow milk production remains officially unreported because of the unknown amounts that are consumed locally at a farmer's home or are sold directly by farmers to local people, especially in developing countries (Haenlein, 2004; Ribeiro & Ribeiro, 2010).

The addition of milk as a product to non-cow farm systems adds value and helps farmers in dealing with the fluctuating prices of meat, hair, and wool. The buffalo, goat, sheep, and camel milking industry is well established in many parts of the world and is proving to be a profitable business. Recently, New Zealand has introduced the development of a red deer dairy farming system. Large dairy companies as well as specialized small and medium enterprises (SMEs) are also interested in using non-cow milks as a diversification strategy for their product portfolios. The regulatory requirements to ensure the safe production of cow milk (and milk products) are well defined in most of the world. However, the same regulatory

limits may not be applicable to non-cow milk and milk products. Thus, the emphasis on species-specific regulatory standards to guarantee the safety and quality of different milks for human consumption is needed (Alichanidis *et al.*, 2016; Bencini *et al.*, 2010; Burgess, 2010; Park & Guo, 2006). Also, understanding the significance of compliance to religious dietary laws (such as Kosher or Halal) will be of importance to the non-cow milk-based dairy companies for gaining acceptance of their products from various consumer groups (Regenstein *et al.*, 2003).

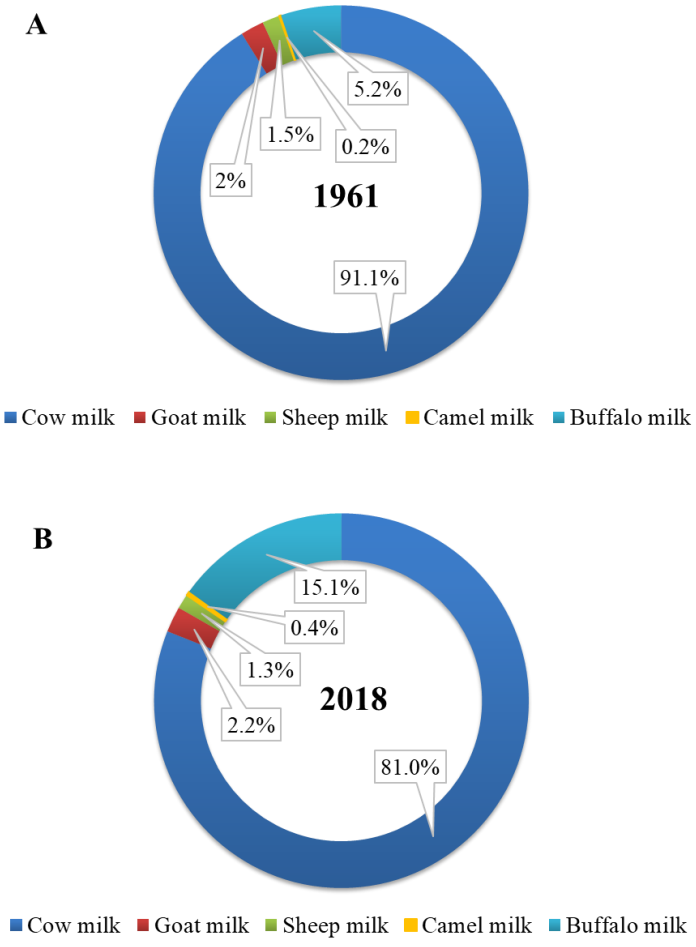


Figure 2.1 Proportion of dairy cow and non-cow milks produced globally in the year (A) 1961 and (B) 2018 (Source: FAOstat, March 2020).

In recent years, the opportunities for non-cow milk production and the manufacture of products have expanded because the numbers of dairy cow are perceived to be reaching their limit from environmental perspectives. Non-cow milks are also believed to have certain nutritional benefits compared with cow milk. For example, goat, sheep, camel, horse, and donkey milk are considered to be relatively more easily digestible, less allergenic, and more similar to human milk than cow milk (Crowley *et al.*, 2017; Park & Haenlein, 2006a; Potočnik *et al.*, 2011). In addition, non-cow milks can be utilized for developing high value specialized dairy products of international as well as regional (local cultural) importance, such as cheese, yogurt, butter, ghee, ice-cream, fermented milk, probiotic dairy drinks, milk tablets and, infant formulas (Balthazar *et al.*, 2017; Faye & Konuspayeva, 2012; Ranadheera *et al.*, 2019). However, relatively little scientific information on the nutritional benefits of non-cow milks is available. In addition, there is a significant gap in scientific knowledge on the detailed compositions, especially the minor components, and the protein and lipid structures in these milks.

2.2 Comparative compositions of cow and non-cow milks

The comparative compositions of milk from different species have been extensively reviewed in previous studies (Barlowska *et al.*, 2011; Claeys *et al.*, 2014; Gantner *et al.*, 2015; Verduci *et al.*, 2019). The milk from different species vary in chemical composition (Table 2.1). Protein, fat, lactose, and minerals are the four major components in all milks, irrespective of the species (Gantner *et al.*, 2015); the composition of milks within the same species varies considerably because of various factors, such as breed, stage of lactation,

milking interval, type of feed, and climate (Claeys *et al.*, 2014; Haenlein, 2004). For example, Li *et al.* (2019) reported recently that the stage of lactation is a key factor responsible for differences in the compositional and physicochemical properties of dairy cow milk in a seasonal calving system in New Zealand.

Nonruminant milks (such as those from horse and donkey) are somewhat similar to human milk (in terms of protein, lactose, and ash contents), compared with dairy cow milk and other ruminant milks (Table 2.1). The compositional comparisons made in this chapter are only between the species mentioned in Table 2.1. Ruminant milks have higher protein and fat contents, compared with human milk and other nonruminant milks (Table 2.1). Human milk contains much higher amounts of total lactose-derived oligosaccharides than milk from other species (Table 2.1). Goat milk is also known to have a relatively higher oligosaccharide content, the composition of which is considered to be similar to that of human milk (Oliveira *et al.*, 2012; Oliveira *et al.*, 2015).

Table 2.1 Composition (g/100 mL) of milk from different mammalian species.

Properties	Ruminants						Nonruminants		Human
	Cow	**Buffalo**	**Goat**	**Sheep**	**Red deer***	**Camel**	**Horse**	**Donkey**	
Total solids	11.8–13.0	15.7–17.2	11.9–16.3	18.1–20.0	20.0–30.5	11.9–15.0	9.3–11.6	8.8–11.7	10.7–12.9
Protein	3.0–3.9	2.7–4.7	3.0–5.2	4.5–7.0	5.9–10.6	2.4–4.2	1.4–3.2	1.4–2.0	0.9–1.9
Fat	3.3–5.4	5.3–9.0	3.0–7.2	5.0–9.0	6.6-19.7	2.0–6.0	0.3–4.2	0.3–1.8	2.1–4.0
Lactose	4.4–5.6	3.2–4.9	3.2–5.0	4.1–5.9	2.6-6.2	3.5–5.1	5.6–7.2	5.8–7.4	6.3–7.0
Ash	0.7–0.8	0.8–0.9	0.7–0.9	0.8–1.0	1.04–1.18	0.69–0.9	0.3–0.5	0.3–0.5	0.2–0.3
Oligosaccharides$^{\Omega}$	0.003-0.006	No data	0.025-0.030	0.002-0.004	No data	No data	No data	No data	0.500–0.800

Source: Adapted and modified from Claeys *et al.* (2014) and Crowley *et al.* (2017).

*Values based on minimum and maximum values found in the literature for different species of red deer; may include values from different stages of lactation (Arman *et al.*, 1974; Krzywiński *et al.*, 1980; Landete-Castillejos *et al.*, 2000; Vithana, 2012; Wang *et al.*, 2017).

$^{\Omega}$Values derived from Martinez-Ferez *et al.* (2006).

2.2.1 Proportions of major proteins

Compared with cow milk and other ruminant milks, horse and donkey milk have a low casein-to-whey-protein ratio, similar to that in human milk. Among the ruminant milks, goat, sheep, and camel milk have a lower casein-to-whey-protein ratio as well as a relatively higher β-casein-to-α_s-casein ratio compared with cow milk (Table 2.2). Thus, these non-cow milks are an attractive alternative as a potential natural ingredient for infant formula (Crowley *et al.*, 2017); a lower casein-to-whey-protein ratio (i.e., a higher proportion of whey proteins) leads to faster digestion of the milk proteins in infant formula than a casein-dominant protein composition (Tari *et al.*, 2018; Ye *et al.*, 2019a). As human milk has the lowest casein-to-whey-protein ratio, has a high β-casein-to-α_s-casein ratio, and contains no β-lactoglobulin (Table 2.2), milk from other species with similar properties are of great interest to the consumer as well as to the dairy industry for the development of specialized dairy products, not only for infants but also for people in other age groups.

β-Lactoglobulin is considered to be one of the major allergens that is responsible for cow milk allergy in children (Selo *et al.*, 1999). Thus, milk from species that lack β-lactoglobulin or have lower β-lactoglobulin-to-α-lactalbumin ratios are of interest for human consumption. Camel milk, like human milk, does not contain β-lactoglobulin (El-Hatmi *et al.*, 2015; Ereifej *et al.*, 2011) or it may be present in trace amounts in different forms (Beg *et al.*, 1987; Beg *et al.*, 1984; Farah, 1986). Llama milk is also known to contain no β-lactoglobulin (Park & Haenlein, 2013; Verduci *et al.*, 2019), but little detailed information on its protein composition is available.

2.2.2 Casein micelle characteristics

Individual caseins (α_{s1}-, α_{s2}-, β-, and κ-caseins) are present in all milks as self-assembled particles known as "casein micelles" (De Kruif *et al.*, 2012). The fundamental structure of the casein micelles in the milk from many species has not been studied in great detail, except in cow milk. Recently, Ingham *et al.* (2018) used small-angle X-ray scattering and reported that the internal structures of the casein micelles of cow, goat, and sheep milk had strong similarities. Slight differences were observed, which may be due to the differences in casein composition, hydration, and physicochemical properties.

Apart from the differences in the proportions of different caseins (Table 2.2), the casein micelles in the milk from different species differ in size, hydration, and mineralization (Table 2.3). Among all mammalian milks, the casein micelles in human milk have the smallest diameter. The casein micelle sizes of goat, sheep, deer, camel, and horse milk are larger than that of human milk as well as cow milk (Table 2.3).

Table 2.2 Protein profile (g/L) of milk from different mammalian species.

Protein fractions	Ruminants						Nonruminants		Human
	Cow	**Buffalo**	**Goat**	**Sheep**	**Red deer***	**Camel**	**Horse**	**Donkey**	
Total casein	24.6–28	32–40	23.3–46.3	41.8–46	~57–70	22.1–26.0	9.4–13.6	6.4–10.3	2.4–4.2
Total whey proteins	5.5–7.0	6	3.7–7.0	10.2–11	~11–13	5.9–8.1	7.4–9.1	4.9–8.0	6.2–8.3
Casein-to-whey protein ratio	82:18	82:18	78:22	76:24	–	73:27–76:24	52:48	56:44	29:71–33:67
Major caseins									
α_{s1}-Casein	8–10.7	8.9	0–13.0	15.4–22.1	–	4.9–5.7$^{\Omega}$	2.4	Present	0.77
α_{s2}-Casein	2.8–3.4	5.1	2.3–11.6	–	–	2.1–2.5$^{\Omega}$	0.2	Present	Absent
β–Casein	8.6–9.3	12.6–20.9	0–29.6	15.6–17.6	–	14.4–16.9$^{\Omega}$	10.66	Present	3.87
κ-Casein	2.3–3.3	4.1–5.4	2.8–13.4	3.2–4.3	–	0.8–0.9$^{\Omega}$	0.24	Present	0.14
Major whey proteins									
β-Lactoglobulin	3.2–3.3	3.9	1.5–5.0	6.5–8.5	–	Absent	2.55	3.3	Absent
α-Lactalbumin	1.2–1.3	1.4	0.7–2.3	1–1.9	–	0.8–3.5	2.37	1.9	1.9–3.4

Source: Adapted and modified from Claeys *et al.* (2014) and Crowley *et al.* (2017).

*Values (g/kg) derived from Arman *et al.* (1974). There are insufficient data for red deer milk in the literature to derive approximate values.

$^{\Omega}$Values derived from Kappeler (1998).

Table 2.3 Casein characteristics of milk from different mammalian species.

Properties	Ruminants						Nonruminants		Human
	Cow	Buffalo	Goat	Sheep	Red deer	Camel	Horse	Donkey	
Casein micelle diameter (nm)	150–182	180	180–301[A]	180–210	–	380	255	100–200	64–80
Hydration (g H_2O/g protein)	1.92–3.7[B]	1.90[C]	1.43-2.05[D]	1.71-1.93[E]	No data	1.70[F]	No data	~1.0[G]	No data

Source: Adapted and modified from Claeys *et al.* (2014).
Values derived from other references are represented by uppercase case letters: A, Nguyen *et al.* (2018), Pierre *et al.* (1998), and Pierre *et al.* (1995); B, Dalgleish (2014), Wang *et al.* (2013), and Dewan *et al.* (1973); C, Ahmad *et al.* (2008); D, Remeuf *et al.* (1989); E, Pellegrini *et al.* (1994); F, Beaucher *et al.* (2013); G, Luo *et al.* (2019).

Sood *et al.* (1979) reported that the loss of micellar calcium from the skim milk casein micelles (when dialyzed against same skim milk sample containing ethylenediaminetetraacetic acid, EDTA) resulted in increased hydration (or swelling) of casein micelles. Based on this, it was considered that the hydration level of the casein micelles was negatively correlated with mineralization of micelles (Remeuf & Lenoir, 1986) i.e. when the mineralization of the casein micelle increases, the degree of hydration of casein micelle decreases. Thus, the lower hydration of goat and sheep milk casein micelles had been related to its higher mineralization than those of cow milk casein micelles (Park, 2007; Park *et al.*, 2007). Similarly, the casein micelles in buffalo milk (Ahmad *et al.*, 2008) and donkey milk (Luo *et al.*, 2019) are considered to be less hydrated and more mineralized than those in cow milk.

It should be highlighted that there is a high degree of variation in the results that have been reported for the casein micelle characteristics within the same species, which may be due to differences in the analytical methods used. In addition, differences in breeds, genetic variants, and phosphorylation sites of the caseins may also add to the variation in the characteristics of the casein micelles within and across species (Crowley *et al.*, 2017).

2.2.3 Milk fat composition

Compared with milk fat from other species (especially ruminants), human milk fat contains lower proportions of saturated fatty acids, higher proportions of monounsaturated fatty acids and polyunsaturated fatty acids, a higher ratio of ω-6 to ω-3 fatty acids, and higher levels of cholesterol (Table 2.4).

In general, horse and donkey milk contain lower proportions of saturated fatty acids and higher proportions of polyunsaturated fatty acids than ruminant milks. In contrast, ruminant milks contain higher proportions of monounsaturated fatty acids, a higher ratio of ω-6 to ω-3 fatty acids, and a higher cholesterol content than horse and donkey milk (Table 2.4). The conjugated linoleic acid content is similar in human and ruminant milks but is lower in nonruminant milks (Table 2.4).

Sheep and goat milk fats are known to be rich in short chain (responsible for the distinct flavour of these milks) and medium chain triacylglycerols (TAGs); similarly, buffalo milk fat contains higher proportions of medium chain TAGs than cow milk, which has high proportions of long chain TAGs (Abd El-Salam & El-Shibiny, 2011; Ceballos *et al.*, 2009; Jenness, 1980; Ruiz-Sala *et al.*, 1996). In contrast, camel milk contains a higher proportion of long chain fatty acids and a lower proportion of short chain fatty acids than cow milk (Kula & Tegegne, 2016). Data for the fat composition of red deer milk are scarce, but this milk is considered to contain 5–10% fewer unsaturated fatty acids and higher proportions of shorter chain and saturated fatty acids than cow milk (Krzywiński *et al.*, 1980). These differences may contribute to the different digestion behaviours of milk fat from different species, as short or medium chain TAGs are considered to be more rapidly hydrolysed by lipases (Jandal, 1996; Park, 2006).

Free long chain saturated fatty acids, such as palmitic acid (C16:0), are not considered to be efficiently absorbed in the body as they form insoluble fatty soaps with calcium in the small intestine (German & Dillard, 2006; Stroebinger, 2016). In this context, the TAG structure is

considered to play a key role. Most of the long chain palmitic acid (C16:0) present in human milk (>70%) is located in the sn-2 position of the TAG structure; this position is considered to be suitable for the digestion and absorption of this fatty acid as well as other nutrients (Gantner *et al.*, 2015; Innis, 2011; Park, 2006). German and Dillard (2006) stated that the location of saturated fatty acids, such as long chain palmitic acid on the sn-2 position of TAGs, makes both the sn-1 and the sn-3 position fatty acids easily hydrolysable by pancreatic lipases into free fatty acids, and produces sn-2 monoacylglycerols, which are easily absorbed in the small intestine; this also makes the milk calcium completely available and absorbable. Donkey milk has the closest proportion of palmitic acid located at the sn-2 position (i.e., 54%) to that of human milk (74%) (Table 2.4). Thus, the modification of the TAG structure in milk from other species may help to deliver better milk fat digestion profiles; this could be an area of future interest.

Table 2.4 Fatty acid profile (% of total fatty acids) and cholesterol content of milk from different mammalian species.

	Ruminants						Nonruminants		Human
Fatty acids	**Cow**	**Buffalo**	**Goat**	**Sheep**	**Red deer**	**Camel**	**Horse**	**Donkey**	
SFA (%)	55.7–72.8	62.1–74	59.9–73.7	57.5–74.6	No data	47–69.9	37.5–55.8	46.7–67.7	39.4–45
MUFA (%)	22.7–30.3	24.0–29.4	21.8–35.9	23.0–39.1	No data	28.1–31.1	18.9–36.2	15.31–35.0	33.2–45.1
PUFA (%)	2.4–6.3	2.3–3.9	2.6–5.6	2.5–7.3	No data	1.8–11.1	12.8 –51.3	14.17–30.5	8.1–19.1
ω-6: ω-3 fatty acids ratio	2.1–3.7	No data	4	1.0–3.8	No data	No data	0.3–3.5	0.9–6.1	7.4–8.1
CLA (%)	0.2–2.4	0.4–1	0.3–1.2	0.6–1.1	No data	0.4-1	0.02–0.1	No data	0.2–1.1
Cholesterol (mg/100 mL milk)	13.1–31.4	4–18.0	10.7–18.1	14–29.0	No data	31.3–37.1	5.0–8.8	No data	14–20
% of C16:0 at sn-2	38	37	36	29	No data	No data	No data	54	74

Source: Adapted and modified from Claeys *et al.* (2014) and Crowley *et al.* (2017).
Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CLA, conjugated linoleic acid, C16:0, palmitic acid on the sn-2 position of milk TAG.

Table 2.5 Fat globule size of milk from different mammalian species.

Property	Ruminants						Nonruminants		Human
	Cow	Buffalo	Goat	Sheep	Red deer	Camel	Horse	Donkey	
Fat globule diameter (μm)	2.8–4.6	4.1–8.7	2.6–3.5	3.0–4.6	–	3.0	2–3	1–10	4

Source: Adapted and modified from Claeys *et al.* (2014) and Crowley *et al.* (2017).

2.2.4 Milk fat globule size

The fat in the milk of all species is present as small spherical droplets, called globules, the diameter of which ranges from 0.2 to 15 μm (Singh, 2006). The size of these fat globules varies among milk from different species; goat, sheep, camel, and equine (horse and donkey) milks have higher proportions of smaller size fat globules compared to cow milk (Table 2.5). The differences in the sizes of the fat globules of milk from different species may influence the digestion of their fat differently (Claeys *et al.*, 2014; Gantner *et al.*, 2015). The TAG core of the fat globules from all species is surrounded, protected, and stabilized by a phospholipid trilayer (along with specific membrane proteins) called the milk fat globule membrane (MFGM) (Lopez *et al.*, 2019; Lopez *et al.*, 2010). The MFGM is unique to milk and its structure is considered to be similar in all milks, although the proportions of different proteins in the MFGM may differ among different species (Nguyen *et al.*, 2017).

In general, the differences in the characteristics of the casein micelles and the fat globules among different milks are considered to play important roles in influencing their coagulation behaviour and nutrient delivery during digestion. This aspect will be discussed in the section on milk digestion.

2.3 Hypoallergenic potential of non-cow milks

More than 20 proteins in cow milk are known to cause allergic reactions; of these, the casein fractions (especially α_{s2}-, α_{s1}-, and κ-caseins as well as, to some extent, β-casein), lactoferrin, serum albumin, and β-lactoglobulin are considered to be the most common cow milk allergens (El-Agamy, 2007; Jenkins *et al.*, 2007; Natale *et al.*, 2004).

There is increasing interest with respect to the suitability of non-cow milks as a hypoallergenic option to cow milk (Hinz *et al.*, 2012). A few studies have reported that horse milk (Businco *et al.*, 2000), donkey milk (Monti *et al.*, 2007; Tesse *et al.*, 2009), camel milk (Ehlayel *et al.*, 2011; El-Agamy *et al.*, 2009), and water buffalo milk (Sheehan & Phipatanakul, 2009) may be potential alternatives in cases of moderate allergenicity to cow milk in children; however, this needs to be further investigated because weak cross-reactivity of non-cow milk proteins with cow milk proteins has been reported (Katz *et al.*, 2008; Restani *et al.*, 2002; Restani *et al.*, 1999). Jenkins *et al.* (2007) conducted a comprehensive study on the cross-reactivity of human and non-human milk proteins and found that the degree of allergenicity of a non-human milk protein is related to its extent of similarity with its human homologs. They found that, compared with cow, goat, and sheep milk proteins, camel and horse milk proteins (i.e., α_{s1}- and β-caseins) are more homologous to their human milk counterparts, which may be a reason for their weak cross-reactivity or less allergenic nature compared with other non-cow milks.

Infante *et al.* (2003) reported that 25% of patients had a negative immunological test for adverse reactions to goat milk proteins; thus, goat milk cannot be considered to be a suitable alternative in cases of cow milk allergy. Similarly, there is also strong evidence of allergenicity or positive cross-reactivities of goat, sheep, deer, and buffalo milk with cow milk (Bellioni-Businco *et al.*, 1999; Restani *et al.*, 1999; Robinson, 2001; Spuergin *et al.*, 1997). In addition, reports concerning selective allergy to goat and sheep milk proteins, but not to cow milk proteins, are also available (Ah-Leung *et al.*, 2006; Martín *et al.*, 2004). Bevilacqua *et al.* (2001) found that goat milk with lower proportions of α_{s1}-casein (and higher

amounts of α_{s2}-casein) was significantly less allergenic in guinea pigs than goat milk with high α_{s1}-casein content (and low α_{s2}-casein content); thus, different proportions of milk proteins may also play a key role in controlling milk protein allergy.

Overall, the scientific evidence indicates that there is little basis for promoting non-cow milk or milk proteins as an alternative to cow milk for people suffering from cow (or cow) milk allergy.

2.4 Milk digestion

2.4.1 Indispensable role of the gastric phase in milk digestion

It is well accepted that milk is a source of nutritionally balanced and highly digestible proteins (Bos *et al.*, 1999; Rutherfurd *et al.*, 2015). Previous studies have reported that the gastric emptying rates of two major fractions of milk protein (i.e., casein and whey protein) differ markedly; this has led to the concept of "slow" digested caseins and "fast" digested whey proteins (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2002; Fruhbeck, 1998; Mahe *et al.*, 1996; Pennings *et al.*, 2011).

The digestion of milk by the stomach enzymes (mainly pepsin and, to some extent, gastric lipases) in the presence of hydrochloric acid is considered to be the first key step, which is followed by further digestion in the small intestine by intestinal proteases and lipases (Mulet-Cabero *et al.*, 2020b). Some human infants may have chymosin like enzyme along with pepsin, which disappears from the gastric fluid by day 11 after birth (Henschel *et al.*, 1987). Chymosin and pepsin belong to the same group of aspartic proteinases that uses aspartic acid

residues in their active centre (Moschopoulou, 2011). Both the enzymes can preferentially hydrolyse the Phe105–Met106 bond of κ-casein, except that pepsin also exhibits unspecific proteolytic activity towards bonds with Trp, Tyr, Leu or Val residues, and thus have higher proteolytic activity relative to its milk clotting activity than chymosin (Guinee & Wilkinson, 1992; Júnior *et al.*, 2015; Moschopoulou, 2011). As the site of action of both chymosin and pepsin is the same, the mechanism of action of chymosin and pepsin is expected to be similar in relation to milk clotting. Chymosin is most stable in the pH range 5.3–6.3, but loses its activity rapidly under acidic conditions, i.e., below pH 3–4, as well as at high alkaline pH values, i.e., above pH 9.8 (Crabbe, 2004). Pepsin has maximum proteolytic activity at pH 2, with an optimum pH range of 2–5, and has activity in the pH range pH 5.5–7.5. Pepsin is irreversibly inactivated at pHs above 7.5 (Piper & Fenton, 1965).

The protein hydrolysis sites of pepsin are different from those of the intestinal proteases (mainly trypsin and chymotrypsin). Pepsin acts preferentially on κ-casein on the casein micelles, leading to the coagulation of the casein fraction of milk proteins under acidic conditions, whereas the whey protein fraction remains soluble (Ye *et al.*, 2016b). Thus, the early role played by the stomach in milk digestion is an essential step in regulating the rate of digestion of the milk proteins in the gastrointestinal tract (Nakai & Li-Chan, 1987). In this respect, it is of great importance to understand the digestive dynamics and coagulation behaviour of milk during gastric digestion, as milk coagulation can influence the delivery rates of proteins, fats, and associated milk constituents.

2.4.2 Evidence of milk coagulation

Human milk is known to form very soft and fragile curds in the infant stomach. Mason (1962) investigated the changes in pH and the extent of protein hydrolysis in the stomach contents collected using a gastric tube at different time intervals from 25 healthy new-born infants (full-term, aged between 5 and 13 days). He reported the presence of casein curds in the stomach contents collected after 30 min of breastfeeding. He also reported that there was negligible protein hydrolysis in these samples. Similarly, recently, de Oliveira *et al.* (2017) studied the gastric digestion of raw and pasteurized human milk in tube-fed preterm infants. The microstructural analysis in their study showed that human milk formed very soft and fragile protein aggregates in the infant's stomach.

Piglets and growing pigs have been regarded as a suitable animal model for human digestion research (Moughan *et al.*, 1992; Moughan *et al.*, 1994; Moughan & Rowan, 1989). Bottle-fed piglets have been used to study the digestion of human milk and infant formulas (Darragh & Moughan, 1998; Moughan *et al.*, 1991; Moughan *et al.*, 1990). Some evidence of clot (or curd) formation by cow milk in pigs or piglets has been reported in the literature. Washburn and Jones (1916) reported that cow skim milk formed a tough or hard clot, whereas cow whole milk formed a more friable and mellow curd in the stomach of baby pigs (28–35 days old), and that, the higher the fat content, the softer was the curd that formed. Braude *et al.* (1970) found that the caseins from homogenized cow milk clotted in the stomach of the 28-day-old pig after 15–30 min of feeding, whereas the "whey" fraction of the milk remained soluble and passed rapidly into the small intestine. Similarly, Decuypere *et al.* (1978) reported the formation of firm casein clots in the stomachs of early weaned pigs (10–29 days

of age) fed dry cow-milk-based food; their gastric chyme had a long retention time and a low buffering capacity and stimulated more gastrin release, compared with the gastric contents of suckling piglets fed pig milk. They believed that these differences were due to the firm casein clot formed by a dry cow-milk-based food in early weaned pigs in comparison with the soft casein aggregate formed from pig milk in suckling piglets.

2.4.3 Clotting characteristics of human milk and cow milk

Cow milk is known to form firm curds (or clots) in the stomach, in comparison with human milk. Nakai and Li-Chan (1987) studied the coagulation characteristics of human and cow milk using an *in vitro* acid precipitation test at 37°C, in which they added 0.2% acidic pepsin solution to 100 mL each of cow milk and human milk at a flow rate of 15 mL/h. They found that human milk formed much finer protein aggregates (or clots) than cow milk and reported that this could be the possible reason for the shorter gastric emptying time for human milk.

The differences in the structures of human and cow milk curds could be related to the differences in their fat and protein compositions. The protein (casein)-to-fat ratio of human milk is very low (Tables 2.1 and 2.2) compared with that of cow milk (as well as of other non-cow milks), which is likely to be a factor that is responsible for its soft (or fragile) curd formation. In addition, the higher β-casein-to-α_s-casein ratio of human milk has been associated with the fine and loose curd formed by human milk in an infant stomach. Lichan and Nakai (1988) performed an *in vitro* coagulation study with untreated cow milk casein, rennin-modified cow milk casein, and human milk casein. The rennin-modified cow milk casein was a β-casein-rich cow milk (similar to β-casein-rich human milk) that was produced

by selectively eliminating the α_{s1}-casein fraction from cow milk by a process involving rennet action. Upon acidification of the different casein solutions to pH 2 or pH 4, Lichan and Nakai (1988) observed that the hardness of the clot formed from these different casein solutions decreased in the order: cow milk casein > rennin-modified cow milk casein (rich in β-casein) > human casein. In another study, Lichan and Nakai (1989) also reported that moderate or partial dephosphorylation of cow milk casein using different phosphatases (calf intestinal alkaline phosphatase and potato acid phosphatase) at pH 4 resulted in the acid-coagulating properties of these modified cow milk casein solutions being similar to those of human milk as well as in a greater rate of proteolysis compared with the firm clots of untreated cow milk casein. However, all these studies were *in vitro* physicochemical studies, and further studies in *in vitro* or *in vivo* digestion models need to be conducted to validate such findings.

Blakeborough *et al.* (1986) studied the digestion of human milk, cow milk, and reconstituted baby formula (based on full cream dry cow milk powder) using 14-day-old piglets; cow milk or baby formula formed firm solid curds, whereas human milk formed a very liquid-like coagulum (little solid material) in the piglet's upper gastrointestinal tract. They also determined the bioavailability of zinc (Zn) from these milk systems; they found that, for cow milk (as well as baby formula), ~55–72 and ~60–66% of the Zn was retained in the curds present in the gastric chyme and the intestinal digesta, respectively, whereas, for human milk, ~43 and 7% of the Zn was retained in the curds present in the gastric chyme and the intestinal digesta, respectively. They suggested that these differences in the distribution and bioaccessibility of Zn in the gastrointestinal tract of piglets fed human milk or cow milk may

have been due to the differences found in the consistency of the casein curds formed by the different milks.

2.4.4 Digestion of milk from different species

2.4.4.1 Protein digestion

The lower protein content, lower casein-to-whey-protein ratio, and higher β-casein-to-α_s-casein ratio of human milk compared with milk from other species have been related to its soft curdling properties *in vitro* as well as *in vivo*, as described earlier. Although none of the non-human milks match the composition of human milk, horse and donkey milk are known to form very weak or fragile gels (or curds or flocs) when acidified or treated with rennet (Charfi *et al.*, 2018; Iannella, 2015; Uniacke-Lowe & Fox, 2011) and thus are expected to form soft or fragile curds in the stomach, in comparison with cow milk, because of their lower casein content. Similarly, some of the ruminant milks, such as goat and camel milk (Gamble *et al.*, 1939; Genene *et al.*, 2019; Kamal *et al.*, 2017; Ould Eleya *et al.*, 1998; Wang *et al.*, 2019), are also considered to form soft curds in the stomach when acidified or treated with rennet (or pepsin), because of their lower casein content or larger casein micelle size compared with cow milk, even though they contain comparatively higher proportions of caseins than equine and human milk. However, no direct comparative *in vitro* or *in vivo* digestion studies between cow and non-cow milks, focusing on their curd formation characteristics in the stomach, have been reported to date.

There are only a few comparative *in vitro* digestion studies on cow and non-cow milks, focusing on their protein or fat digestion. Jasińska (1995) compared the degrees of hydrolysis

by pepsin and trypsin of micellar caseins obtained from cow, human, goat, and horse skim milks; the peptic hydrolysis rates of the micellar caseins from cow, human, goat, and horse milk were 23–42 (differed for different breeds of cow), 80, 65, and 43%, respectively. The tryptic hydrolysis rates of the micellar caseins from cow, human, goat, and horse milk were 76–90, 100, 96, and 92%, respectively. The higher susceptibility of human and goat milk was believed to be due to the smaller micellar aggregates and the presence of higher proportions of β-casein in their micellar structures, when compared with cow milk (which had higher proportions of α_{s1}-casein).

Recently, Hodgkinson *et al.* (2018) studied the *in vitro* static gastric digestion of cow and goat whole milks (at pH 3.0) and reported that, after both 20 and 60 min of digestion, goat milk caseins were digested faster than cow milk caseins (based on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) image analysis), possibly because of the relatively soft or fragile coagulum formed by goat milk. Tagliazucchi *et al.* (2018) also studied the *in vitro* static gastrointestinal digestion of cow, goat, sheep, and camel skim milks (as per the INFOGEST protocol) and reported that the extent of free amino groups generated during the gastric digestion was higher for goat, sheep, and camel milk proteins, indicating that the proteins in these non-cow milks were hydrolysed faster than cow milk proteins during the gastric step. However, after the intestinal step, they reported that only the goat milk proteins were hydrolysed faster than the milk proteins from the other species, all of which had similar hydrolysis rates. Tagliazucchi *et al.* (2017) and Rutella *et al.* (2016) reported similar findings in their previous studies, i.e., that the degree of hydrolysis of goat skim milk proteins during the gastric and intestinal steps was much higher than that of cow skim milk

proteins. The authors stated that the higher degree of hydrolysis of goat milk proteins observed in all studies was probably due to the higher susceptibility of goat milk proteins to pepsin.

Maathuis *et al.* (2017) investigated the comparative protein digestibilities and qualities (based on bioaccessible nitrogen and amino acids) of human milk, cow-milk-based infant formula, and goat-milk-based infant formula using the tiny-TIM model (a dynamic *in vitro* infant gastrointestinal model). They found that the protein digestibilities and qualities of all diets were similar; however, the rates of protein digestion were slower during the first 60 min of digestion for the cow-milk-based formula than for the human milk and the goat-milk-based formula. They hypothesized that the differences in the clotting characteristics of different milks would have led to differences in their gastric emptying, as they found that the curds formed from the cow-milk-based formula were retained for a longer duration in the gastric compartment of tiny-TIM compared with those from the human milk and the goat-milk-based infant formula. Similarly, Ye *et al.* (2019a) investigated the *in vitro* dynamic gastric digestion of goat- and cow-milk-based formulas in a mini version of the human gastric simulator (HGS), simulating infant gastric digestion. The authors found that the goat-milk-based infant formula formed smaller protein aggregates in the mini-HGS, leading to faster hydrolysis of its proteins compared with those from the cow milk formula. Based on the above-mentioned studies it appears that the differences in the structures of the curds formed from milk of different species during gastric digestion may be a key factor that is responsible for their different digestion behaviours.

In contrast, Almaas *et al.* (2006) did not find any differences in the digestion of caseins and α-lactalbumin from cow and goat skim milks (with high and low α_{s1}-casein content) after static gastrointestinal digestion using human gastric juice (HGJ) and human duodenal juice (HDJ). They also did not find any differences between goat milk with high and low α_{s1}-casein content after digestion with HGJ and HDJ. However, they observed (using SDS-PAGE image analysis) that goat milk β-lactoglobulin was rapidly digested during both gastric digestion and intestinal digestion, compared with cow milk β-lactoglobulin. El-Zahar *et al.* (2005) studied the hydrolysis of isolated β-lactoglobulin from sheep and cow milk by porcine pepsin and found that β-lactoglobulin from sheep milk was hydrolysed faster because of its slightly different tertiary structure and higher surface hydrophobicity. As β-lactoglobulin is considered to be one of the major allergens (as it is absent in human milk), the higher degree of hydrolysis by pepsin of the β-lactoglobulin in goat and sheep milk may be a possible reason that these non-cow milks are better tolerated by some people than cow milk.

Vithana *et al.* (2012) studied the comparative *in vitro* gastrointestinal digestion of raw cow and deer skim milks. They found that, after gastric digestion, nearly 49 and 27% of the deer and cow milk caseins remained undigested (SDS-PAGE image analysis), respectively, whereas, after intestinal digestion, the caseins from both species were completely digested. This indicated that, during the gastrointestinal digestion, deer milk caseins were digested at a faster rate than cow milk caseins. It was hypothesized that the higher amounts of caseins retained in the gastric phase for deer skim milk may have been due to the higher protein content (as well as casein content) of the deer milk used in their study, indicating that the inherent composition of milk also has a key role to play during gastric digestion. Vithana *et*

al. (2012) also found that α-lactalbumin was hydrolysed faster in deer milk than in cow milk. However, β-lactoglobulin from both species was found to be resistant to both gastric and duodenal digestion.

In contrast to the above studies, some studies have reported no differences or faster hydrolysis of cow milk proteins than of goat milk proteins. For instance, Inglingstad *et al.* (2010) reported (based on SDS-PAGE image analysis) that 69 and 82% of the caseins remained undigested after hydrolysis by HGJ of cow and goat skim milks, respectively; however, after further treatment with HDJ, almost all of the caseins from the milk of both species were digested. They found that the β-lactoglobulin and α-lactalbumin from both species were highly resistant to HGJ and that, after hydrolysis with HDJ, ~64% of the β-lactoglobulin from both species remained undigested and 91 and 65% of the α-lactalbumin from the cow and goat skim milks, respectively, remained undigested. Mros *et al.* (2017) reported no differences in the protein digestion of cow, goat, and sheep skim milks following hydrolysis by pepsin and pancreatin.

Similarly, Milan *et al.* (2018) reported that whole goat-protein fortified milk, compared to whole cow-protein fortified milk, was digested and metabolized similarly (despite the differences in their inherent nutrient composition) in young adults (aged 18-28 years). However, they dissolved paracetamol in fortified milk drinks before giving it to the participants for consumption (plasma paracetamol levels were used as a marker for gastric emptying). It has to be noted that depending on the type of paracetamol used, it may have a buffering action during the gastric digestion in the stomach (Mills, 1989) and thus, careful

consideration needs to be made while conducting human digestion studies to draw any firm conclusions.

Vaisman *et al.* (2006) investigated the gastric emptying times in humans of camel and cow milk using a scintigraphic technique and reported that the poor coagulation properties of camel milk (as observed during acid or rennet coagulation) did not provide any comparative advantage over cow milk in terms of gastric emptying. It should be noted that the soft or fragile curd formed from non-cow milks (such as camel, goat, horse, and donkey milk) during acid or rennet coagulation provides only an indication of how these non-cow milks may behave in the human stomach during gastric digestion. The gastric digestion process is a complex and dynamic phenomenon, and in-depth comparative *in vitro* and *in vivo* studies on cow and non-cow milks that simulate the gastric digestion in humans need to be undertaken, to draw any definite conclusions.

Not only protein composition and (or) casein micelle structure, but also different processing temperature and time combinations may induce differences in the curd structure in the stomach, which may influence the rate of delivery of proteins to the small intestine and their subsequent absorption. For instance, Ye *et al.* (2016b) studied the dynamic gastric digestion behaviour of raw and heated (90°C for 20 min) cow skim milks using an HGS. The HGS is a dynamic stomach model that is capable of simulating the stomach contraction forces and the flow of gastric fluids that occur *in vivo* (Kong & Singh, 2010). Ye *et al.* (2016b) found that raw milk formed a "closely knitted" tight clot, whereas heated milk formed fine and loose protein aggregates (Figure 2.2), leading to slow hydrolysis of caseins from raw milk,

compared with heated milk. This was because, in raw milk, only the caseins were involved in clot formation, whereas, in heated milk, both the caseins and denatured whey proteins were involved in clot formation (Ye *et al.*, 2019b). Heating at 90°C for 20 min would have led to complex formation between fully denatured whey proteins and caseins via sulfhydryl groups and disulphide linkages (Figure 2.3), hindering the formation of a firm clot (Dannenberg & Kessler, 1988; Schorsch *et al.*, 2001).

Kaufmann (1984) reported that ultrahigh-temperature-treated (UHT) milk led to the formation of soft coagulates in the mini-pigs stomach, leading to higher levels of amino acids and urea in their blood serum compared to that of pasteurized and raw milk, which formed stronger coagulum. Thus, these differences in gastric restructuring induced by heating are expected to be a key possible reason for higher postprandial utilization of dietary nitrogen from defatted UHT milk (140°C for 5 s) compared to defatted pasteurized milk (72°C for 20 s) as well as defatted microfiltered milk in humans (Lacroix *et al.*, 2008).

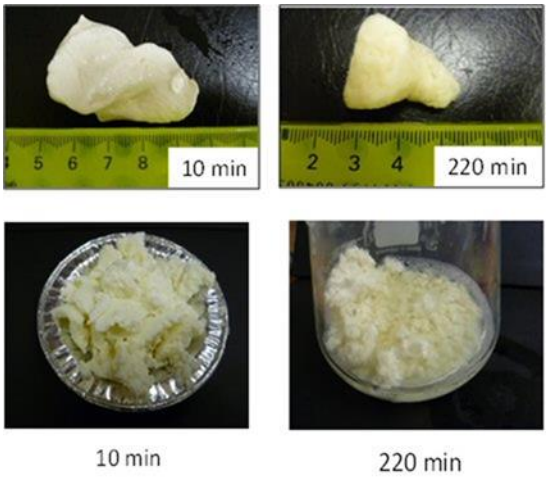


Figure 2.2 Images of clots formed during the gastric digestion of 200 g of unheated (top row) and heated (bottom row) cow skim milk at different digestion times. Source: Adapted from Ye et al. (2016b).

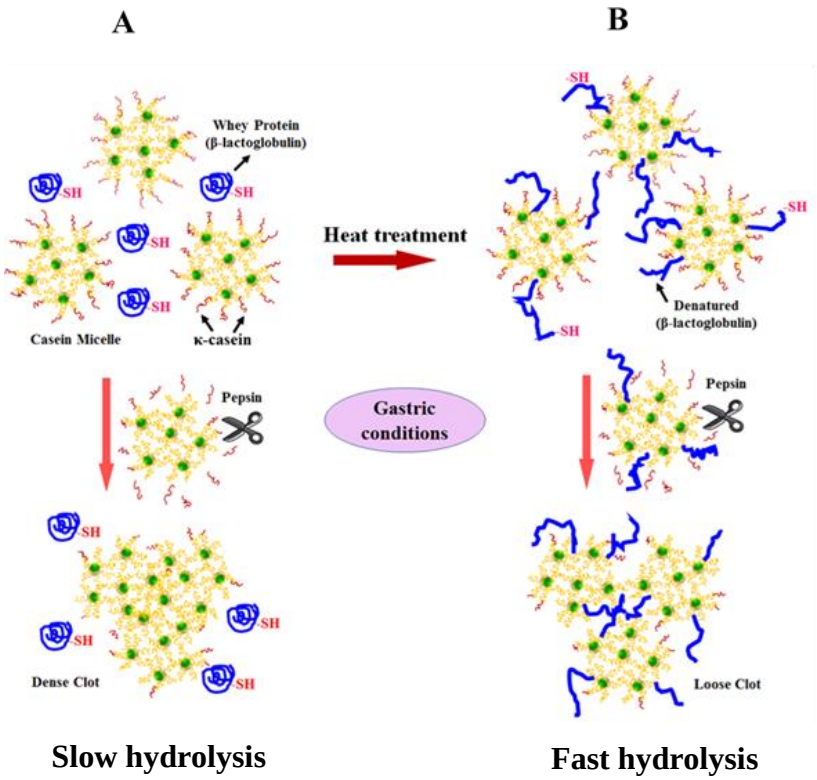


Figure 2.3 Schematic diagram of the possible mechanism of events during the formation of protein curds from (A) raw milk (unheated) and (B) heated milk during gastric digestion. Source: Adapted from Ye et al. (2019b).

Doan (1938) published a comprehensive review based on studies on the gastric digestion of processed (boiled, evaporated, or acidified) and raw cow milk in the early 1900s, and reported that boiled, evaporated, or acidified milks were emptied rapidly from the human stomach because of the finer or softer curd that formed. It was suggested that the modification of raw cow milk using different processing conditions may be a potential option in the development of dairy-based baby foods or beverages with properties similar to those of human milk.

To date, no studies on the impact of different heating or processing conditions on the digestion behaviours of non-cow milks have been reported in the literature. It should be noted that the commercial processing or technological conditions needed for non-cow milks may be different from those needed for cow milk. In addition, the impact of different processing conditions on the digestion behaviours of non-cow milks may be different from that on cow milk because of the differences in their composition and structures.

2.4.4.2 The influence of the protein network on fat digestion – the whole milk matrix

During the gastric digestion of whole milk, the fat globules are known to be physically entrapped within the protein clot that is formed. Thus, the nature or structure of the protein network formed will influence the rate of release and the digestion of fat by gastrointestinal lipases (Mulet-Cabero *et al.*, 2019; Ye *et al.*, 2016a, 2017; Ye *et al.*, 2019b). Previous studies have shown that the nature or structure of the protein network formed is, in turn, dependent on the protein composition (casein-to-whey-protein ratio), the protein-to-fat ratio, and the impact of different processing conditions (Mulet-Cabero *et al.*, 2020c). For instance, Mulet-Cabero *et al.* (2020c) studied the *in vitro* gastrointestinal digestion of model systems based on different casein-to-whey-protein ratios using a semi-dynamic gastric model, and reported that the viscosity or firmness of the coagulum formed increased as the casein-to-whey-protein ratio increased in the model protein systems, leading to slower gastric emptying, and slower digestion and absorption of nutrients. They also found that the addition of increasing amounts of fat to the casein-rich protein models produced more fragmented clots with a significant decrease in their firmness. This indicates that the presence of fat hindered the aggregation of proteins, which may, in turn, influence the digestion rates of nutrients.

Ye *et al.* (2016a) studied the gastric digestion of raw (unheated) and heated (90°C for 20 min) cow whole milks and reported that the release of fat globules was dependent on the disintegration characteristics of the protein clot and that the release of fat globules was higher from the finer aggregates of protein clots formed from the heated whole milk than from the firm clots formed from the raw whole milk (Figure 2.4).

Similarly, Ye *et al.* (2019b) studied the comparative *in vitro* and *in vivo* (in rats) gastric digestions of raw (non-homogenized), pasteurized (homogenized), and UHT (homogenized) cow whole milks, and reported that the UHT milk had faster rates of protein hydrolysis as well as release of fat globules during gastric digestion, compared with the raw and pasteurized milks; the differences were attributed to the smaller or fragmented protein aggregates formed from the UHT milk proteins in comparison with the aggregates from the other milks.

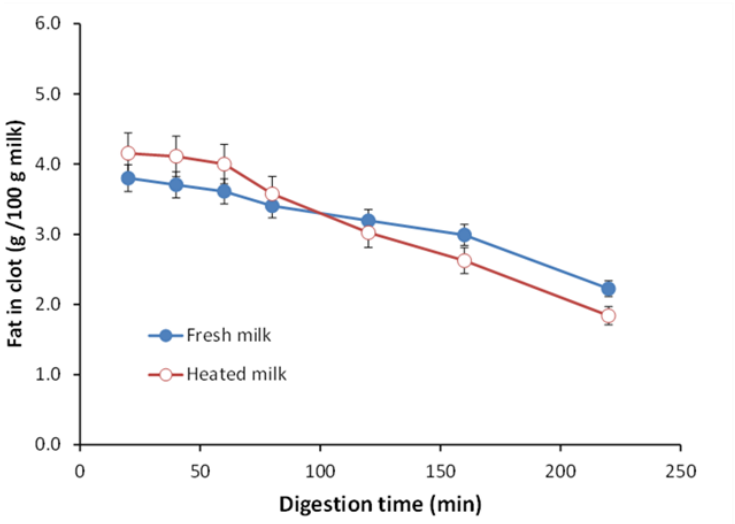


Figure 2.4 Changes in the fat content (g/100 g milk) in clots obtained from (●) unheated (raw) and (○) heated cow whole milk during gastric digestion. Source: Adapted from Ye et al. (2016a).

In another gastric digestion study, Ye *et al.* (2017) reported that the release of fat globules was relatively higher in homogenized milk (20/5 MPa (primary/secondary pressure), 20°C) as well as heated, homogenized milk (20/5 MPa, 20°C + 90°C for 20 min) because of the fine and crumbled structure of the coagulum formed in these milks compared with the firm coagulum formed from raw cow whole milk (Figure 2.5). Similar results have been reported by Mulet-Cabero *et al.* (2019) for processed cow whole milks.

The coalescence of fat globules entrapped within the protein network as well as those present in the liquid phase of the gastric chyme has also been reported (Mulet-Cabero *et al.*, 2019; Ye *et al.*, 2017; Ye *et al.*, 2019b), which is expected to be due to the hydrolysis of the proteins present at the surface of the milk fat globule (present naturally in the MFGM or adsorbed proteins because of processing treatments).

Figure 2.5 Images of clots formed during the gastric digestion of raw (unheated), homogenized, and heated homogenized cow whole milk during 20 and 160 min of gastric digestion. Source: Adapted from Ye et al. (2017).

As the milk from different species are known to vary in fat content, protein-to-fat ratio, fat globule size, and structure, there may be differences in the consistency of the coagulum formed from milk of different species during gastric digestion, which may impact their overall digestion behaviour differently.

2.4.4.3 Gastrointestinal digestion of fat

Little information is available on the gastric digestion of milk fat, irrespective of species. Lipolysis during the gastric phase was previously considered to be of less relevance during the overall digestion process as gastric lipolysis accounts for only 10–25% of the overall lipid digestion in adults (Mulet-Cabero *et al.*, 2020a). Therefore, most of the studies reported in the literature on fat digestion have focused mainly on intestinal digestion. However, it is now

widely suggested that gastric lipases should be incorporated in *in vitro* digestion studies as their preliminary role may facilitate further breakdown of lipids by intestinal lipases (Mulet-Cabero *et al.*, 2020a). Also, in contrast to adults, gastric lipases play a significant role in infants because of their high postprandial gastric pH (Ménard *et al.*, 2018).

It is hypothesized that, the smaller the fat globule size, the higher will be the fat digestibility, because the higher surface area of smaller fat globules will help in rapid digestion via gastrointestinal lipases (Bourlieu *et al.*, 2015; Claeys *et al.*, 2014; Crowley *et al.*, 2017; Gantner *et al.*, 2015). Meena *et al.* (2014) investigated the digestion of milk fat by pancreatic lipase in standardized raw cow, buffalo, camel, and goat whole milks. The authors found that the amount of free fatty acids released followed the order: goat ~ camel > cow > buffalo. The higher digestibility of goat and camel whole milks was believed to be due to the small size of their fat globules, as the fat globule sizes of the different milks were in the order: buffalo (3.9–7.7 μm) > cow (1.6–4.9 μm) > goat (1.1–3.9 μm) ~ camel (1.1–2.1 μm). In addition to the fat globule size, the outer surface of the fat globule and its structure (i.e., the fat globule interface) have a crucial role to play in the digestion of fats. For example, the presence of adsorbed proteins (caused by processing such as heating and homogenization) at the interface of fat globules may result in providing easy access of lipases to the TAG core of the fat globules and thus in influencing the digestion of milk fat (Bourlieu *et al.*, 2015).

Some studies have also shown the influence of differences in the milk fat composition among different milks on their digestibility. For instance, Alférez *et al.* (2001) studied the fat digestibility and metabolism in faeces samples of male albino rats that were fed diets

containing lyophilized goat and cow whole milks. They found that, compared with the rats on the cow-milk-based diet, the digestive utilization of fat was higher, and the levels of cholesterol were lower, in the rats on the goat-milk-based diet. The authors believed that the differences may have been due to the greater amounts of medium chain TAGs and the smaller fat globule sizes of the goat milk fat compared with the cow milk fat used in their study. Similarly, Teng *et al.* (2020) studied the *in vitro* gastric digestion of raw (non-homogenized) and homogenized cow and sheep milk, and reported that the TAGs from both raw and homogenized sheep milk were digested by rabbit gastric lipases more rapidly than those from cow milk; this was due to the presence of higher levels of medium chain fatty acids at the sn-1 or sn-3 position of the TAG structure in sheep milk compared with cow milk, emphasizing that the structural characteristics of TAGs have an important role to play in their gastric digestion.

Overall, the digestibilities of the protein and fat in milk are likely to be functions of the unique compositions, protein profiles, fat compositions, casein micelle and fat globule structures, interfacial properties, mineral distributions, and physicochemical properties, all of which are likely to be affected to different degrees by the processing conditions, depending on the animal species. Although there are very few studies on the impact of the processing conditions and the milk composition of non-cow milks in the literature, the principles of cow milk protein coagulation and its impact on fat digestion are expected to also be applicable to non-cow milks. However, as cow and non-cow milks vary in protein composition (proportion of different proteins) as well as protein-to-fat ratio, it is likely that there will be differences in the structure and consistency of the protein curd (or clot) formed from different milks,

which may lead to further differences in the release of fat globules from the clot matrix of different milks. It should also be noted that the gastric and intestinal digestion conditions of infants (as well as the elderly) are different from those of adults in terms of acid secretions and enzyme (proteases and lipases) activities (Ménard *et al.*, 2018; Mulet-Cabero *et al.*, 2020a; Shani-Levi *et al.*, 2017). Thus, relevant dynamic *in vitro* models need to be used to study the digestion of milks in different age groups, and *in vitro* results need to be ultimately corroborated based on *in vivo* observations.

2.5 Concluding remarks

As non-cow milk and milk products are highly regarded as a potential source of human nutrition, they can be utilized to develop specialized dairy products for people in all age groups. Non-cow milks are of great interest to people as well as industries, because of their perceived better nutritional properties compared with cow milk. However, most of these presumptions are based on anecdotal reports and only little scientific research has been conducted to understand the nutritional and physicochemical properties of non-cow milks. One widely perceived notion is the formation of soft curds in the human stomach for some non-cow milks (such as goat, camel, horse, and donkey milk). Because of this, these milks are considered to be better digested and tolerated by people of different age groups. However, to date, no direct scientific studies have been reported and there is a knowledge gap. As cow and non-cow milks vary in composition and structure of the casein micelles and fat globules, they are likely to behave differently in the gastrointestinal tract, possibly affecting the kinetics of digestion and the bioavailability of nutrients. Because of differences in milk composition and the structure of the casein micelles (or fat globules), there may be

differences in the curds formed by the milk of each species in the stomach, which may further affect the delivery rates of macronutrients further down the gastrointestinal tract. Furthermore, different commercial processing conditions such as pasteurization or UHT (or other heat treatments) may influence the digestion behaviours of non-cow milks differently. Thus, in-depth scientific studies need to be conducted to understand the impact of compositional as well as structural differences in milk from different species (in their natural form as well as processed forms) on their dynamic digestion behaviours, especially focusing on their differences in curd formation as well as their disintegration properties in the stomach. Such studies will often involve *in vitro* digestion models, which where possible should be dynamic and sophisticated enough to at least include the effects of key variables known to influence food digestion. Further, the physiological relevance of such phenomena needs to be investigated in animal and human studies focusing on different age groups or people in need of targeted personalized nutrition (such as infants, the elderly, athletes or malnourished people).

Chapter 3: Common materials and methods

The common materials, methods and analytical techniques used in the experiments are summarized in this chapter. For ease of clarity, any modifications to these protocols or other protocols used specifically for any particular study have been described in specific chapters.

3.1 Common materials

3.1.1 Milk samples from different mammalian species

Pooled raw whole milk samples from different mammalian species (Figure 3.1) were obtained under chilled conditions from different farms/suppliers within New Zealand. The milking breeds for cow (*Bos taurus*), water buffalo (*Bubalus bubalis*), goat (*Capra aegagrus hircus*), sheep (*Ovis aries*), and deer (*Cervus elaphus scoticus)* were predominantly Friesian × Jersey, Swamp × Riverine (Riverine backcrossed), Saanen, *East Friesian, and Red deer,* respectively.

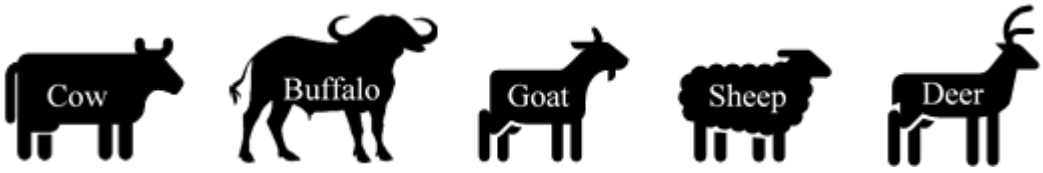


Figure 3.1 Different kinds of mammalian milk samples used in the thesis.

3.1.2 Pepsin for *in vitro* studies

Pepsin from porcine gastric mucosa-powder (EC 3.4.23.1; product number-P7000, batch number - SLBR2349V) was purchased from Sigma Aldrich Co. LLC., Saint Louis, MO,

USA. This particular batch of pepsin had an enzymatic activity of 479 U/mg solid as per the specification received from Sigma. The average pepsin activity was also determined in-house as outlined in the supplementary material of the harmonized INFOGEST protocol (Minekus *et al.*, 2014) and it was found to be 325 ± 13 U/mg solids.

3.1.3 Water

Milli-Q water (water purified by treatment with a Mill-Q apparatus; Mill-Q Synthesis, Millipore Corp., SAS – 67120, Molsheim - France) was used for the preparation of all solutions wherever needed.

3.1.4 Chemicals

All chemicals were obtained from Sigma Aldrich Co. LLC (St. Louis, MO) unless otherwise specified.

3.2 Common methods

3.2.1 Milk processing and storage

Skim milk (raw) sample was prepared by low-speed centrifugation of raw whole milk at 3,500 x *g*, 20 min, 4°C in a ThermoFisher Scientific Multifuge Heraeus 3SR+ centrifuge (Thermo Electron LED GmbH, Osterode, Germany) (Figure 3.2) and was then decanted to remove the fat layer. For pasteurized skim milk, pasteurized whole milk (72°C, 15 s) was cooled to 4°C and then separated into skim milk by low speed centrifugation as stated above. Raw whole milk was pasteurized using a steam jacketed pan (MERCER) and continuously

agitated with a spoon to ensure uniform temperature of all the milk in the pan. The milk was then rapidly cooled using an ice batch in Massey FoodPilot. The rate of increase and decrease in temperature for heating and cooling during pasteurisation was ~20°C/min and ~12°C/min, respectively. To avoid microbial growth, 0.02% (wt/wt) sodium azide (Merck KGaA, Darmstadt, Germany) was added to all samples. For all the analyses, the skim milk samples were stored in a cold room at 4°C until further use.

Figure 3.2 ThermoFisher Scientific Multifuge Heraeus 3SR+ centrifuge.

3.2.2 Milk characterization techniques

The analytical techniques used to characterize the physicochemical and microstructural properties of milks are discussed in this section.

3.2.2.1 Milk composition

The chemical composition of the samples was analysed by Nutrition Laboratory (Massey University, Palmerston North, New Zealand) as per the AOAC procedures. Total solids, fat,

total protein (total nitrogen × 6.38), and ash were determined using air oven-drying method 990.19 (AOAC, 2005), Mojonnier method 989.05 (AOAC, 2005), Dumas method 968.06 (AOAC, 2005) or Kjeldahl method 991.20 (AOAC, 2005), and gravimetric method 945.46 (AOAC, 2005), respectively. The available carbohydrate content was determined by the difference method, i.e., 100 – (moisture + total protein + fat + ash). The total calcium (Ca) and inorganic phosphorus (P_i) contents of the samples were analysed on an RX Daytona Plus analyser (Randox Laboratories, Crumlin, UK) using Randox reagents CA 8309 and PH 8328, respectively (Li *et al.*, 2019). The total Ca content of the samples was analysed by colorimetric method using Arsenazo III reagent. Arsenazo III specifically binds to Ca forming a coloured complex at 660 nm. The amount of calcium present in the sample is directly proportional to the intensity of the coloured complex formed. The total P_i content of the samples was analysed by UV method using ammonium molybdate reagent. P_i reacts with ammonium molybdate in the presence of sulphuric acid to form a phospho-molybdate complex, which is measured at 340 nm.

3.2.2.2 pH measurement

pH of all the samples was measured by using a CyberScan pH 510 pH/mV/°C Meter (Eutech Instruments, Fisher, Malaysia). pH meter was calibrated with standard solutions of pH 4.0, 7.0 and 10.0 before measuring the pH of milk samples and reagents.

3.2.2.3 Determination of milk fat globule diameter

The volume-weighted (D_{43}) and surface-weighted (D_{32}) average fat globule diameter (μm) of raw whole milks were measured by static light scattering technique as per Ménard *et al.*

(2010) with slight modifications, using a Malvern MasterSizer 2000 Hydro MU (Malvern Instruments Ltd, Malvern, Worcestershire, UK) with two laser sources (Figure 3.3). The refractive indexes used were 1.458 and 1.460 for milk fat at 633 and 466 nm, respectively, and 1.33 for water. The absorption coefficient at both wavelengths was 0.0001 (Ménard *et al.*, 2010; Michalski *et al.*, 2001). After many initial trials, all the raw whole milk samples were diluted ten times and gently mixed with a EDTA-SDS buffer solution containing 2% sodium dodecyl sulphate (SDS, ≥99.0% (GC), Sigma Aldrich Co. LLC., Saint Louis, MO, USA) and 50 mM EDTA, pH 6.7, to disrupt the casein micelles and remove any flocs prior to the measurements (Ye *et al.*, 2002). Prior to use for dilution, the EDTA-SDS buffer was filtered through 0.45 μm syringe filters (diameter of 25 mm, glass fibre + PVDF, Axiva Sichem Biotech, Axiva, Delhi, India) to remove any large particles. A small amount of sample was added to the measurement cell (with around 800 mL of water) to reach around 10% obscuration. A general purpose (spherical) analysis model was used and the pump speed of 2000 rev/min was maintained. The experiments were performed at room temperature.

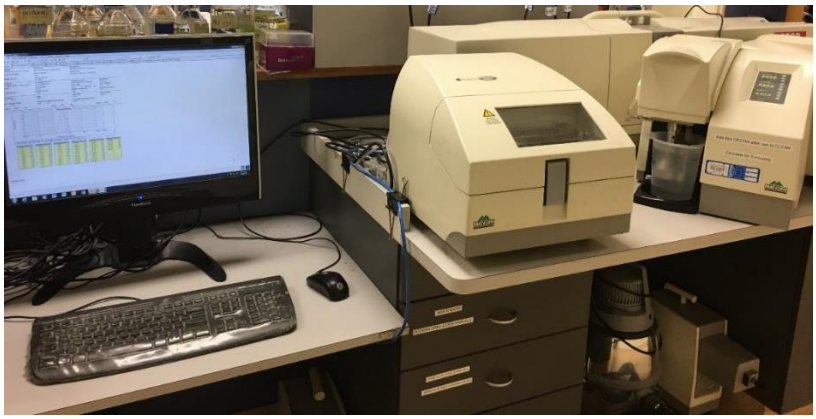


Figure 3.3 Malvern MasterSizer 2000 Hydro MU (Manual Unit).

3.2.2.4 Determination of casein micelle diameter

The mean hydrodynamic diameter (nm) i.e. the Z-average diameter (an intensity-based calculated value) of the casein micelles was measured by a dynamic light scattering technique using a Zetasizer Nano ZS (Figure 3.4), Model ZEN 3600, Serial No- MAL500836, Red Badge (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The milk samples were diluted 100 times with a calcium imidazole buffer solution (pH 6.7) containing 20 mM imidazole, 5 mM $CaCl_2$, and 30 mM NaCl (Anema, 1997; Anema & Li, 2003a, 2003b). In the experiments, the viscosity of the calcium imidazole buffer is taken as 0.89 cp and dielectric constant as 79 following Ménard *et al.* (2010). The diluted milk samples were filtered using a 0.45 μm syringe filter (diameter of 25 mm, glass fibre + PVDF, Axiva Sichem Biotech, Axiva, Delhi, India) to remove large particles just prior to analysis. The samples were equilibrated for about 120 s and then measured at 25°C in a particle sizing cell using back scattering technology at a detection angle of 173°. A general purpose (normal resolution) analysis model was used. The calcium imidazole buffer was used as a common diluent for easier comparative analysis of all skim milk samples.

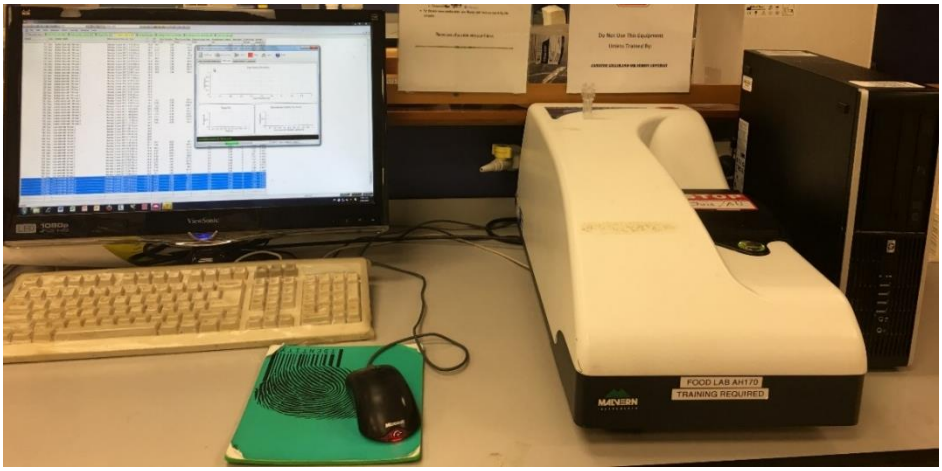


Figure 3.4 Zetasizer Nano ZS (Model ZEN 3600).

3.2.2.5 Sodium-Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Frozen skim milk samples (-25°C) were thawed overnight at 4°C and were used next day for SDS-PAGE analysis (Laemmli, 1970)**.** The SDS-PAGE gels were prepared using a resolving gel and a stacking gel mounted on a vertical Mini-Protean II dual cell unit (Bio-Rad Laboratories, Richmond, CA, USA). The recipe for the preparation of resolving and stacking gel is shown in Table 3.1. Sample buffer and electrode buffer were prepared as per the recipes in Table 3.2.

3.2.2.5.1 Sample preparation for SDS-PAGE

Different volumes (or weights) of sample were diluted with SDS reducing buffer (sample buffer) to result into a final equal protein concentration in each lane of the gels. The mixture was heated in a boiling water bath for 5 min and the samples were cooled to room temperature.

3.2.2.5.2 Running of gels

The electrode buffer was added to the chamber containing the gel system. The samples were then loaded onto SDS-PAGE gels previously prepared on the dual cell unit. Molecular weight markers of range (2-250 kD) were also loaded on the SDS-PAGE gels. The electrophoresis was then carried out at a constant voltage of 200 mV for a period of approximately 1 h on the power unit (Bio-Rad power supply Unit, model no- PowerPac Basic, Bio-Rad, Kaki Bukit View, Techview, Singapore). After the bromophenol blue dye had reached the bottom of the gel, the power was turned off; gels were carefully removed and transferred into transparent plastic containers and were sequentially stained and destained.

Table 3.1 Recipe for preparing two SDS-PAGE gels.

Gel constituents	Amount of constituents used for two gels
Resolving Gel Mixture (16% concentration)	
Milli-Q water	2.02 mL
1.5 M Tris-HCl buffer, pH 8.8[#]	2.5 mL
10% SDS	100 µL
Acrylamide (30%) [$]	5.3 mL
TEMED^	5 µL
10% APS^	50 µL
Stacking Gel Mixture (4% concentration)	
Milli-Q water	3.05 mL
0.5 M Tris-HCl buffer, pH 6.8*	1.25 mL
10% SDS	50 µL
Acrylamide (30%)	0.65 mL
TEMED^	5 µL
10% APS^	25 µL

[#]1.5 M Tris-HCl buffer (pH 8.8) was prepared by dissolving 4.5375 g of Tris in about 10 mL of Milli-Q water, adjusted to pH 8.8 and finally volume made up-to 25 mL.

*0.5 M Tris-HCl buffer (pH 6.8) was prepared by dissolving 1.5 g of Tris in about 10 mL of Milli-Q water, adjusted to pH 6.8 and finally volume made up-to 25 mL.

[$]30% Acrylamide/Bis-acrylamide solution, 37.5:1 (30%T, 2.6%C); T- denotes the total percentage concentration of both monomers (acrylamide plus Bis-acrylamide) in g per 100 mL. C- denotes the percentage by weight of the cross-linker (Bis) relative to the total monomer.

^APS and TEMED are added to the gel mixture just before being poured into the casting apparatus.

Table 3.2 Recipe for preparing sample buffer and electrode buffer for SDS-PAGE.

Buffer constituents	Amount of constituents used for buffer
25 mL Sample Buffer (SDS reducing buffer)	
Glycerol	3.125 g
0.5 M Tris-HCl, pH 6.8	3.125 mL
10% SDS	5 mL
Bromophenol blue	2.5mg
β-Mercaptoethanol[@]	1.25 mL
Milli-Q water	For making up total volume to 25 mL
500 mL Electrode/Tank Buffer, pH 8.3 ± 0.2 (freshly made)	
Tris base	1.512 g
Glycine	7.2 g
SDS	0.5 g
Milli-Q water	500 mL

[@]Sample buffer is prepared without β-mercaptoethanol and stored. A 5% β-mercaptoethanol is added fresh to the sample buffer and is then used for dilution of milk samples and heated at 95-100°C for 5 min before SDS-PAGE analysis.

3.2.2.5.3 Staining and destaining of the gels

Recipe for staining and destaining solutions is given in Table 3.3. The gels were stained by dispensing sufficient quantities of Coomassie brilliant blue staining solution into the container to cover the entire gel. The container was set on a platform rocker and allowed to stain for 60 min. After the gels were adequately stained, the staining solution was drained and nearly 100 mL of destaining solution was added and the container was again kept on the rocker for 1 h. After 1 h, the destaining solution was drained off and replenished with 100 mL of fresh destaining solution. The container was again allowed to destain on the rocker for 6 h and the procedure was repeated twice at further 6 h intervals.

Table 3.3 Recipe for preparing staining and destaining solutions for SDS-PAGE.

Constituents	Constituents % (wt/vol)
Coomassie Brilliant Blue – Staining Solution	
Coomassie brilliant blue R (250)	0.3
Isoproponol	20
Glacial acetic acid	10
Mill-Q water	70
Filter the solution through Whatman filter paper grade 4 and store in a dark bottle.	
Destaining Solution	
Isoproponol	10
Glacial acetic acid	10
Mill-Q water	80

3.2.2.5.4 Scanning and quantification of the protein bands

The gels were scanned (and the intensities of the protein bands were quantified where needed) using a Bio-Rad Molecular Imager Gel Doc XR+ imaging system (Bio-Rad Laboratories, Universal Hood II, Hercules, CA, USA) attached to a computer with Bio-Rad Image Lab software (Version 5.2.1) linked to it. The protein bands of non-cow milks were identified by comparing with cow milk counterparts.

3.2.2.6 Confocal scanning laser microscopy (CSLM)

A Leica SP5 upright confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) at the Manawatu Microscopy & Imaging Centre (Massey University, Palmerston North) was used for high-resolution imaging of microstructure of the samples (Figure 3.5). 1.0% (wt/vol) solution of Fast Green (dye content 90%) in water was used to stain protein and 0.1% (wt/vol) solution of Nile Red dye in acetone was used to stain fat (He-Neon laser with an excitation

line at 633 nm). A procedure similar to reported by Ye *et al.* (2016a) was followed. For examining liquid samples, a drop of sample was placed on a microscopic slide (plain, 90° corners, ground edges, 25 mm x 75 mm, 1.0-1.2 mm thick, Citotest Labware Manufacturing Co. Ltd, Haimen, China), 10 uL of the needed staining dye solution was gently mixed using a pipette tip with the sample on the slide, then covered with a cover slip (22 mm x 22 mm, 0.13-0.16 mm thick, Biolab Scientific, Melbourne, Victoria, Australia) and examined under a 63x-oil immersion objective (numerical aperture=1.4). Care was taken to avoid areas near the sides or close to the top of the microscope slide while imaging. Multiple fields were viewed, and typical micrographs are presented.

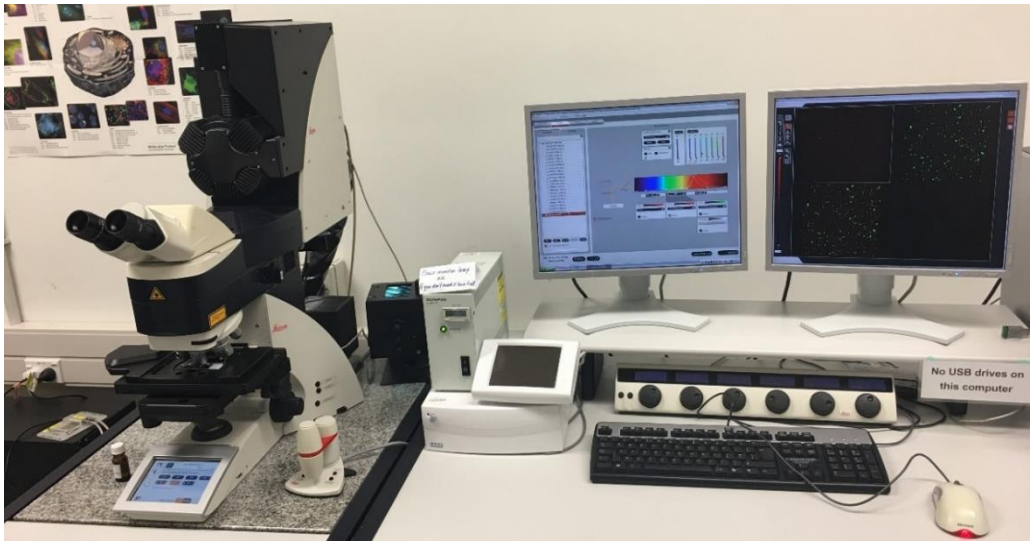


Figure 3.5 Leica SP5 upright confocal microscope.

3.2.2.7 Transmission electron microscopy

The structure of the casein micelles and (or) fat globules in the samples was investigated using a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI Company, Czech

Republic) equipped with an Olympus SIS VELETA camera (Germany) (Figure 3.6). Samples were submitted to Manawatu Microscopy & Imaging Centre (MMIC, Massey University, Palmerston North, New Zealand) for specimen preparation for imaging. Agarose tubes (5-7 mm length) were prepared from 3.0% low temperature agarose and the liquid milk samples (5-50 µL) were injected into the tube using an auto-pipette. The ends were sealed with agarose to form an enclosed capsule and samples were placed into a 3.0% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for at least 24 h at 4°C followed by three times washing in 0.1 M sodium cacodylate buffer, pH 7.2 (45 min for each wash). The agarose embedded samples were then fixed in 1.0% osmium tetra oxide and placed in 0.1 M sodium cacodylate buffer for 1 h at room temperature, overnight at 4°C and for another hour at room temperature. The samples were then washed in buffer again as mentioned above. The dehydration process was then carried out through a graded acetone series (25%, 50%, 75%, 95%, 100%, 100%, 100%; 30 min for each series). Samples were then put into 50:50 resin: acetone and placed on the stirrer overnight at 4°C. This was replaced by fresh 100% resin (Procure 812, ProSciTech, Australia) for 8 h on the stirrer at 4°C. This step was repeated four more times (overnight in 100% resin, 8 h in 100% resin, overnight in 100% resin, 8 h in 100% resin) at 4°C. Samples were then embedded in moulds with fresh resin and cured in a 60°C oven for 48 h, after which the block was then trimmed down to the selected area and cut using a diamond knife (Diatome Ltd., Switzerland) at 100 nm. These were stretched with chloroform and mounted on a grid using a Coat-Quick "G" pen (Daido Sangyo Co. Ltd., Japan). Grids were then stained in saturated uranyl acetate in 50% ethanol for 4 min, followed by washing with 50% ethanol and Milli-Q water and then stained in lead citrate for further 4 min. This was followed by a wash in Milli-Q water.

The stained specimens were then examined under a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI Company, Czech Republic) equipped with an Olympus SIS VELETA camera (Germany). Multiple fields were viewed, and typical micrographs are saved as TIFF image and presented.

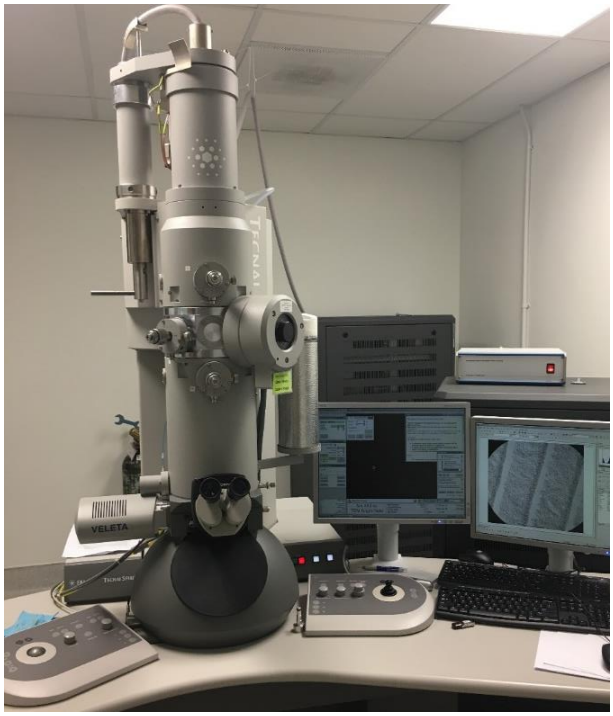


Figure 3.6 Tecnai G2 Spirit BioTWIN transmission electron microscope.

Chapter 4: Composition, structure, and gelation of skim milk of different species (cow, goat, sheep, red deer, and water buffalo) using glucono-δ-lactone (GDL) and pepsin[2]

4.1 Abstract

Dynamic low-amplitude oscillatory rheology was used to study the gelation properties of skim milk gels made at 37°C, using glucono-δ-lactone (GDL) alone (acid gels) or a combination of GDL and porcine pepsin ('combination gels'). The protein contents of the skim milk increased in the order: goat milk < cow milk < buffalo milk < sheep milk < deer milk, whereas the average casein micelle diameters increased in the order: cow milk < buffalo milk < goat milk < sheep milk ≃ deer milk. The gelation pHs (4.55–4.73) of all milks were close to the isoelectric pH (4.6) of casein, except for buffalo milk, which had a significantly higher gelation pH (5.72). The storage moduli (G') of the acid gels increased with time in the milk of all species except for buffalo milk, for which there was a double peak in G'. The final storage moduli after 6 h (G'_{final}) increased in the order: goat milk < cow milk < sheep milk < deer milk < buffalo milk. In general, for the combination gels, the G'_{final} values and the gelation pHs increased to variable extents, except for goat milk. Confocal scanning laser microscopy showed that goat milk and cow milk formed gels with more open protein

[2] Part of the contents presented in this chapter has been published as a peer-reviewed paper: Roy, D., Ye, A., Moughan, P. J., & Singh, H. (2020). Gelation of milks of different species (dairy cattle, goat, sheep, red deer, and water buffalo) using glucono-δ-lactone and pepsin. *Journal of Dairy Science, 103(7), 5844-5862.*

networks compared with the dense clustered protein networks of the high protein content milks (buffalo, sheep, and deer milk).

4.2 Introduction

The formation and the physical, rheological, and microstructural properties of acid-induced milk gels have been widely studied (Donato *et al.*, 2007; Lucey *et al.*, 1998a, 1998b); however, all of these studies were based on heated or unheated (raw) cow milk. Ould Eleya *et al.* (1998) studied the comparative rheological properties of goat, sheep, and cow skim milks in relation to their initial physicochemical compositions. They reported that sheep milk formed stronger gels and that goat milk formed weaker gels compared with cow milk. Wang *et al.* (2019) reported similar findings for the rheological and structural properties of acidified skim milks made from goat and cow milk. A few studies on the microstructural, textural, and rheological properties of set yogurts prepared from goat, sheep, and cow milk have reported similar findings (Domagała, 2009; Miocinovic *et al.*, 2016; Nguyen *et al.*, 2018). Abd El-Salam *et al.* (1996) used thrombolastography to study the acid-induced gelation of buffalo milk and reported that its gelation pH was higher than that of cow milk. There have been no reported studies on the rheological and structural properties of deer milk. The differences in the acid-induced gelation behaviours of different milks could be due to differences in the casein concentration, casein composition, casein micelle size, and casein-to-whey-protein ratio, all of which vary from species to species. This indicates that, although the principles of the acid-induced gelation of cow milk can be applied to non-cow milks, some adaptations may be necessary because of the compositional and physicochemical differences in milk from different species.

The digestion of milk proteins in the human starts with curd formation in the stomach by the combined action of hydrochloric acid and the enzyme pepsin. This is due to coagulation of the casein proteins in the presence of acid and the enzymes in the stomach (Ye *et al.*, 2016b). Gamble *et al.* (1939) used curd tension as a property to study the softness and hardness of goat and cow milk curds formed using hydrochloric acid and pepsin as coagulants and found that goat milk formed 31–54% softer curds compared with milk from different breeds of cow. They reported that the milk composition, the buffering capacity, the hydrogen-ion concentration, the existence of soluble and insoluble forms of calcium and phosphates, and the physical nature of the curd formed by the coagulation of milk in the stomach may be important factors in its digestibility. In this respect, it is important to understand the compositional as well as structural differences among various milks and their impact on the gelation properties of the curds formed from these milks. There is very little available information on the comparative acid-induced gelation properties of cow and non-cow milks. In addition, no rheological and structural studies on 'combination gels' (formed using both acid and pepsin) of cow and non-cow milks have been reported. It was hypothesized that as milk from different species have different composition and structures (potentially different casein micelle sizes and/or fat globule sizes), they are likely to produce gels with different properties. Thus, the aim of this study was to compare the physicochemical properties of cow, buffalo, goat, sheep, and deer milk under similar conditions and in the same study, focusing especially on the properties of the gels formed by using glucono-δ-lactone (GDL) alone as well as in combination with porcine pepsin.

4.3 Material and methods

4.3.1 Materials

The dairy cow, water buffalo, goat, sheep, and red deer milk were supplied by Massey University No.4 dairy farm (Palmerston North, New Zealand), Whangaripo Buffalo Cheese Co. (Auckland, New Zealand), Dairy Goat Co-operative (Hamilton, New Zealand), Spring Sheep Milk Co. (Hamilton, New Zealand), and Landcorp Farming Limited (Wellington, New Zealand), respectively. Bulk raw whole milk samples from different species were obtained under chilled conditions between January and May 2017 as described in Section 3.1.1 (Chapter 3). Other experimental materials needed were as per the protocol described in Section 3.1 (Chapter 3).

4.3.2 Methods

4.3.2.1 Milk processing and storage

Milk processing and storage was performed as per the method described in Section 3.2.1 (Chapter 3).

4.3.2.2 Skim milk composition

The chemical composition of skim milk was analysed as per the protocol described in Section 3.2.2.1 (Chapter 3).

4.3.2.3 Casein micelle diameter

The casein micelle size of the skim milks was determined as per the protocol described in Section 3.2.2.4 (Chapter 3).

4.3.2.4 Transmission electron microscopy of milk

Transmission electron microscopy of casein micelles and fat globules in milk was performed as per the procedure described in Section 3.2.2.7 (Chapter 3).

4.3.2.5 Identification of skim milk proteins

Identification of skim milk proteins was performed using SDS-PAGE as per the method mentioned in Section 3.2.2.5 (Chapter 3).

4.3.2.6 Skim milk gel formation using GDL alone and together with porcine pepsin

Skim milk gels from the different species were prepared using GDL only (referred to as 'acid gels') and in combination with porcine pepsin (referred to as 'combination gels') at 37°C. As curd formation in the human stomach is mainly initiated by pepsin and HCl, the gel rheological study was chosen to conduct at human body temperature (37°C) using GDL and pepsin. Porcine pepsin was used in the study as its amino acid composition is very similar to human pepsin (Mills & Tang, 1967), which has resulted into its wide use in *in vitro* gastric digestion studies. GDL was used to obtain a gradual decrease in pH. It should be noted that relatively low pepsin concentrations (or activities) were used in this study (compared to the pepsin concentrations or activities present in the human stomach) to allow gradual monitoring of gel formation.

Preliminary experiments were conducted to select the optimum amount of GDL that would be required to achieve ~pH 4 after 6 h at 37°C for the different milks. The pH change with time was monitored using a 'pH-stat', TitraLab TIM 856 Titration Manager Unit (Radiometer

Analytical, Lyon, France), attached to a computer to record the data. The amounts of GDL required (%, wt/vol) to achieve a pH of approximately 4.0 after 6 h at 37°C were 2.2, 4.2, 2.0, 3.1, and 4.15% for cow, buffalo, goat, sheep, and deer raw skim milk, respectively.

The protocols for acid gel (GDL only) and combination gel (GDL along with pepsin) formation were similar, the only difference being that, for preparing the combination gels, different amounts of freshly prepared pepsin solution were also added to the prewarmed milk samples along with the required concentration of GDL. For each analysis, different stock solutions of pepsin were freshly prepared with Milli-Q water and were stirred for 1 h at room temperature to dissolve the pepsin in water (Table 4.1). Then, 150 μL of the pepsin stock solution was added along with the respective amount of GDL required for the skim milk (100 mL) of a particular species. The pHs of the pepsin stock solutions ranged from 4 to 4.5; however, the addition of the pepsin stock solution either alone or together with GDL did not have any significant impact on the pH change with time.

The different amounts of pepsin powder used (calculated) per 100 mL of skim milk were ≃ 0.01, 0.02, 0.03, 0.04, and 0.05 mg, and were coded as P0.01, P0.02, P0.03, P0.04, and P0.05, respectively, as shown in Table 4.1. These amounts of pepsin and defined concentrations of GDL for the milk from different species are referred to as GDL + P0.01, GDL + P0.02, GDL + P0.03, GDL + P0.04, and GDL + P0.05.

Table 4.1 Pepsin concentration and pepsin activity of stock solution per 100 mL of skim milk.

Coding	Pepsin stock solution: Pepsin powder (mg)/100 mL Milli-Q water	Pepsin stock solution (µL)/ 100 mL milk	Pepsin powder concentration (mg)/100 mL milk	Pepsin activity (units/100 mL milk)
P0.01	7	150	0.0105 ≃ 0.01	≃ 5.03
P0.02	14	150	0.0195 ≃ 0.02	≃ 9.34
P0.03	20	150	0.0305 ≃ 0.03	≃ 14.61
P0.04	27	150	0.0405 ≃ 0.04	≃ 19.40
P0.05	34	150	0.0495 ≃ 0.05	≃ 23.71

4.3.2.7 Gelation properties of acid and combination skim milk gels

The gelation process and the changes in storage modulus (*G'*) of the acid and combination gels were monitored using dynamic low-amplitude oscillatory rheology in AR-G2 magnetic bearing rheometer (TA Instruments, Crawley, West Sussex, UK) (Figure 4.1) as described by Lucey *et al.* (1998a). The changes in pH during acidification were monitored by pH-stat (Figure 4.2), simultaneous to the rheological measurements in the rheometer.

The skim milk samples (100 mL) were equilibrated for 1 h in a water bath pre-set at 37°C. The rheometer and pH-stat sample containers were prewarmed to 37°C prior to the measurements. The required level of GDL or GDL together with pepsin was then added to the prewarmed milk sample and the mixture was stirred for 2 min on a magnetic stirrer (Code-F203A0160, Velp Scientifica, Europe) at maximum speed. Then, 20 mL of this acidified milk was transferred into the cup-and-bob geometry (cup diameter of 30.36 mm, bob diameter of 27.93 mm, and bob length of 42.09 mm) of the rheometer, and 50 mL of the same

stirred milk was quickly transferred into the sample beaker of the pH-stat. The test conditions, i.e., 0.1 Hz oscillation frequency, 0.01 applied strain, and 37°C temperature, were maintained during the entire 6 h experiment (generally referred to as a 'time sweep' experiment). A light mineral oil was added to the surface of the sample to prevent evaporation. The time difference between the addition of GDL and the measurement starting time in the rheometer and the pH-stat was recorded to determine the actual gelation time and gelation pH. Gelation was defined as the point at which the storage modulus [expresses the measure of energy stored per deformation cycle and indicates the solid-like properties (Ozcan, 2013)] of the gel was ≥1 Pa (Lucey *et al.*, 1998a). The loss tangent (tan δ) is the ratio of the loss modulus (***G"***) to the storage modulus (*G'*), i.e., tan δ = *G"*/*G'*. The total pepsin to protein ratio (wt/wt) in the combination gel studies (based on theoretical calculations) ranged from approximately 1: 62200 to 1: 979000 (depending on the amount of pepsin added and protein content of different milk samples).

Figure 4.1 Cup-and-bob geometry of AR-G2 magnetic bearing rheometer.

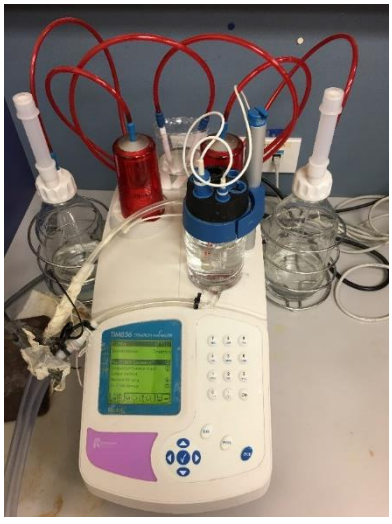


Figure 4.2 pH-stat (TitraLab TIM 856 Titration Manager Unit).

4.3.2.8 Confocal scanning laser microscopy of skim milk gels

A Leica SP5 upright confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) at the Manawatu Microscopy and Imaging Centre (Massey University, Palmerston North, New Zealand) was used for high-resolution imaging of the microstructures of the gels. A 1.0% (wt/vol) solution of Fast Green (dye content 90%) was used to stain the protein (helium–neon laser with an excitation line at 633 nm). A procedure similar to that reported by Lucey *et al*. (1998c) was followed. A prewarmed skim milk sample (50 mL at 37°C) was stirred for 2 min with a few drops of Fast Green solution to disperse the dye and was further mixed for 2 min after the addition of the required level of GDL and (or) pepsin. A few drops of this mixture were placed on a double-concave microscope slide (clear glass, ground edges, 26 mm x 76 mm, 1.2–1.3 mm thick; Sail, Sailing Medical-Lab Industries Co. Ltd, Suzhou, China), covered with a coverslip, placed in a petri dish, and held in a temperature-controlled incubator (INFORS HT Ecotron, Infors AG CH, Bottmingen, Switzerland) at 37°C for 6 h. The skim milk gels formed were immediately examined with a 63x oil immersion objective (numerical aperture = 1.4). Multiple fields were viewed, and typical micrographs are presented.

4.3.2.9 Statistical analysis

Each experiment was carried out in duplicate for at least 2 different batches of milk. The results from all experiments were analysed statistically using SAS (Statistical Analysis System) for Microsoft Windows (SAS 9.4, SAS Institute Inc. 2013, Cary, NC) and are reported as mean ± SD (standard deviation). One-way ANOVA followed by Tukey's test

was used to determine the significant differences between samples at $P < 0.05$, using the procedure of the general linear model of SAS/STAT.

4.4 Results and discussion

4.4.1 Chemical compositions of different milks

The chemical compositions of the cow, buffalo, goat, sheep, and deer skim milks are shown in Table 4.2. The dry matter (total solid) and protein contents of the non-cow milks were significantly different ($P < 0.05$) from those of cow milk, with red deer milk having the highest total solids and protein content. Sheep milk and buffalo milk had the next highest total solids contents after deer milk, followed by cow milk and goat milk. Similarly, sheep milk had the next highest protein content followed by buffalo, cow, and goat milk. Among all the milks, goat milk had the lowest total solids and protein contents.

Deer milk had considerably higher ash content ($P < 0.05$) than the cow, goat, and buffalo milk, all of which had similar ash contents. The ash content of sheep milk was comparable with that of both deer milk and the other milks. The total calcium and inorganic phosphorus contents of the deer, sheep, and buffalo milk were significantly higher than those of cow milk, with deer milk having the highest concentrations. Goat milk had a slightly lower ($P < 0.05$) amount of calcium and a similar inorganic phosphorus content compared with cow milk.

These results are consistent with those reported by Barlowska *et al.* (2011) and Claeys *et al.* (2014). Overall, all the non-cow milks, except goat milk, were higher in total solids, protein,

calcium, and inorganic phosphorus than cow milk. In contrast, Park (2006) and Ceballos *et al.* (2009) reported that goat milk had higher protein and mineral contents than cow milk. Such differences in the composition of goat milk or other non-cow milk and cow milk can be explained by differences in breed, diet, lactation stage, health of the animal, and region (Claeys *et al.*, 2014; Haenlein, 1996). Higher protein and mineral contents in milk are important factors that affect the firmness of acid gels (Anema, 2008a; Gastaldi *et al.*, 1997; Kamal *et al.*, 2017).

Table 4.2 Physicochemical properties of cow, water buffalo, goat, sheep, and red deer skim milks.

Physicochemical properties	Cow	Water buffalo	Goat	Sheep	Red deer
Dry matter (%)	10.16 ± 0.01^{c}	12.09 ± 0.14^{b}	8.32 ± 0.05^{d}	13.06 ± 0.00^{b}	15.63 ± 0.54^{a}
Protein (%)	4.11 ± 0.04^{d}	5.28 ± 0.04^{c}	3.11 ± 0.06^{e}	6.87 ± 0.06^{b}	9.79 ± 0.33^{a}
Fat (%)	0.14 ± 0.01^{c}	0.31 ± 0.01^{a}	0.19 ± 0.01^{bc}	0.18 ± 0.03^{c}	0.27 ± 0.04^{ab}
Carbohydrate (by difference, %)	5.12 ± 0.01^{b}	5.53 ± 0.04^{a}	4.13 ± 0.03^{e}	4.98 ± 0.04^{c}	4.28 ± 0.03^{d}
Ash (%)	0.81 ± 0.04^{b}	0.99 ± 0.06^{b}	0.88 ± 0.01^{b}	1.03 ± 0.05^{ab}	1.29 ± 0.14^{a}
Calcium (mg/100 mL)	122.04 ± 2.55^{c}	214.4 ± 26.1^{b}	101.59 ± 3.13^{d}	199.00 ± 26.90^{b}	330.80 ± 23.00^{a}
Inorganic phosphorus (mg/100 mL)	86.10 ± 0.88^{c}	143.86 ± 7.49^{b}	87.18 ± 5.26^{c}	148.10 ± 22.60^{b}	208.04 ± 9.31^{a}
Casein micelle size, Z-average diameter (nm)	164.93 ± 3.16^{d}	175.67 ± 1.27^{c}	182.42 ± 2.45^{b}	188.10 ± 0.98^{a}	190.30 ± 1.46^{a}

[a–e]Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SD.

4.4.2 Casein micelles in different milks

The Z-average diameters (nm) of the casein micelles varied significantly among the milk from the various species (Table 4.2). Those of cow milk were the smallest (~165 nm) whereas sheep milk (~188 nm) and deer milk (~190 nm) which were similar had the largest casein micelle diameters among the milks, followed by goat milk (~182 nm) and buffalo milk (~176 nm). It has been reported by others that goat, sheep (Park *et al.*, 2007), and buffalo (Abd El-Salam & El-Shibiny, 2011) milks have larger average casein micelle diameters than cow milk. There are no available reports on the casein micelle size of deer milk. It is evident that all the non-cow milks had significantly ($P < 0.05$) larger average casein micelle diameters than cow milk. Nguyen *et al.* (2018) also used dynamic light scattering and found that the average casein micelle sizes for cow, goat, and sheep milk were 171, 180, and 182 nm, respectively, similar to the results obtained in this study.

The primary results obtained for the casein micelle size distribution using dynamic light scattering are typically reported as an intensity distribution (Figure 4.3). As reported in Table 4.2, the Z-average diameter (nm) of casein micelles of cow, sheep, deer, goat, and buffalo milk was ~165 nm, 188 nm, 190 nm, 182 nm, and 176 nm, respectively. However, the casein micelle size distribution in cow, sheep, deer, goat, and buffalo milk was wide and ranged from approximately 91-396 nm, 91-459 nm, 91-531 nm, 68-615 nm, and 91-459 nm, respectively (Figure 4.3). The results indicated that milk from all the species contained different sizes of casein micelles. Relatively larger % of bigger casein micelle sizes were found to be present in sheep, deer, and goat milk.

Genotypic variants within a species can be a factor in the variation in casein micelle size (Pierre *et al.*, 1995; Pierre *et al.*, 1998). For example, Pierre *et al.* (1995) studied the casein micelle size in goat milk from animals with different α_{s1}-casein genotypes and suggested that these genotypes may have a role in the casein micelle size regulation. They found that the mean diameter of casein micelles in milk without α_{s1}-casein was 280 nm; milk with lower and higher amounts of α_{s1}-casein had diameters of 237 and 199 nm, respectively.

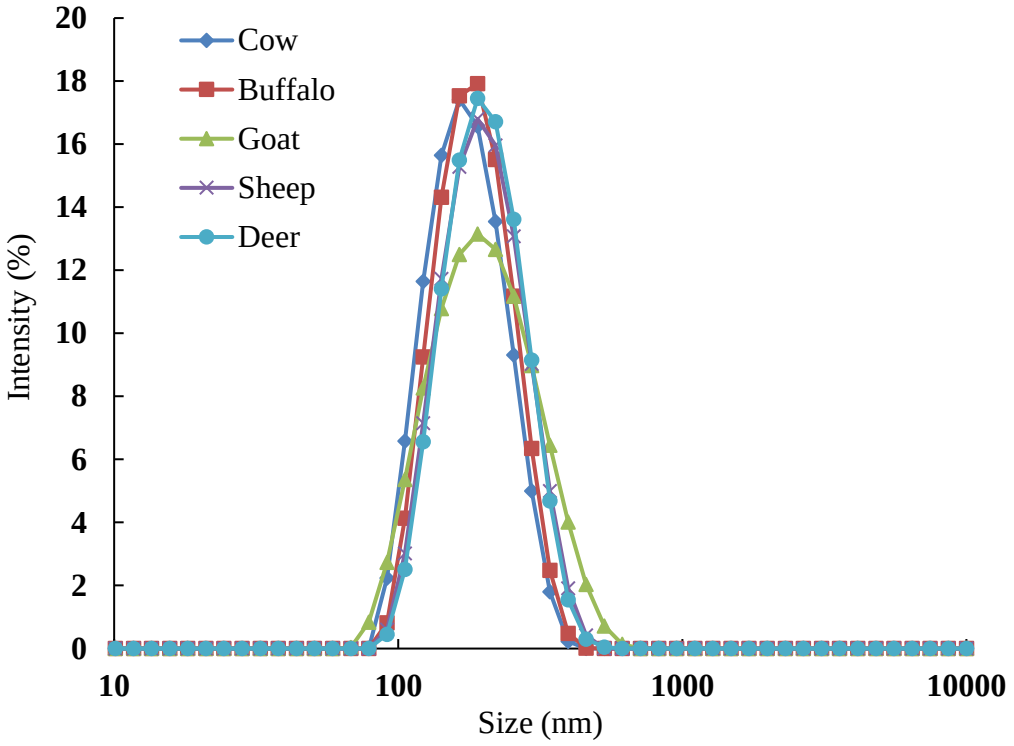


Figure 4.3 Casein micelle size distribution of different skim milks. Mean values are depicted.

Transmission electron micrographs of the casein micelles (Figure 4.4) from the milk of different species also showed qualitatively that larger casein micelles were present in the

non-cow milks than in cow milk. The dark and light electron-dense areas in the micrographs correspond to micelles with high density and low density, respectively (Pierre *et al.*, 1995). In the milk from all species, both larger and smaller casein micelles were present, with some having smooth outlines and some having rough (fibrous) outlines. These different aspects of the micelles may correspond to different native micelle structures in the milk or may have been induced during the sample preparation for transmission electron microscopy (Pierre *et al.*, 1995).

In cow milk, the casein micelles were relatively small, were not perfectly spherical, and had a rough periphery (Figure 4.4A). Liu *et al.* (2017) have reported similar results for cow skim milk at its natural pH. The casein micelles of the buffalo milk (Figure 4.4B) were relatively smaller than those of the deer, sheep, and goat milk (Figures 4.4E, 4.4D, and 4.4C, respectively), but appeared to be larger with sharper external outlines than the cow milk casein micelles. A greater proportion of large casein micelles were observed in the deer, sheep, and goat milk than in the cow milk; in addition, they were more spherical and had a comparatively smoother outer surface.

Data relating to the whole milk from the different species such as fat content, fat globule size distribution, and their microstructures were also investigated and are provided in the Annexure 1 as the main focus of this chapter was on skim milk.

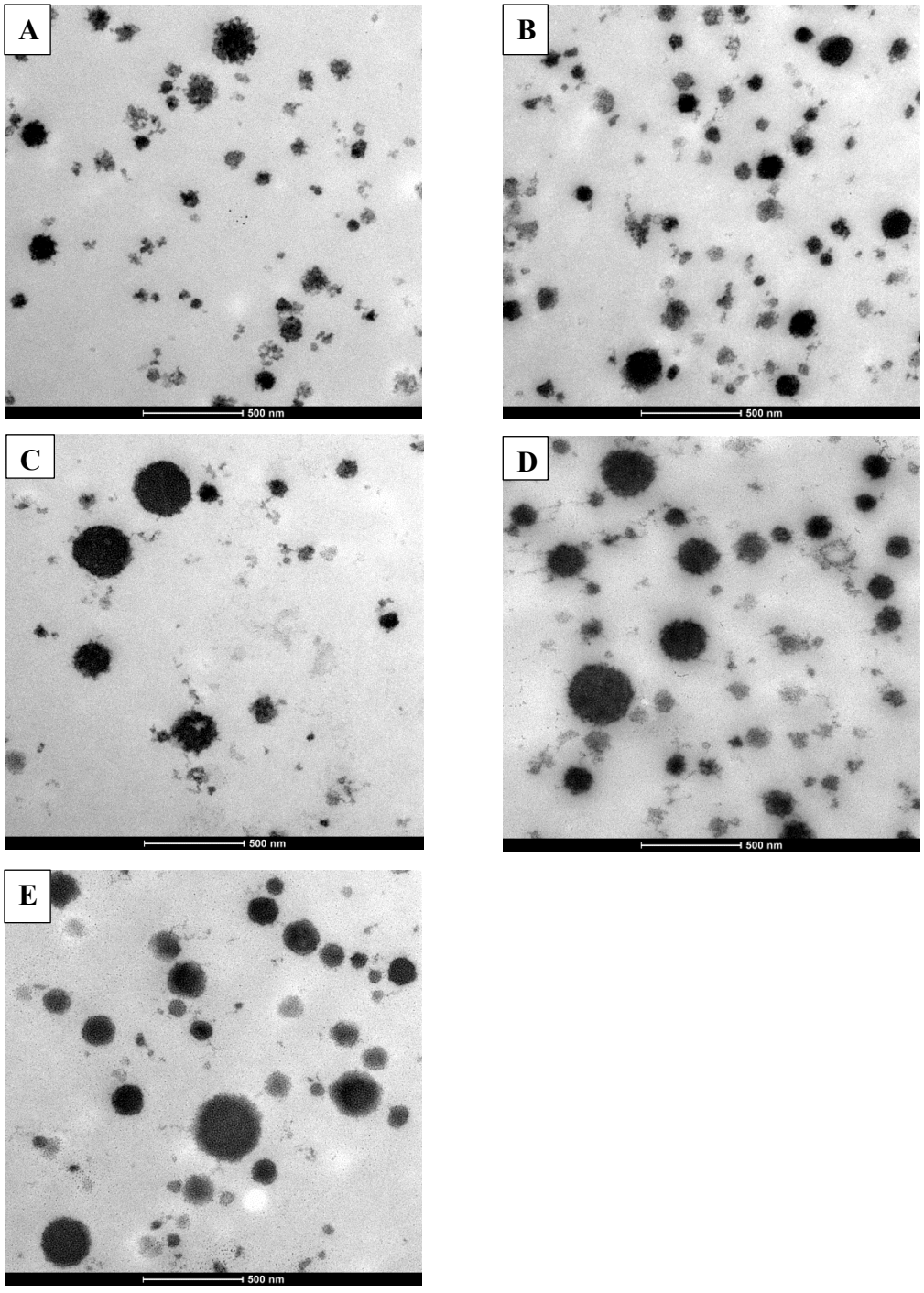


Figure 4.4 Transmission electron micrographs showing casein micelles from skim milk of different species at 43,000x magnification: A, cow; B, water buffalo; C, goat; D, sheep; E, red deer. Scale bar represents 500 nm.

4.4.3 Protein composition by SDS-PAGE

SDS-PAGE showed that the α_{s1}-casein and α_{s2}-casein bands of the goat, sheep, and buffalo milk had slightly lower electrophoretic mobility than those of cow milk (Figure 4.5). The goat, sheep, and buffalo milk had lower proportions of α_{s1}-casein than cow milk, as indicated by the band intensities (Figure 4.5, B, C, and E, respectively) and approximate quantification (Table 4.3). Among all the milks, goat milk appeared to have the lowest amount of α_{s1}-casein and a relatively higher amount of α-lactalbumin (α-La).

As the casein components of deer milk had significantly different and higher electrophoretic mobilities than those of cow milk and the milk from the other species, it was difficult to identify the casein components (Figure 4.5, D). Deer milk seemed to have lower proportions of α_{s2}-casein than cow, goat, and sheep milk. For deer milk, it was difficult to segregate α_{s1}-casein and κ-casein from β-casein on the SDS-PAGE gel. The κ-casein, α-La, and β-lactoglobulin (β-Lg) bands of deer milk had lower mobility on the SDS-PAGE gel than those of the milk from the other species. Among all of the milks, deer milk had extremely low proportions of α-La compared with its β-Lg content. Ha *et al.* (2014) also reported that red deer milk had the lowest α-La content, in comparison with milk from other species (cow, goat, and sheep).

As the protein profile of deer milk, using SDS-PAGE, was different from those of the other milks, this needs to be further investigated. Using starch gel electrophoresis, Ha *et al.* (2014) also found that the migration of the proteins in red deer milk was different from the migrations of cow, goat, and sheep milk proteins. They were not able to identify the

concentration of several proteins, including β-casein, because of a difference in the elution profile on a C18 reversed phase HPLC column; this was probably due to the different hydrophobic interaction characteristics of the deer milk proteins compared with cow, goat, and sheep milk proteins (Ha *et al.*, 2014).

Claeys *et al.* (2014) reported that goat, sheep, and buffalo milk have higher β-casein contents than cow milk, which was also evident from the SDS-PAGE gels in this study (Figure 4.5). They stated that goat, sheep, and, to some extent, buffalo milk have lower total-casein-to-total-whey-protein ratios, whereas rein(deer) milk has a higher total-casein-to-total-whey-protein ratio, than cow milk. The different proportions of the milk protein components have an effect on the coagulation properties of the milk. In general, higher amounts of casein, specifically, α_{s1}-, β-, and κ-casein, compared with whey proteins, have been shown to be positively correlated with the acid or rennet coagulation properties of milk (Ketto *et al.*, 2017; Wedholm *et al.*, 2006).

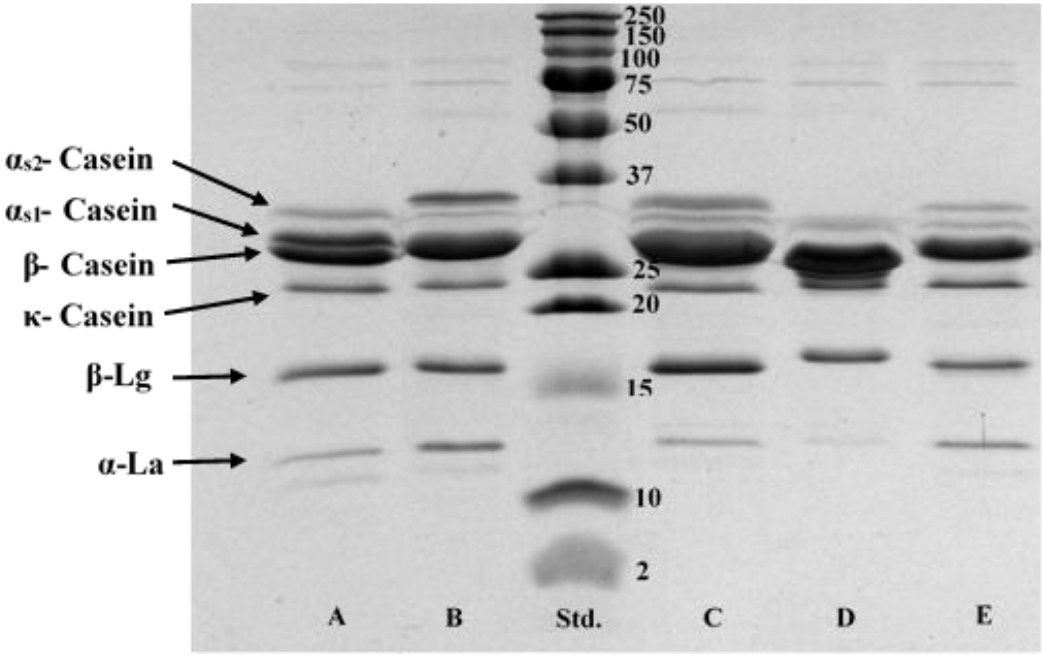


Figure 4.5 Protein fractions of skim milk from various species analyzed using SDS-PAGE: A, cow; B, goat; C, sheep; D, red deer; E, water buffalo. Std, protein molecular weight standard; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

Table 4.3 Protein fractions of skim milk from various species analysed from quantitative SDS-PAGE.

Protein Composition	Skim milk from different Species				
	Cow	**Buffalo**	**Goat**	**Sheep**	**Deer**
Total Protein %*	4.11 ± 0.04^{d}	5.28 ± 0.04^{c}	3.11 ± 0.06^{e}	6.87 ± 0.06^{b}	9.79 ± 0.33^{a}
Total Caseins (%) **: Total Whey Proteins (%) **	79.05: 20.95	77.65: 22.35	72.25: 27.75	76.55: 23.45	85.3: 14.7
Total Caseins/ Total Whey ratio	3.77 ± 0.016^{b}	3.47 ± 0.014^{c}	2.60 ± 0.064^{e}	3.26 ± 0.039^{d}	5.82 ± 0.448^{a}
α_{s2}-casein %*	0.290 ± 0.009^{b}	0.322 ± 0.015^{b}	0.384 ± 0.024^{b}	0.601 ± 0.083^{a}	_
α_{s1}-casein %*	1.02 ± 0.011^{a}	0.206 ± 0.022^{b}	0.051 ± 0.011^{b}	0.244 ± 0.121^{b}	_
β-casein %*	1.50 ± 0.017^{c}	2.80 ± 0.007^{b}	1.51 ± 0.000^{c}	3.96 ± 0.117^{a}	_
κ-casein %*	0.440 ± 0.012^{b}	0.773 ± 0.003^{a}	0.300 ± 0.019^{c}	0.457 ± 0.092^{b}	_
(α_s + κ-casein)/β-casein ratio	1.166 ± 0.023^{a}	0.465 ± 0.005^{b}	0.487 ± 0.010^{b}	0.330 ± 0.043^{c}	_
(α_s + β-casein)/κ-casein ratio	6.39 ± 0.20^{ab}	4.30 ± 0.02^{b}	6.5 ± 0.55^{ab}	10.75 ± 2.34^{a}	_
β-Lactoglobulin (β-Lg) %*	0.618 ± 0.003^{d}	0.718 ± 0.015^{c}	0.527 ± 0.011^{e}	1.346 ± 0.019^{a}	1.237 ± 0.069^{b}
α-Lactalbumin (α-La) %*	0.140 ± 0.006^{c}	0.330 ± 0.004^{a}	0.257 ± 0.007^{b}	0.134 ± 0.005^{c}	0.065 ± 0.006^{d}
β-Lg/α-La Ratio	4.43 ± 0.20^{c}	2.18 ± 0.07^{d}	2.06 ± 0.10^{d}	10.06 ± 0.22^{b}	19.0 ± 1.08^{a}
Total Caseins %*	3.25 ± 0.003^{d}	4.09 ± 0.004^{c}	2.25 ± 0.015^{e}	5.26 ± 0.015^{b}	8.35 ± 0.093^{a}
Total Whey Proteins %*	0.861 ± 0.003^{d}	1.18 ± 0.004^{c}	0.863 ± 0.015^{d}	1.61 ± 0.015^{a}	1.44 ± 0.094^{b}

*Units – g per 100g skim milk; ** Units – g per 100g protein

a-e Values within each row with different superscripts are significantly different ($P < 0.05$); minimum of duplicate measurements was performed, and values are reported as mean ± SD.

4.4.4 Acid skim milk gel formation

The changes in the rheological properties of the acidified milks as a function of time are shown in Figure 4.6 and Table 4.4. To obtain similar pH–time profiles to reach a pH of ~4.0 after 6 h at 37°C (Figure 4.6A), the amount of GDL added varied for the different milks (probably because of the difference in their buffering capacities).

In this study, the gelation pH of all milks (pH 4.55–4.73) was close to the isoelectric point of casein (pH 4.6) except that for buffalo milk, which had a significantly higher gelation pH of 5.72 (Table 4.4). Abd El-Salam *et al.* (1996) also reported that buffalo milk, heated at 70°C for 15 s, started to gel at a much higher pH (5.5–5.9) than heated cow milk, which generally had a gelation pH of ~5.1–5.2. Buffalo milk had the shortest gelation time, because of its higher gelation pH (Table 4.4). There was no significant difference ($P < 0.05$) in the gelation pH of deer milk (4.66) and cow milk (4.73), whereas the gelation pH of goat milk (4.59) and sheep milk (4.55) were slightly lower than that of cow milk. This also resulted in a longer gelation time for sheep milk (88 min) than for cow milk (74 min), even though the total solids and protein contents of sheep milk were higher than those of cow milk. Such differences in the gelation properties may reflect differences in the relative proportions of caseins (α_s-, β-, and κ-casein) present in these milks. The gelation time for deer milk was also lower than that for cow milk, possibly because of its higher total protein, total solids, and total ash (mineral) contents.

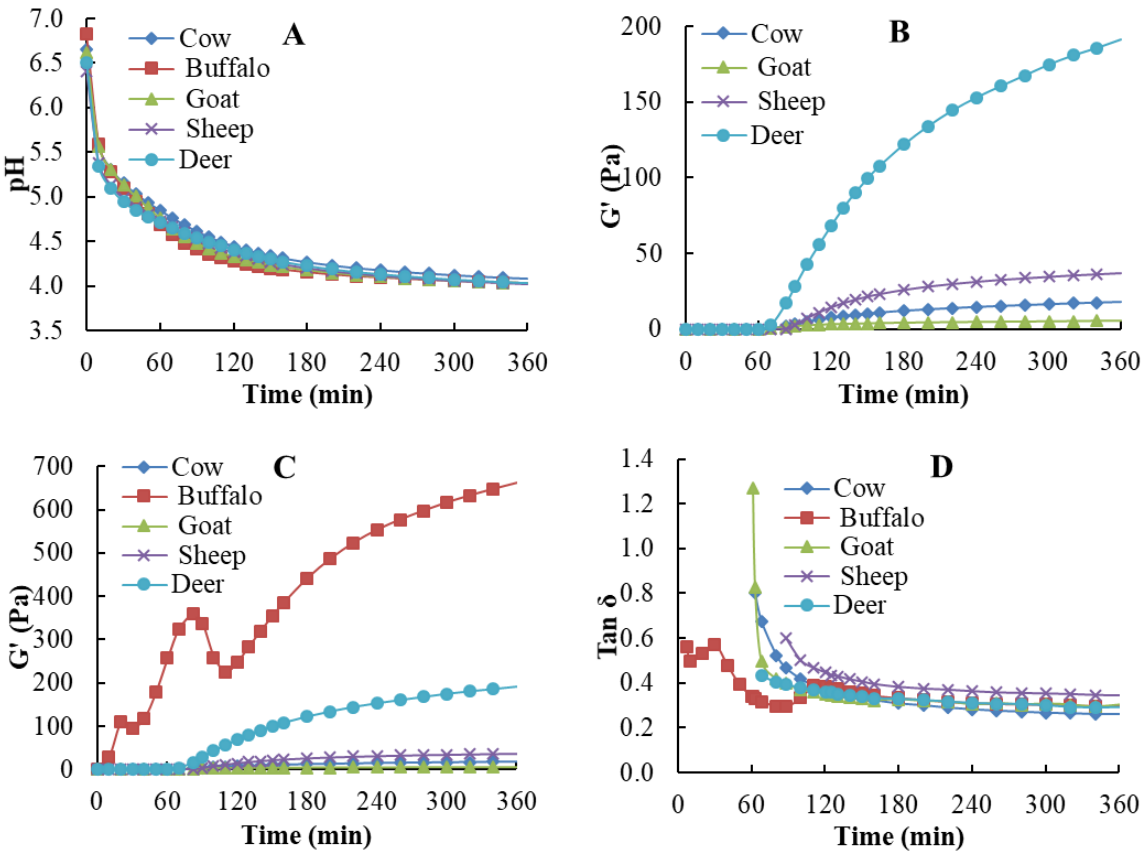


Figure 4.6 Gelation properties of acid skim milks as a function of time at 37°C: A, changes in milk pH of different milks; B, changes in G' values of cow, goat, sheep, and red deer milks; C, changes in G' values of different milks, including water buffalo milk; D, changes in tan δ values of different milks. Values depicted are mean ± SD.

Table 4.4 Effect of glucono-δ-lactone (GDL) on the properties of milk gels formed at 37°C from different skim milks.

Gelation properties	Cow	Water Buffalo	Goat	Sheep	Red deer
Initial milk pH[1]	6.66 ± 0.01[b]	6.83 ± 0.04[a]	6.62 ± 0.00[b]	6.41 ± 0.01[d]	6.50 ± 0.00[c]
Gelation[2] pH	4.73 ± 0.06[b]	5.72 ± 0.00[a]	4.59 ± 0.01[cd]	4.55 ± 0.02[d]	4.66 ± 0.00[bc]
Gelation[2] time (min)	74.40 ± 0.00[b]	7.20 ± 0.00[d]	75.40 ± 0.69[b]	88.35 ± 2.88[a]	68.28 ± 0.00[c]
Gelation[2] loss tangent, tan δ	0.58 ± 0.00[a]	0.56 ± 0.01[a]	0.44 ± 0.03[b]	0.60 ± 0.04[a]	0.44 ± 0.01[b]
Final storage modulus, G'_{final} (Pa)[3]	18.06 ± 1.64[d]	661.70 ± 45.26[a]	5.51 ± 0.35[e]	37.27 ± 0.90[c]	191.40 ± 1.41[b]
Final loss tangent, tan δ_{final} [3]	0.26 ± 0.01[c]	0.30 ± 0.00[b]	0.30 ± 0.01[b]	0.34 ± 0.00[a]	0.29 ± 0.00[b]
Maximum in tan δ ($\tan \delta_{max}$, if any)[4]	None	Two peaks for $\tan \delta_{max}$: A, $\tan \delta_{max}$ = 0.57 ± 0.01 at pH 5.12 ± 0.02 at 28.3 ± 1.4 min; B, $\tan \delta_{max}$ = 0.39 ± 0.00 at pH 4.29 ± 0.03 at 115.29 ±7 min	None	None	None
Final pH[3]	4.09 ± 0.08[a]	4.03 ± 0.03[a]	4.03 ± 0.01[a]	4.02 ± 0.01[a]	4.04 ± 0.00[a]

[a–e] Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SD.
[1] Natural pH of the different skim milks.
[2] Point at which gels had a $G' \geq 1$ Pa.
[3] Measured 6 h after addition of GDL.
[4] Point after gelation at which the value of tan δ reached a maximum and thereafter decreased.

Lucey *et al.* (1998a) stated that the primary reason for the formation of an acid gel in raw milk is the reduction in the net negative charge on the caseins as the isoelectric pH is approached. The native whey proteins do not play any part in the acid gel structure of a raw milk system and remain soluble (Anema, 2008b; Anema *et al.*, 2004). A combination of hydrophobic interactions and colloidal calcium phosphate (CCP) is primarily responsible for maintaining the structural integrity of the casein micelles (Anema, 2009). As the pH is reduced, the CCP is progressively solubilized so that essentially all of the inorganic phosphate (along with calcium associated with CCP) is soluble at below about pH 5.0–5.2, whereas all of the calcium (including casein bound calcium) is soluble at below about pH 4.6–4.9 (Anema, 2008b, 2009; Dalgleish & Law, 1989).

Gastaldi *et al.* (1997) and Anema (2008a) reported that as casein is responsible for structure formation in a raw milk gel, an increase in gel firmness would be expected because of the increase in the number of casein particles (casein concentration) and interactions between them within a particular unit area.

The final storage modulus at the end of 6 h (G'_{final}) of the acid-induced milk gels from the various species followed the trend in protein and total solids concentrations (i.e., goat milk gel < cow milk gel < sheep milk gel < deer milk gel), except for the buffalo milk gel. Although buffalo milk had lower protein content than sheep milk and deer milk and lower mineral content than deer milk, it formed gels with higher G' values compared with sheep milk and deer milk. The exceptional behaviour of buffalo milk towards acid gelation is difficult to explain and needs to be further investigated.

Any specific relationship between the casein micelle size and the final storage modulus was not observed. Ekstrand *et al.* (1980) and Glantz *et al.* (2010) reported that casein micelle size is an important factor that influences the rennet coagulation properties of milk and that milk with smaller casein micelle size has a shorter coagulation time and forms stronger gels. A similar relationship was reported by Horne (2003) for the gel strength and casein micelle size of rennet gels; however, for acid gels, he reported that the maximum complex modulus (G*) values were independent of the micellar size. Along with casein micelle size, milk protein composition, mineral content, and pH also play a crucial role in determining the coagulation properties of milk.

Similar to the observations in this chapter on the gelation properties of goat, sheep, and cow milk, previous studies (Domagała, 2009; Ould Eleya *et al.*, 1998; Wang *et al.*, 2019) also found that goat milk formed less firm acid-induced gels, and that sheep milk formed gels that were firmer than those of cow milk when acidified with starter cultures or GDL. They also reported that such differences could be attributed to the various total solids, total protein, and total casein contents, the proportions of the casein fractions, and the size of the casein micelles. Bell and Vlahopouloua (1995) reported that goat milk forms weaker gel structures with higher tan δ values than does cow milk, even at higher protein concentration, which could be related to the fact that not only the total protein content but also the protein composition influence acid gel formation.

Lucey and Singh (1997) reported that raw cow milk forms weak gels and that the pH at gelation is generally ~4.8. They also reported that raw cow milk gels generally have G' <20

Pa (at 30°C) and that, after gelation, tan δ decreases to ~0.25 during the ageing of the gel (Lucey *et al.*, 1998a). Similar results for the gelation pH, *G*', and tan δ values were obtained in this study of the raw cow milk gel at 37°C (Table 4.4).

G' gradually increased with time for all skim milks, except buffalo skim milk (Figure 4.6C). For buffalo skim milk, *G*' increased rapidly just after gel formation, but soon reached a maximum (at pH 5.28) and then decreased, but subsequently increased rapidly again to a maximum (at pH 4.46) and then decreased, after which it kept on increasing until 6 h (Figure 4.6C). Horne (2003) studied the effect of the rate and the temperature of acidification of raw cow skim milk on acid gelation and observed double peaks in the *G*' versus time profile and minimum–maximum phenomena at high GDL concentrations (4–6%) and high temperatures (45°C), resulting in rapid acidification rates. He reported that, if the temperature is sufficiently high, aggregation begins at a higher pH (5.0–5.2) and the loss of CCP may not be complete at this pH. The ongoing loss of CCP weakens the internal structure of the casein micelles and thus the overall network structure, producing the maximum–minimum feature in the gelation profiles at around pH 5.0–5.2. The continued decrease in casein micelles charge with the decrease in pH then allows the internal bonds to reform; thus, the casein interactions are stronger, which causes the stiffness to increase again to a maximum (at pH 4.3) near the isoelectric point of casein (pH 4.6) (Horne, 2003). Thus, as aggregation in the raw cow, goat, sheep, and deer milk did not occur at a pH higher than 5.0–5.2 in this study, the minimum–maximum phenomena in *G*' were not observed; in contrast, it was observed in buffalo milk as its gelation started at a higher pH, i.e., pH 5.72 (Table 4.4). Further, Anema (2009) reported that both the $G'_{shoulder}$ and maximum in tan δ (tan δ_{max}) are a consequence of

the residual CCP in the acidified milk. Gastaldi *et al.* (1997) also reported that the gelation properties in a GDL-acidified milk can be attributed to the differences in casein micelle composition and the CCP content of the casein particles.

A decrease in *G*' may also be an indication of spontaneous breakdown of the gel network, i.e., syneresis (Mellema *et al.*, 2002), but no visible syneresis was observed in the buffalo milk. The minimum–maximum pattern in *G*', coupled with the high tan δ_{max} values, in the buffalo milk gels may represent rapid rearrangements in the gel network due to breakage or relaxation of bonds that might have tendency towards syneresis or microsyneresis. The rheometer cup was examined carefully at the end of all gelation experiments to observe any signs of syneresis such as shrinkage or whey separation. No such syneresis was observed, not even in the case of the buffalo milk gel.

Lucey *et al.* (2000) reported that trends in tan δ are very useful for understanding changes in gel networks. They also reported that tan δ_{max} could occur in any milk that forms gels at pH ≥5.3 followed by a continuous decrease in pH due to acid production. A similar phenomenon was observed in this study for buffalo milk. There were 2 maxima in tan δ for buffalo milk, whereas such maxima in tan δ were not observed for the raw milk from the other species (Figure 4.6D). In the skim milk from all other species, tan δ decreased at gelation and continued to decrease throughout the gelation period (Figure 4.6D). In the buffalo skim milk gel, tan δ decreased to a value of <0.5 immediately after gelation (pH 5.72), but then increased to a maximum (tan δ_{max}) of 0.57 at pH 5.12, decreased again to a minimum of 0.3

and then increased again to a maximum of 0.39 at pH 4.29, after which it kept on decreasing (Figure 4.6D).

Lucey *et al.* (2000) reported that a high value for the tan δ_{max} (say ≥0.4) within a short time might indicate that the milk gels are more prone to microstructural rearrangements and syneresis. For the buffalo milk, the tan δ_{max} values were close to or greater than 0.4, which may indicate the susceptibility of the buffalo milk gel towards rearrangements and syneresis. The minimum–maximum phenomena in *G*' and tan δ have also been observed in different gel systems, such as milk protein concentrate–pectin gels (Matia-Merino & Singh, 2007), milk protein concentrate–gelatin gels (Pang *et al.*, 2015), and milk protein concentrate–lactose gels (Meletharayil *et al.*, 2016).

4.4.5 Microstructure of acid skim milk gels

The microstructures of the acid skim milk gels from cow, buffalo, goat, sheep, and deer milk, observed by confocal microscopy (Figure 4.7), showed that the protein clusters in the goat milk gel were less compact, sparser, and more open than those in the other milk gels. The interconnectivity between the protein strands in the goat milk gel was less pronounced, with larger irregular void spaces (Figure 4.7C). The cow milk gel structure (Figure 4.7A) was similar to the goat milk gel structure, but had more dense clusters of aggregated protein particles, probably because of the higher protein content of cow milk. Overall, the microstructures of the cow and goat milk gels represented weaker structures with fewer cross-links, which is consistent with the low final *G*' values obtained for these milks. In contrast, the sheep milk gel had more dense protein clusters and less porosity than the cow and goat

milk gels, probably because of its higher protein concentration (Figure 4.7D). The microstructure of the sheep milk gel had more contact points between aggregated particles, indicating its higher gel strength compared with the goat and cow milk gels. These microstructural observations are in agreement with the findings of Wang *et al.* (2019) on acidified skim milk gels from cow milk and goat milk. Domagała (2009) and Nguyen *et al.* (2018) have also reported similar observations for unstirred goat, sheep, and cow yogurts.

The protein network in the confocal micrograph of the deer milk gel was the most dense and compact, with the least porosity (Figure 4.7E), compared with all the other milk gels studied in this work, which could have been due to its highest protein concentration. The micrograph of the buffalo milk gel appeared to be similar to that of the cow milk gel but had thin strands of interconnected protein network (Figure 4.7B). There are no reports in the literature on the microstructure of acid-induced deer and buffalo skim milk gels.

The microstructures of the acid skim milk gels formed from the different milks appeared to follow their final *G*' values – the higher was the final *G*' value (Figure 4.6C), the more interconnected, with less porosity, was the protein network (Figure 4.7). However, the buffalo milk gel network was an exception; despite having a higher *G*' value than all the other milk gels, its microstructure did not appear to be highly interconnected.

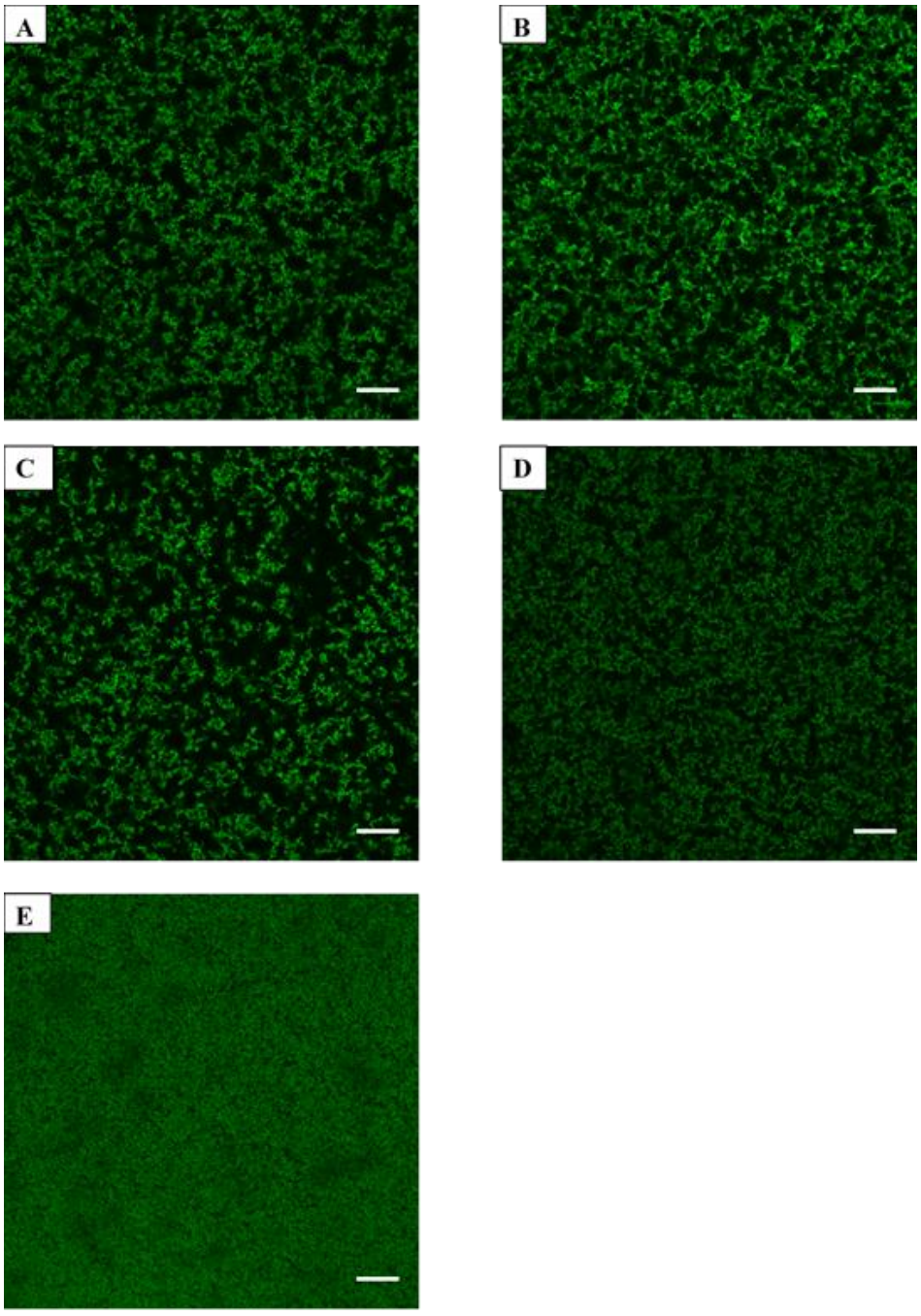


Figure 4.7 Confocal microscopy images of acid gels made from different skim milks using glucono-δ-lactone: A, cow milk; B, water buffalo milk; C, goat milk; D, sheep milk; E, red deer milk. Scale bar represents 25 μm.

4.4.6 Gel formation by a combination of GDL and pepsin

For combination gel formation, GDL was used along with increasing levels of pepsin, i.e., P0.01, P0.02, P0.03, P0.04, and P0.05 (Table 4.1). The addition of different levels of pepsin alone did not result in gel formation for any of the milk species at their natural pH values. Also, the addition of various levels of pepsin along with GDL did not have a significant effect on the pH change with time in the milk from all species (Figure 4.8). The data obtained from the rheological analysis of the combination gels are shown in Figure 4.9 and Table 4.5.

The specific activity of pepsin is similar to that of chymosin; however, pepsin exhibits unspecific proteolytic activity towards bonds other than the Phe105–Met106 bond of κ-casein and thus has more proteolytic activity relative to its milk clotting activity than chymosin (Guinee & Wilkinson, 1992; Júnior *et al.*, 2015; Moschopoulou, 2011). Chymosin is most stable in the pH range 5.3–6.3, but loses its activity rapidly under acidic conditions, i.e., below pH 3–4, as well as at high alkaline pH values, i.e., above pH 9.8 (Crabbe, 2004). Pepsin has maximum proteolytic activity at pH 2, with an optimum pH range of 2–5, and has activity in the pH range pH 5.5–7.5. Pepsin is irreversibly inactivated at pHs above 7.5 (Piper & Fenton, 1965).

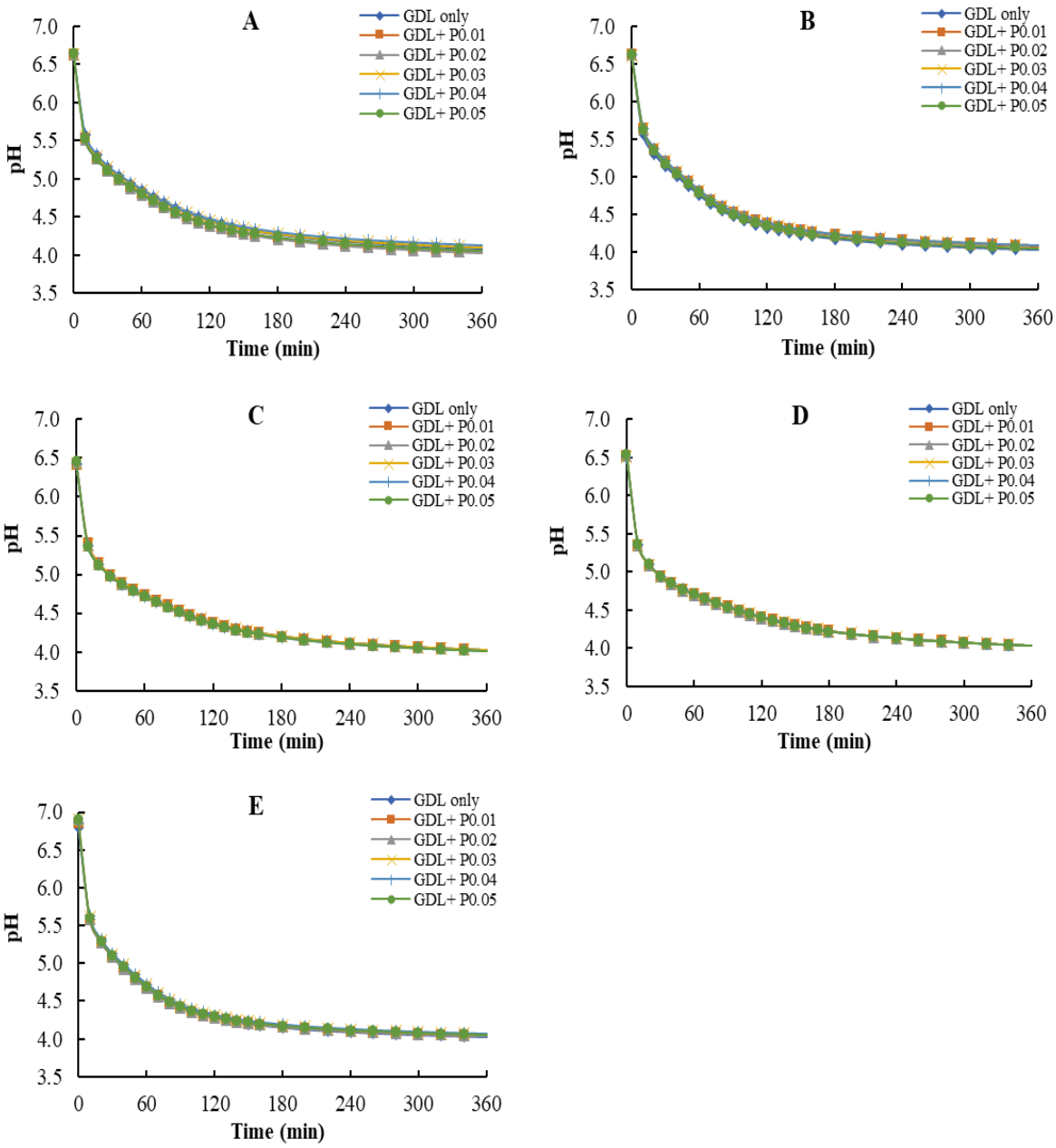


Figure 4.8 Changes in pH during gelation with glucono-δ-lactone (GDL) and various levels of pepsin added at 37°C: A, cow milk; B, goat milk; C, sheep milk; D, red deer milk; E, water buffalo milk. Mean values are depicted.

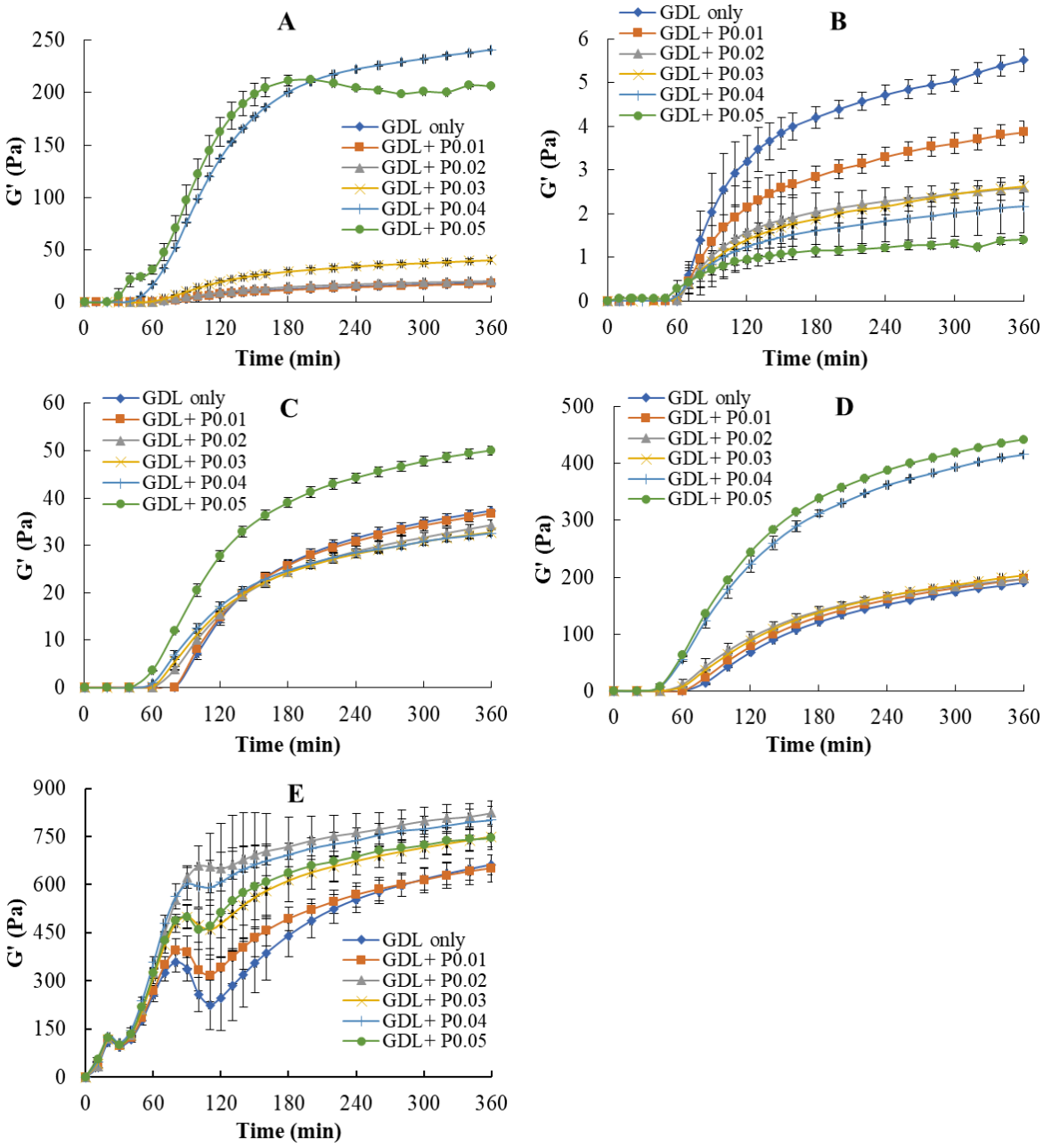


Figure 4.9 Effect of glucono-δ-lactone and pepsin on G' of raw skim milks: A, cow milk; B, goat milk; C, sheep milk; D, red deer milk; E, water buffalo milk. Values depicted are mean ± SD.

Table 4.5 Effect of glucono-δ-lactone (GDL) and pepsin on the properties of cow, goat, sheep, deer, and buffalo skim milk gels formed at 37°C.

Parameters	Milk	GDL only	GDL + P0.01	GDL + P0.02	GDL + P0.03	GDL + P0.04	GDL + P0.05
Gelation pH (point at which gels had a *G*' ≥1 Pa)	Cow	4.73 ± 0.06^{d}	4.69 ± 0.01^{d}	4.70 ± 0.00^{d}	4.85 ± 0.01^{c}	5.02 ± 0.02^{b}	5.15 ± 0.03^{a}
	Goat	4.59 ± 0.01^{a}	4.59 ± 0.01^{a}	4.55 ± 0.00^{b}	4.51 ± 0.00^{c}	4.49 ± 0.01^{c}	4.32 ± 0.01^{d}
	Sheep	4.55 ± 0.02^{d}	4.56 ± 0.00^{d}	4.63 ± 0.00^{c}	4.69 ± 0.01^{b}	4.71 ± 0.00^{b}	4.77 ± 0.01^{a}
	Deer	4.67 ± 0.00^{c}	4.70 ± 0.04^{bc}	4.77 ± 0.01^{b}	4.75 ± 0.03^{b}	4.92 ± 0.01^{a}	4.92 ± 0.00^{a}
	Buffalo	5.72 ± 0.00^{d}	5.71 ± 0.00^{e}	5.71 ± 0.00^{e}	5.81 ± 0.00^{b}	5.82 ± 0.00^{a}	5.80 ± 0.00^{c}
Gelation time (min) (point at which gels had a *G*' ≥1 Pa)	Cow	74.40 ± 0.00^{a}	72.87 ± 2.16^{ab}	68.29 ± 0.29^{b}	60.49 ± 0.01^{c}	42.53 ± 0.03^{d}	27.63 ± 2.00^{e}
	Goat	75.40 ± 0.70^{f}	81.5 ± 0.98^{e}	89.31 ± 0.98^{d}	94.39 ± 0.11^{c}	99.48 ± 0.69^{b}	130.35 ± 0.27^{a}
	Sheep	88.35 ± 2.88^{a}	85.3 ± 0.834^{a}	73.41 ± 0.057^{b}	66.79 ± 3.69^{bc}	61.19 ± 0.325^{c}	53.38 ± 0.057^{d}
	Deer	68.28 ± 0.00^{a}	62.51 ± 5.66^{ab}	49.28 ± 0.99^{c}	52.33 ± 3.44^{bc}	32.33 ± 0.01^{d}	32.32 ± 0.01^{d}
	Buffalo	7.20 ± 0.00^{a}	7.20 ± 0.00^{a}	7.20 ± 0.00^{a}	6.20 ± 0.00^{b}	6.20 ± 0.00^{b}	6.20 ± 0.00^{b}
Final storage modulus, G'_{final} (Pa) (measured 6 h after addition of GDL)	Cow	18.06 ± 1.64^{d}	17.87 ± 0.05^{d}	20.24 ± 0.07^{d}	40.11 ± 2.32^{c}	240.9 ± 1.17^{a}	206.10 ± 2.20^{b}
	Goat	5.51 ± 0.35^{a}	3.87 ± 0.35^{ab}	2.59 ± 0.39^{bc}	2.63 ± 0.21^{bc}	2.12 ± 0.85^{bc}	1.4 ± 0.13^{c}
	Sheep	37.27 ± 0.90^{b}	36.73 ± 1.27^{bc}	34.29 ± 1.41^{bc}	32.75 ± 0.58^{c}	32.55 ± 0.85^{c}	50.03 ± 1.358^{a}
	Deer	191.40 ± 1.41^{e}	197.60 ± 0.40^{d}	197.20 ± 0.60^{d}	203.90 ± 0.82^{c}	415.90 ± 2.40^{b}	442.20 ± 1.13^{a}
	Buffalo	661.70 ± 45.30^{ab}	651.20 ± 58.80^{b}	824.00 ± 51.30^{a}	750.50 ± 35.40^{ab}	800.50 ± 14.30^{ab}	745.16 ± 6.84^{ab}
Final tan δ (measured 6 h after addition of GDL)	Cow	0.261 ± 0.006^{b}	0.263 ± 0.003^{b}	0.267 ± 0.004^{ab}	0.275 ± 0.009^{ab}	0.286 ± 0.006^{a}	0.289 ± 0.004^{a}
	Goat	0.302 ± 0.010^{a}	0.305 ± 0.005^{a}	0.317 ± 0.007^{a}	0.311 ± 0.006^{a}	0.303 ± 0.006^{a}	0.307 ± 0.001^{a}
	Sheep	0.344 ± 0.000^{a}	0.342 ± 0.001^{a}	0.342 ± 0.002^{a}	0.341 ± 0.001^{a}	0.341 ± 0.004^{a}	0.334 ± 0.005^{a}
	Deer	0.292 ± 0.000^{cd}	0.295 ± 0.000^{ab}	0.297 ± 0.001^{a}	0.294 ± 0.000^{bc}	0.290 ± 0.000^{d}	0.294 ± 0.000^{b}
	Buffalo	0.297 ± 0.002^{a}	0.297 ± 0.006^{a}	0.297 ± 0.003^{a}	0.293 ± 0.002^{a}	0.295 ± 0.005^{a}	0.296 ± 0.004^{a}

[a–f] Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SD.

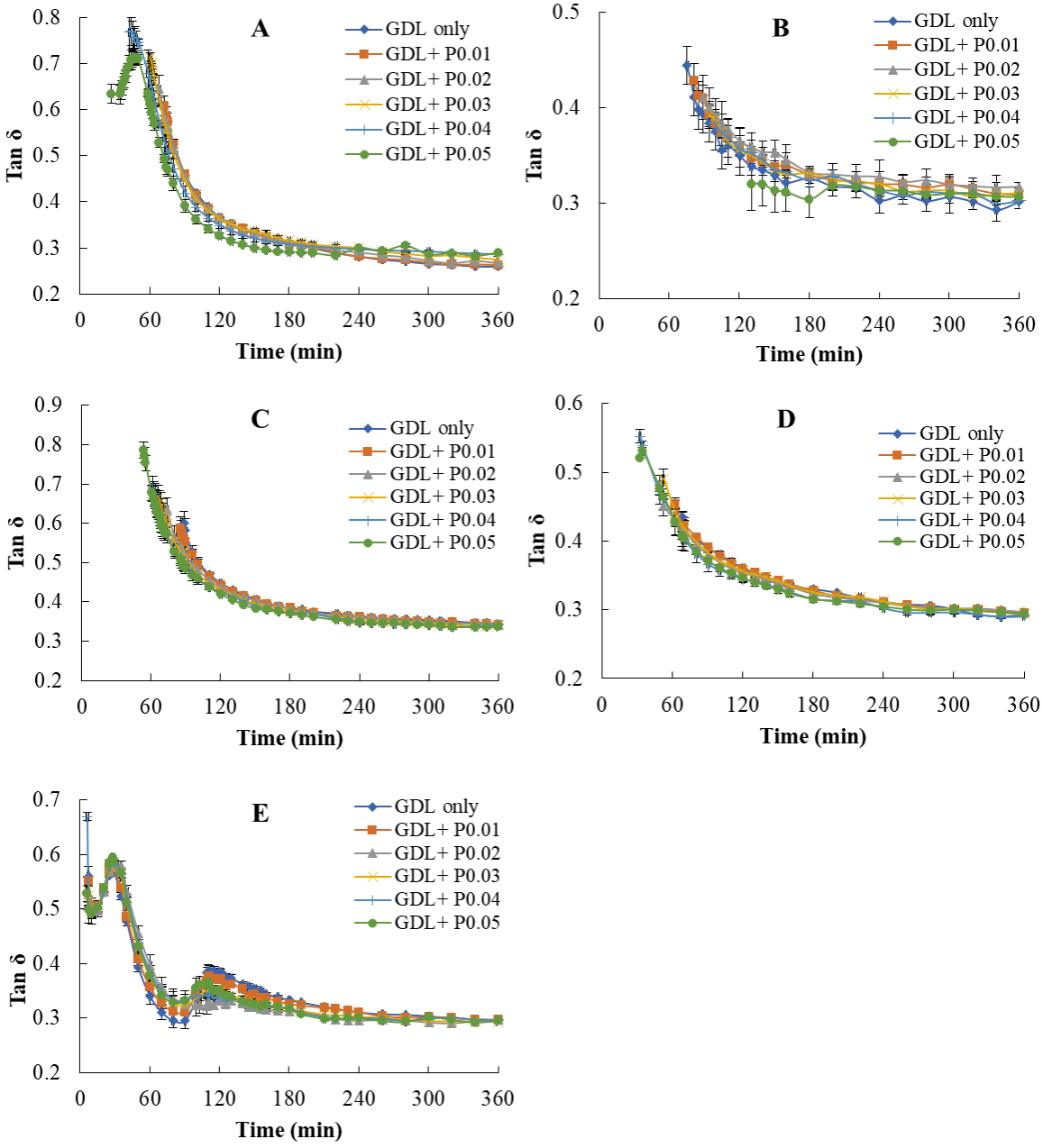


Figure 4.10 Effect of glucono-δ-lactone and pepsin on tan δ of raw skim milks: A, cow milk; B, goat milk; C, sheep milk; D, red deer milk; E, water buffalo milk. Values depicted are mean ± SD.

It was observed that the different milks responded differently to the various pepsin concentrations (or pepsin activity). In the cow skim milk combination gels, increasing the pepsin concentration decreased the gelation time and simultaneously increased the gelation pH and the final *G*' value (Figure 4.9A and Table 4.5); however, at the highest level of pepsin addition (P0.05, i.e., 0.05 mg of pepsin per 100 mL of skim milk), a $G'_{shoulder}$ was observed at a pH of 4.85–4.98 together with a slow decrease in the gel strength from pH 4.26 (192 min) onwards. The $G'_{shoulder}$ observed in the GDL + P0.05 cow milk combination gel could have been due to the residual CCP (Horne, 2003) because gelation occurred at a higher pH (5.15) than for the GDL-only cow milk gel (pH 4.73). The observed decrease in the gel strength in the GDL + P0.05 combination gel from cow milk could have been due to the proteolytic activity of pepsin for caseins other than κ-casein or to rearrangements in the gel structure at such a low pH. A tan δ_{max} of 0.71 (Figure 4.10A) was also observed at a pH of 4.9 for the same gel, which indicates the tendency for the gel to undergo further rearrangements and syneresis (Lucey *et al.*, 2000). The final tan δ ranged from 0.26 to 0.29 under all experimental conditions for the cow milk gels, with a slightly higher tan δ value at higher levels of pepsin, indicating the susceptibility of the gel systems to bond relaxation or deformation at higher pepsin concentrations (Table 4.5).

The combination gels from goat skim milk showed an opposite gelation profile to those from cow skim milk. With increasing pepsin concentration, the goat milk combination gels took longer to reach a *G*' value of 1, compared with the acid-only gel. Because of this, the gelation time increased and the gelation pH and the final *G*' value decreased (Figure 4.9B and Table 4.5). G'_{final} decreased remarkably with increasing concentrations of pepsin along with GDL,

leading to a *G*' value of ~1.4 Pa at the highest concentration of pepsin, compared with 5.51 Pa for the GDL-only gel. It was evident that gel formation was delayed with increasing pepsin concentration in goat milk.

Awad *et al.* (1998) studied the proteolytic activities of chymosin and porcine pepsin on cow, goat, and buffalo caseins and reported that, in general, porcine pepsin had higher proteolytic action on casein than did chymosin, especially on goat casein. This may have been reflected in this study; goat milk was more susceptible than cow milk to proteolysis by pepsin. It may be possible that pepsin is readily able to break some of the other protein bonds in goat milk, compared with cow milk, and thus cause a delay in gel formation. No trend in the maximum in tan δ was observed in the goat milk gels; however, the final tan $\delta \simeq 0.31$ was higher in the goat milk gels than in the cow milk gels, which also indicates the more viscous or liquid like characteristics of goat milk gel systems compared with the cow milk gel systems (Figure 4.10B). The final *G*' values obtained for the combination gels from goat milk were in the range 1.4–3.87 Pa, which represents very weak gels; thus, any conclusions with respect to its gelation trends need to be regarded as tentative.

Similar to the goat milk gels, there was a slight decrease in the *G*' value for the sheep milk gels at medium pepsin concentrations (GDL + P0.03 and GDL + P0.04); however, at the highest pepsin concentration (GDL + P0.05), an increase in the final *G*' value was observed (Figure 4.9C), even though the gelation time decreased and the gelation pH increased for the sheep milk combination gels (Table 4.5). This behaviour is difficult to explain. The final tan δ values for the sheep milk gels ranged from 0.33 to 0.34 (Figure 4.10C), which were

relatively higher than the reported values for the goat milk and cow milk gels (Table 4.5), which might be an indicator of rapid bond relaxation and a more viscous behaviour in the networks of the sheep milk gel systems. As the proteins in goat milk and sheep milk are considered to be similar (Masoodi & Shafi, 2010), it may be that sheep milk proteins, like goat milk proteins, are also more susceptible to proteolysis by pepsin.

The trends observed in the gelation profile of the deer milk combination gels (i.e., a decrease in the gelation time and simultaneous increases in the gelation pH and the final *G*' value with increasing pepsin concentration) were similar to those for the cow milk combination gels except that, at the highest pepsin concentration (P0.05), there was no $G'_{shoulder}$ or decrease in gel strength (Figure 4.9D and Table 4.5). The final tan δ values of ≃ 0.29 were slightly lower than the values reported for the goat and sheep milk gels and were closer to the values reported for the cow milk gels, which indicates that deer skim milk combination gels were less susceptible to bond rearrangements and had more of a solid (or elastic) like characteristic than sheep milk and goat milk combination gels.

In the buffalo milk combination gels, the gelation time decreased slightly, and the gelation pH increased slightly with increasing pepsin concentration (Figure 4.9E and Table 4.5). The gelation time ranged from 6.2 to 7.2 min under all gelation conditions, indicating rapid coagulation of the buffalo milk. It was difficult to define any particular pattern of increase or decrease in the final *G*' value with increasing pepsin concentration because the rapid gel formation resulted in a double peak pattern in the buffalo milk combination gels, similar to the GDL-only gels (Figure 4.9E). All the buffalo milk gels showed similar double tan δ_{max}

values, ranging from 0.57 to 0.58 at pHs from 5.11 to 5.18, and from 0.34 to 0.39 at pHs from 4.27 to 4.36 (Figure 4.10E). The first peak at tan δ_{max} of 0.57–0.58 represents the tendency of the bonds to relax (or break) and indicates the possibility that microstructural rearrangements were taking place at such high gelation pHs. The unusual behaviour of buffalo milk towards acid or combination gels needs to be further investigated.

4.4.7 Microstructure and protein composition of combination gels

The microstructure and protein composition of the combination gels after 6 h (GDL + P0.01, ……., GDL + P0.05) in comparison to acid gel (GDL only) are depicted in Figure 4.11 and Figure 4.12. The microstructure of the combination gels appeared to be only slightly more open (especially for GDL + P0.05) compared to that of the acid gels (Figure 4.11). The SDS-PAGE analysis revealed that in milk gels from all the species κ-casein was hydrolysed (Figure 4.12). The extent of hydrolysis of κ-casein was more rapid with increasing pepsin concentration as indicated by the increase in intensity of the para-κ-casein band. However, as the microstructural and SDS-PAGE analysis was done on gel samples after 6 h, it was not possible to relate the changes occurring in milk gels from different species during the process of gelation.

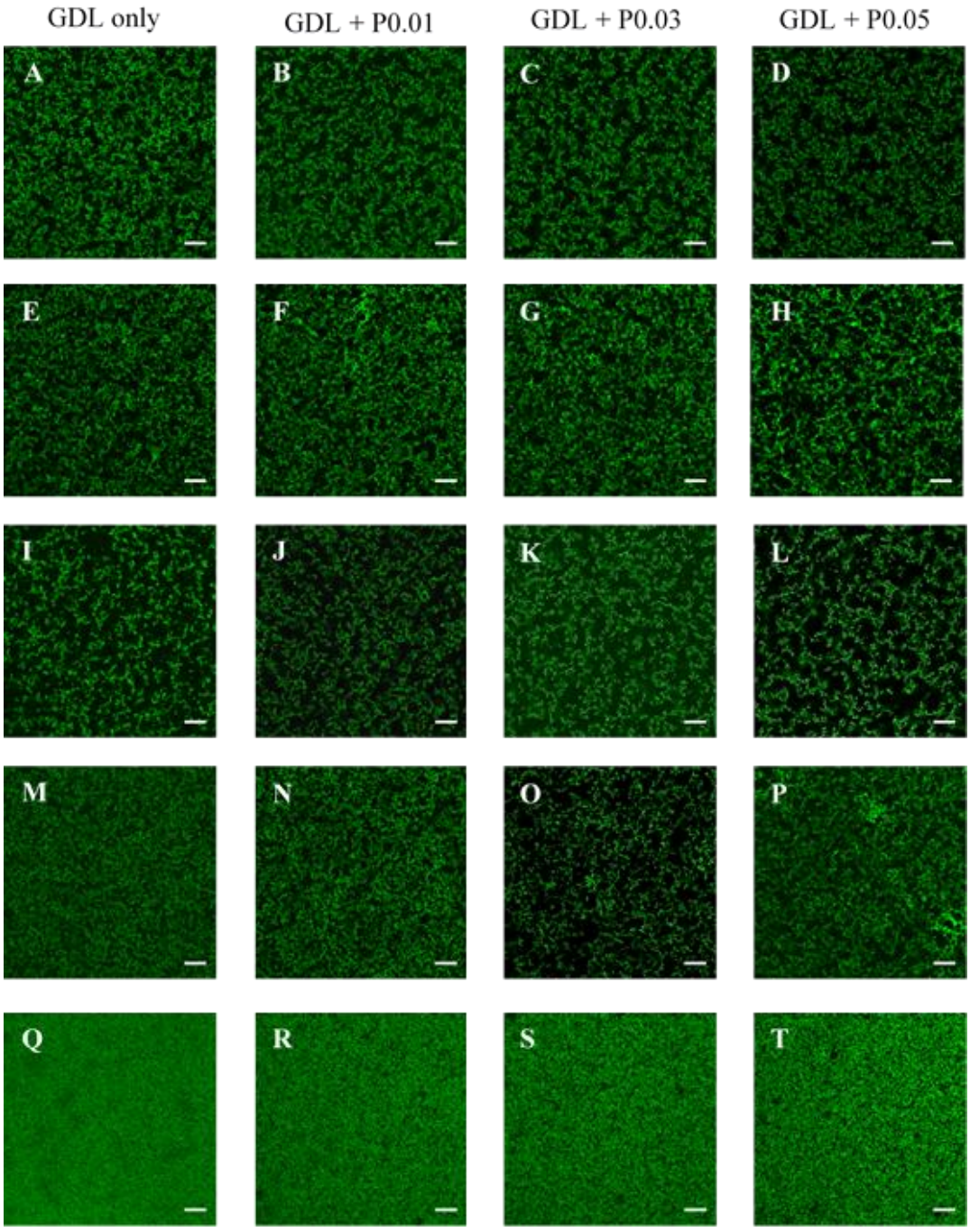


Figure 4.11 Confocal microscopy images of acid and combination gels made from different skim milk using glucono-δ-lactone (GDL) alone and along with pepsin (scale bar = 25μm). A, B, C and D represents cow milk gels. E, F, G and H represents buffalo milk gels. I, J, K and L represents goat milk gels. M, N, O and P represents sheep milk gels. Q, R, S and T represents deer milk gels.

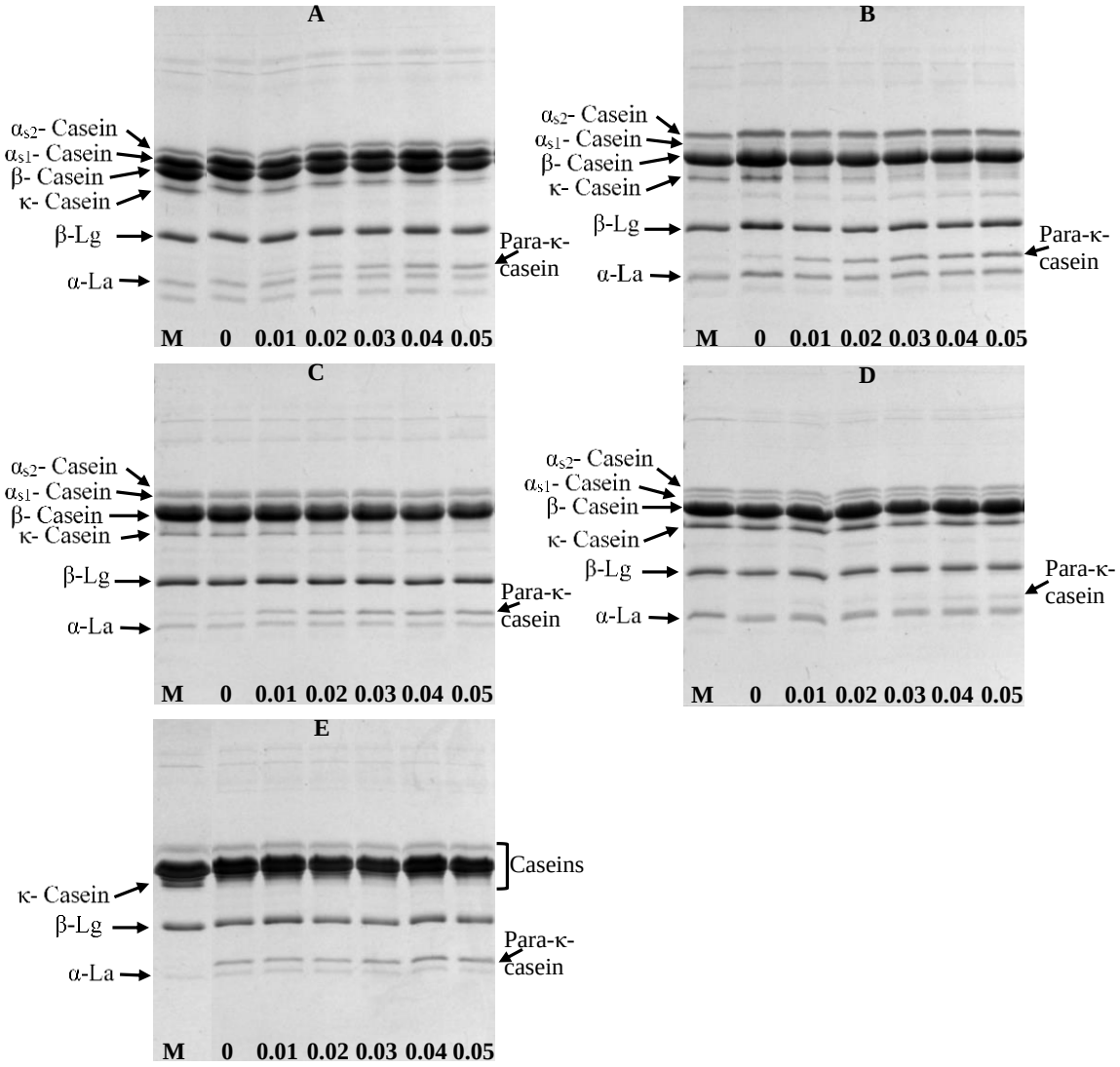


Figure 4.12 Protein compositions of skim milk and gels from various species analysed using SDS-PAGE, A- cow, B- goat, C- sheep, D- water buffalo, E- red deer. M represents raw skim milk before gelation; 0, 0.01, 0.02, 0.03, 0.04, and 0.05 represents amount of pepsin (mg/100 mL of skim milk) added along with GDL for gel formation.

A simplified illustration of the trends in the gelation time and the final storage modulus (G'_{final}) of the combination gels of the milk from all species with pepsin concentration is depicted in Figure 4.13.

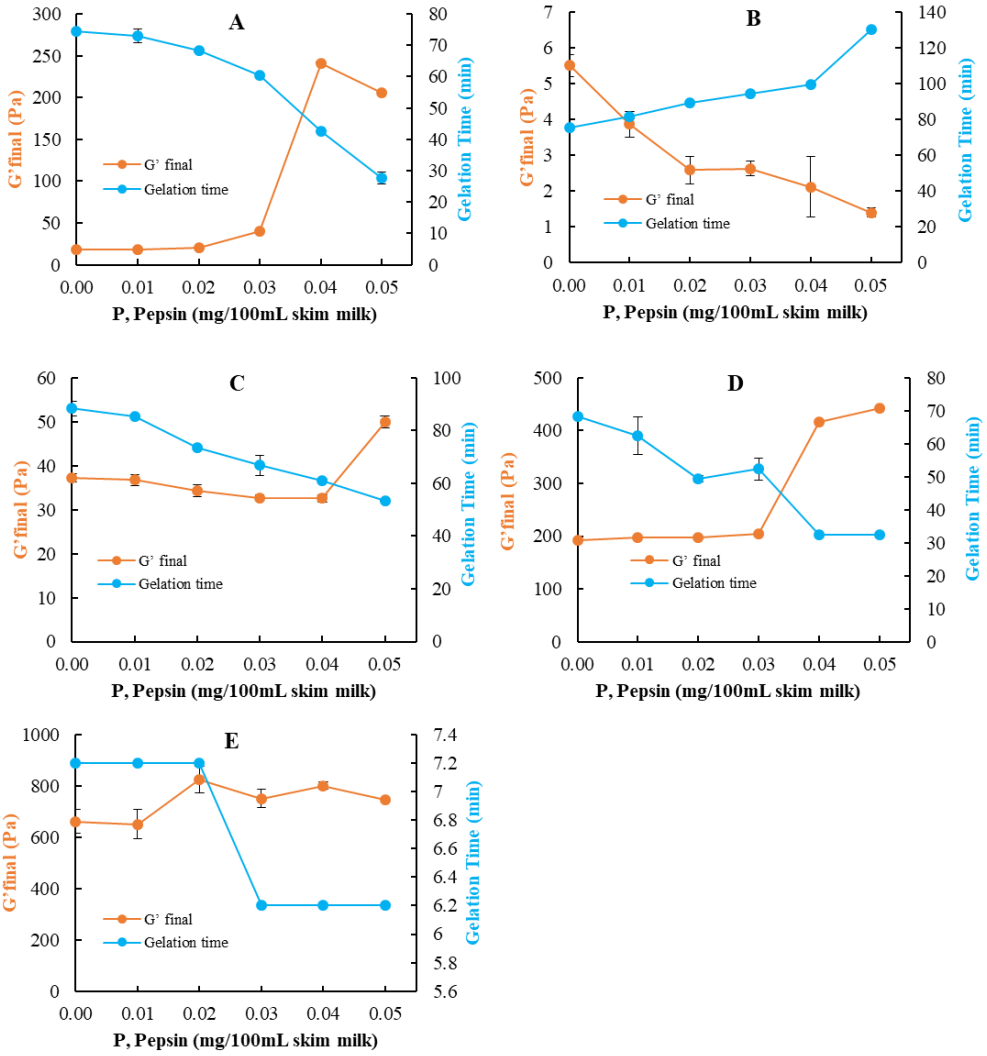


Figure 4.13 Relationship between pepsin concentration (mg/100 mL skim milk), gelation time, and final storage modulus (G'_{final}) of combination gels: A, cow milk; B, goat milk; C, sheep milk; D, red deer milk; E, water buffalo milk. Mean ± SD is reported.

The gelation behaviour of combination gels is a complex phenomenon as both pepsin-induced and acid-induced casein aggregation are occurring simultaneously in these gels. Initially, in the combination gels, as the pH is decreased, the electrostatic repulsion and the steric stabilization of the casein micelles are reduced. In addition, pepsin is active enough at pH ~6.0 to partially hydrolyse the κ-casein, leading to the early onset of gelation at a higher pH, as observed in this study. As the pH is reduced, the CCP is gradually solubilized and inorganic phosphate is completely solubilized below pH 5.0–5.2. In the later stages of gelation, when sufficient acid has been produced, i.e., at pH ~4.6–4.9, pH-induced coagulation takes over and all the calcium is also solubilized in this pH range. Thus, there is a transition from a pepsin-induced coagulation to an acid-induced coagulation and this may be different for different milks because of the differences in their CCP contents, casein micelle sizes, and structures. As pepsin is still present in the combination gel system and has sufficient proteolytic activity in the pH range 4–5, it may be able to hydrolyse some of the other proteins in the system and this may again be different for different milks because of the differences in their total protein contents and the proportions of the various protein components. At higher pepsin concentration along with GDL, casein aggregation begins at a higher pH, resulting in a shorter gelation time and a higher gel strength at the end of gelation, compared with GDL-only gels. This may be the phenomenon occurring in the cow milk and deer milk combination gels. However, the phenomenon in the goat, buffalo, and, to some extent, in sheep milk combination gels is more complex and thus needs further investigation.

4.5 Conclusions

Milk from different species vary in their physicochemical and rheological properties. Sheep, deer, and buffalo milk have higher total solids and protein contents, which result in stronger gel formation on acidification compared with cow milk. In contrast, goat milk contains less protein and forms less firm gels on acidification compared with cow milk. Buffalo milk is an exception as, despite having lower protein and total solids contents than deer milk and sheep milk, it forms stronger gels than deer and sheep milk. Milk from different species also responds differently to the action of pepsin. Goat milk showed a decrease in gel firmness with the addition of the smallest concentration of pepsin; in contrast, deer milk and buffalo milk show increases in gel strength with increasing pepsin concentration. Such differences can be attributed to the differences in the relative proportions of the different caseins in the milks. However, the rheological properties of the milk from different species cannot be determined by a single universal factor, such as protein content or casein composition; it is a complex association among various parameters such as casein micelle size, CCP content, proportions of different caseins and whey proteins, mineral content, genetic variants, and other factors. It is possible that different milks may respond differently to the enzymes and the acid concentration in the human stomach, which may lead to the formation of curds with different structures. Thus, in the next chapters the curd formation and gastric digestion behaviour of milk from different species have been investigated.

Chapter 5: Structural changes in cow, goat, and sheep skim milk during dynamic *in vitro* gastric digestion[3]

5.1 Abstract

Using a human gastric simulator, the dynamic gastric digestion of goat and sheep skim milk were compared with that of cow skim milk, focusing particularly on their physical characteristics. The gastric contents were analysed for changes in dry matter and microstructure, and the extent of protein digestion. The study revealed that the skim milk from all species formed a curd within the first 15 min of gastric digestion, at which time the pH was ~6.1–6.3. Compared with cow skim milk, the dry matter contents of the clots formed from goat and sheep skim milk were lower and higher, respectively, which was due to the differences in their total solids and protein contents. Microstructural analysis showed that, as the digestion progressed, the clot structure became more cohesive along with a decrease in moisture content, which in turn affected the breakdown and hydrolysis of caseins by pepsin; this phenomenon was similar for milk from all species. However, the extent of moisture retained in the sheep skim milk clot appeared to be lower than those of the cow and goat skim milk clots. In addition, the relative firmness of the sheep milk clot was higher than those of the cow and goat milk clots at the end of gastric digestion. The pattern of protein hydrolysis

[3] The contents presented in this chapter has been published as a peer-reviewed paper: Roy, D., Ye, A., Moughan, P. J., and Singh, H. (accepted for publication, 2020). Structural changes in cow, goat and sheep skim milk during dynamic *in vitro* gastric digestion. *Journal of Dairy Science.*

by pepsin was similar for the milk of all species, despite the differences in the proportions of different proteins.

5.2 Introduction

Goat milk contains lower amounts of casein (especially lower α_{s1}-casein) and larger casein micelles than cow milk; these characteristics are considered to be responsible for the weak consistency of goat milk gels upon acid or enzymatic coagulation (Bell & Vlahopouloua, 1995; Ould Eleya *et al.*, 1998; Storry *et al.*, 1983). On the contrary, higher amounts of caseins and minerals in sheep milk are considered to be responsible for its stronger acid or rennet gel than goat or cow milk (Domagała, 2009; Ould Eleya *et al.*, 1998; Storry *et al.*, 1983). It was shown in the previous chapter (Chapter 4) that goat milk forms weaker acid gels whereas sheep milk forms stronger acid gels compared to cow milk. Such observations have led to a widely perceived notion that goat milk may form soft or loose curd in the human stomach, leading to easier digestion compared with the firm curd formed by cow milk (El-Agamy, 2007; Jenness, 1980; Maathuis *et al.*, 2017). However, little is known about the coagulation characteristics of goat and sheep milk in the human stomach. To date, no direct comparative studies on the coagulation behaviour of cow, goat, and sheep milk in the stomach have been reported, although a few *in vitro* studies have reported the comparative proteolysis profiles of proteins in these milks. For instance, Jasińska (1995) observed that the *in vitro* peptic as well as tryptic hydrolysis rates of micellar caseins from goat skim milk were higher than cow skim milk (based on protein content). The higher proteolysis rate of goat milk was attributed to the smaller size of its micellar aggregates (or complexes) and the presence of a higher proportion of β-casein in its micellar structures when compared with cow milk. Similarly,

Hodgkinson *et al.* (2018) reported that lower proportions of caseins remained undigested in goat whole milk than in cow whole milk during the first 60 min of static *in vitro* gastric digestion at pH 3.0 (based on SDS-PAGE analysis); they speculated that this could be due to the fragile nature of the coagulum formed by goat milk in comparison with cow milk.

All of the previous studies on the milk of different species were carried out using static *in vitro* digestion models. It is now well accepted that, to understand the physicochemical modification as well as digestive dynamics of a complex structured fluid such as milk, the use of dynamic gastric digestion models is essential (Mulet-Cabero *et al.*, 2019; Ye *et al.*, 2019b). A few recent studies have focused on understanding the structural changes that occur in the milk matrix during dynamic gastric digestion (Ye *et al.*, 2016a, 2016b, 2017; Ye *et al.*, 2019b). All the previous dynamic *in vitro* gastric digestion studies were based on cow milk. To date, no dynamic gastric digestion studies have been reported for goat milk and sheep milk.

It was hypothesized that, as the milk from cow, goat, and sheep vary in composition and physicochemical properties, there may be differences in the curd (or clot) formation and disintegration in the stomach. Therefore, in this study, the dynamic comparative coagulation as well as nutrient release behaviours of cow, goat, and sheep skim milk using the Human Gastric Simulator (HGS) was investigated for the first time. The HGS is widely recognized as a dynamic stomach model that efficiently simulates the physiologically relevant mechanical forces that develop *in vivo* (Ferrua & Singh, 2015; Mulet-Cabero *et al.*, 2020b).

5.3 Materials and methods

5.3.1 Materials

Pooled cow (Jan – May 2018), goat (Jan – May 2018), and sheep (Jan – April 2019) raw whole milk were obtained under chilled conditions from the Massey University No. 4 dairy farm (Palmerston North, New Zealand), Dairy Goat Co-operative (Hamilton, New Zealand), and Neer Enterprises Limited (Carterton, New Zealand), respectively. Other experimental materials needed were as per the protocol described in Section 3.1 (Chapter 3).

5.3.2 Methods

5.3.2.1 Milk processing and storage

Milk processing and storage was performed as per the method described in Section 3.2.1 (Chapter 3).

5.3.2.2 Skim milk composition

The chemical composition of skim milk was analysed as per the protocol described in Section 3.2.2.1 (Chapter 3).

5.3.2.3 Casein micelle diameter

The casein micelle size of the skim milks was determined as per the protocol described in Section 3.2.2.4 (Chapter 3).

5.3.2.4 *In vitro* gastric digestion

A HGS (Ferrua & Singh, 2015; Kong & Singh, 2010) was used for running simulated gastric digestion experiments with some modifications for a liquid food such as milk (Figures 5.1, 5.2, and 5.3). The oral digestion fluid phase, normally containing amylase, was not included as milk contains no starch (Minekus *et al.*, 2014). Simulated gastric fluid (SGF, pH 7) was prepared according to the salt composition suggested in Minekus *et al.* (2014), but the pH was different. The ratio of total milk to total SGF was considered to be 1:1 for a liquid food diet, meaning that, in total, 200 mL of SGF was gradually added during the simulated gastric digestion of 200 g of milk (Minekus *et al.*, 2014; Wisén & Johansson, 1992). The 200 mL of SGF was divided into 2 solutions: Solution A, i.e., 176 mL of SGF (inclusive of the amount of 6 M HCl needed to drop the pH of the different skim milk to pH 2 in 4 h); Solution B, i.e., 24 mL of SGF (pH 7), which was used to make the pepsin solution. Thus, the combined pH of Solution A + Solution B was 1.40 ± 0.10, to achieve a gradual decrease in pH of the different milks to 2 over a period of 240 min of digestion, i.e., 4 h. A separate batch of 25 mL of SGF at pH 2 was prepared; this was added to the HGS at the beginning of the gastric digestion of all milk samples to simulate the fasting residual volume of the stomach (Grimm *et al.*, 2018; Kong & Singh, 2008; Maltby *et al.*, 1986). The amount of pepsin used for the raw cow skim milk was based on 2000 U/mL of the final digestion mixture (Minekus *et al.*, 2014). The first batch of cow skim milk sample had a protein concentration of 4.48% (g/100 g milk), which meant that the overall pepsin-to-protein ratio was 94.25 U/mg cow skim milk protein. On this basis, the amount of pepsin used for all batches of cow, goat and sheep skim milk was 94.25 U/mg milk protein to achieve an overall equal pepsin-to-protein ratio

(theoretical) for obtaining comparative protein digestion profiles. The total pepsin to protein (caseins + whey protein) ratio was approximately 1:3.45 (wt/wt) in this study (theoretical).

Figure 5.1 Setting up of the HGS (Human Gastric Simulator).

Figure 5.2 Picture of the internal compartment of the HGS.

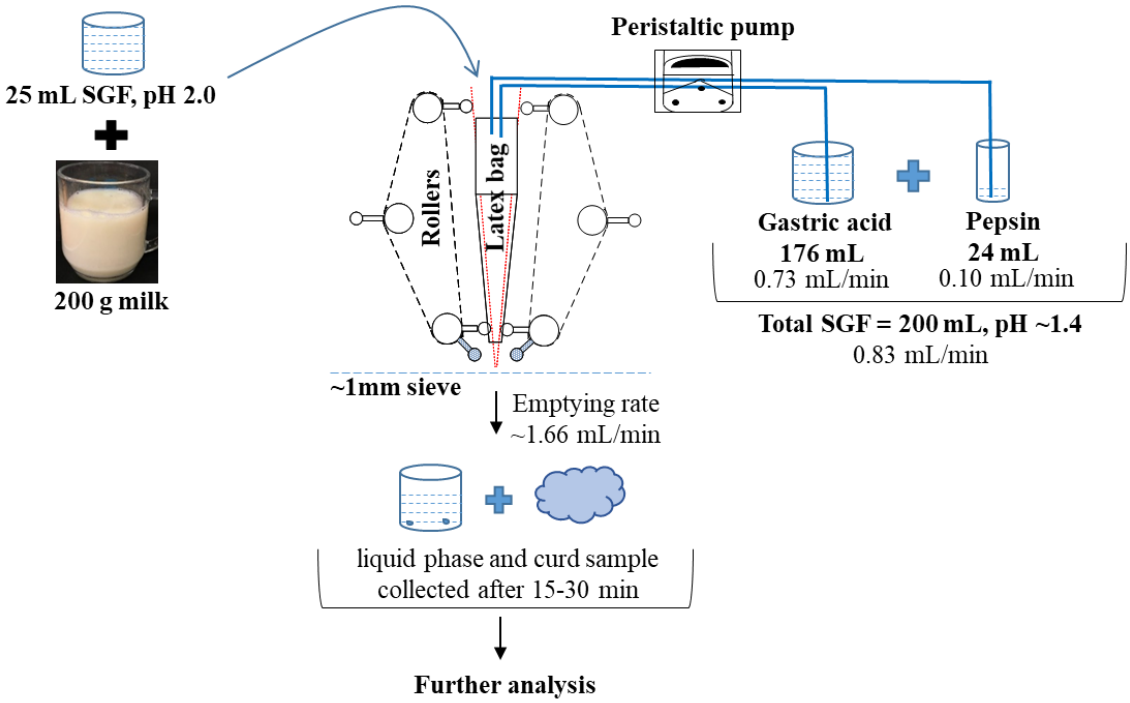


Figure 5.3 Simplified representation of the in vitro gastric digestion method (not to scale).

The HGS was pre-warmed and maintained at 37 ± 0.5°C throughout the experiment using a heater and a thermostat. The contraction frequency was set to 3 times per min for simulating the actual stomach contraction in humans. A 200 g pre-warmed milk sample at ~37°C was added to the HGS and was allowed to mix for a minute with the fasting residual volume (25 mL of pre-warmed SGF, pH 2). Then, 200 mL of SGF [176 mL of SGF (with HCl) at 0.73 mL/min, and 24 mL of pepsin solution at 0.10 mL/min] was added gradually to the HGS at 0.83 mL/min during the 240 min of gastric digestion. Every 30 min (30, 60, 90, 120, 150, 180, 210, and 240 min), the chyme was emptied out (emptied liquid chyme) from the bottom of the HGS after sieving through a 1 mm pore size sieve to mimic human gastric sieving, equaling a gastric emptying rate of ~1.66 mL/min i.e. ~50 mL every 30 min. The emptying rate was theoretically calculated for emptying 200 g milk + 200 mL SGF i.e. a total of ~400 mL of gastric contents in 4 h = 1.66 mL/min, though the emptied liquid chyme at 210 and 240 min was <50 mL due to coagulation of a part of liquid milk (i.e. caseins) during gastric digestion experiments. The gastric digestion time of 4 h was selected for all the milk based on the consideration that the complete emptying of a milk protein-based meal from the stomach would take about 3-6 h (Boutrou *et al.*, 2013; Chabance *et al.*, 1998). For analysis of the clot samples at each time interval (including a 15 min gastric digestion sample), the total sample was removed from the HGS and filtered through a mesh with a pore size of 1.0 mm diameter to obtain both the coagulated mass (curd or clot) and the liquid phase (liquid chyme) for further analysis. NaOH (10 N) was added to the liquid chyme samples to increase the pH to 8, to inhibit pepsin activity, and the samples were immediately stored at –20°C for further analysis. The coagulated phase at different digestion timepoints was broken down into smaller pieces, mixed with the help of a spatula, diluted with ~100 mL of Milli-Q water

to raise the pH to ~5.5-6.5 and then immediately frozen at –80°C to reduce the pepsin activity. The determination of pH, wet and dry weights, microstructure, and texture were conducted immediately on fresh samples after stopping the gastric digestion at different time intervals with minimal disturbance to the clot structure. Additional gastric digestion experiments were performed for the microstructure and texture analyses.

5.3.2.5 pH measurement

The initial natural pH of the milk samples and the pH of the liquid chyme (or emptied liquid chyme) samples from the HGS at each time point were measured using a CyberScan pH 510 pH/mV/°C meter (Eutech Instruments, Fisher, Malaysia). The pH of the coagulated phase was also measured, by inserting the pH probe inside the clots (close to the centre). All the pHs referred to correspond to the pH of the liquid chyme, unless specified otherwise.

5.3.2.6 Wet and dry weights of the clots

The weights of the clots obtained after gastric digestion of the milks at different time intervals were measured immediately to monitor their fresh wet weights. A representative sample (~1 g) of the fresh wet clot (after being manually broken with a spatula to obtain a homogeneous mixture) obtained at different time intervals was dried (along with the respective skim milk sample) at 105°C for 16 h in an air oven (Contherm Scientific Ltd, Hutt City, New Zealand), so that the dry weights could be determined.

5.3.2.7 Protein content of the clots

The total protein (total nitrogen × 6.38) contents of the clots (freeze dried) were determined using the Dumas method 968.06 (AOAC, 2005).

5.3.2.8 Identification of milk proteins by SDS-PAGE

Reducing SDS-PAGE was performed on the milk clots (freeze-dried and ground powder) as well as on the liquid chyme samples, as described by Ye *et al.* (2016b). The samples were treated with sample buffer to visualize the time-dependent hydrolysis of the proteins in the gastric chyme samples. The detailed method is described in the Section 3.2.2.5 (Chapter 3).

5.3.2.9 Confocal scanning laser microscopy

A Leica SP5 upright confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) at the Manawatu Microscopy and Imaging Centre (Massey University, Palmerston North, New Zealand) was used for high-resolution imaging of the microstructure of the clot samples at 15, 90 and 240 min of digestion. A procedure similar to that of Ye *et al.* (2017) was followed. A 1.0% (wt/vol) solution of Fast Green (dye content 90%) in water was used to stain protein (helium–neon laser with an excitation line at 633 nm). At different time points, the clot samples were collected, immediately cut into thin slices using a surgical blade, and stained with Fast Green solution for 10 min. The stained samples were then placed on double concave microscope slides (clear glass, ground edges, 26 mm x 76 mm, 1.2–1.3 mm thick; Sail, Sailing Medical-Lab Industries Co. Ltd, Suzhou, China), covered with coverslips, and examined with a 63x oil immersion objective (numerical aperture = 1.4). Care was taken to avoid areas near the sides or close to the top of the microscope slide while imaging. Multiple

fields were viewed, and typical micrographs are presented. The imaging was completed within 40 min of stopping the digestion at the respective times.

5.3.2.10 Texture analysis of the clots

The clots remaining at the end of digestion, i.e., after 240 min, were observed immediately for texture using penetration tests. Physical examination of the clots revealed that their outside layer was relatively soft compared with their inner core. Thus, the analysis was conducted by penetrating the clot samples from outside at different locations, to obtain the overall force that was required to penetrate a particular clot sample. Another experiment was also conducted; the clot was cut into 2 halves and the force required to penetrate into the different areas of the core inside the clot was measured. A TA-XTplus texture analyzer (Stable Micro Systems, Surrey, UK) was fitted with a 5.0 kg load cell and a 2.0 mm diameter lightweight stainless-steel flat probe. For each test, Exponent software (version 6.1.15.0, Stable Micro Systems, Surrey, UK) was used for the TA-XTplus. The set-up was calibrated for load weight and height before starting the measurement. The probe was moved downwards until contact with the sample just above the surface was detected. The experiment was run, and the sample was penetrated to a specified depth of 5.0 mm at a constant speed of 2.0 mm/s. On completion of the downward stroke, the probe was moved up at the same speed to above the surface of the sample. The measurements were taken at room temperature (about 22°C). The value of the maximum force (N) was obtained from the force–time curve of each measurement.

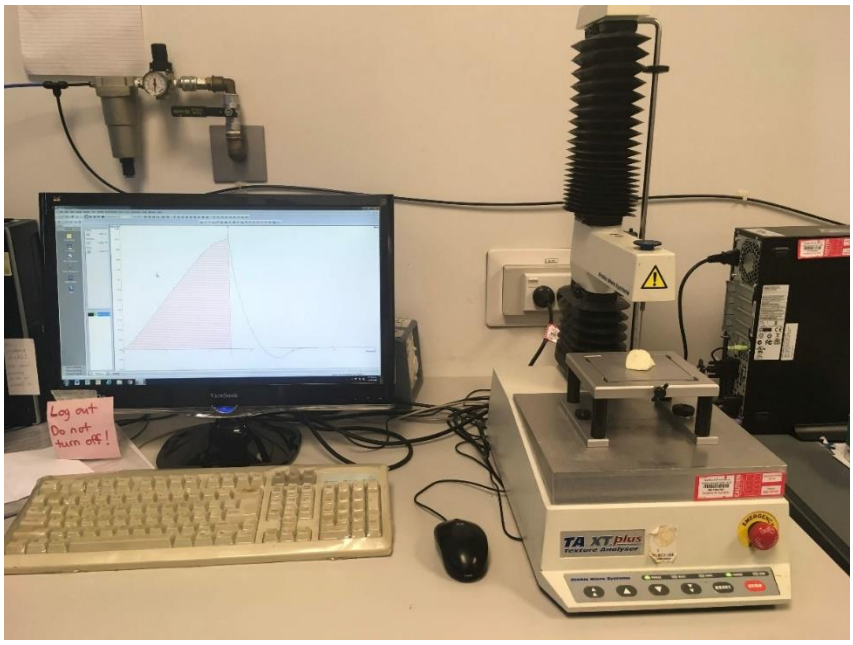


Figure 5.4 TA-XTplus texture analyzer (penetration test).

5.3.2.11 Statistical analysis

Experimental data were analysed by running analysis of variance (ANOVA) tests using Minitab statistical software (version 18.1, Minitab Inc., State College, PA). The changes in pH, wet weight, dry weight, moisture content, and protein were analysed using 2-way ANOVA with milk, digestion time, and their interaction (milk x time) as fixed effects. Another 2-way ANOVA was used to analyse the force required to penetrate the milk clots with milk, clot section (outside and core), and their interaction (milk x section) as fixed effects. If significant interaction effects were found, then further analysis was conducted with Tukey pairwise comparison test. Chemical composition and casein micelle size of milk were analysed by using 1-way ANOVA with Tukey test. The minimum level of statistical significance was $P < 0.05$. Statistical analysis was carried out on results from at least 2

different batches of milk and values (or data points) are expressed as mean ± SD (standard deviation).

5.4 Results and discussion

5.4.1 Chemical compositions of different skim milk

The chemical compositions of the cow, goat, and sheep skim milk are shown in Table 5.1. Sheep milk was significantly higher ($P < 0.05$) and goat milk was significantly lower ($P < 0.05$) than cow milk in total solids, crude protein, and calcium contents. Sheep milk also had higher amounts of phosphorus compared to cow and goat milk, both of which had similar phosphorus contents. The interspecies differences in the nutrient compositions observed were consistent with those reported in previous studies (Barlowska *et al.*, 2011; Stergiadis *et al.*, 2019) as well as in the previous chapter (Chapter 4). Considerable variability in milk composition within and between species exist in the literature, which is expected to be due to the differences in region, breed, feed, milking interval, stage of lactation, and time of the year (Claeys *et al.*, 2014).

5.4.2 Casein micelle sizes of different skim milk

The Z-average diameter (nm) of the casein micelles (Table 5.2) of cow milk was significantly ($P < 0.05$) smaller (~157–158 nm) than those of sheep milk (~195–198 nm) and goat milk (~192–201 nm), in agreement with the results of previous studies (Nguyen *et al.*, 2018; Park *et al.*, 2007) as well as those reported in Chapter 4. No significant differences ($P > 0.05$) in the average casein micelle sizes of the raw and pasteurized milk from the same species were observed (Table 5.2). A low degree of denaturation of the whey proteins under pasteurization

(72°C, 15 s) conditions (Guinee *et al.*, 1998; Rynne *et al.*, 2004) would have led to a smaller amount of complexation between the denatured whey proteins and the casein micelles (Anema & Li, 2003a, 2003b; Kethireddipalli *et al.*, 2011); this appeared to have no impact on the casein micelle size of the pasteurized milk compared with its raw milk counterpart.

Table 5.1 Chemical compositions of cow, goat, and sheep milk.

Properties	Cow skim milk	Goat skim milk	Sheep skim milk
Dry matter (%)	10.31 ± 0.30^{b}	8.66 ± 0.16^{c}	12.03 ± 0.31^{a}
Protein (%)	4.63 ± 0.22^{b}	3.38 ± 0.07^{c}	6.22 ± 0.36^{a}
Fat (%)	0.13 ± 0.01^{b}	0.20 ± 0.04^{a}	0.19 ± 0.02^{a}
Carbohydrates (by difference, %)	4.70 ± 0.06^{a}	4.21 ± 0.05^{c}	4.50 ± 0.07^{b}
Ash (%)	0.85 ± 0.02^{b}	0.87 ± 0.01^{b}	0.96 ± 0.03^{a}
Calcium (mmol/L)	37.70 ± 1.31^{b}	27.42 ± 1.52^{c}	44.42 ± 3.39^{a}
Inorganic phosphorus (mmol/L)	31.89 ± 0.95^{b}	30.18 ± 2.59^{b}	40.78 ± 0.17^{a}

$^{a–c}$Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SD.

Table 5.2 Average casein micelle sizes of raw and pasteurized cow, goat, and sheep milk (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk).

Casein micelle size	Cow		Goat		Sheep	
	RCSM	PCSM	RGSM	PGSM	RSSM	PSSM
Z-average diameter (nm)	158.0 ± 3.5^{b}	156.6 ± 2.5^{b}	192.0 ± 5.1^{a}	200.9 ± 9.6^{a}	198.0 ± 2.8^{a}	195.3 ± 3.0^{a}

$^{a–b}$Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SD.

5.4.3 pH changes during gastric digestion of different skim milk

The changes in the pH profiles of the liquid chyme in the HGS as a function of time are shown in Figure 5.5. The initial pH in the HGS represents the pH of the residual gastric fluid, i.e., pH 2 (at 0 min). With the ingestion of milk, the pH was immediately increased to ~6.7 (at 2 min). Then, with the continuous addition of SGF (+ HCl) and pepsin solution, the pH of the liquid chyme in the HGS decreased gradually for all milk samples. Raw cow skim milk and raw goat skim milk had similar gradual decreases in pH with digestion time; the pH of the liquid chyme at the end of 4 h, i.e., 240 min, was ~2.0 for both milk samples. Raw sheep skim milk had a slightly higher buffering capacity than raw goat and cow skim milk, as indicated by the slower decrease in pH, especially from 150 min onwards ($P < 0.05$), and the pH of its liquid chyme at 240 min was ~2.3. This was possibly due to the higher protein and mineral (calcium and phosphorus) contents (Park, 1991; Salaün *et al.*, 2005) of raw sheep skim milk compared with raw goat and cow skim milk. These results were consistent with those reported by Vithana (2012); they observed that the titrated buffering capacity of raw sheep whole milk was significantly higher than that of raw cow and goat whole milk, both of which had similar buffering capacities. Some small but statistically significant differences ($P < 0.05$) in the decrease in pH were observed for pasteurized cow, goat, and sheep skim milk compared with their raw milk counterparts, especially from 60 min onwards. The pH at the end of 240 min for all pasteurized skim milk was in the range ~2.6–2.8. In contrast to the pH of the liquid chyme, the pHs at the centre of the clots from the different milks towards the end of digestion were higher, i.e., pH 4.0–5.0, indicating the higher buffering capacity of the aggregated (or coagulated) casein phase.

The dissolution of colloidal calcium phosphate with decreasing pH would release both calcium ions and phosphate ions from the curd which may also have a crucial role in buffering capacity. The phosphate ions are relatively unstable and may have a tendency to exist as hydrogen phosphate ions and thus sequester hydrogen ions and cause an increase in pH. In addition, higher pH of the curd could be due to the hindered diffusion of gastric acid into the curd and as well as a relatively high concentration of phosphate or hydrogen phosphate ions.

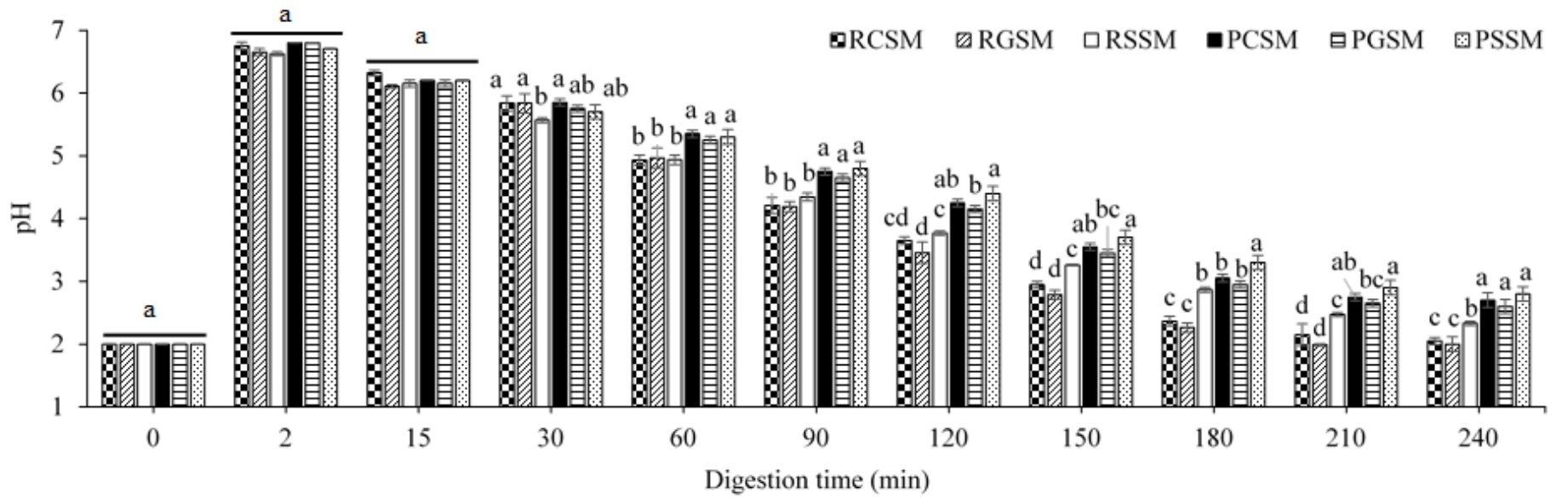


Figure 5.5 Changes in pH of skim milk during gastric digestion in the HGS (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk). Different letters (a-d) above bars represent significant differences among milk samples at a given digestion time point ($P < 0.05$); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD.

5.4.4 Coagulation behaviour of different skim milk

The photographs of the clots and liquid chyme obtained during the gastric digestion of 200 g of raw and pasteurized skim milk at different times are shown in Figures 5.9 and 5.10.

The initial signs of milk coagulation were detected within the first 6–7 min of digestion for all milk (as visually observed), soon after which all milk separated into a clot and a liquid phase (Figures 5.6, 5.7, and 5.8). Both the raw and the pasteurized skim milk from cow, goat, and sheep formed a clot (or curd) within the first 15 min of gastric digestion, at which time the pH of the liquid chyme was around pH 6.1–6.3. Ye *et al.* (2016b) and Wang *et al.* (2018) have reported similar coagulation pHs for the clots (or curds) formed from raw cow skim milk and reconstituted low-heat cow skim milk powder during dynamic gastric digestion. No other reports on the coagulation behaviours of goat and sheep milk during dynamic digestion are available.

Figure 5.6 Internal chamber of the latex stomach before adding milk and SGF.

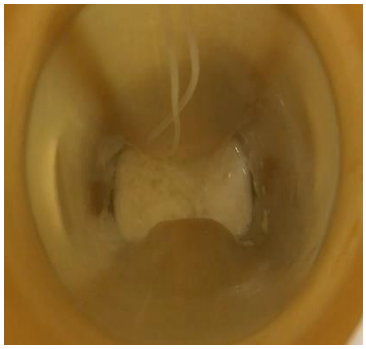


Figure 5.7 Signs of milk coagulation few minutes after starting gastric digestion.

Figure 5.8 An example of the separated curd and liquid phase inside the HGS.

The formation of the milk clot at around pH 6.0 is primarily due to the action of pepsin on the κ-casein located at the surface of the casein micelles (Ye *et al.*, 2016b). Pepsin has been shown to cause faster initial rate of hydrolysis for κ-casein than α_s- and β-caseins at pH 6.0 (Tam & Whitaker, 1972). Pepsin hydrolyses the Phe_{105}–Met_{106} bond of κ-casein but also have the ability to hydrolyse bonds with Trp, Tyr, Leu or Val residues in milk proteins (Agudelo *et al.*, 2004; Moschopoulou, 2011). At the start of the *in vitro* gastric digestion, the pepsin concentration in the HGS was low, but sufficient to quickly hydrolyse κ-casein to para-κ-casein and glycomacropeptide, resulting in destabilization of the casein micelles and their aggregation at ~pH 6.0. This phenomenon has been shown to occur in the milk from all species (Jollès, 1966; Miranda & Pelissier, 1983). The clot formed from raw milk is mainly due to coagulation of the caseins, whereas whey proteins do not play a functional role in raw milk clot formation (Ye *et al.*, 2016b). Pasteurization (72°C, 15 s) would be expected to cause small degree of denaturation of whey proteins (Guinee *et al.*, 1998; Rynne *et al.*, 2004), but this did not appear to affect the initial clot formation as both raw and pasteurized milk from all the species formed a clot within 15 min at pH ~6.1-6.3 ($P > 0.05$, Figures 5.5, 5.9 and 5.10). As the digestion progressed (and the pH decreased), the concentration and the activity of the pepsin in the HGS increased, which led to higher degree of hydrolysis of all the caseins (Miranda & Pelissier, 1983; Tam & Whitaker, 1972).

Initially, when the clot was formed within the first 15 min (pH 6.1 – 6.3), it was loose and soft, and had an open structure; however, as the digestion progressed, the clot became smaller, more cohesive, and firmer. This was due to the combined action of pepsin, mechanical forces in the stomach, and the gradual decrease in pH by stomach acid. The liquid

chyme in the gastric phase was expected to consist mainly of water-soluble components of the milk such as whey proteins, lactose, and minerals. The separated liquid phase at <60 min of digestion was opaque and contained some fine aggregated particulate matter. This was due to the loose structure of the clot at the beginning of digestion leading to the loss of particulate matter from the clot. As the digestion progressed and the pH reached near the isoelectric point of casein i.e. pH 4.6 (approximately about 90 min onwards), the clot became stronger and denser, leading to less transfer of aggregated casein particles to the liquid phase. During the later stages of digestion, caseins would have mainly transferred as very fine dissolved particles (as well as peptides) from the outer surface of the clot to the liquid chyme.

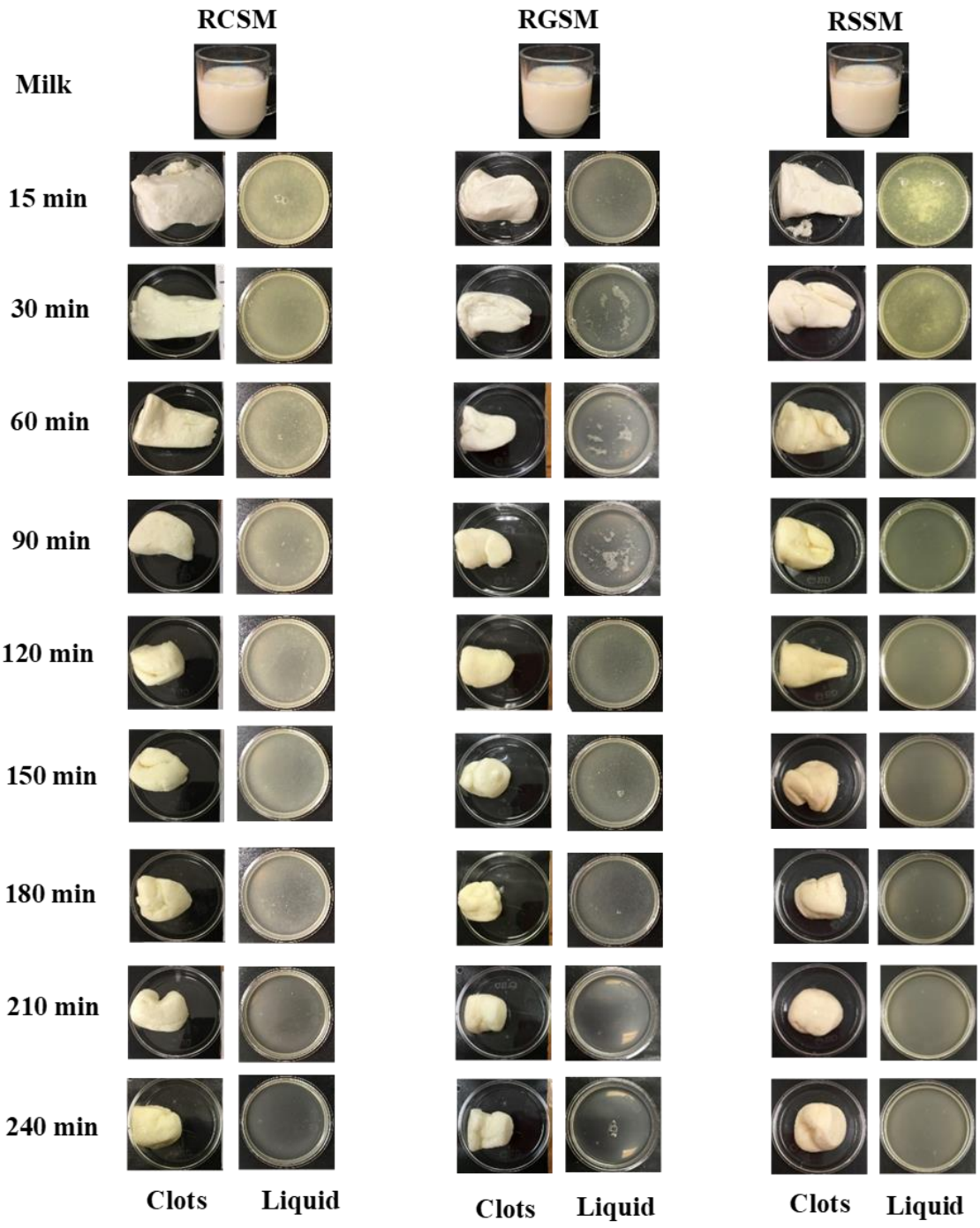


Figure 5.9 Photographs of the clots and liquid chyme obtained during the gastric digestion of 200 g of raw milk at different times in the HGS (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk).

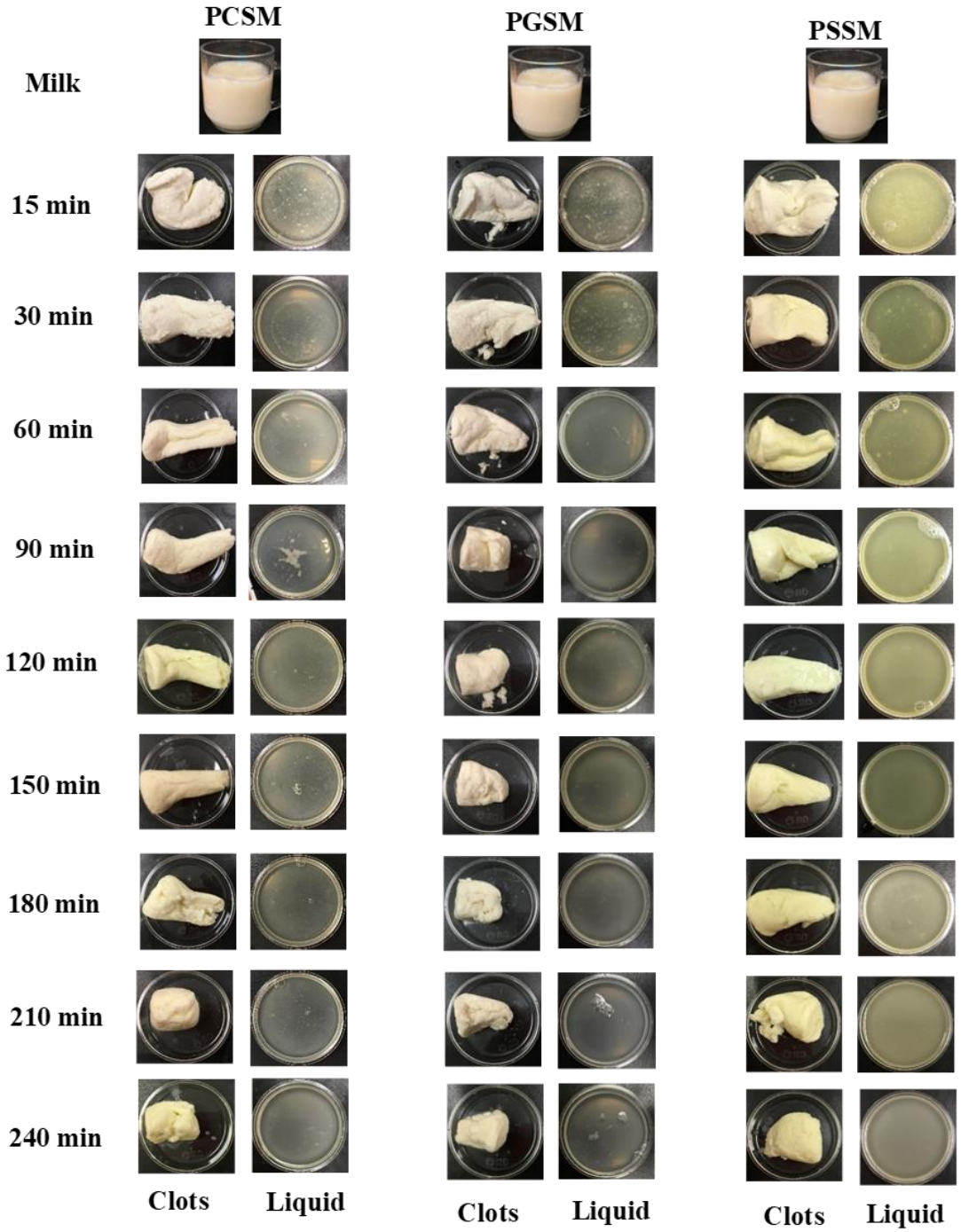


Figure 5.10 Photographs of the clots and liquid chyme obtained during the gastric digestion of 200 g of pasteurized milk at different times in the HGS (PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk).

5.4.5 Wet and dry weights of the clots

The wet weights of the clots obtained during the gastric digestion of 200 g of raw and pasteurized skim milk from different species as a function of time are shown in Figure 5.11. The wet weight of the clots obtained from both raw and pasteurized milks followed the order: goat < cow < sheep (Figure 5.11). In general, the wet weights of the clots from all species decreased with digestion time.

The dry weights of the clots obtained during the gastric digestion of 200 g of different raw and pasteurized milk as a function of time are shown in Figure 5.12. In general, the dry weights of the raw and pasteurized milk clots from the different species progressively decreased with the digestion time. The dry matter of the clots obtained from both raw and pasteurized milk during gastric digestion followed the order: goat < cow < sheep ($P < 0.05$), and there was no significant difference ($P > 0.05$) in the dry weights of the clots formed from the raw and pasteurized milk within a species towards the end of digestion. The clots formed from goat milk had the lowest dry weights, and those formed from sheep milk had the highest dry weights, compared with those formed from cow milk. This was mainly dependent on the total protein (casein) as well as total solids contents of the milk.

The changes in the dried weight of the clot reflected the loss of matter, which was because of the removal (or squeezing out) of the entrapped liquid and loose curd particles from the clot (mainly during the early stages of digestion by the continuous mechanical contraction of the stomach) as well as hydrolysis of the coagulated proteins by pepsin (especially during the later stages of digestion). A similar phenomenon was reported by Ye *et al.* (2016b) while

studying the dynamic gastric digestion of raw cow skim milk using HGS; they also reported that both the stomach mechanical contraction and the pepsin activity plays a key role in the gradual decrease of the dried matter content of the milk during gastric digestion.

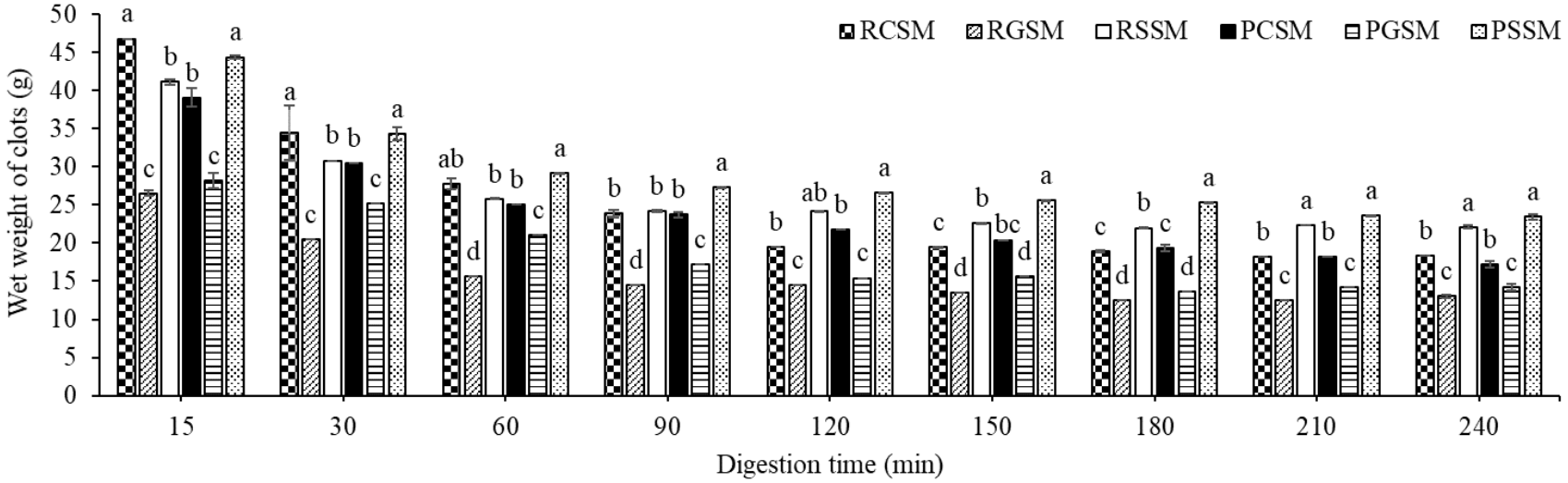


Figure 5.11 Changes in the wet weights of the clots during the gastric digestion of 200 g of skim milk in the HGS (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk). Different letters (a-d) above bars represent significant differences among milk samples at a given digestion time point ($P < 0.05$); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD of results from at least 2 different batches of milk.

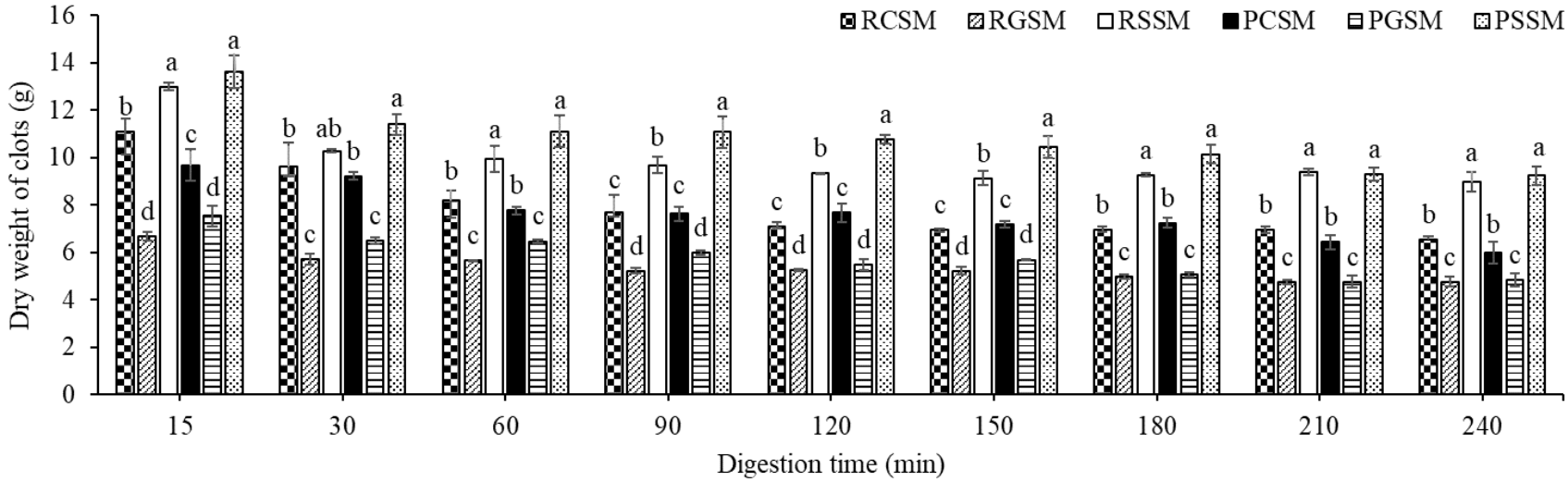


Figure 5.12 Changes in the dry weights of the clots during the gastric digestion of 200 g of skim milk in the HGS (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk). Different letters (a-d) above bars represent significant differences among milk samples at a given digestion time point (P < 0.05); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD.

5.4.6 Moisture content of the clots

Figure 5.13 shows the calculated overall moisture content (grams of water retained per gram of dry matter) of the clots obtained at different digestion times (calculated based on the wet and dry weight of the clots obtained). It should be noted that the moisture content of the clots could be different between the outer layer and the inner core of the clot, as the outer layer of the clot was in direct contact with the surrounding liquid phase. Given the dynamic nature of the clot throughout the digestion, it was difficult to segregate the moisture content of the outer and inner layers; therefore, the moisture content referred to here was based on the total dry matter of the clot.

The moisture contents of both the raw and the pasteurized skim milk clots from all 3 species decreased considerably with time after they had initially formed within 15 min. The moisture content of the raw cow skim milk clots decreased gradually until 120 min of digestion, after which it remained fairly constant. Similarly, the moisture contents of the raw goat and sheep skim milk clots decreased rapidly until 90 and 60 min of digestion, respectively, after which the decrease was very slow (Figure 5.13). There were no significant differences between the moisture content of the raw goat and cow skim milk clots at almost all digestion time points ($P > 0.05$). The raw sheep milk clots had considerably lower moisture content than the raw cow milk clots ($P < 0.05$); this was more evident during the initial stages of digestion i.e. till 90 min of digestion. This lower initial moisture content of the raw sheep milk clots may be related to their lower initial wet weights (Figure 5.11), despite the higher total solids contents of sheep milk compared with cow milk (Table 5.1 and Figure 5.12). The pasteurized skim

milk from all species had similar overall comparative patterns for moisture retained in the clots (Figure 5.13).

The calculated moisture content of the clots formed from both the raw and the pasteurized cow, goat, and sheep skim milk were in the ranges ~3.2–1.8, 3.0–1.7, and 2.3–1.5 g water/g dried clot, respectively, for 15–240 min of digestion (Figure 5.13). These changes in moisture content of the raw cow skim milk clots (3.2–1.8 g water/g dried clot) were in line with the previous reported moisture content of clots (2.7–1.4 g water/g clot dry matter) during the gastric digestion of raw cow skim milk using an HGS (Ye *et al.*, 2016b). The core hydration (excluding the κ-casein layer) of cow milk casein micelles is considered to be 2–3 g water/g protein (Dalgleish, 2014); the results obtained in this study for the cow skim milk clots were close to this range (though the clot moisture content obtained in this study may not a true representation of the hydration of the casein micelles due to the presence of other materials in the casein clot).

There are no previous reports on the moisture content of the casein clots formed from goat milk and sheep milk during gastric digestion. However, the hydration values of goat milk and sheep milk casein micelles have been reported to be lower i.e. 1.43-2.05 and 1.71–1.93 g water/g dry weight of casein micelles, respectively than the hydration values for cow milk (Pellegrini *et al.*, 1994; Remeuf & Lenoir, 1986; Remeuf *et al.*, 1989). Sood *et al.* (1979) reported that the loss of micellar calcium from the skim milk casein micelles (when dialyzed against same skim milk sample containing ethylenediaminetetraacetic acid, EDTA) resulted into increased hydration (or swelling) of casein micelles. Thus, it is considered that the

hydration level of the casein micelles is negatively correlated with mineralization of micelles (Remeuf & Lenoir, 1986) i.e. when the mineralization of the casein micelle increases, the degree of hydration of casein micelle decreases; this is expected to be due to the decrease in the void spaces within the casein micelles that can be filled up with water along with an increase in the extent of binding of the proteins in the casein micelle. Goat and sheep milk casein micelles are more mineralized (amount of colloidal calcium per g of casein) than cow milk casein micelles (Park, 2007; Park *et al.*, 2007). Thus, the lower moisture content observed for sheep milk clots in this study could be due to the higher mineralization of its casein micelles. The sheep milk used in this study had higher total calcium and inorganic phosphorus than cow and goat milk.

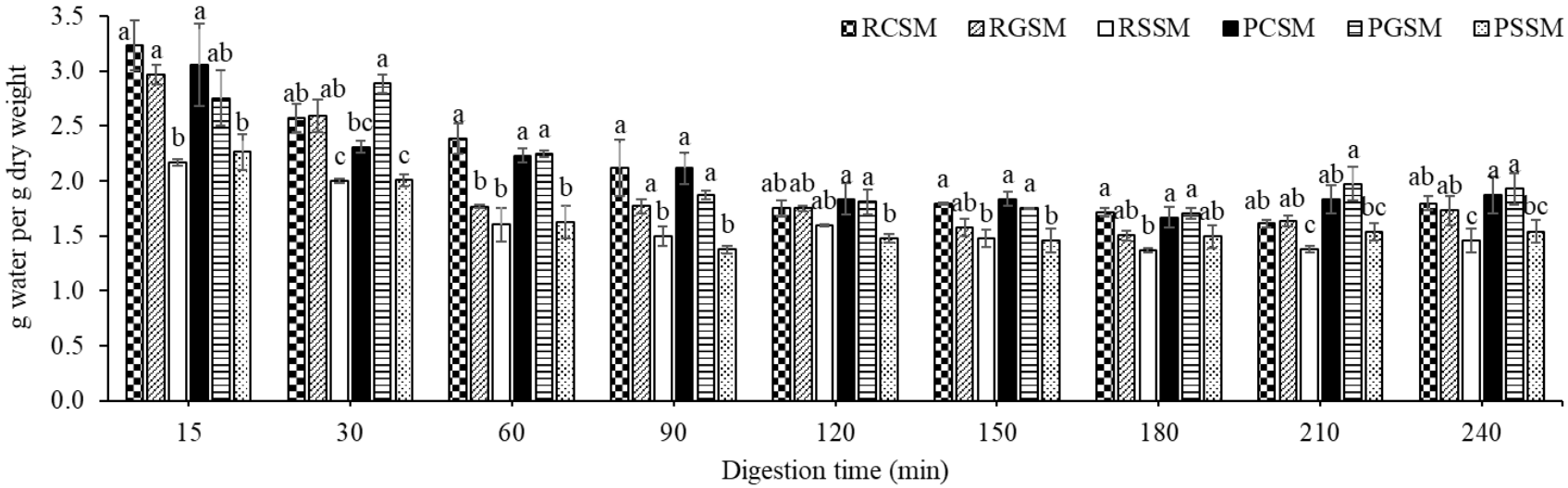


Figure 5.13 Changes in the moisture content of the raw and pasteurized skim milk clots collected during gastric digestion in HGS (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk). Different letters (a-c) above bars represent significant differences among milk samples at a given digestion time point (P < 0.05); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD.

5.4.7 Protein content of the clots

The quantities of protein retained (g/200 g milk) in the dried clots from the different milks during digestion are shown in Figure 5.14. Skim milk clots were composed mainly of caseins with some minor amounts of entrapped water-soluble components such as lactose, whey proteins, and minerals. Thus, in general, the higher was the protein (casein) content of the milk, the higher was the amount of protein retained in the clot; the protein contents of the clots formed from the raw and pasteurized milk from the different species during digestion were in the following order: goat < cow < sheep ($P < 0.05$) (Figure 5.14). The total protein content of the dried clots decreased gradually during digestion, with most of the decrease occurring during the initial stages of digestion for all the milk, i.e., up to 90 min (when the pH was ~4.2-4.8). The decrease in protein content during this initial period was due mainly to the loss of fine casein particles and entrapped liquid (dissolved) whey proteins from the clots due to the less firm nature of the clot. After 90 min (as the pH dropped below the isoelectric point of casein i.e. pH 4.6), the clot became stronger and more cohesive. Thus, the decrease in the protein content of the clot after 90 min was slow and mainly due to the hydrolysis of caseins by pepsin.

As expected, the trend of the decrease in protein content of the clots (Figure 5.14) with the digestion time was similar to that of the dry matter content of the clots (Figure 5.12), although the protein content generally remained lower than the dry matter content during gastric digestion. This difference may reflect the loss of entrapped lactose, whey proteins and soluble salts from the clot as well as dissolution and removal of micellar calcium phosphate from the casein clots because of a decrease in pH to below 4.6 (Dalgleish & Law, 1989).

The comparative percentage protein retained (g/100 g milk protein) in the dried clots from the different milks during digestion is shown in Figure 5.15. At the end of 240 min of digestion, approximately 59%, 58%, and 64% of the milk protein remained in the raw cow, goat, and sheep skim milk clots, respectively. However, there were no significant differences ($P > 0.05$) between the percentage protein retained by the raw cow, goat, and sheep milk clots at 240 min (Figure 5.15). The percentage protein retained in the pasteurized milk clots was slightly lower than that in their respective raw milk clots, but the differences were not significant ($P > 0.05$). For instance, approximately 53%, 55%, and 59% of the protein remained in the pasteurized cow, goat, and sheep skim milk clots at 240 min, respectively.

As the raw sheep skim milk had higher pH ($P < 0.05$) towards the later stages of gastric digestion than the raw goat and cow skim milk (150 min onwards, Figure 5.5), another experiment was conducted (results not shown) to adjust the pH profile for sheep milk to achieve a pH of 2 with SGF (pH ~1.3, added extra HCl). This was to explore whether sheep milk with the adjusted pH profile had a greater disintegration or hydrolysis of the clots. However, there was no significant difference in the dry matter and protein contents retained in the raw sheep milk clots with adjusted and unadjusted pH profiles. This indicated that the lower moisture content and the more tightly knitted clots formed by sheep skim milk, in comparison with the skim milk from the other species, might have been a factor in its lower disintegration rate.

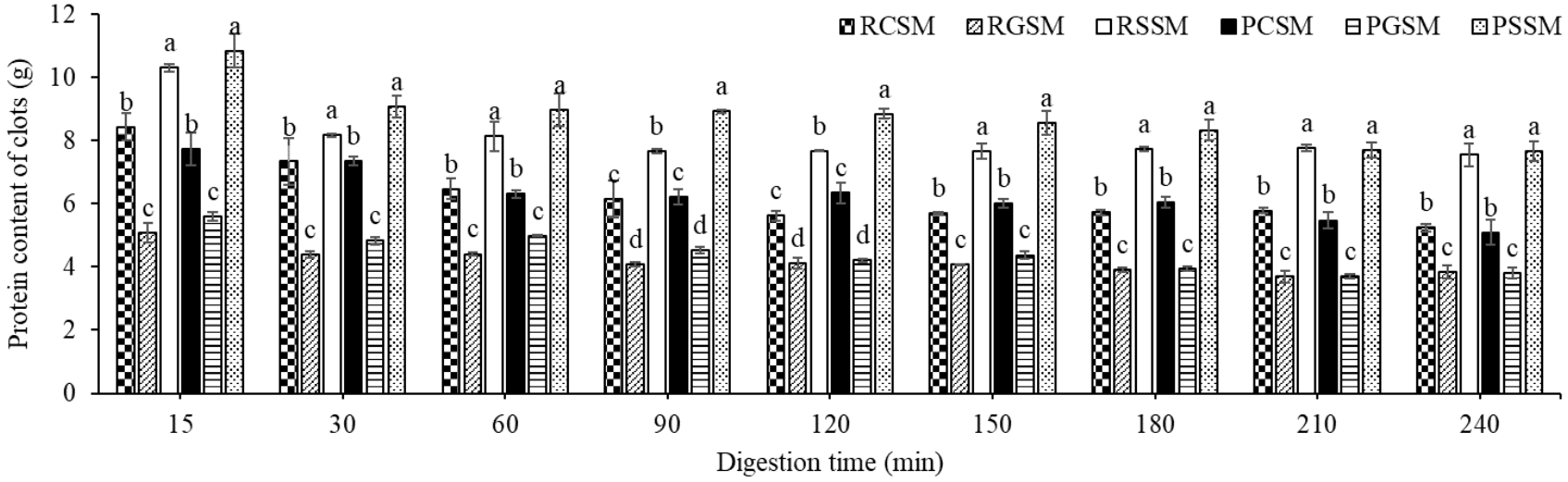


Figure 5.14 Changes in the protein content of the clots during the gastric digestion of 200 g of skim milk in the HGS (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk). Different letters (a-d) above bars represent significant differences among milk samples at a given digestion time point (P < 0.05); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD of results from at least 2 different batches of milk.

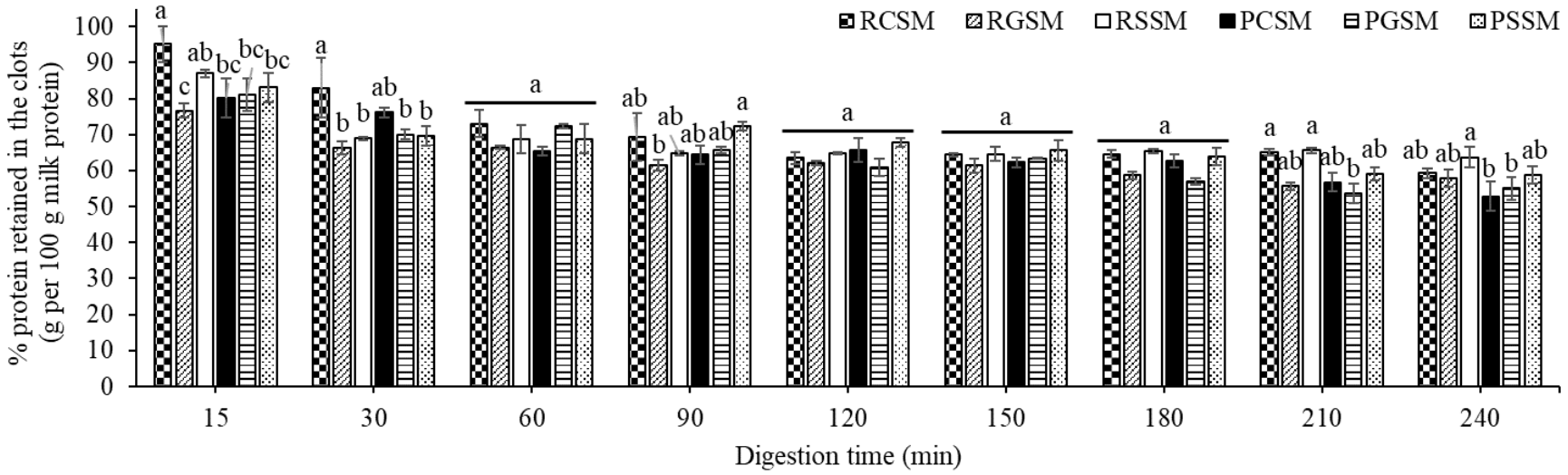


Figure 5.15 Changes in the percentage protein retained in the clots (expressed as g/100 g milk protein) during the gastric digestion of skim milk in the HGS (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk). Different letters (a-c) above bars represent significant differences among milk samples at a given digestion time point (P < 0.05); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD.

5.4.8 Protein composition of the clots

During digestion in the gastric environment, the milk samples generated a clot and a liquid fraction. The protein compositions of the milk clots and the liquid chyme obtained at different digestion times were determined using SDS-PAGE under reducing conditions (Figures 5.16 and 5.17). Analysis of the raw cow skim milk clots (Figure 5.16, RCSM) showed that the intensity of the κ-casein band decreased markedly within 30 min of digestion, whereas the intensities of the other casein bands (especially α_{s1}-, α_{s2}-, and β-caseins) at 30 min were much higher and remained fairly constant during further digestion. In addition, a 15 kDa band (para-κ-casein band derived from the hydrolysis of κ-casein) appeared at 30 min of digestion and the intensity of this band did not change with the digestion time. A faint β-lactoglobulin band was also observed in all clots, which was possibly due to the entrapped stomach liquid in the pore spaces of the clots. Some other intense bands (such as at ~20, 16.5, 12, and 10 kDa) were also observed at 30 min of digestion; the intensities of these bands increased only slightly with the digestion time. However, these bands also appeared to be present in the undigested cow milk, as indicated by their extremely faint intensities (Figure 5.16, RCSM). This means that, as the milk clotted, these bands concentrated in the clotted fraction of the milk and their intensities increased with further enzymatic hydrolysis. Miranda and Pelissier (1983) observed the presence of γ_1-casein (20.5 kDa), γ_2-casein, and γ_3-casein (11.5 kDa) bands in raw cow skim milk and its chyme sediments in rats. γ-Casein is known to be a natural product of the hydrolysis of α_s- and β-caseins by the milk proteolytic enzyme plasmin (Bastian & Brown, 1996). Thus, the ~20 and 12 kDa bands observed in this study could have been γ-casein bands. Ye *et al.* (2016b) also observed the presence of a ~20 kDa band in cow skim milk clots. The results obtained for the raw cow skim milk clot are in agreement with previous studies on cow skim milk (Miranda & Pelissier, 1983; Ye *et al.*, 2016b).

The protein hydrolysis profile of the raw sheep milk clots showed a very similar pattern to that of the raw cow milk clots with a few exceptions (Figure 5.16, RSSM). A ~16.5 kDa band and a new band at ~19 kDa appeared in the sheep milk clots only after 120 min of digestion. Also, the ~10 kDa band that was present in the cow milk clots appeared to be absent from the sheep milk clots. Relatively higher amounts of α-lactalbumin were entrapped in the sheep milk clots than in the cow milk clots, as indicated by its band intensities. The protein digestion profile of the raw goat milk clots (Figure 5.16, RGSM) also showed a similar pattern to that of the raw cow and sheep milk clots. However, both the ~16.5 kDa band and the ~10 kDa band appeared to be absent from the raw goat milk clots. Also, the α-lactalbumin band intensities of the raw goat milk clots appeared to be higher than those of the raw cow and sheep milk clots. In contrast, the β-lactoglobulin band intensities of the raw goat milk clots were considerably lower than those of the raw cow and sheep milk clots.

The pasteurized milk clots from all species (Figure 5.16, PCSM, PGSM, and PSSM) followed a similar trend to their respective raw milk clots; however, the intensities of the whey protein bands (β-lactoglobulin, lactoferrin, serum albumin, and immunoglobulin) associated with the pasteurized milk clots increased markedly, especially for goat milk and sheep milk. In addition, towards the end of digestion, greater amounts of peptides appeared to be generated by the pasteurized goat milk clots than by the pasteurized sheep and cow milk clots. Also, in the pasteurized goat milk clots, new bands appeared at ~19 kDa and 16.5 kDa at ~90 and 30 min of digestion, respectively, the intensities of which increased thereafter. These bands were absent from the raw goat milk clots. These results indicated that pasteurization may have had a greater impact on the hydrolysis of goat milk proteins than of cow milk proteins.

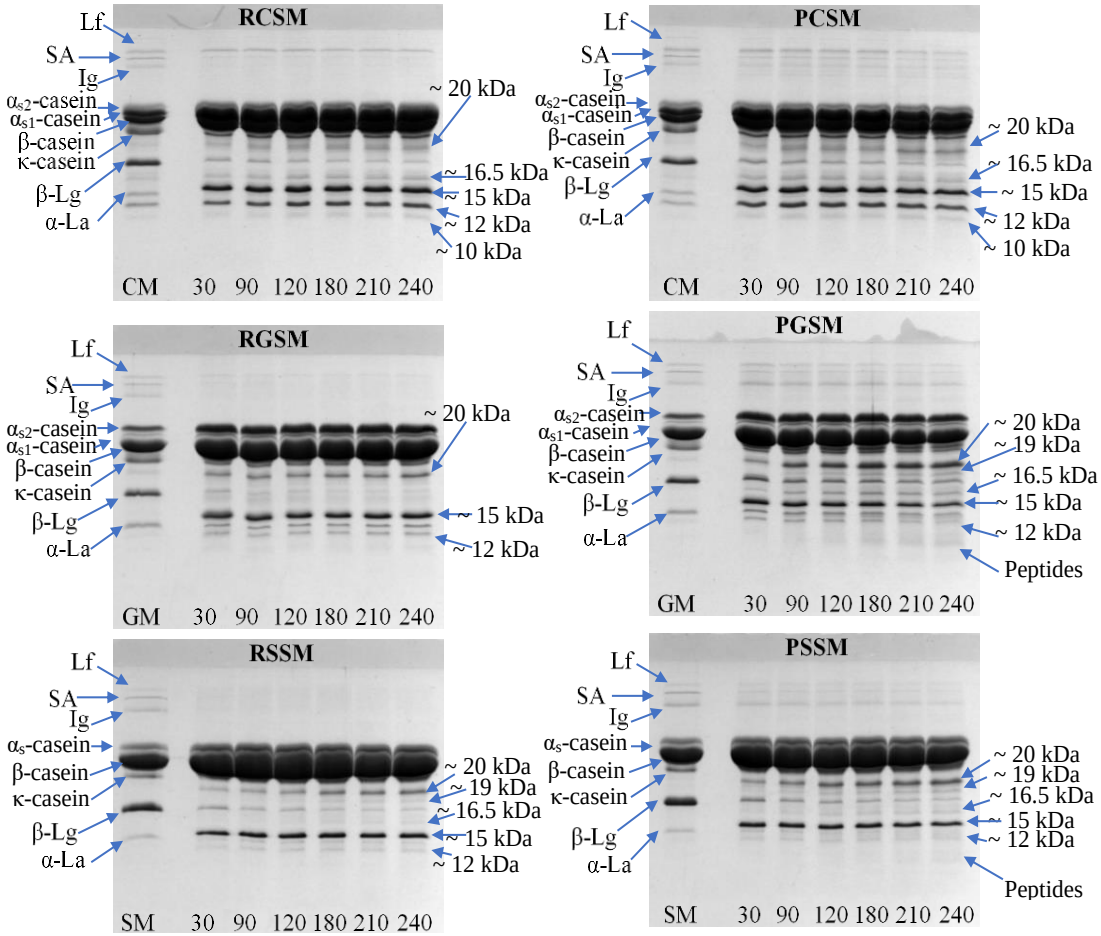


Figure 5.16 SDS-PAGE patterns of the freeze-dried clots (~36 mg of protein in each lane) collected during the gastric digestion of raw and pasteurized skim milk in the HGS at different times (30, 90, 120, 180, 210, and 240 min) (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk; CM, cow skim milk; GM, goat skim milk; SM, sheep skim milk; Lf, lactoferrin; SA, serum albumin; Ig, immunoglobulin; β-Lg, β-lactoglobulin; α-La, α-lactalbumin).

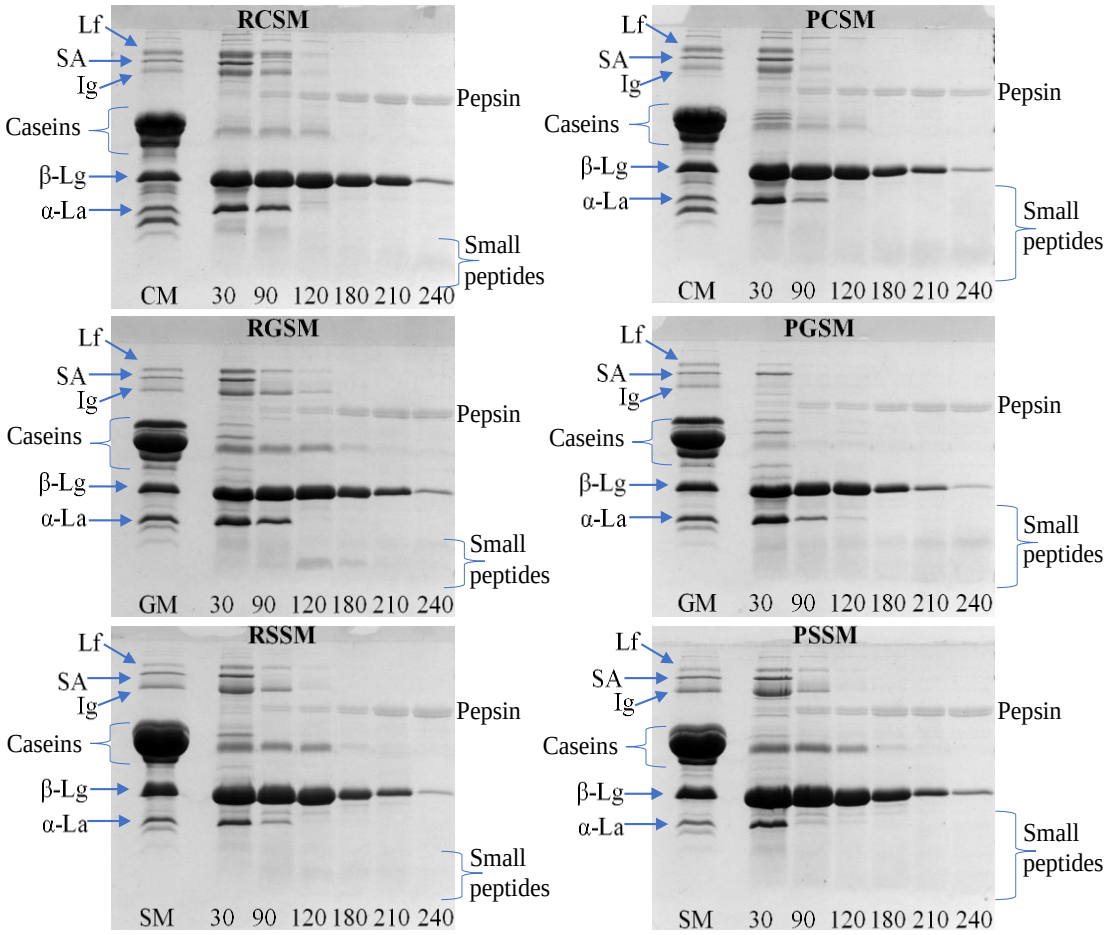


Figure 5.17 SDS-PAGE patterns of the liquid chyme (~69 µg of protein in each lane) collected during the gastric digestion of raw and pasteurized skim milk in the HGS at different times (30, 90, 120, 180, 210, and 240 min) (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk; PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk; CM, cow skim milk; GM, goat skim milk; SM, sheep skim milk; Lf, lactoferrin; SA, serum albumin; Ig, immunoglobulin; β-Lg, β-lactoglobulin; α-La, α-lactalbumin).

Figure 5.17 shows the SDS-PAGE patterns of the protein compositions of the liquid chyme. Analysis of the liquid chyme of raw cow skim milk (Figure 5.17, RCSM) showed the presence of lactoferrin, serum albumin, immunoglobulins, β-lactoglobulin, and α-

lactalbumin at 30 min of digestion. The intensities of the lactoferrin, serum albumin, immunoglobulin, and α-lactalbumin bands decreased rapidly and these bands disappeared completely within 120 min of digestion (i.e., pH ~3.5–4.4). The intensity of the β-lactoglobulin band decreased gradually with digestion time (especially after 120 min onwards) and only a faint β-lactoglobulin band was present at 240 min. In addition, a smaller amount of caseins was present in the liquid chyme at 30 min, which could have been due to the more open casein network of the clot initially, leading to the delivery of very fine casein particles in the liquid chyme; the intensity of these casein bands decreased rapidly during the digestion. Some small peptides were observed at all digestion points in the liquid chyme which could be the hydrolysis products of both caseins and whey proteins. It should also be noted that the products of casein hydrolysis (those observed in the clots, Figure 5.16) were not observed in the liquid chyme, which indicated that the hydrolysed products of the caseins remained bound within the clots (Figures 5.16 and 5.17), either via hydrophobic interactions or because of tight physical entrapment within the clot. Similar observations were made for the liquid chyme of the pasteurized cow milk (Figure 5.17, PCSM).

SDS-PAGE experiments were run on equal protein basis at all digestion timepoints, thus the dilution of the liquid chyme at different digestion time due to continuous addition of SGF did not affect the results for different milks. The disappearance of α-lactalbumin within 120 min (pH ~3.5–4.4) of gastric digestion in this study was due to its susceptibility to pepsin hydrolysis below pH 4 due to the change in its protein conformation (Miranda *et al.*, 1989). Native β-lactoglobulin is considered to be very resistant to digestion (Kitabatake & Kinekawa, 1998; Reddy *et al.*, 1988) by human gastric enzymes as well as commercial porcine pepsin. Under the conditions of this study,

β-lactoglobulin band intensity began to gradually decrease especially after 120 min onwards (when overall pH was <4) indicating its hydrolysis by pepsin. This is in agreement with previous observations of Peram *et al.* (2013) and Sarkar *et al.* (2009), who reported that ~20-35% of native β-lactoglobulin was hydrolysed by porcine pepsin after 2h of *in vitro* static incubation at pH 1.5; they also reported that a higher pepsin to substrate (β-lactoglobulin) ratio may have been the reason for the observed degradation of native β-lactoglobulin in their studies.

The protein hydrolysis profiles of the raw and pasteurized goat milk (Figure 5.17, RGSM and PGSM) and sheep milk (Figure 5.17, RSSM and PSSM) liquid chyme were observed to be similar to those of the raw and pasteurized cow milk liquid chyme (Figure 5.17, RCSM and PCSM), despite the differences in protein composition of the different milks. It should be noted that the theoretical pepsin-to-protein concentration used during gastric digestion in this study was kept the same for all milk, considering the differences in their initial protein contents.

5.4.9 Microstructure of the clots

The microstructures of the wet clots formed from the raw and pasteurized milk of all species at 15, 90, and 240 min of digestion were observed by confocal scanning laser microscopy (Figures 5.18 and 5.19). At 15 min, the protein networks in the clots from the raw milk of all species appeared to be loose with void spaces. These void spaces were filled with liquid entrapped within the protein network. As the digestion progressed, the clots became more cohesive and expelled the entrapped liquid; consequently, the protein network became tighter with fewer void spaces (Figure 5.18). The protein network in the 240 min clot appeared to be smooth, indicating that it was much stronger and connected

towards the end of digestion than at the beginning, i.e., at 15 min, of digestion. The decrease in the wet weight of the clots observed during the early stages of gastric digestion (Figure 5.11) could be attributed to these changes in the cohesiveness and denseness of the microstructure of the clots (Figure 5.18). The microstructural observations further support the results reported for the change in the moisture content of the clots during digestion, i.e., greater moisture content of the clots at 15 min than at 90 and 240 min (irrespective of the milk species). Although there were slight apparent differences in the microstructures of the clots formed from raw cow, goat, and sheep milk, it was not possible to draw any definite conclusions. Ye *et al*. (2016b) also observed in that the clot from raw cow skim milk had a closely knitted protein network with numerous aqueous pore spaces (at 20 min), which as the digestion progressed became denser and fused into a smooth block with little porosity at a long digestion times (220 min).

The microstructures of the pasteurized milk clots from all species at 15, 90, and 240 min of digestion followed a similar pattern to those of the raw milk clots (Figure 5.19). However, the clots formed by the pasteurized milk at 90 and 240 min of digestion (Figure 5.19) appeared to have slightly more open protein networks with a large number of pores compared with those of raw milk clots (90 and 240 min, Figure 5.18), especially for goat milk and sheep milk.

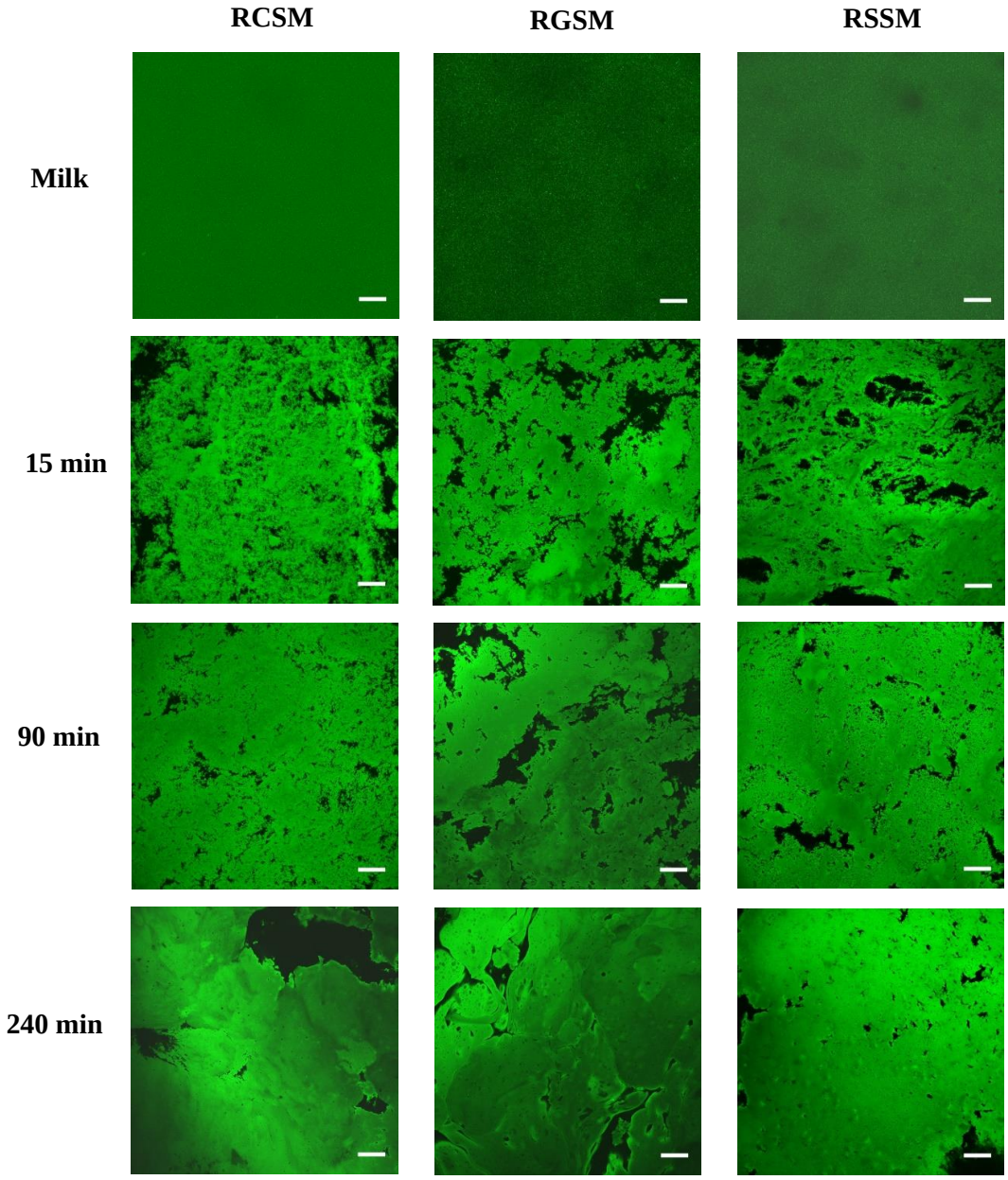


Figure 5.18 Confocal micrographs of the clots obtained during the gastric digestion of different raw skim milk in the HGS at different times (RCSM, raw cow skim milk; RGSM, raw goat skim milk; RSSM, raw sheep skim milk). Scale bars represent 25 µm.

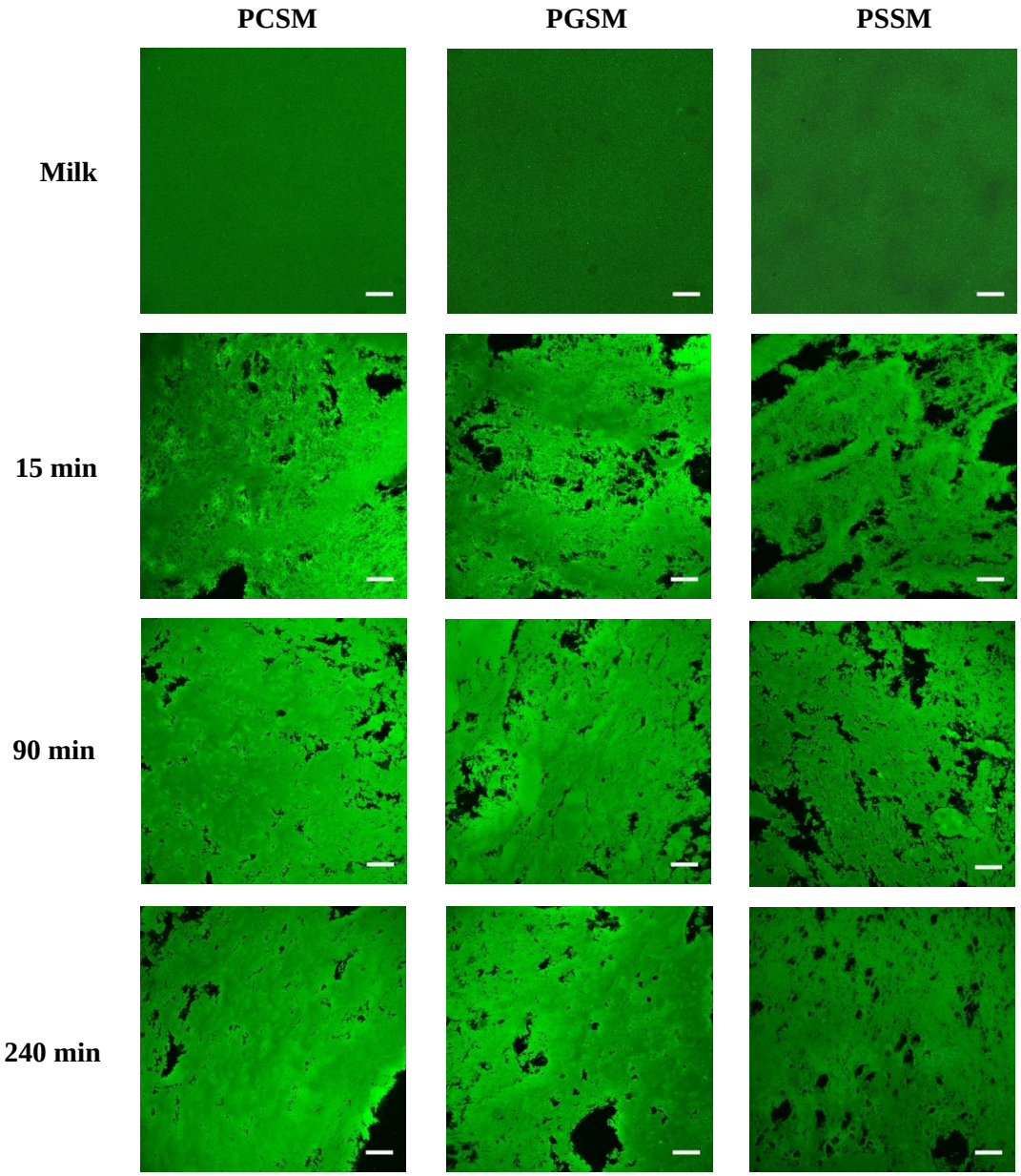


Figure 5.19 Confocal micrographs of the clots obtained during the gastric digestion of different pasteurized skim milk in the HGS at different times (PCSM, pasteurized cow skim milk; PGSM, pasteurized goat skim milk; PSSM, pasteurized sheep skim milk). Scale bars represent 25 µm.

5.4.10 Texture analysis of the residual clots

It should be noted that the texture analysis results are an indicative measurement only, given the heterogeneous and dynamic nature of the clots during gastric digestion. The forces required to penetrate the clots from outside for each species after 240 min of digestion are shown in Figure 5.20.

There were no significant differences ($P > 0.05$) in the mean force required to penetrate the raw goat and sheep skim milk clots from outside, when compared with the raw cow skim milk clots. However, there was a significant difference ($P < 0.05$) between the mean forces required to penetrate the raw goat and sheep skim milk clots from outside (Figure 5.20, OUTSIDE). The mean forces required to penetrate the pasteurized skim milk clots from outside were observed to be significantly lower than those required to penetrate their raw milk counterparts, except for sheep milk for which the values were lower but not significantly ($P > 0.05$) different (Figure 5.20, OUTSIDE). In addition, the pasteurized sheep skim milk clots were significantly ($P < 0.05$) firmer than the pasteurized cow and goat skim milk clots, for both of which the firmness was similar (Figure 5.20, OUTSIDE).

The lower hardness observed for the pasteurized milk clots possibly indicated the slightly more open structure of these clots. Pasteurization (72°C, 15 s) would be expected to cause a small degree of denaturation of whey proteins (Guinee *et al.*, 1998). The denatured whey proteins are known to form complexes with the κ-casein at the casein micelle surface that can interfere with the aggregation of casein micelles during coagulation by chymosin (Kethireddipalli *et al.*, 2010, 2011). This could be a reason for the lower firmness of the pasteurized milk clots observed in this study. Both chymosin and pepsin belong to the same group of aspartic proteinases that hydrolyses the Phe_{105}–Met_{106} bond

of κ-casein and has activity in the pH range 6-7 (Crabbe, 2004; Moschopoulou, 2011; Piper & Fenton, 1965). As the site of action of both chymosin and pepsin is the same, the mechanism of action of chymosin and pepsin would be expected to be similar in relation to milk clotting.

Another penetration experiment was conducted to analyse the firmness of the inner core of the clots; the clots were cut into 2 halves and the force required to penetrate the inner core was measured (Figure 5.20, CORE). The results indicated that the inner cores of the raw and pasteurized sheep skim milk clots were firmer than those of the raw and pasteurized cow (as well as goat) skim milk clots (Figure 5.20, CORE). It should be noted that the inner cores (Figure 5.20, CORE) of the raw and pasteurized milk clots from all species were significantly ($P < 0.05$) firmer than their outside layers (Figure 5.20, OUTSIDE), except for the pasteurized goat milk clots for which the force required to penetrate the inside core was not significantly ($P > 0.05$) different from that required to penetrate the outside layers (Figure 5.20).

The relatively higher firmness observed for the sheep milk clots could have been due to their lower moisture content, as observed earlier (Figure 5.13, 240 min). Sheep milk is also known to form stronger gels (with glucono-δ-lactone or enzymes) primarily because of its higher casein content compared to goat milk and cow milk (Ould Eleya *et al.*, 1998; Storry *et al.*, 1983), which have contributed to the formation of a stronger clot by sheep milk in the HGS.

The greater degree of softness of the outside surface of the clot was due to its continuous contact with the surrounding liquid in the HGS and reduced penetration of liquid to the

inner core due to its firm texture. This supports the previous findings of Ye *et al.* (2016b) and Mulet Cabero (2018), who reported that the proteins in the outer layer were hydrolysed faster than the proteins in the inner core of the cow milk clots because of the lower pepsin diffusion to the core (inside) due to the compact and dense nature of the clot.

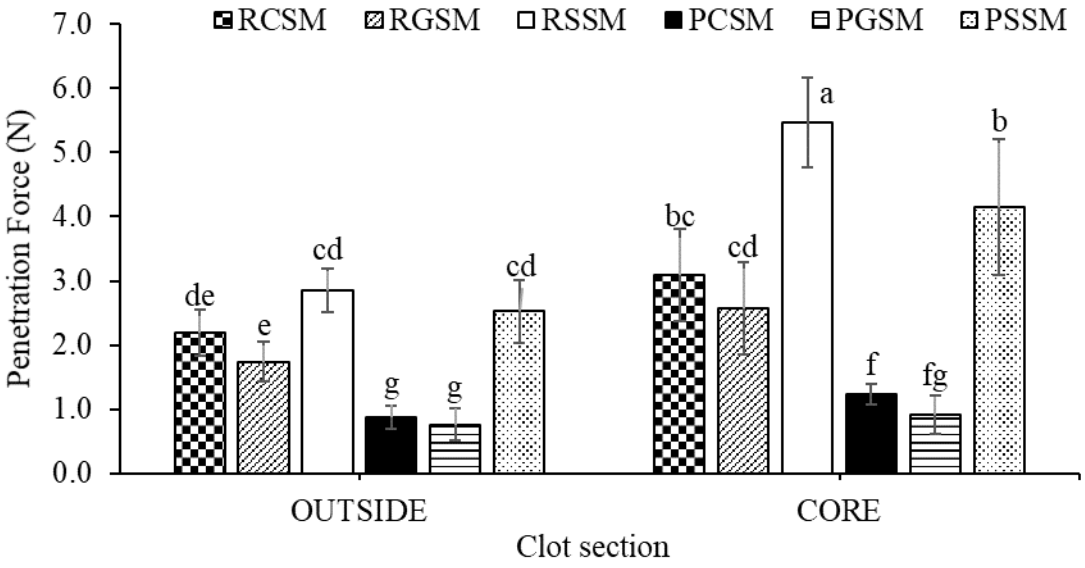


Figure 5.20 Force (N) required to penetrate the skim milk clots remaining in the HGS at 240 min (OUTSIDE represents the measurements taken on complete clot when penetrated from outside; CORE represents the measurements taken on half cut clot when penetrated from inside i.e., at the core). Different letters (a-g) above bars indicate significant differences (P < 0.05). Each data point represents mean ± SD.

5.5 Conclusions

The study provided newer insights and an improved understanding about the coagulation behaviour of non-cow milks. Raw skim milk from cow, goat, and sheep separated into a clot and a liquid phase in the HGS. The amount of the clot formed at the beginning of the gastric digestion was directly proportional to the protein and total solids contents of the different skim milk. The macro- and microstructures of the clots formed from all skim milk underwent similar dynamic changes throughout digestion. Compared with the raw

cow and goat skim milk, the raw sheep skim milk had relatively firmer and less hydrated clots. Although the goat and sheep skim milk protein compositions (concentrations of α_{s1}-, α_{s2}-, and β-caseins as well as whey proteins) varied compared with the cow skim milk composition, the hydrolysis patterns (SDS-PAGE) of the different proteins appeared to be similar for all milk. The gastric digestion behaviours of the pasteurized milk from all species also appeared to be similar to those of their raw milk counterparts, with only slight differences in the rates of protein hydrolysis. The pasteurized goat milk clot appeared to have a higher rate of casein hydrolysis compared with its raw milk counterpart and compared with the pasteurized milk from other species, as indicated by SDS-PAGE. Further studies are necessary to elucidate how fat globules are affected during the gastric digestion of milk. Thus, in the next chapter, the impact of intra-gastric coagulation on the *in vitro* digestion of fat globules of different mammalian whole milk was investigated.

Chapter 6: Impact of gastric coagulation on the kinetics of release of fat globules from milk of different species[4]

6.1 Abstract

The behaviour of fat globules during the gastric digestion of raw and pasteurized (non-homogenized) cow, goat, and sheep whole milks was studied using a human gastric simulator. Microstructural and physicochemical analysis revealed that, initially, the coagulation of the milks in the human gastric simulator resulted in the majority of the milk fat globules being entrapped within the curd. As the digestion progressed, the proportion of fat globules entrapped within the aggregated protein matrix (curd) decreased; there was also some flocculation as well as coalescence of the fat globules within the curd. The liberation of the entrapped fat globules from the curd to the liquid phase of the chyme was strongly dependent on the disintegration and hydrolysis of the structured casein network. Surprisingly, the fat globules released (or already present) into the liquid phase of the chyme were not as extensively coalesced as those remaining within the curd. These phenomena were observed to be similar for the raw and pasteurized whole milk of all species. The pasteurized whole milk from all species formed relatively less structured coagula compared with their raw milk counterparts, leading to a greater extent of protein breakdown and, thus, higher proportions of fat release from the pasteurized milk curds.

[4] Part of the contents presented in this chapter has been submitted to be published as a peer-reviewed paper: Roy, D., Ye, A., Moughan, P. J., and Singh, H. (under review). Impact of gastric coagulation on the kinetics of release of fat globules from milk of different species. *Targeted Journal - Food and Function.*

6.2. Introduction

Milk is nature's most complex oil-in-water liquid emulsion (Singh & Gallier, 2017). Milk fat is one of the most complex components of milk in terms of both its composition and its structure (Gallier & Singh, 2020; Lopez *et al.*, 2019). The majority of the milk fat (approximately 95–98%) is composed of triglycerides, and the remaining proportion consists of diacylglycerides, phospholipids, cholesterol, free fatty acids, monoacylglycerides, and other minor components (Jensen, 2002). The triglycerides in the milk of all species are present as small spherical droplets, called fat globules (Singh, 2006), the diameter (size) of which varies among the milk of different species (Claeys *et al.*, 2014; Gantner *et al.*, 2015). The fat globules are surrounded, protected, and stabilized by a complex phospholipid trilayer (along with specific membrane proteins and cholesterol) called the milk fat globule membrane (MFGM), which is unique to milk (Lopez *et al.*, 2019; Lopez *et al.*, 2010). The complex structure of the milk fat globule is considered to be similar in the milk of all species studied to date, although the proportions of different fatty acids and MFGM components may differ among the different species (Gallier & Singh, 2020; Nguyen *et al.*, 2017).

Some recent studies have reported that the changes in the structure of the matrix surrounding the milk fat during gastric digestion may have an impact on the release and digestion behaviour of milk fat globules. Ye *et al.* (2016a) studied the gastric digestion of raw (unheated) and heated (90°C for 20 min) cow whole milk using a dynamic human gastric simulator (HGS). They reported that the fat globules were embedded within the protein clot formed in the HGS, and that the release of the fat globules was dependent on the structure and disintegration characteristics of the clots; consequently, the release of fat globules was greater from the less structured clots formed from heated whole milk

than from the firm clots formed from raw whole milk. Similar observations have been reported using *in vitro* digestion assays for differently processed whole milks (Ye *et al.*, 2017; Ye *et al.*, 2019b). Therefore, it appears that the bioaccessibility of the fat globules from milk is influenced by the coagulum structure in which the fat globules are entrapped in the stomach.

The milk of different species are known to vary in fat globule size and composition, which have been reported to be responsible for their different digestion behaviours (Alférez *et al.*, 2001; Claeys *et al.*, 2014; Gantner *et al.*, 2015; Meena *et al.*, 2014; Teng *et al.*, 2020). However, little attention has been given to the structural changes that occur in the whole milk matrix during gastric digestion, and how they influence the digestion and delivery of fat globules in the gastrointestinal tract.

It was hypothesized that the coagulation of the proteins from the milk of different species plays a crucial role in influencing the state and liberation of the fat globules during gastric digestion. Therefore, the comparative structural changes in raw and pasteurized (non-homogenized) whole milk of different species using an *in vitro* dynamic gastric digestion model, i.e., the HGS was investigated. This study was built on the previous chapter (Chapter 5). Moreover, this is the first study to report on the dynamic *in vitro* gastric digestion behaviours of goat and sheep milk fat globules.

6.3. Materials and methods

6.3.1 Materials

Pooled raw cow (January–May 2018), goat (January–May 2018), and sheep (January–April 2019) whole milks were obtained under chilled conditions from Massey University No. 4 dairy farm (Palmerston North, New Zealand), Dairy Goat Co-operative (Hamilton, New Zealand), and Neer Enterprises Limited (Carterton, New Zealand), respectively. Other experimental materials needed were as per the protocol described in Section 3.1 (Chapter 3).

6.3.2 Methods

6.3.2.1 Milk processing and storage

Some of the raw whole milk was pasteurized (non-homogenized) at 72°C for 15 s and cooled to 4°C. Sodium azide (0.02% wt/wt) (Merck KGaA, Darmstadt, Germany) was added to all samples to prevent microbial growth and the milk samples were stored at 4°C until further use.

6.3.2.2 Whole milk composition

The chemical composition of whole milk was analysed as per the protocol described in Section 3.2.2.1 (Chapter 3).

6.3.2.3 Fat globule size

The fat globule diameter of the skim milks was determined as per the protocol described in Section 3.2.2.3 (Chapter 3).

6.3.2.4 Casein micelle size

The casein micelle diameter of the skim milks was determined as per the protocol described in Section 3.2.2.4 (Chapter 3).

6.3.2.5 *In vitro* gastric digestion

In vitro gastric digestion procedure and the HGS set-up was similar to that of Chapter 5 (Section 5.3.2.4) with an exception that the combined pH of Solution A (176 mL of SGF containing HCl) + Solution B (24 mL of SGF, pH 7) was in the range 1.0–1.3 to achieve a gradual decrease in pH of the different milks over a period of 240 min of digestion, i.e., 4 h.

Gastric lipase digests approximately 10-30% of the ingested lipid (Pafumi *et al.*, 2002). However, gastric lipase was not included, because a suitable alternative to human gastric lipase with similar activity and specificity (such as rabbit gastric lipase), as suggested by Minekus *et al.* (2014) was commercially unavailable when this study was started.

6.3.2.6 pH measurement

The pH of the milks and chyme was measured as described in the Section 5.3.2.5 (Chapter 5).

6.3.2.7 Wet and dry weights of the curds

The wet and dry weight of the curds was measured as described in the Section 5.3.2.6 (Chapter 5).

6.3.2.8 Protein and fat contents of the curds

The total protein (total nitrogen × 6.38) and fat contents of the curds (freeze dried and ground powder) were analysed by the Dumas method 968.06 (AOAC, 2005) and the Mojonnier method 989.05 (AOAC, 2005), respectively.

6.3.2.9 Confocal laser scanning microscopy (CLSM)

A Leica SP5 upright confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) at the Manawatu Microscopy and Imaging Centre (Massey University, Palmerston North) was used for high-resolution imaging of the microstructure of the curd and liquid chyme samples at 15, 90, and 240 min of digestion. A 1.0% (wt/vol) solution of Fast Green (dye content 90%) in water was used to stain protein and a 0.1% (wt/vol) solution of Nile Red in acetone was used to stain fat (He–Ne laser with an excitation line at 633 nm). A procedure similar to that reported by Ye *et al.* (2016a) was followed. At different digestion timepoints, curd samples were collected and immediately cut into thin sections using a surgical blade. The thinly sliced curd samples as well as the liquid chyme samples were then stained with a 1:1 mixture of both dyes for about 10 min. Just before staining, the mixture of dyes was centrifuged at 13,000 rev/min (Microcentrifuge MiniSpin plus, Eppendorf AG, Hamburg, Germany) and filtered through a 0.22 µm filter (13 mm, PVDF, Thermo Fisher Scientific) to remove any crystals formed in the dye mixture. The stained samples were then placed on double concave microscope slides (clear glass, ground edges, 26 mm x 76 mm, 1.2–1.3 mm thick; Sail, Sailing Medical-Lab Industries Co. Ltd, Suzhou, China), covered with coverslips, and examined with a 63x-oil immersion objective (numerical aperture = 1.4). Care was taken to avoid areas near the sides or close to the top of the microscope slide while imaging.

Multiple fields were viewed, and typical micrographs are presented. The imaging was completed within 40 min of stopping the digestion at the respective times.

6.3.2.10 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Reducing SDS-PAGE was performed on the milk curds (freeze dried and ground powder) as well as on the liquid chyme samples to visualize the time-dependent hydrolysis of the proteins in the gastric chyme samples, as described by Ye *et al.* (2016a). The detailed method is described in the Section 3.2.2.5 (Chapter 3).

6.3.2.11 Statistical analysis

The experimental data were subjected to analysis of variance (ANOVA) tests using Minitab statistical software (version 18.1, Minitab Inc., State College, PA, USA). The changes in pH, wet weight, dry weight, protein, fat, and fat globule sizes during gastric digestion were analysed using three-way ANOVA with species (cow, goat, and sheep), processing (raw and pasteurised), and digestion time (30 to 240 min), and their interaction as fixed effects. The changes in texture of the curds obtained after 240 min of gastric digestion were analysed using three-way ANOVA with species (cow, goat, and sheep), processing (raw and pasteurised), and section (CORE and OUTSIDE), and their interaction as fixed effects. If statistically significant interaction effects were found, then further analysis was conducted using the Tukey pairwise comparison test. The correlations between protein and fat lost from the curds were also determined. Differences in physicochemical composition were analysed using one-way ANOVA and the Tukey test. The probability level for statistical significance was $P < 0.05$. Statistical analysis was carried out on the results from at least two different batches of milk and values (or data points) are expressed as mean ± SD (standard deviation).

6.4. Results and discussion

6.4.1 Chemical and physicochemical properties of different whole milks

The chemical composition, fat globule size, and casein micelle size of the cow, goat, and sheep whole milks are reported in Table 6.1. Compared with cow milk, sheep milk was significantly ($P < 0.05$) higher in dry matter, protein content, and calcium content, whereas goat milk had significantly ($P < 0.05$) lower proportions of these components. Sheep milk also had the highest content of total inorganic phosphorus, whereas the inorganic phosphorus levels were similar for cow milk and goat milk. Goat milk had the lowest fat content, and sheep milk and cow milk had similar fat contents. The interspecies differences in the nutrient compositions observed are consistent with those reported in previous chapters (Chapters 4 and 5). However, the milk composition within and between species may vary considerably because of different regions, feeds, breeds, stages of lactation, milking intervals, and time of the year (Barlowska *et al.*, 2011; Claeys *et al.*, 2014).

Cow milk had the highest volume weighted (D_{43}) fat globule mean diameter (~4.3 μm) ($P < 0.05$) followed by sheep milk (~4.1 μm) and goat milk (~3.9 μm) (Table 6.1). Similarly, the surface weighted (D_{32}) fat globule mean diameter of cow milk (~3.6 μm) was higher ($P < 0.05$) than that of sheep milk (~3.5 μm) and goat milk (~3.0 μm). These results are in line with previous studies, which have reported that goat and sheep milk have smaller fat globule sizes than cow milk (El-Zeini, 2006; Gantner *et al.*, 2015; Park *et al.*, 2007). The Z-average diameters (nm) of the casein micelles (Table 6.1) of goat milk (~196 nm) and sheep milk (~197 nm) were significantly ($P < 0.05$) larger than those of cow milk (~157 nm), which is in agreement with results reported previously (Nguyen *et al.*, 2018; Park *et al.*, 2007).

Table 6.1 Physicochemical compositions of cow, goat, and sheep whole milks.

Composition/Property	Cow milk	Goat milk	Sheep milk
Dry matter (%)	15.61 ± 0.08^{b}	11.96 ± 0.60^{c}	18.13 ± 0.71^{a}
Protein (%)	4.85 ± 0.01^{b}	3.40 ± 0.06^{c}	6.35 ± 0.53^{a}
Fat (%)	5.70 ± 0.06^{a}	3.66 ± 0.26^{b}	6.27 ± 0.53^{a}
Carbohydrate (by difference, %)	4.20 ± 0.03^{a}	4.03 ± 0.26^{a}	4.56 ± 0.41^{a}
Ash (%)	0.86 ± 0.02^{b}	0.86 ± 0.03^{b}	0.94 ± 0.05^{a}
Calcium (mg/100 mL)	150.51 ± 1.95^{b}	108.40 ± 3.47^{c}	180.81 ± 17.32^{a}
Inorganic phosphorus (mg/100 mL)	92.97 ± 3.50^{b}	95.84 ± 4.82^{b}	144.80 ± 31.10^{a}
Protein-to-fat ratio	0.85 ± 0.01^{b}	0.93 ± 0.05^{ab}	1.02 ± 0.09^{a}
Fat globule size			
D_{43} (μm)	4.33 ± 0.09^{b}	3.84 ± 0.06^{c}	4.08 ± 0.04^{a}
D_{32} (μm)	3.61 ± 0.07^{b}	3.00 ± 0.04^{c}	3.49 ± 0.03^{a}
Casein micelle size, Z-average diameter (nm)	157.25 ± 2.92^{b}	196.43 ± 8.53^{a}	196.60 ± 3.05^{a}

$^{a–d}$Values within each row with different superscripts are significantly different (P <0.05). Values are reported as mean ± SD.

6.4.2 Changes in the intragastric pH

The changes in the pH of the liquid chymes from the raw and pasteurized whole milk of different species as a function of the digestion time in the HGS are shown in Figure 6.1. The pHs of the raw whole milk of different species were adjusted to achieve a pH of 2 at the end of the 240 min of digestion (i.e., 4 h). As the raw whole milk of the different species varied slightly in buffering capacity, the total amounts of 6 M HCl added to reach pH ~2 at the end of 240 min were ~4.2, ~2.2, and ~3.2 mL for the cow, goat, and sheep milk, respectively; such differences were probably due to the differences in protein content, mineral content, and disintegration behaviour of the curds formed from the different milks.

The pasteurized whole milk of all species had a slightly slower drop ($P < 0.05$) in pH (especially from 60 min onward) compared with their raw milk counterparts (Figure 6.1), which indicated that the pasteurized whole milk of the different species would have needed a greater amount of 6 M HCl compared with their respective raw whole milks to drop the pH to 2 during the 4 h of gastric digestion. The relatively slower drop in pH of the pasteurized milks was probably due to their more disintegrated curd structure compared with their raw milk counterparts (as visually observed, Figures. 6.2 and 6.3), which would have led to more dissolved proteins in the liquid chyme and, thus, a slower decrease in pH. It has to be noted that the open curd structure of pasteurised milks may allow easier diffusion of gastric acid into the curds and subsequently faster release and mixing of phosphate ions which may have then sequestered hydrogen ions to form hydrogen phosphate and resulted in an increase in pH. In contrast to the pH of the liquid chyme, the core pH of the curds (aggregated proteins) formed from all the whole milks at the end of 240 min of gastric digestion remained higher i.e. ~pH 3.0-5.0.

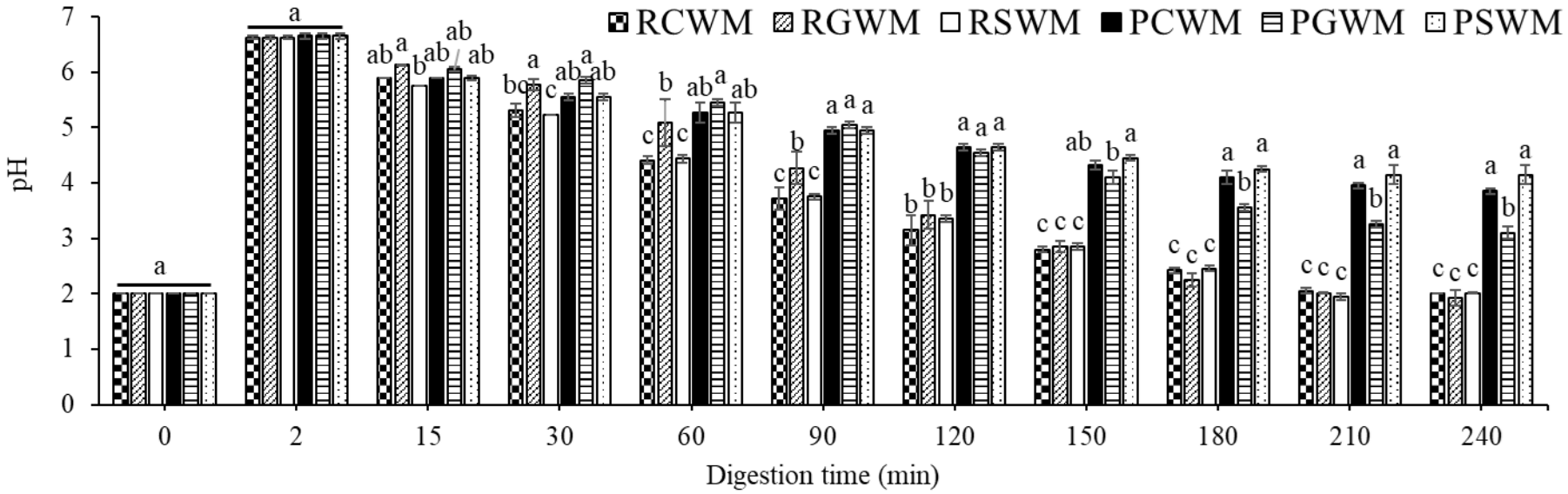


Figure 6.1 Changes in pH of the liquid chymes from the whole milks during gastric digestion in the HGS (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). Different letters (a–c) above the bars represent significant differences among the milk samples at a given digestion timepoint (P < 0.05); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD.

6.4.3 Intragastric coagulation of casein micelles in the whole milks

Visual signs of curd formation were observed within the first 6–8 min of gastric digestion in the HGS for all whole milk samples, soon after which both the raw and the pasteurized cow, goat, and sheep whole milks separated into a curd and a liquid phase at approximately 10–11 min (pH ~6.0), 8–9 min (pH ~6.2), and 10–12 min (pH ~6.0), respectively. Similar pHs of coagulation have been reported in previous studies (Ye *et al.*, 2016a, 2017) during the gastric digestion of cow whole milk. No reports on the coagulation behaviours of goat and sheep whole milks during gastric digestion are available. In the previous study on the gastric digestion of skim milk from different species (Chapter 5), it was found that the casein micelles in goat and sheep skim milks coagulated at pH ~6.1–6.3 under similar experimental conditions (Chapter 5). The formation of the curd at pH ~6 in the whole milk of all species was due to destabilization and aggregation of the casein micelles, caused by the hydrolysis of the Phe_{105}–Met_{106} bond of κ-casein into para-κ-casein and caseinomacropeptide by pepsin (Jollès, 1966; Miranda & Pelissier, 1983; Tam & Whitaker, 1972). The curds and the liquid phases obtained during gastric digestion in this study are shown in Figures 6.2 and 6.3. The liquid in the gastric phase consisted mainly of the water-soluble components, such as whey proteins and lactose.

Initially, the curd that was formed had significant amounts of entrapped liquid (Figure 6.4), but this was expelled from the curd, leading to a more cohesive mass as the digestion progressed. At the same time, the curd disintegrated because of the mechanical action of the stomach as well as the increased proteolysis (caused by the activity of pepsin with the decrease in pH) during gastric digestion. The curds from the pasteurized whole milk of all species appeared to be more fragmented visually than those from their raw milk

counterparts (Figures 6.2 and 6.3). This may have been due to the small extent of heat-induced covalent interactions between the denatured whey proteins and the κ-caseins at the surface of the casein micelles in the milks under pasteurization conditions (72°C, 15 s) (Dalgleish, 1990; Dannenberg & Kessler, 1988; Guinee *et al.*, 1998; Kethireddipalli *et al.*, 2010, 2011). Such interactions may have hindered the formation of a strong protein network in the pasteurized milk curd compared with the raw milk curd (Kethireddipalli *et al.*, 2010, 2011).

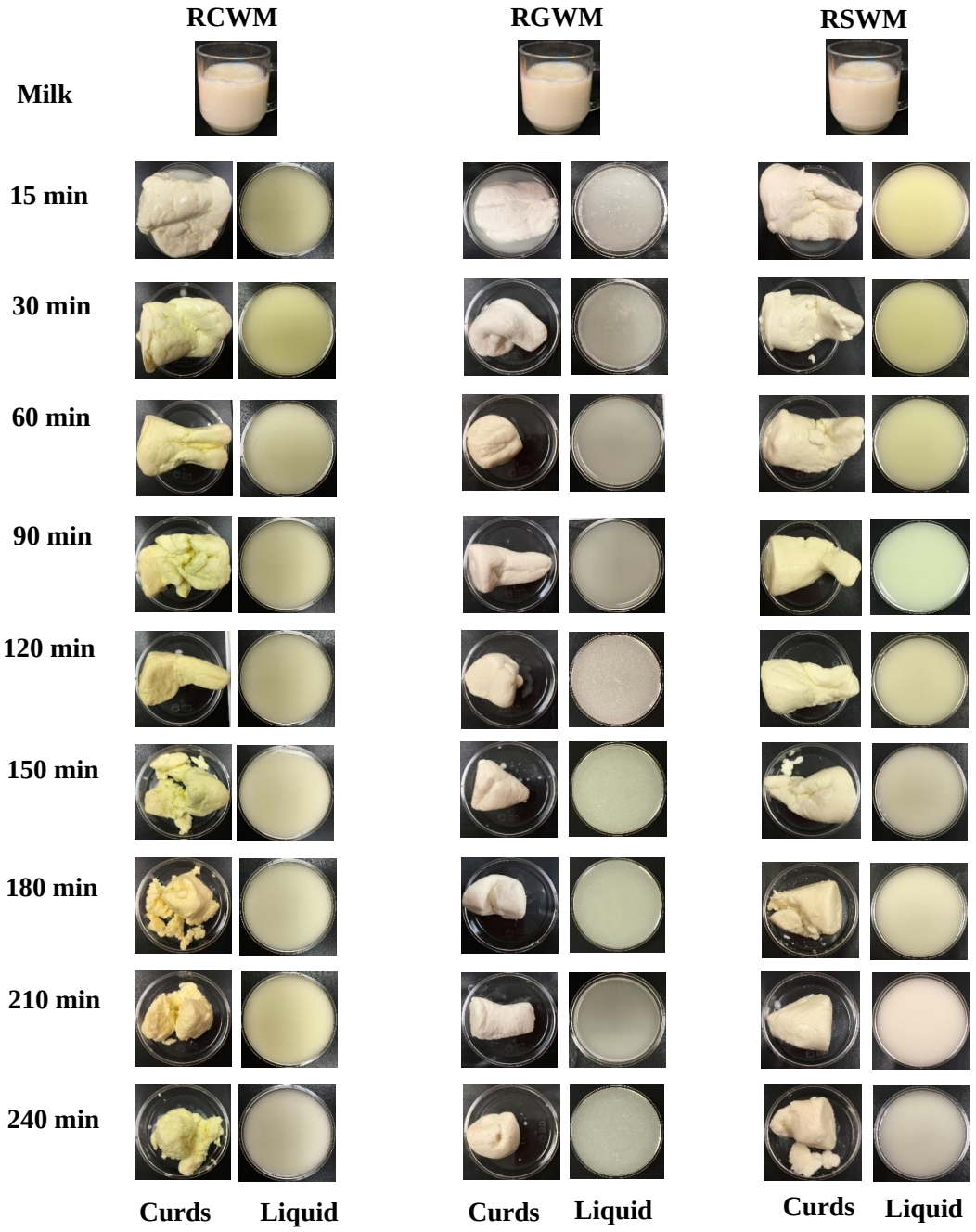


Figure 6.2 Photographs of the curds and liquid chymes during the gastric digestion of 200 g of raw whole milk at different digestion times in the HGS. RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk.

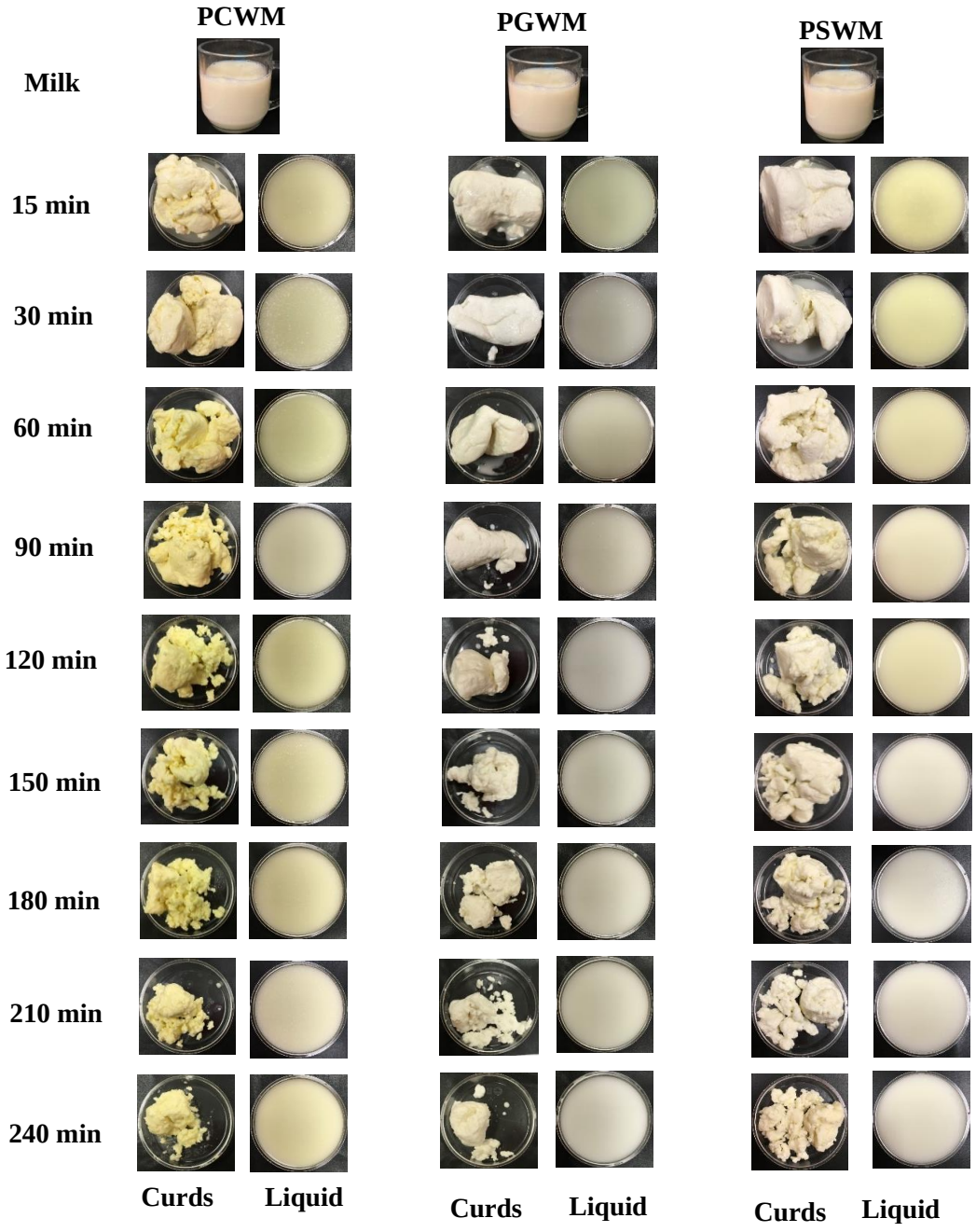


Figure 6.3 Photographs of the curds and liquid chymes during the gastric digestion of 200 g of pasteurized whole milk at different digestion times in the HGS. PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk.

6.4.4 Wet weight and dry weight of the curds

The wet and dry weights of the curds obtained during the gastric digestion of 200 g of raw and pasteurized whole milk of different species as a function of time are shown in Figures 6.4A and 6.4B.

The rates of disintegration of the curds varied in the milk of different species. In general, the wet weight of the curds decreased as the digestion progressed because of the contraction forces of the stomach as well as hydrolysis by pepsin with the decrease in pH (Figure 6.4A). The higher wet weights of the curds formed from raw cow and sheep whole milks was expected to be due to their higher total solids as well as higher protein content. The final wet weight of the curds formed by the raw whole milk of different species at the end of the 240 min of digestion followed the order: goat ≃ cow < sheep (Figure 6.4A). Although both the raw cow milk and the raw sheep milk had higher total solids (and protein) content than the raw goat milk, the raw cow and goat milk had similar amounts of wet curd remaining in the stomach at the end of digestion, and the amount of curd remaining from the raw sheep milk was still higher than those from the raw goat and cow milk. The final wet weights of the curds remaining from the pasteurized milks within a species were lower than the wet weights remaining from their raw milk counterparts (Figure 6.4A), indicating that pasteurization significantly affected the rates of curd breakdown. This faster breakdown of the pasteurized milk curds from the milk of all species may have been due to the loose and fragmented curds formed from the pasteurized milks, as visually observed and reported in previous section and Figures 6 2 and 6.3. The dry weights of the raw and pasteurized curds (Figure 6.4B) from the milk of the different species also followed a similar trend throughout the digestion to the wet weights of their curds, as described above.

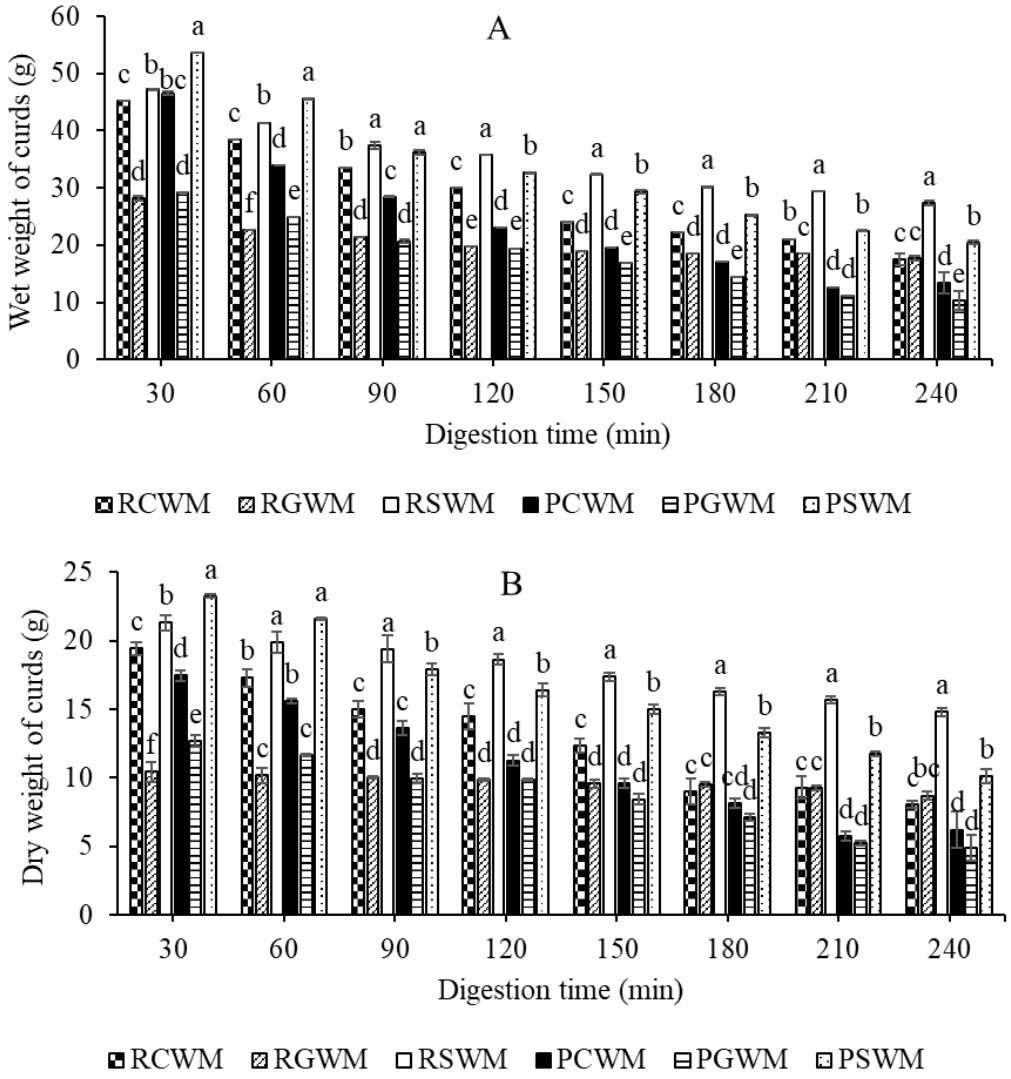


Figure 6.4 Changes in the weight of the curds produced during the gastric digestion of 200 g of different milks in the HGS: (A) wet weights of the curds; (B) dry weights of the curds. RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk. Different letters (a–f) above the bars represent significant differences among the milk samples at a given digestion timepoint (P < 0.05); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD.

6.4.5 Modification of fat globules under gastric digestion conditions

6.4.5.1. Structural changes in the fat globules retained in the curd

To understand the distribution of the fat globules, the fat contents of the gastric curds at different digestion times were analysed. It was found that approximately 80–90% of the fat (g/100 g milk fat) was present in the curd during the first 30 min of gastric digestion of the different milk samples (Figure 6.5). As the digestion progressed, the proportion of fat associated with the curd decreased, but this occurred to variable extents for the different milks (Figure 6.5). At the end of the 240 min of digestion, ~41, ~69, and ~59% of the milk fat remained in the raw cow, goat, and sheep whole milk curds, respectively ($P < 0.05$). However, only about 32–38% of the milk fat remained in the pasteurized milk curds from all species ($P > 0.05$) after 240 min of gastric digestion (Figure 6.5).

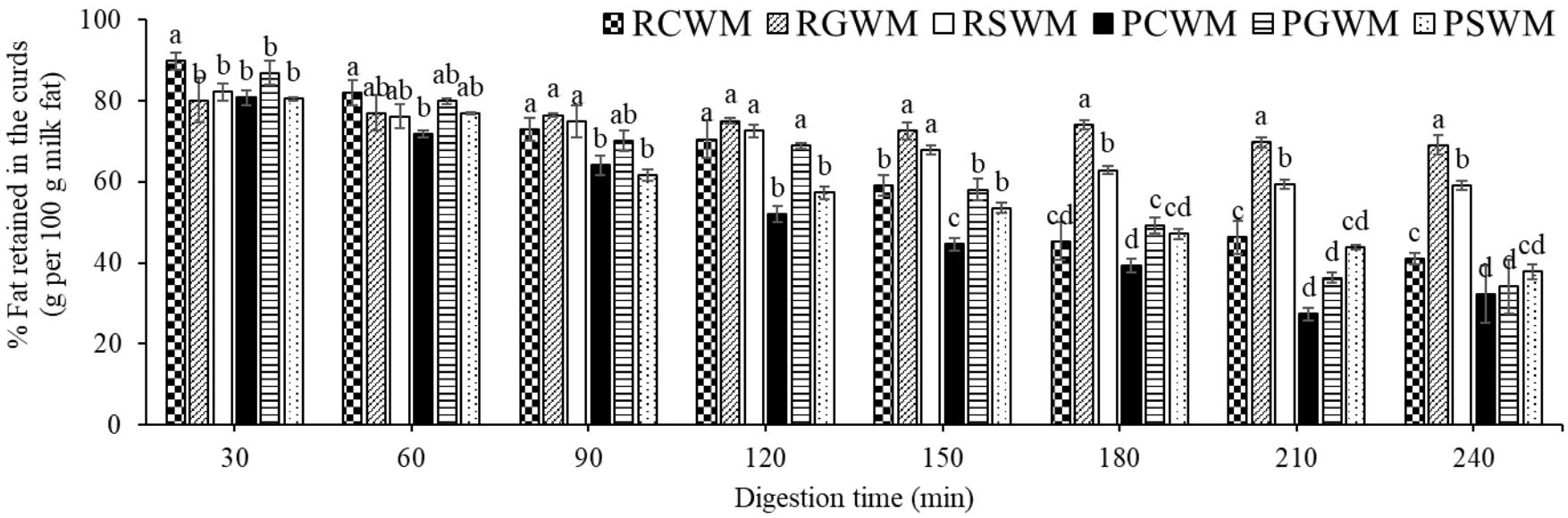


Figure 6.5 Changes in the percentage fat retained (g/100 g milk fat) in the curds during gastric digestion in the HGS (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). Different letters (a–d) above the bars represent significant differences among the milk samples at a given digestion time point ($P < 0.05$); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD.

The CLSM analysis of the curds from the different whole milks at 15, 90, and 240 min of gastric digestion provided further insights into the retention and the state of the fat globules within the coagulated network (Figures 6.6 and 6.7). The fat globules were uniformly distributed in the raw and pasteurized milk of all species before digestion. As the casein micelles coagulated during the initial stages of digestion (i.e., 15 min), a proportion of the fat globules appeared to be physically entrapped (or embedded) in the casein network or the spaces (or pores) of the surrounding casein network; some of these fat globules were much larger in size than those present in the undigested milk, indicating the occurrence of fat globule coalescence. As the digestion progressed, the size of the entrapped fat globules appeared to increase, and many lost their globular (or spherical) structure. At 240 min of digestion, much bigger fat globules with distorted (or irregular) shapes were observed (non-globular fat) (Figures 6.6 and 6.7). These observations were similar for the raw and pasteurized milk of all species. However, the extent of coalescence appeared to be relatively greater (especially at 240 min) in the pasteurized whole milk curds from all species (Figures 6.7).

The enhanced coalescence of the entrapped fat globules within the curd during gastric digestion possibly arises from the hydrolysis of MFGM proteins by the increased activity of pepsin (Le *et al.*, 2012; Ye *et al.*, 2011) with an increase in the digestion time (because of the decrease in pH). The continuous shearing action of the HGS (because of the simulated contraction movements) as well as the higher entrapped fat phase volume in the gastric curd may have enhanced the frequency of contact between the fat globule surfaces, leading to rupture/removal of MFGM and, hence, promoting coalescence. The interfacial changes occurring at the milk fat globule surface was not observed directly; however, the changes in fat globule size and shape are an indication that the MFGM may

have been ruptured or damaged (Lopez *et al.*, 2007). The broken MFGM fragments or components may have been present in the curd or in the liquid chyme during the gastric digestion.

Previous studies on the microstructure of rennet gels, or cooked curd (temperature range 33–53°C), or firm cheese (before ripening) made from whole milk have revealed the existence of different structures of the fat globules in the coagulated protein matrix, such as small spherical intact individual fat globules surrounded by MFGM (especially observed in low-fat cheeses), aggregated fat globules (clumps or clusters of spherical fat globules), larger spherical coalesced fat globules (generated from the fusion of individual fat globules), and non-spherical fat, which is not protected by the MFGM (Lopez *et al.*, 2007; Luo *et al.*, 2017; Michalski *et al.*, 2004; Ong *et al.*, 2011a; Ong *et al.*, 2011b; Rogers *et al.*, 2010). Interestingly, all these forms of fat were observed in the gastric curds in this study (Figures 6.6 and 6.7). The increase in the size of the fat globules (coalescence) in the cheese matrix during its manufacturing process has been attributed mainly to the shear applied during the renneting, cooking, and pressing stages of cheesemaking (Lopez *et al.*, 2007; Luo *et al.*, 2017; Michalski *et al.*, 2004; Ong *et al.*, 2011a; Ong *et al.*, 2011b; Rogers *et al.*, 2010); these conditions/steps during cheesemaking cause an increase in the fat phase concentration (close packing of the fat globules making them more susceptible to collision and fusion) and disruption of the MFGM, which consequently induces partial/complete coalescence of the fat globules in the cheese curd (Lopez *et al.*, 2007; Luo, 2017; Luo *et al.*, 2017; Michalski *et al.*, 2004; Ong *et al.*, 2011a; Ong *et al.*, 2011b; Rogers *et al.*, 2010). As the initial stages of cheesemaking (i.e., rennet coagulation, whey removal, and pressing) can be closely related to the early stages of milk digestion in the stomach, a similar phenomenon may be applicable for the changes occurring in the

entrapped fat globules in the curd during gastric digestion in the HGS, as observed in this study.

The relatively higher degree of coalescence of the fat globules observed in the pasteurized whole milk curds (Figure 6.7) could have been due to the increased susceptibility of the MFGM in the pasteurized milks. In the milk of all species, temperatures as low as 60°C can induce changes in the MFGM structure, such as increased diffusion and coalescence of lipid domains present at the surface, association of whey proteins with the surface, and denaturation of MFGM proteins (Corredig & Dalgleish, 1997; Et-Thakafy *et al.*, 2017; Jukkola & Rojas, 2017; Ye *et al.*, 2004). Such heat-induced structural changes may alter the native MFGM structure and, thus, may lead to its increased susceptibility to destabilization and coalescence within the curd matrix under gastric digestion conditions. In addition, the more fragmented curds observed in the pasteurized whole milks compared with the raw whole milks (Figures 6.2 and 6.3) would have led to relatively easier diffusion of pepsin and gastric juices into the curds of the pasteurized whole milks, leading to enhanced hydrolysis of the MFGM proteins. In turn, this enhanced coalescence of the entrapped fat globules within the protein network may have hindered the strong association between the aggregated protein strands (Luo *et al.*, 2017).

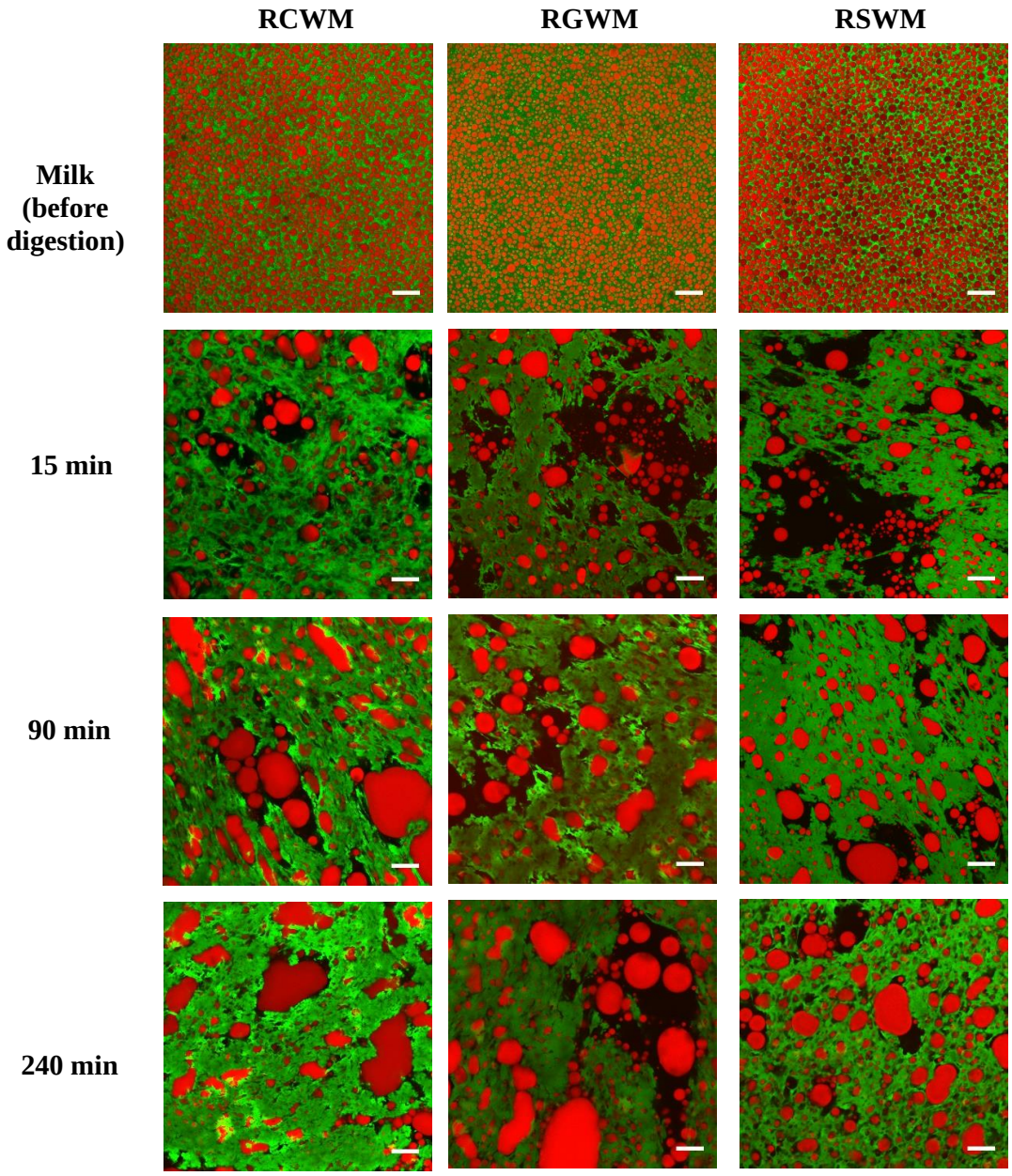


Figure 6.6 Confocal micrographs of the curds obtained during the gastric digestion of different raw whole milks in the HGS at different times (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk). Scale bars represent 25 µm.

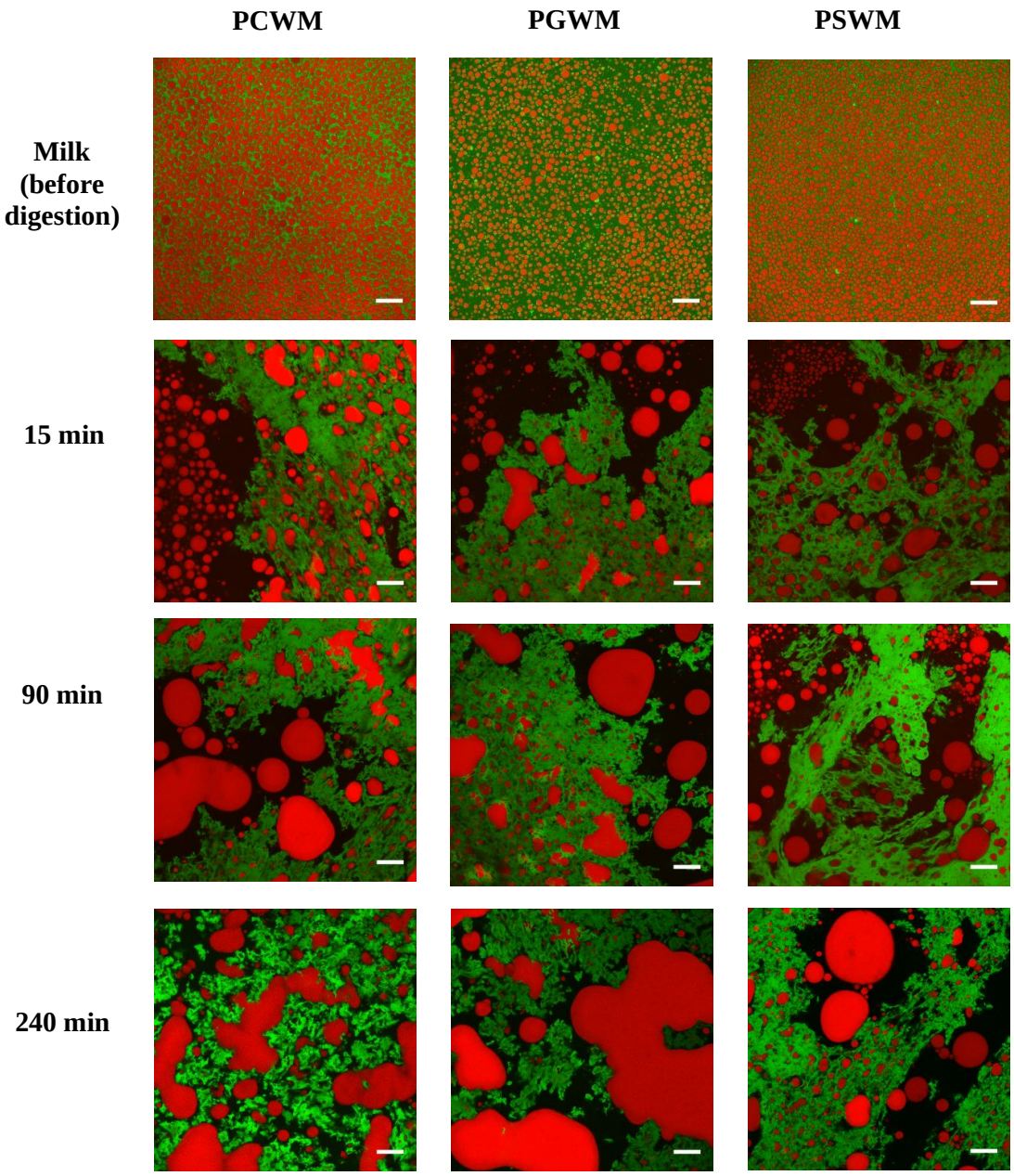


Figure 6.7 Confocal micrographs of the curds obtained during the gastric digestion of different pasteurized whole milks in the HGS at different times (PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). Scale bars represent 25 µm.

6.4.5.2. Structural changes in the fat globules of the liquid chymes

The changes in the microstructure and size distribution of the fat globules in the liquid chymes from the different milks at different digestion times were also studied. Only a few larger (or coalesced) fat globules (in comparison with those present in the undigested milk) were observed in the liquid chymes from the milk of all species at 90 and 240 min (more evident for the pasteurized milks) (Figures 6.8 and 6.9). The particle size measurements also indicated only minor shifts in the fat globule size distribution of the liquid chymes from the different milks during gastric digestion (Figure 6.10). A small increase in the proportion of larger fat globules (in the range ~20–630 μm) with a simultaneous decrease in the proportion of fat globules in the range ~0.7–17 μm (first peak) was observed in the liquid chymes from all species (Figure 6.10). The mean fat globule sizes (D_{43} or D_{32} diameter) of the liquid chymes from the milk of the different species at the end of the 240 min of digestion were not significantly different ($P > 0.05$) (Table 6.2).

At the beginning of the gastric digestion (30 min), only about 10–20% of the fat globules from the milk of the different species were in the liquid phase of the chyme (as 80–90% of the fat globules were entrapped within the curd). With the progressive breakdown of the curd (aggregated protein network) during gastric digestion, the fat globules in the curd were released into the liquid chyme. However, it was evident that the extensive coalescence of fat globules observed within the curd matrix (Figures 6.6 and 6.7) was not observed in the liquid chyme (Figures 6.8 and 6.9). It seems that the fat globules already present (or released) in the liquid chyme were able to maintain their structural integrity and that the larger coalesced fat globules were not able to move out of the curd easily;

only the smaller fat globules were able to move out of the curd structure (Figures 6.8 and 6.9).

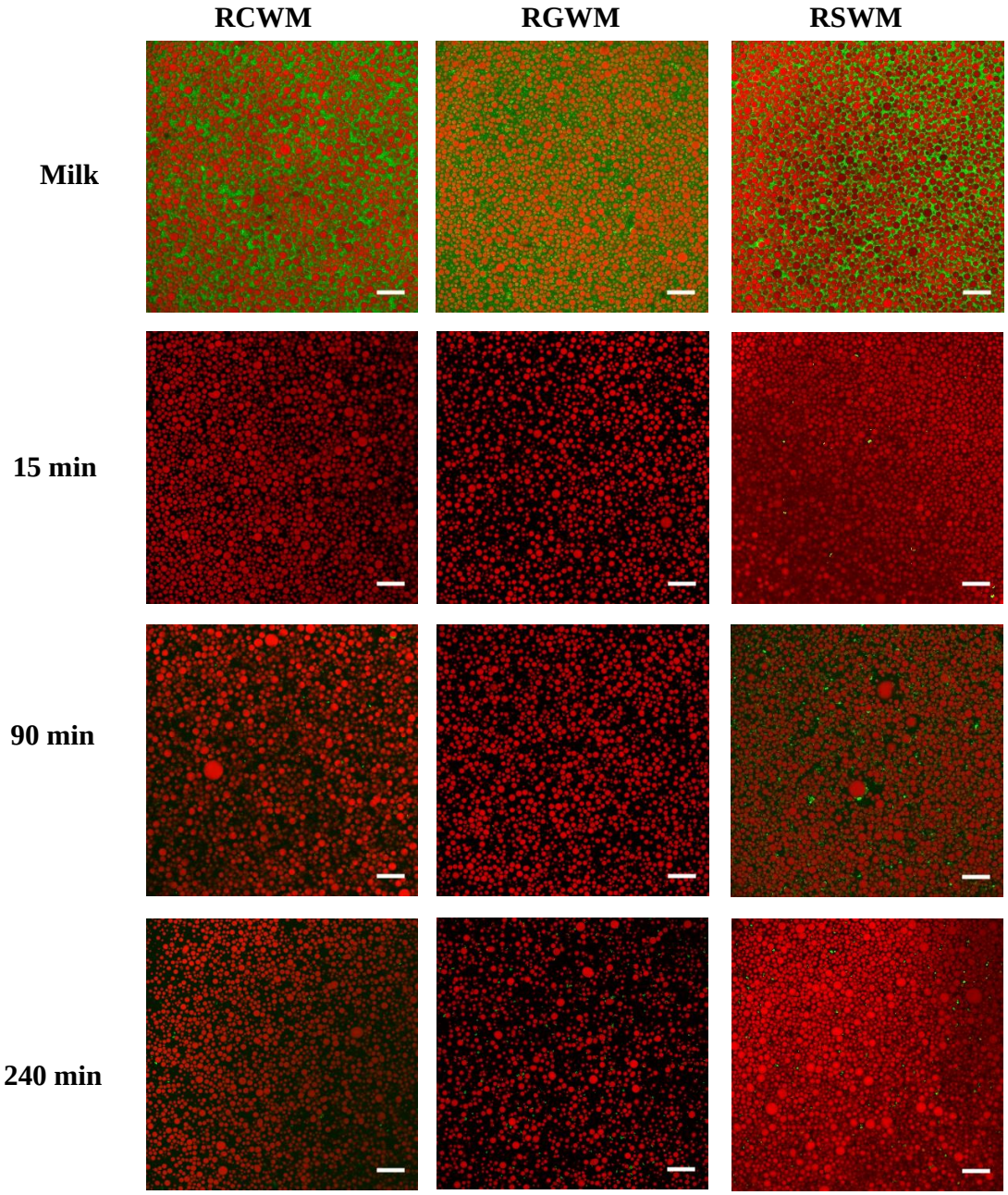


Figure 6.8 Confocal micrographs of the liquid chymes during the gastric digestion of different raw whole milks in the HGS at different times (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk). Scale bars represent 25 µm.

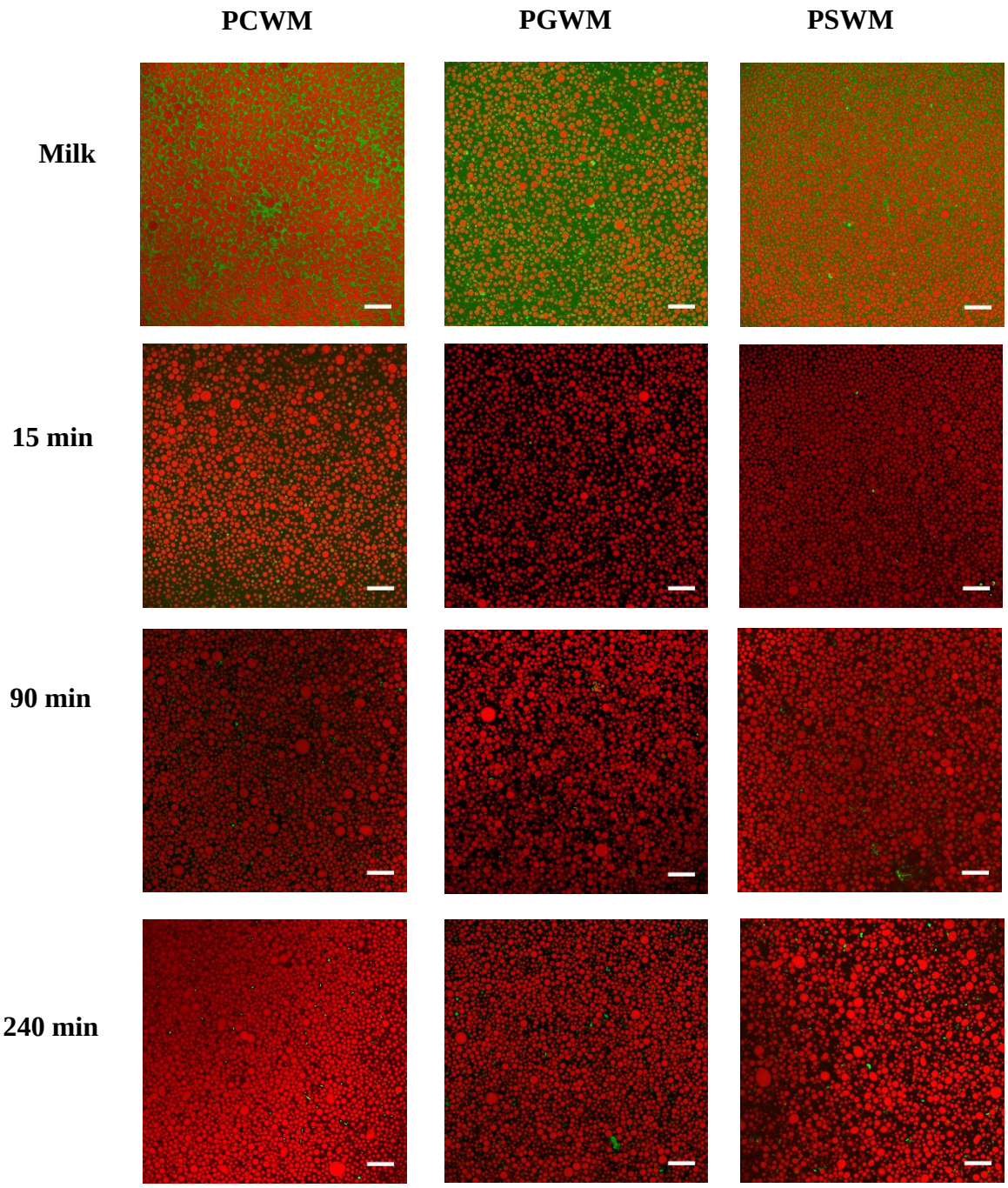


Figure 6.9 Confocal micrographs of the liquid chymes during the gastric digestion of different pasteurized whole milks in the HGS at different times (PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). Scale bars represent 25 µm.

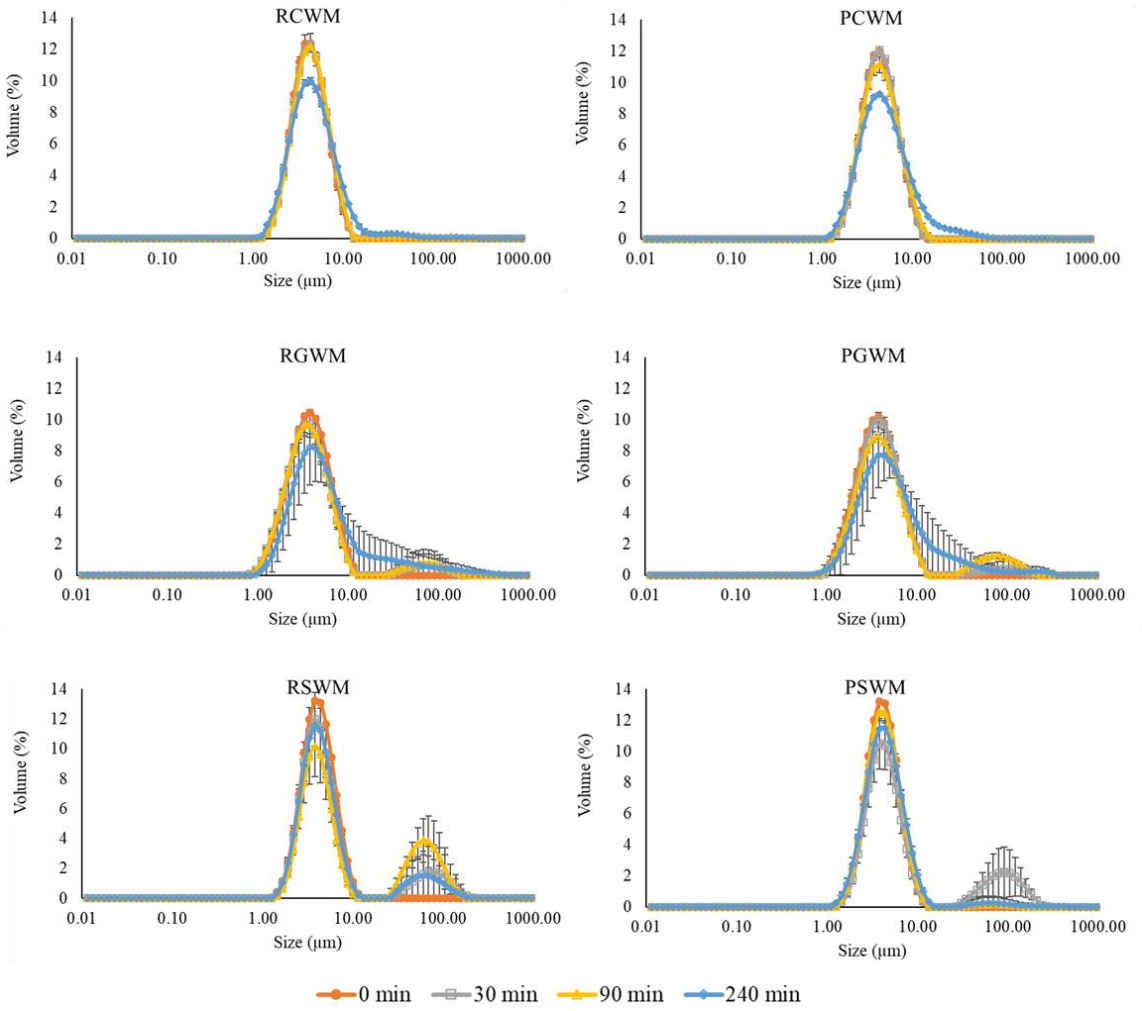


Figure 6.10 Fat globule size (µm) distribution of the liquid chymes from different whole milks dissociated with EDTA-SDS buffer during gastric digestion (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). '0' min represents the fat globule size of the whole milk before gastric digestion. Each data point represents mean ± SD.

Table 6.2 Fat globule sizes (μm) of the liquid chymes from the whole milk of different species at different digestion times (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk).

Fat globule size (μm)	Milk	Digestion time (min)			
		0 min (before digestion)	30 min	90 min	240 min
D_{43}* (volume weighted mean diameter)	RCWM	4.26 ± 0.02^{C}	4.37 ± 0.00^{C}	4.44 ± 0.00^{C}	6.27 ± 1.10^{C}
	RGWM	3.83 ± 0.07^{C}	7.44 ± 4.21^{BC}	8.52 ± 5.4^{BC}	13.65 ± 10.81^{ABC}
	RSWM	4.09 ± 0.01^{ABC}	14.39 ± 10.72^{C}	21.88 ± 8.74^{AB}	12.21 ± 7.33^{ABC}
	PCWM	4.40 ± 0.08^{C}	4.48 ± 0.00^{C}	4.56 ± 0.05^{C}	6.89 ± 1.40^{C}
	PGWM	3.85 ± 0.00^{C}	$6.81 \pm 0.3.93^{BC}$	12.99 ± 0.49^{ABC}	10.99 ± 7.85^{ABC}
	PSWM	4.10 ± 0.01^{C}	21.45 ± 10.08^{A}	5.31 ± 2.73^{C}	6.63 ± 2.32^{BC}
D_{32}* (surface weighted mean diameter)	RCWM	3.59 ± 0.03^{ABCD}	3.66 ± 0.00^{ABCD}	3.71 ± 0.00^{ABCD}	3.78 ± 0.04^{ABCD}
	RGWM	3.00 ± 0.05^{D}	2.98 ± 0.15^{D}	3.05 ± 0.22^{CD}	4.28 ± 1.37^{AB}
	RSWM	3.50 ± 0.00^{ABCD}	4.05 ± 0.67^{ABCD}	4.82 ± 0.69^{A}	3.95 ± 0.45^{ABCD}
	PCWM	3.65 ± 0.07^{ABCD}	3.73 ± 0.00^{ABCD}	3.67 ± 0.02^{ABCD}	3.94 ± 0.04^{ABCD}
	PGWM	2.98 ± 0.00^{BCD}	3.20 ± 0.16^{BCD}	3.36 ± 0.04^{ABCD}	4.22 ± 0.61^{ABC}
	PSWM	3.50 ± 0.00^{ABCD}	$4.48 \pm 0.0.66^{A}$	3.59 ± 0.12^{ABCD}	3.63 ± 0.20^{ABCD}

*Values are reported as mean ± SD. Letters (A-D) represent significant differences ($P < 0.05$) across different digestion times and different milks.

Lopez *et al.* (2007) reported that the whey expelled during the pressing of cheese had a lower fat globule size than the fat globules observed in the curds during cheesemaking. Michalski *et al.* (2004) also found that less fat was retained in cheese curd made from milk with smaller fat globules than from milk with larger fat globules, indicating that smaller globules are easily lost into the whey. These studies suggest that smaller fat globules are readily able to migrate out of the coagulated protein network (in cheese). Previous studies have also reported that, when cheeses were prepared from differently sized fat globules (separated via microfiltration), milk enriched with greater numbers of larger native fat globules had higher susceptibility to coalescence than milk enriched with smaller fat globules (Logan *et al.*, 2017; Luo *et al.*, 2017; Michalski *et al.*, 2004). The greater susceptibility of larger fat globules to structural changes could be because of their relatively lower Laplace pressure, which is due to their larger diameter (Walstra, 2003), and, thus, lower resistance to deformation or disruption under mechanical pressure than smaller fat globules (Lopez *et al.*, 2007; Luo *et al.*, 2017). Moreover, the large coalesced fat globules may be unable to move as freely as the smaller fat globules within the pores of the surrounding protein network and thus remain in closer contact with each other inside the pore spaces, resulting in increased coalescence (Luo *et al.*, 2017).

Ye *et al.* (2011) studied the static *in vitro* gastric digestion of raw cow whole milk and cream (dispersed in water or skim milk). They found that the fat globule size of cream dispersed in water did not change during gastric digestion, whereas that of raw cow whole milk or cream dispersed in skim milk increased, mainly because of flocculation as no obvious coalescence was observed. It was speculated that MFGM phospholipids and some intact or partially hydrolysed MFGM proteins may have remained at the fat–water interface, providing sufficient stability towards coalescence. In view of these observations

from previous static *in vitro* gastric digestion experiments, the results from this study confirm that the dynamic shear forces in the HGS (along with pepsin activity) caused breakdown of the interfacial layers of fat globules trapped within the curd structure, and that the fat globules remaining (or released) in the gastric chyme were stabilized by MFGM phospholipids or hydrolysed MFGM proteins.

6.5. Modification of proteins during digestion

6.5.1. Total protein retained in the curds

The dynamic changes in the protein content of the curd during gastric digestion are depicted in Figure 6.11. Of the protein (g/100 g milk protein), about 69–79% remained in the curds from the different milk samples during the first 30 min of gastric digestion, but this proportion decreased progressively with an increase in digestion time (Figure 6.11). At the end of the 240 min of gastric digestion, ~33, ~58, and ~55% of the total protein remained in the raw cow, goat, and sheep whole milk curds, respectively, whereas, significantly ($P < 0.05$) lower proportions of protein, i.e., ~26, ~30, and ~33%, remained in the pasteurized cow, goat, and sheep whole milk curds, respectively (Figure 6.11). These results indicated that the protein retention in the pasteurized milk (non-homogenized) curds from the milk of all species was significantly lower ($P < 0.05$) than for their raw milk counterparts (especially from 150 min onwards) and this was even more evident for the goat and sheep milk (Figure 6.11). This demonstrated that pasteurization of raw whole milk significantly affected the breakdown and hydrolysis of the protein curds compared with their raw milk counterparts, which was expected to be due mainly to the more loose or disintegrated curds formed from pasteurized (non-homogenized) whole milk (Figures 6.2 and 6.3). The greater impact of pasteurization conditions on the

curd properties of goat and sheep milk could have been due to the lower casein-to-whey protein ratio as well as to the lower heat stability of the whey proteins in both goat milk and sheep milk compared with cow milk (Claeys *et al.*, 2014; Montilla *et al.*, 1995; Raynal-Ljutovac *et al.*, 2007). Such differences may have resulted in greater amounts of denatured whey proteins and in turn greater association between the caseins and the denatured whey proteins, leading to more disintegrated curds and, thus, greater proteolysis of the pasteurized goat and sheep milk curds during the gastric digestion.

It was also found that the percentage protein retained in the raw cow whole milk curds was significantly ($P < 0.05$) lower than that retained in the raw goat and sheep whole milk curds (Figure 6.11), which could have been due to the tendency of the raw cow whole milk curds to break down into much smaller aggregates, as observed in Figure 6.2. Some previous studies have shown that a lower protein (casein)-to-fat ratio increases the softness of the curd (Mulet-Cabero *et al.*, 2020c) and that larger native milk fat globules give rise to softer rennet gels (Luo *et al.*, 2017; Michalski *et al.*, 2002). However, in contrast, other previous studies have also reported that small casein micelles in combination with large native milk fat globules produce firmer rennet gels (Logan *et al.*, 2014; Logan *et al.*, 2015). It was hypothesized that the lower protein-to-fat ratio in combination with the larger fat globule sizes of the raw cow whole milk (despite its smaller casein micelle size) (Table 6.1) may have been responsible for the increased susceptibility of its curd to a greater degree of breakdown during gastric digestion, compared with the raw milk of the other species in this study. However, there needs to be further investigation. The changes in the protein and fat contents of the curds of different milks in the HGS (expressed as g per 200 g of milk) is shown in Annexure 2 (Figure A2.1).

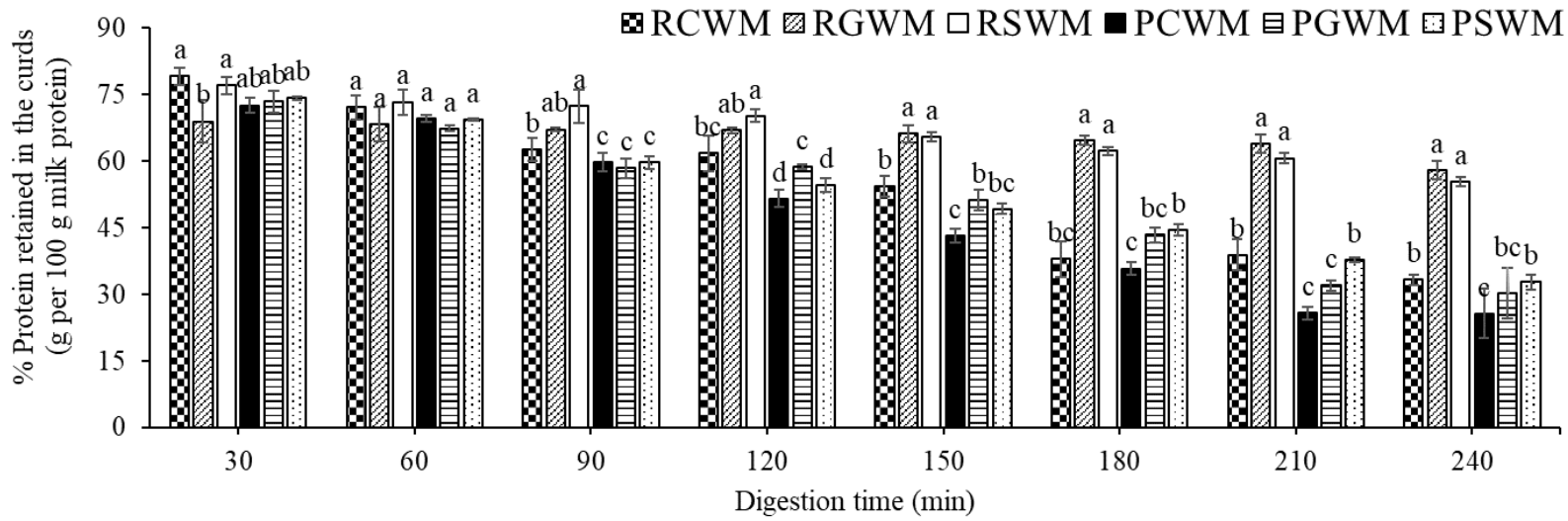


Figure 6.11 Changes in the percentage protein (g/100 g milk protein) retained in the curds during gastric digestion in the HGS (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). Different letters (a–d) above the bars represent significant differences among the milk samples at a given digestion time point (P < 0.05); differences within a particular milk sample across different digestion times are not represented. Each data point represents mean ± SD.

6.5.2. Protein hydrolysis during gastric restructuring and breakdown of caseins – SDS-PAGE of the curds

To understand the formation of the curd and the breakdown (or hydrolysis) of the protein network during gastric digestion, the protein compositions of the different whole milks and their respective curds obtained at different digestion times were qualitatively analysed using reducing SDS-PAGE (run on an equal protein basis) (Figure 6.12, RCWM, RGWM, and RSWM). In the curds from the raw milk of all species, the intensity of the κ-casein band decreased markedly after 30 min of digestion compared with the undigested whole milk samples. At the same time, a para-κ-casein band (above the α-lactalbumin band), which is derived from the hydrolysis of κ-casein by pepsin (Miranda & Pelissier, 1983; Ye *et al.*, 2016a), was observed in the 30-min raw milk curd samples. The appearance of the para-κ-casein band (at ~15–16 kDa) has also been reported in previous studies (Egito *et al.*, 2007; Mohamed Ahmed *et al.*, 2010; Pontual *et al.*, 2012; Ye *et al.*, 2016a, 2016b; Ye *et al.*, 2019b). The intensities of the other casein bands (α_{s1}-, α_{s2}-, and β-casein) decreased very slowly as the digestion progressed. A faint β-lactoglobulin band was also observed in all curds, the band intensity of which decreased gradually as the digestion progressed. No α-lactalbumin band was observed in the raw cow and sheep whole milk curds. However, some small amounts of α-lactalbumin were found in the raw goat whole milk curds, the band intensities of which decreased as the digestion progressed. The small amounts of whey proteins observed in the curds may have been due to the entrapped liquid chyme in the curds, which was drained out or hydrolysed slowly during digestion. The curds from the pasteurized whole milk of all species (Figure 6.12, PCWM, PGWM, and PSWM) followed a similar trend to their respective raw whole milk curds, except that the caseins from the pasteurized milk curds appeared to be much more hydrolysed during the gastric digestion. This further

strengthens the previous observations, i.e., the pasteurized milk curds had lower protein contents than their raw milk counterparts (Figure 6.11). The greater impact of pasteurization on the hydrolysis of the curd proteins was due to the greater ability of pepsin (as well as gastric fluid) to diffuse into the more open and disintegrated curds (Figure 6.3) (Ye *et al.*, 2016a)

6.5.3. Changes in the protein compositions of the liquid chymes – SDS-PAGE of the liquid chymes

The protein compositions and the hydrolysis profiles of the liquid chymes (run on an equal protein basis) at all digestion timepoints are shown in Figure 6.13. In the SDS-PAGE of the liquid chymes from the raw whole milk of the different species (Figure 6.13, RCWM, RGWM, and RSWM), compared with the raw milks, native β-lactoglobulin and α-lactalbumin (the whey proteins) were found to be present with intense bands at 30 min of digestion. The intensity of the native α-lactalbumin band decreased rapidly and disappeared from the chyme within about 120 min of gastric digestion (pH ~3.4). This was probably due to the change in its protein conformation (at pH <4), leading to its increased susceptibility to hydrolysis by pepsin (Miranda *et al.*, 1989). The intensity of the native β-lactoglobulin band decreased gradually with increasing digestion time and only a small amount of intact β-lactoglobulin remained in the chyme emptied at 240 min of digestion. Some previous studies (Peram *et al.*, 2013; Sarkar *et al.*, 2009) have reported approximately 20–35% hydrolysis of native β-lactoglobulin by porcine pepsin (pepsin:β-lactoglobulin ratio, 1:3) after 2 h of *in vitro* static incubation at pH 1.5; they have also reported that a higher pepsin-to-substrate (β-lactoglobulin) ratio may be the reason for the observed degradation of native β-lactoglobulin. Smaller amounts of intact caseins were also found to be present in the 30-min liquid chyme. That casein bands were observed in

the liquid chyme may have been because of the delivery of very fine casein particles to the chyme when the curd lost its mass. Some smaller amounts of peptides were also emptied with the liquid chyme at all digestion points. Similar observations were noted for the protein profiles of the liquid chymes from the pasteurized whole milk of the different species (Figure 6.13, PCWM, PGWM, and PSWM).

The SDS-PAGE results further indicated that the whey proteins, being soluble in the liquid phase, would have emptied rapidly and mainly in intact native form, especially during the early stages of digestion, i.e., up to 2 h (120 min), whereas the caseins coagulated in the stomach, were digested slowly, and would have emptied mainly as fine aggregates as well as peptides into the small intestine during gastric digestion. These results are in accordance with previous studies, which reported that caseins are evacuated from the stomach mainly as peptides, whereas whey proteins empty mainly in their intact forms (Mahe *et al.*, 1996; Miranda & Pelissier, 1983). Boutrou *et al.* (2013) studied the kinetics of release of milk-derived peptides in the jejunum of humans who ingested reconstituted lyophilized casein or whey protein powder (prepared from microfiltered/ultra-filtered raw skim milk). They found that the caseins slowly delivered a wide range of medium-sized peptides during 6 h of digestion, whereas fewer peptides but of larger size were released from the whey proteins until 3 h of gastric digestion, at which time the whey proteins were entirely emptied from the stomach.

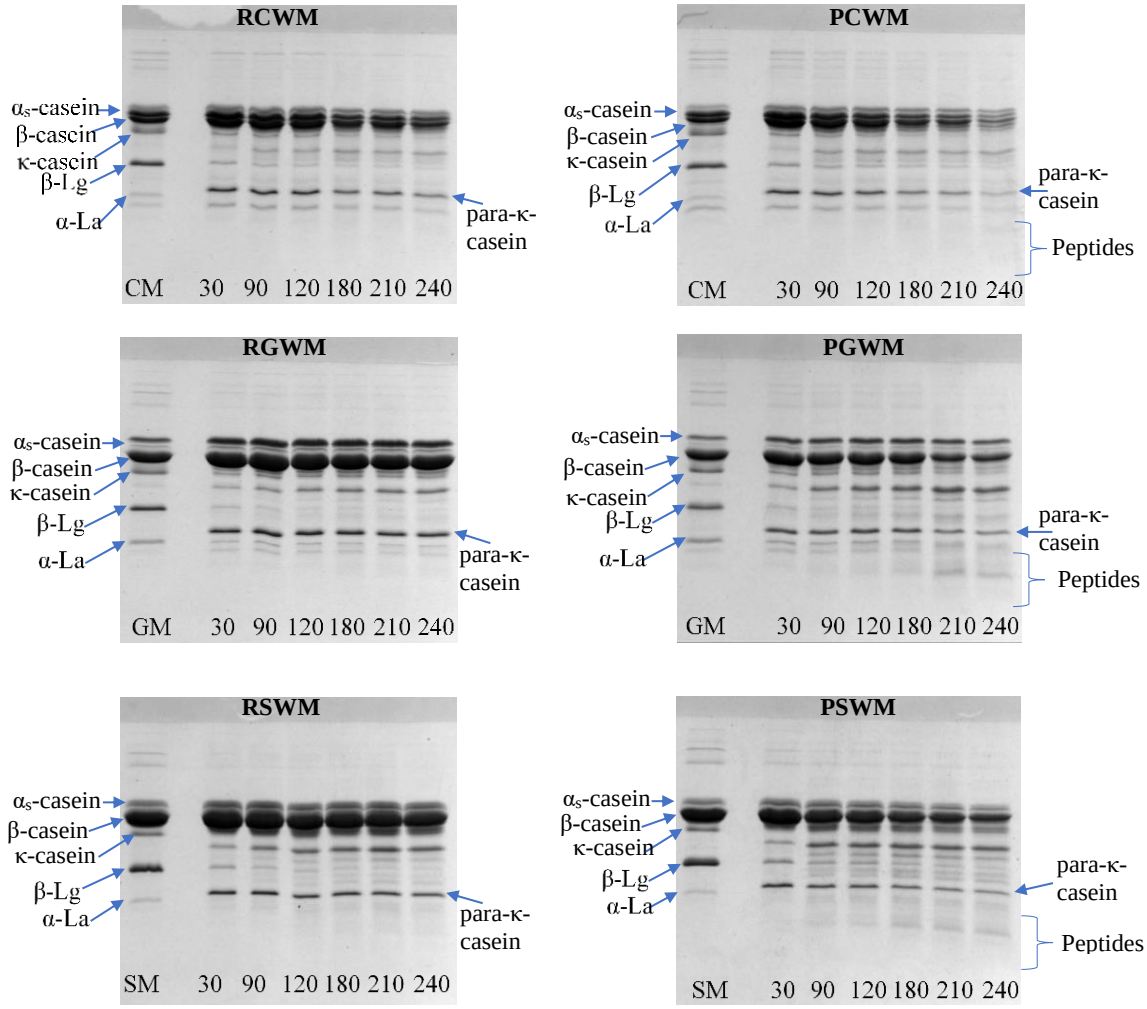


Figure 6.12 SDS-PAGE patterns of the curds (~18 mg of protein in each lane) remaining in the HGS at different times (30, 90, 120, 180, 210, and 240 min) during the gastric digestion of raw and pasteurized whole milks (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk; CM, cow whole milk; GM, goat whole milk; SM, sheep whole milk).

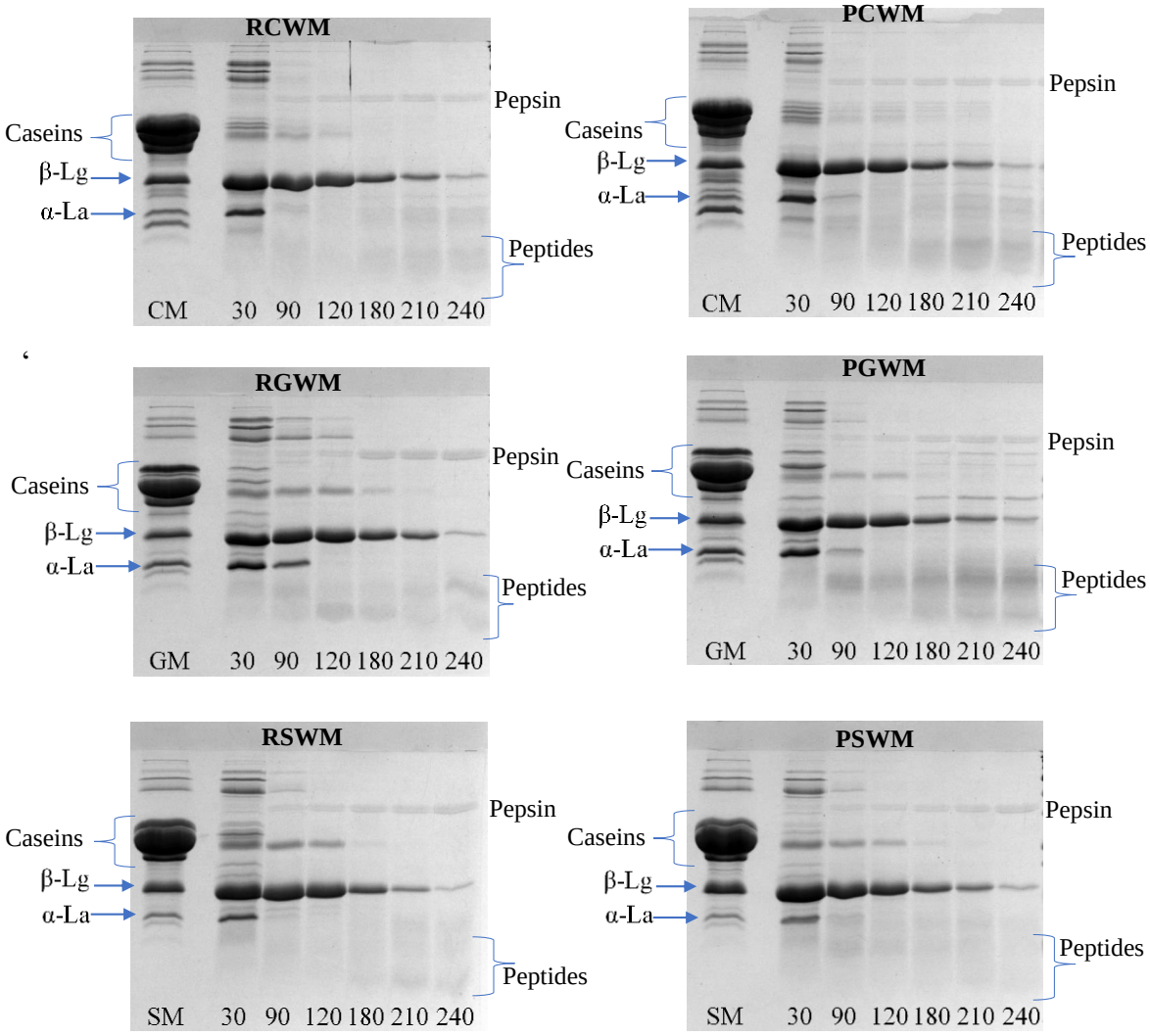


Figure 6.13 SDS-PAGE patterns of the liquid chymes (~69 µg of protein in each lane) collected from the HGS at different times (30, 90, 120, 180, 210, and 240 min) during the gastric digestion of raw and pasteurized whole milks (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk; CM, cow whole milk; GM, goat whole milk; SM, sheep whole milk).

6.6. Relationship between fat globule release and curd breakdown

The protein and fat retention data (Figures 6.5 and 6.11) also suggested that the changes in the protein and fat contents of the curd during gastric digestion may have been interrelated (irrespective of the different types of milk and the different processing treatments), as the changes in protein retention and fat retention in the curd followed similar trends (Figures 6.5 and 6.11), and that the dynamic changes occurring in the protein content of the curd during gastric digestion may have had an influence on the release of the fat globules entrapped in the protein (casein) network. Thus, the interrelationship between the amounts of protein and fat lost progressively from the curd matrix during the gastric digestion of the different milks was further analysed (Figure 6.14). It was found that, irrespective of the species and the processing treatment, there was a strong positive linear correlation (Pearson correlation coefficient, r = 0.978) between the amounts of fat and protein lost from all curd samples throughout the gastric digestion. When a least squares linear regression model was fitted for the mean protein and fat lost from all curd samples at all digestion timepoints (irrespective of species and processing treatment) (Figure 6.14), an equation with a slope of 1.0653 (R^2 = 0.9557) for the protein loss (predictor) was obtained (Figure 6.14). As the slope was nearly equal to 1, it showed that the average fat lost from the curd samples was positively proportional to the loss of protein from the curd samples. This suggested that the movement of protein from the curd into the liquid phase, because of breakdown and hydrolysis of casein aggregates, allowed the release of fat globules into the liquid phase. Thus, the rate of fat release was dependent on the rate of breakdown of the curd structure, which, in turn, was dependent on the rate of protein hydrolysis. For instance, the loose curd structure of the pasteurized milk led to the loss of greater amounts of protein from the curd, which, in

turn, led to the release of greater amounts of fat from the pasteurized whole milk curds compared with their raw milk counterparts (Figures 6.5 and 6.11).

These results are in agreement with previous studies (Ye *et al.*, 2016a, 2017), which reported that the fat retained in raw and heated cow whole milk curds was linearly dependent on the fat-free matter or dry matter remaining in the curds during dynamic gastric digestion. Guo *et al.* (2014) studied the gastric digestion of soft and hard whey protein emulsion gels and found that the gastric digestion of the proteins and the release of oil droplets from the gels were primarily dependent on the gel structure, i.e. a 'hard' gel structure and a 'soft' gel structure had slower and faster rates of disintegration of the protein matrix as well as oil droplet release, respectively. Sarkar *et al.* (2015) also demonstrated that the rate of lipid digestion in an oil-in-water emulsion embedded within gelatin (i.e., a gelatin-gelled emulsion) was directly related to the microstructure (pore size) of the gel (allowing the diffusion of enzymes) and the gradual breakdown of the gelatin (protein) network by proteases. Although there are no direct reports on the intragastric coagulation of goat and sheep whole milks, the linear regression equation and the Pearson correlation coefficient (r) values obtained for each milk in this study (Figure 6.15) indicated that there was a strong relationship between the protein and fat lost from the milk curds of both species (raw as well as pasteurized whole milks) and that this was a similar behaviour to that of cow whole milk. There was also a strong correlation between the dry matter and fat lost from the milk curds of all the species (Annexure 2, Figure A2.2).

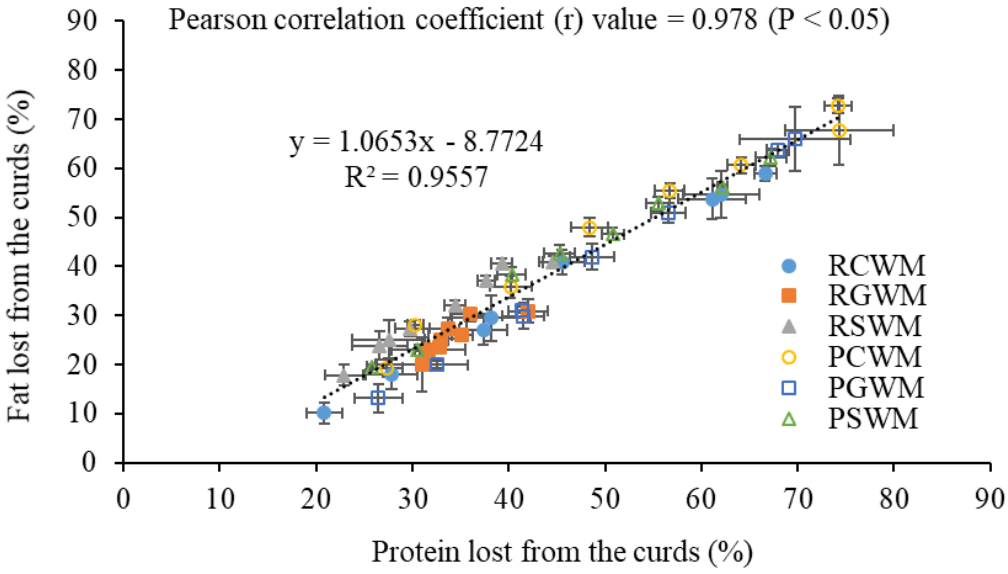


Figure 6.14 Relationship between the fat and protein contents lost from the curds of different whole milks during gastric digestion in the HGS from 30 to 240 min. The Pearson correlation coefficient value (r) (P < 0.05) as well as the regression line and the equation for the overall graph are depicted. Each data point represents the mean ± SD of results from at least two different batches of milk (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk).

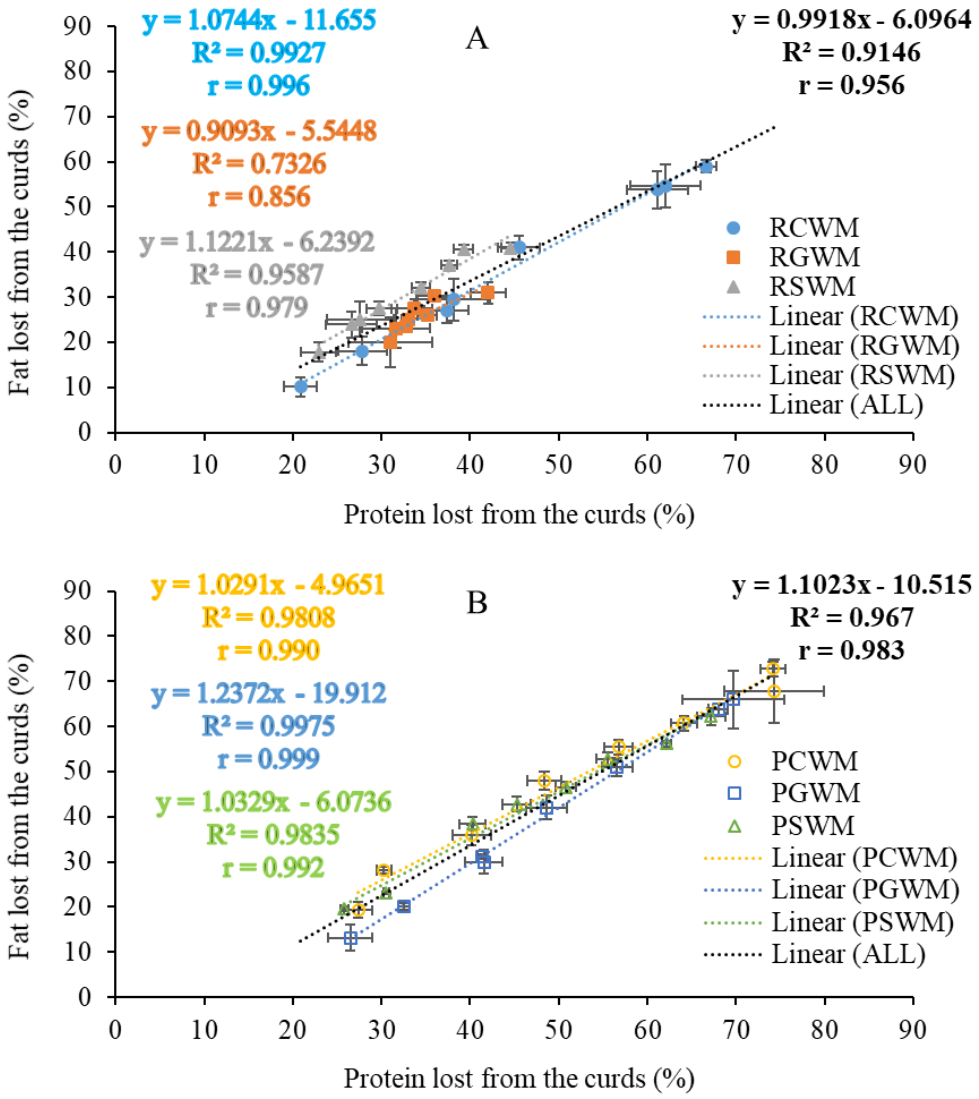


Figure 6.15 Relationship between the fat and protein contents lost from the curds of different whole milks during gastric digestion in the HGS from 30 to 240 min: (A) raw whole milk curds; (B) pasteurized whole milk curds (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). Each data point represents the mean ± SD of results from at least two different batches of milk. The Pearson correlation coefficient value (r) ($P < 0.05$) as well as the regression line and the equation for each milk and the overall graph are depicted.

6.7 Texture analysis of the residual curds

The force required to penetrate the curds from the milk of each species remaining at the end of the 240 min of digestion is reported in Figure 6.16. As seen from the visual images (Figures 6.2 and 6.3), as the curds formed by the pasteurized whole milks were very fragile and fragmented, sections of the curds were taken only when there was a sufficient amount to conduct the penetration test. Although this might not be a very accurate representation, this measurement was done for comparison with the previous study on understanding the curd formation and disintegration characteristics of skim milk from different species during digestion (Chapter 5). The results from the texture analysis reported in this study are only indicative.

The texture analysis results indicated that the curds formed from the raw and pasteurized cow and goat whole milks were of similar hardness and relatively softer than the curds formed from the raw and pasteurized sheep whole milks (Figure 6.16, OUTSIDE). The curd hardness of the pasteurized whole milks was similar to that of their raw milk counterparts except for the pasteurized goat whole milk curd, which had significantly lower hardness than its raw milk counterpart (Figure 6.16, OUTSIDE). The relatively greater hardness of the sheep whole milk curds was expected to be due to the lower hydration of sheep milk caseins, as observed in the previous study on the gastric digestion of skim milk from different species (Chapter 5).

The force required to penetrate the inner core of the curds remaining from the milk of different species is depicted in Figure 6.16, CORE. The forces required to penetrate the inner core of the raw cow and goat whole milk curds were similar but lower than that of the raw sheep whole milk curd. However, the forces required to penetrate the core inside

the pasteurized whole milk curds from the milk of all species were similar and there were no significant differences between them (Figure 6.16, CORE). In addition, the force required to penetrate the core of the whole milk curds was no different from the force required to penetrate the outer surfaces of the whole milk curds, except for the raw sheep whole milk curd, for which the force required to penetrate the inner surface of the curd was relatively higher (Figure 6.16).

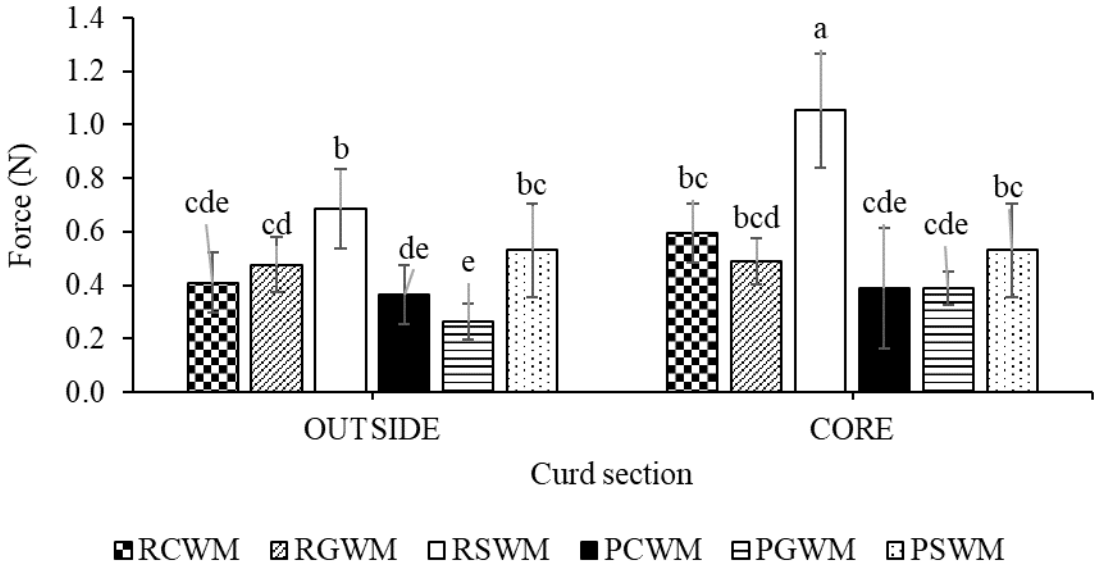


Figure 6.16 Force (N) required to penetrate the whole milk curds remaining in the HGS at 240 min (OUTSIDE represents the measurements on the complete curd when penetrated from outside; CORE represents the measurements on half-cut curds when penetrated from inside, i.e., at the core) (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). Different letters (a–e) above the bars indicate significant differences (P < 0.05). Each data point represents mean ± SD.

The penetration test results for the raw and pasteurized whole milk curds observed in this study indicated that the hardness of the whole milk curds was much lower than that of the skim milk curds in the previous study using the same method (Chapter 5), indicating that

milk fat had a crucial role in influencing the degree of curd softness and in turn the disintegration dynamics during the gastric digestion of milk.

Based on the results and discussion in this chapter, a schematic illustration to summarize the possible changes in the casein micelles and fat globules of whole milk under dynamic gastric digestion conditions (in which gastric lipase has very little to no activity) is given in Figure 6.17.

6.8. Conclusions

This study provided a deeper understanding of how the curd-forming properties of different mammalian milks in the gastric environment provide controlled digestion and delivery of nutrients (such as proteins, fat, and micronutrients). Both the raw and the pasteurized (non-homogenized) whole milk of different species formed a curd in the HGS, which physically entrapped (or integrated) the majority of the milk fat globules within its aggregated casein network at the beginning of the digestion. The gradual breakdown and hydrolysis of the surrounding casein network by pepsin and the mechanical shearing of the HGS were the fundamental mechanisms behind the controlled liberation of fat globules from the structured curd throughout the gastric digestion. These mechanisms were found to be similar in the milk of all species used in this study. The study also emphasizes the importance of developing dynamic *in vitro* digestion models to better mimic the structural changes occurring in complex food systems (such as milk) in the gastrointestinal tract. Overall, the knowledge gained in this study, from understanding the structural changes in a natural emulsion system such as milk, may help in designing bioinspired macro- and microstructures for the controlled digestion and

delivery of macro- or micronutrients (as well as lipophilic medicinal drugs) in the gastrointestinal tract.

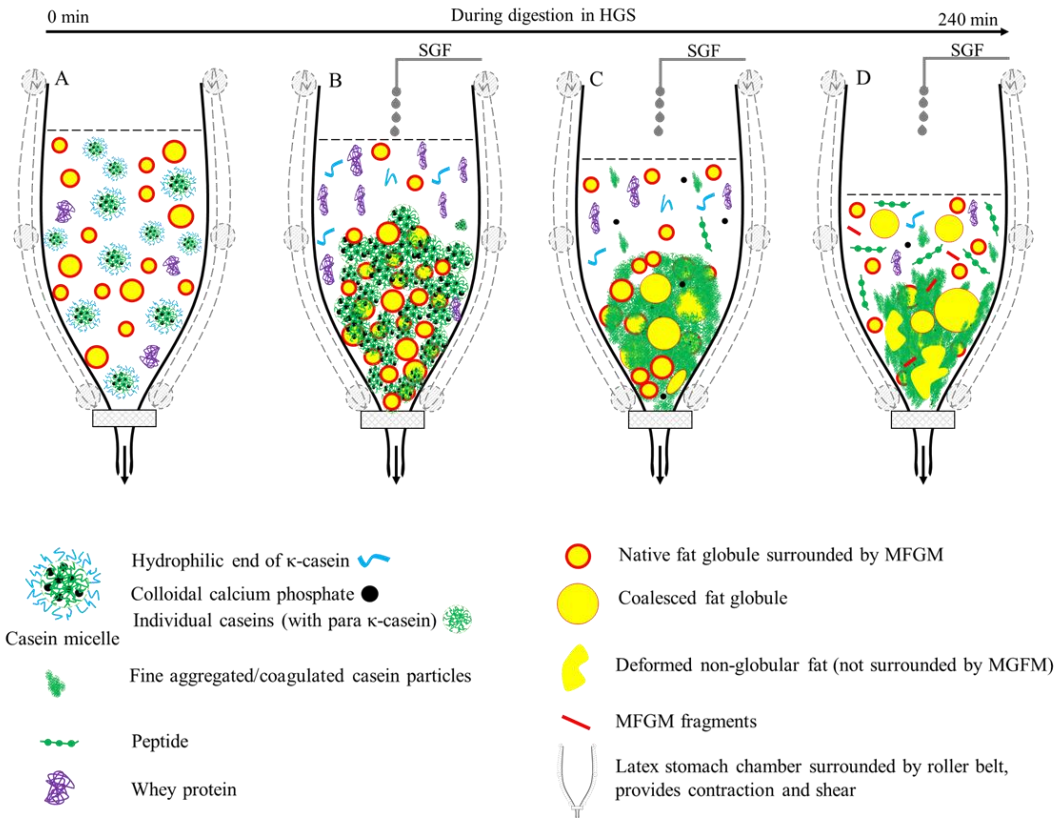


Figure 6.17 Schematic diagram illustrating the possible changes in the casein micelles and the fat globules during the dynamic gastric digestion of whole milk in the stomach chamber of the HGS: A, whole milk before the beginning digestion (pH ~6.7, proteins and fats are homogeneously dispersed in the milk matrix); B, intragastric coagulation of the casein micelles (at pH ~6) because of cleavage of the hydrophilic part of κ-casein by pepsin, with the majority of the fat globules being closely entrapped (or packed) in the aggregated casein network (curd); C, increase in denseness of the coagulated casein network (loss of colloidal calcium phosphate around pH <4.6 and expulsion of the entrapped liquid); some fat globules coalesce in the curd because of the rupture of the MFGM by the combined action of shear and pepsin; mostly smaller fat globules move to the liquid phase; D, at longer digestion times, curd proteins are hydrolysed and broken down by pepsin (and shear); they then move into the liquid chyme, and the loss of fat from the curd is directly proportional to the loss of protein; coalescence of the globules increases and non-globular fat is observed. SGF, simulated gastric fluid (containing pepsin, HCl, and salts); MFGM, milk fat globule membrane. Not to scale; the drawing is highly schematic, and sizes and quantities are not proportional.

Chapter 7: *In vivo* gastric digestion of cow, goat, and sheep whole milk using the piglet model

7.1 Abstract

The aim was to investigate the formation and disintegration behaviour of curd during the gastric digestion of cow, goat and sheep whole milk, using the bottle-fed piglet as an animal model. Cow and non-cow milks separated into a curd (aggregated caseins) and liquid (soluble whey) phase in the piglet's stomach. During digestion, the liquid phase emptied faster, while the curds were slowly digested and remained longer in the stomach. Throughout digestion, the protein and lipid content of the curds remained higher than that of the liquid phase and the firmness of the curds increased as digestion progressed. Initially, the majority of the fat globules were entrapped within the curd network but as the protein curd was hydrolysed, the fat globules were released into the liquid chyme. The extent of coalescence of fat globules in the curd was higher than that in the liquid chyme. The mechanisms of milk coagulation in the stomach were similar for the different milks.

7.2 Introduction

As demonstrated in Chapters 5 and 6, milk proteins and lipids undergo significant changes in their physicochemical and microstructural properties during dynamic *in vitro* gastric digestion. Such changes may have an impact on the gastric emptying rates of different nutrients (amino acids and fatty acids), which may influence their subsequent rates of absorption in the small intestine. To date little research has been carried out on the underlying mechanisms and physiological relevance of such phenomena.

Several studies have reported solid curd formation from cow or pig milk in piglets or minipigs (Braude *et al.*, 1970; Cranwell *et al.*, 1976; Decuypere *et al.*, 1978; Meisel & Hagemeister, 1984; Washburn & Jones, 1916). Blakeborough *et al.* (1986) found that the stomach chyme and intestinal digesta of piglets fed cow milk, or cow milk based baby food, was semi-solid in consistency with hard casein curds, whereas stomach chyme from piglets fed human milk had a liquid consistency with little curd formation. Animal models are useful to allow in-depth investigation of the intra-gastric changes in a food matrix, such as phase separation, microstructural changes, textural attributes, particle sizes, etc. which are difficult to accurately observe in studies involving humans. The objective here was to investigate the mechanisms of the gastric digestion of whole milk in three different species (cow, goat and sheep), under physiological conditions using the bottle-fed (suckled) piglet as an animal model (Moughan *et al.*, 1992; Moughan *et al.*, 1994; Moughan & Rowan, 1989). The study also investigated the impact of curd formation and its disintegration on the overall gastric emptying of the different mammalian milks. The aim was to build upon the knowledge gained in previous work which involved *in vitro* measures of the gastric digestion of different mammalian milks (Chapters 5 and 6) and to relate the *in vitro* and *in vivo* observations.

Bottle-fed piglets which are exclusively milk-fed have been used previously to study the digestion of human milk and infant formulas (Moughan *et al.*, 1991; Rutherfurd *et al.*, 2006a) and the model has been validated (Darragh & Moughan, 1998). The bottle-fed piglet was chosen here to allow a comparison of gastric digestion between cow, goat, and sheep whole milk.

7.3. Materials and methods

7.3.1 Diets

Spray-dried cow, goat and sheep whole milk powders were purchased from Davis Food Ingredients (Palmerston North, New Zealand), Dairy Goat Co-operative (Hamilton, New Zealand) and Spring Sheep Milk Co. (Hamilton, New Zealand), respectively. A vitamin and mineral premix formulated for piglets was procured from Nutritech International Limited, Auckland, New Zealand. Raw pooled whole milk from cow, goat and sheep was procured during November 2018 – January 2019 from the Massey University No.4 dairy farm (Palmerston North, New Zealand), Dairy Goat Co-operative (Hamilton, New Zealand) and Phoenix Goats (Palmerston North, New Zealand), and Neer Enterprises Ltd (Carterton, New Zealand), respectively.

7.3.2 Animal study

7.3.2.1 Experimental design

All procedures involving animals were approved by the Massey University Animal Ethics Committee (MUAEC protocol no. 18/97). Sixty male Large White X (Landrace X Large White) piglets [7-8 days of age; mean body weight (BW) on arrival was 3 kg (range 1.9-4.2 kg)] were obtained from a local commercial farm (Aorere Farms Partnership, Whanganui, New Zealand). The piglets were ear tagged, weighed and housed individually in purpose-built plastic metabolism crates with toys in a temperature-controlled room maintained at 28 ± 2^{o}C with a 16:8 h light: dark cycle from the day of arrival (Day 1) at the Massey University Animal Physiology Unit, Palmerston North, New Zealand. The piglets were allocated at random the three dietary treatments on Day 1 (arrival day) such that there was a total of 20 piglets per diet (reconstituted milk powder/fresh milk diet).

7.3.2.2 Feeding frequency

The piglets were weighed on arrival and then every two days, and their daily ration was adjusted to ensure an intake of 345 g reconstituted whole milk powder diet (liquid) per kg bodyweight per day (Darragh & Moughan, 1995; Rutherfurd *et al.*, 2006b). For the first 6 days of the study (acclimatization period) the piglets were trained to drink from a bottle with a rubber teat and fed their daily ration across 17 meals with a 1 h interval from 06:00 h to 22:00 h. The aim was to have the piglets consuming >80% of their daily ration (Darragh & Moughan, 1995) prior to the commencement of the experimental period which started on day 7. Any piglet that did not learn to adequately bottle feed and consumed <80% of their daily ration over the first 6 days was withdrawn from the study and replaced with a spare piglet raised on the same diet. From day 7 onwards, the amount of food received by the piglets at each meal was gradually increased by decreasing the frequency of feeding to reach the target meal intake on the last experimental day (day 15). Thus, from day 7-10, the daily ration was given as 7 meals at 2.5 h intervals from 06:30 h to 21:30 h, and from day 11-14 the daily ration was fed as 5 meals with a 3.5 h interval from 06:30 h to 20:30 h. The total intake of each piglet was carefully observed during the entire period.

7.3.2.3 Diet schedule

The piglets were fed the reconstituted whole milk powder diet (including the vitamin and mineral supplement) from day 1 to day 12. From day 13 onwards the piglets received their respective fresh whole milk diet to start adapting them to any changes in taste when shifting from a reconstituted whole milk powder diet to a fresh whole milk diet. Fresh milk was not fed to the piglets from the start of the study due to the limited supply of fresh goat and sheep milk.

During the first 7 days of feeding (Day 1 to Day 7), the piglets received iso-caloric and iso-volumetric amounts of each diet on a body weight basis. Then, for the next 5 days (i.e. Day 8 to Day 12), the piglets received equal amounts of protein (i.e. 2 g protein per kg BW in each single meal) and equal volumes of diet per kg body weight. From day 13 to Day 15, the piglets received their respective fresh milk volumes based equal amounts of protein per kg BW as for the previous days (as protein is considered to be the main milk component influencing curd formation in the stomach). On day 14, the piglets were fed their fresh whole milk for the first three meals of the day (i.e. at 06:30 h, 10:00 h and 13:30 h). During the last two meals (i.e. at 17:00 h and 20:30 h), the piglets were fed 10% dextrose solution made in cooled boiled water (equivalent energy related to the previous three meals). This was to ensure a minimum 18 h gap between the last milk meal on the pre-sampling day (day 14) and the milk meal on the sampling day (day 15, last day), to minimize the presence of milk components remaining from the pre-sampling day(s) in the piglet's stomach. On day 15, the piglets received only one meal of fresh milk in the morning (2 g protein per kg BW, Table 7.1), following which the piglets were euthanized at set times post-feeding. Some piglets didn't receive any milk as they were '0' time piglets used to check if there was any remaining food from the previous day meals in the stomach.

Table 7.1 Calculated amounts of cow, goat, and sheep raw whole milk diet ingested in the last meal per kg BW.

Intake (g/kg BW)	Cow	Goat	Sheep
Fresh milk	55.3	63.1	31.9
Protein	2.0	2.0	2.0
Fat	2.2	2.0	2.0
Lactose	2.5	2.5	1.3
Dry matter	7.2	7.1	5.6
Gross energy (kcal/kg BW)	41.9	38.6	34.3

7.3.2.4 Euthanasia

On the sampling day (day 15, last day), at time zero (piglets fed no milk meal) and 30, 90, 150, and 210 min after completion of the fresh milk by the piglets (4 piglets per species and time, n=60), each piglet was anaesthetized with zolazepam/tiletamine, ketamine and xylazine by intramuscular injection. Immediately after sedation, the piglets were euthanized by an intracardial injection (0.3 mL/kg BW) of sodium pentobarbitone. The time period was calculated from the time the piglet finished suckling the milk until the time of death. The piglets ingested their milk quickly (2-3 min).

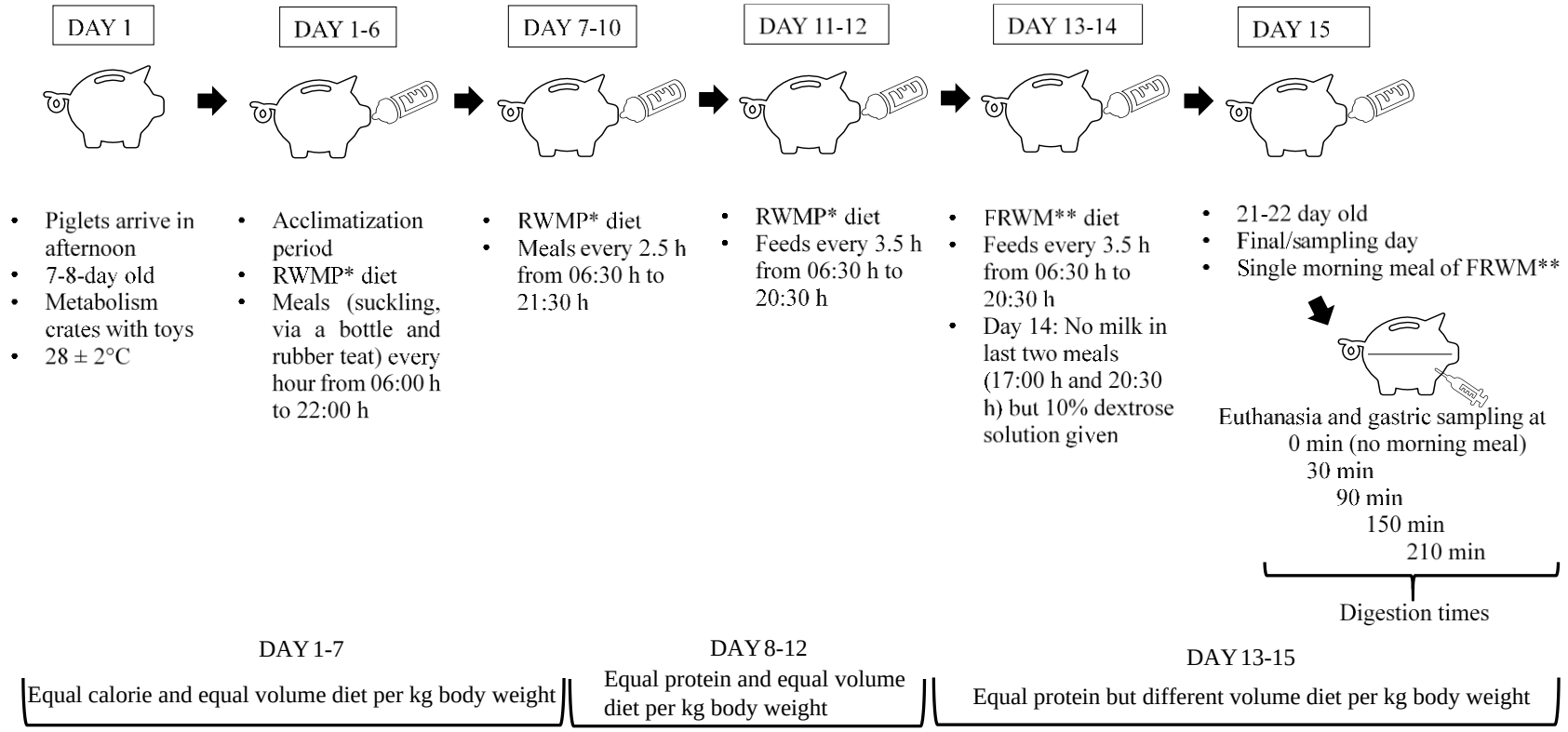
7.3.2.5 Sample collection

Following euthanasia, the abdomen of each animal was opened, and the digestive tract was immediately secured with clamps at the oesophagus, pyloric sphincter, ileo-caecal valve and rectum prior to dissecting out the whole gastrointestinal tract (GIT). The stomach was removed, the small intestine was gently uncoiled (proximal, distal and terminal ileum), the caecum was removed and the colon including rectum was uncoiled (proximal and distal). Any blood was washed off with deionized water and each GIT

section was dried using absorbent paper towels. The chyme and digesta samples were then collected from each section.

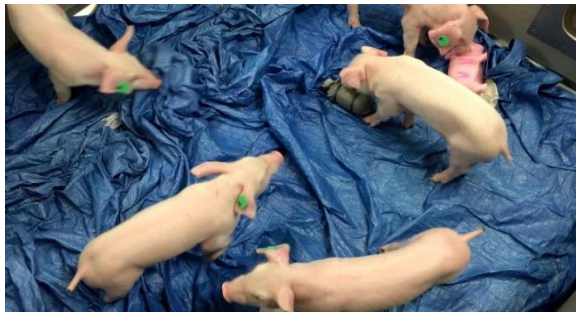
The stomach was dissected laterally from the oesophageal to the duodenal end with a single incision through the middle of the superior face of the stomach and the chyme was removed and collected. The stomach was weighed full and again empty to determine the amount of contents at each time point (0, 30, 90, 150, and 210 min). The gastric curd (or curd) and the liquid chyme were collected separately, and their pH was measured. A weighed container was used to collect the liquid part of the gastric chyme after sieving through 1 mm sieve. Visual images were taken of the gastric curd. The total time between euthanasia and completion of sampling for each pig was ~15 min. Samples for immediate analysis (i.e., rheology, microscopy, and particle size) were stored on ice and analysed or processed as soon as possible. Remaining stomach cyme (curd and liquid samples) were stored on dry ice until the collection process and then at -20°C and finally freeze dried. The freeze-dried samples were stored at -20°C.

A pictorial overview of the procedure adopted for the trial is given in Figures 7.1 and 7.2.

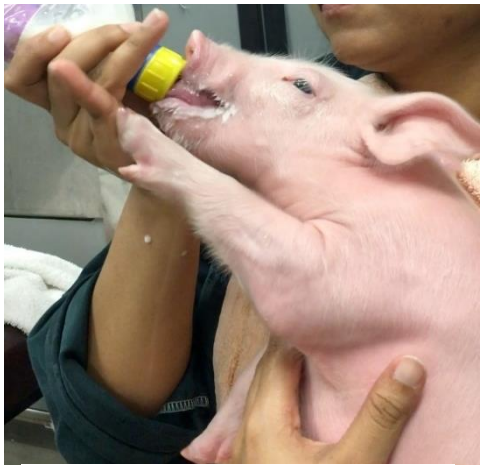


**RWMP- Reconstituted Whole Milk Powder diet (cow, goat and sheep milk-fed groups) with vitamin and mineral premix.*
***FRWM- Fresh Raw Whole Milk diet (cow, goat and sheep milk-fed groups)*

Figure 7.1 The in vivo piglet study.



Piglet socialising.



Training piglet to suckle using a bottle with rubber teat.

Figure 7.2 Photographs from the piglet study.

7.3.3 Analysis

7.3.3.1 Chemical composition of milks

The chemical composition (dry matter, crude fat, crude protein (total nitrogen × 6.38), ash, and total calcium and inorganic phosphorus) of each milk was determined as per the protocols described in Section 3.2.2.1 (Chapter 3). The lactose content of the milk was determined using a spectrophotometric enzymatic kit (Catalog no. - 10176303035) from R-Biopharm (R-Biopharm AG, Darmstadt, Germany). The gross energy content of the milk was measured using a LECO AC500 bomb calorimeter (LECO Corporation, St. Joseph, MI, USA).

7.3.3.2 Particle size distribution of the milk fat globules

Particle size distribution was undertaken as described in the protocol given in Section 3.2.2.3 (Chapter 3).

7.3.3.3 Casein micelle size

Casein micelle size was determined as described in the protocol given in Section 3.2.2.4 (Chapter 3).

7.3.3.4 pH

The pH of the milk samples, and the pH of the gastric chyme (curd as well as the liquid phase) samples at each digestion point was measured using a CyberScan pH 510 pH/mV/°C meter (Eutech Instruments, Fisher, Malaysia)**.** The initial pH of the piglet stomach contents represented the pH of the time '0' piglets that had received no meal. Please note that all the pH values given in this paper correspond to the pH of the liquid chyme, unless specified otherwise.

7.3.3.5 Confocal scanning laser microscopy (CSLM)

The proteins and fat globules were observed using CSLM as described in the protocol given in Section 6.3.2.9 (Chapter 6).

7.3.3.6 Transmission Electron Microscopy

The liquid samples (5–50 μL) were injected into agarose tubes (5–7 mm length, prepared from 3% low-temperature gelling agarose) using an auto-pipette, while the curd samples were trimmed to the correct size and shape. Both the liquid and curd samples were then processed as described in Section 3.2.2.7 (Chapter 3).

7.3.3.7 Identification of milk proteins by tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (tricine- SDS-PAGE)

Reducing tricine-SDS-PAGE was performed on the freeze-dried gastric curd and liquid phase samples from each piglet along with freeze dried milk samples. Finely ground freeze-dried powder (15 mg) was mixed with 1 mL tricine sample buffer (0.2 M Tris–HCl buffer, pH 6.8; 40% glycerol, 2% SDS, 0.04% Coomassie brilliant blue G-250, β-mercaptoethanol (0.05%, vol:vol)), heated in a boiling water bath for 5 min, cooled to room temperature, and then centrifuged at 6600 rev/min for 3 min. Immediately, a small volume of this mixture was loaded on to 16.5% Criterion tris-tricine gels (Ref-3450064, Bio-Rad) to achieve a protein concentration of 20 µg in each well. The gels were run at a constant voltage of 150 mV for 85 min using a criterion cell (Bio-rad Laboratories Pty Ltd., Auckland, New Zealand), after which the gels were removed, stained, destained, and scanned as per the method described in the Section 3.2.2.5 (Chapter 3).

7.3.3.8 Rheological analysis

An AR-G2 magnetic bearing rheometer (TA Instruments, Crawley, West Sussex, UK) fitted with a 40 mm diameter parallel steel plate geometry was used. Gastric curd (~5 g) was placed in the rheometer geometry and the samples were pressed to the systems default gap of 2000 µm. After pressing, ~2 g of the sample moved out of the geometry, which was removed and then the analysis was conducted on the remaining ~3 g of the sample. Time sweep tests were performed on the gastric curds at a frequency of 1 Hz, strain of 0.5, for 20 min at 37°C (Mulet-Cabero *et al.*, 2019). The complex modulus (G*, Pa = oscillation stress/ % strain) values obtained after 15 min of measurement are reported (Mulet-Cabero *et al.*, 2019). G* is a measure of resistance to deformation of a viscoelastic sample (in this case, it is the curd formed in the stomach); the higher the G* value, the

higher the rigidity (Kulkarni & Shaw, 2016). Rheological measurements were completed within an hour of sampling the gastric curds.

7.3.3.9 Chemical analysis of the stomach chyme (curd and liquid)

The stomach was weighed full and empty to determine the weight of the contents at each sampling time point (0, 30, 90, 150, 210 min). This provided data on wet weight of the total stomach contents at each timepoint. The curd and the liquid were then separated. A weighed container was used to collect the liquid part of the stomach chyme after sieving through a 1 mm sieve (to mimic the gastric sieving effect) and the weight of the liquid part was recorded. A similar method has been used previously by Meisel and Hagemeister (1984) while studying the impact of different commercial processing on cow milk in minipigs. The weight of the wet curd was determined by deducting the weight of the liquid from the weight of the total stomach content. Milk samples as well as stomach chyme samples were freeze-dried, weighed and then finely ground (to pass through a 1 mm sieve). These samples were analysed for dry matter content by drying overnight at 105°C. The crude protein (total nitrogen × 6.38) and fat content of the freeze-dried chyme were analysed using the Dumas method 968.06 (AOAC, 2005) and Mojonnier method 989.05 (AOAC, 2005), respectively. The protein and fat contents of the chyme were then expressed on a dry matter basis (oven dry).

7.3.3.10 Statistical Analysis

Statistical analyses were in general performed using the MIXED Model procedure of SAS (SAS/STAT version 9.4; SAS Institute Inc., Cary, NC, USA). The chemical composition of milk (DM, crude protein, crude fat, ash, lactose, calcium, phosphorus, protein to fat ratio, and gross energy) from the different species was compared using one-way ANOVA.

Gastric emptying rates were calculated after making a correction for amounts of dry matter and chemical components present in the stomach from previous meals (time 0). The relative retentions and total amounts retained (wet weight, DM, moisture, crude protein, and crude fat) post-feeding were determined according to a power exponential model:

$$\text{Relative retention or amount retained}_{\text{Time}} = \alpha_0 \exp - (\kappa \times \text{time})^{\beta}$$

where α_0 is the proportion remaining at time 0 (100% for relative retention, or absolute amount retained for 100 g milk ingested). The parameters κ (slope of the curve) and β (index for the shape of the curve, results not shown), and $T_{1/2}$ [half gastric emptying time, $T1/2 = (1/\kappa) \times (\log(1/0.5))(1/\beta)$] were estimated using the Proc NLIN procedure of SAS. For all analysed response variables, fitted curves of the reduced (i.e. no difference between species) or full non-linear model were compared using the F-test. For all variables, the full non-linear model better described the responses, unless otherwise specified.

For pH, rheological properties, particle size response variables and relative retention of the solid and liquid phases of the chyme, models containing species, time (30 to 210 min as either categorical or numerical variable), and species by time interaction as fixed effects were used. When the interaction effect was not statistically significant, it was removed from the model.

The model diagnostics for each response variable were tested after combining the PROC UNIVARIATE and the ODS GRAPHICS procedures of SAS before comparing the means. When a response variable did not fulfil the model assumptions of normality and

homoscedasticity, a transformation of the raw data was conducted. When the F-value of the model was significant ($P < 0.05$), the means were compared using the adjusted Tukey's test. Results are reported as mean ± SEM (standard error mean).

7.4 Results and discussion

7.4.1 Chemical composition of different whole milks

The comparative chemical composition (per 100 g or mL of milk) of fresh cow, goat and sheep whole milks are reported in Table 7.2. Sheep milk was significantly higher ($P < 0.05$) in total solids (dry matter), protein, fat, mineral (Ca and P), and gross energy content compared to both goat and cow milk. Goat milk had significantly lower amounts ($P < 0.05$) of total solids, protein, and fat, calcium, and gross energy content when compared to both cow and sheep milk. The phosphorus content of goat milk was similar to that of cow milk. There were no statistically significant differences in the ash content and protein to fat ratio of milk from different species. The lactose content of cow milk was the highest followed by sheep and goat milk. The differences in chemical composition was similar to that observed in the previous chapters and results reported in literature (Barlowska *et al.*, 2011; Claeys *et al.*, 2014; Stergiadis *et al.*, 2019). As the milk from different species varied in dry matter, the comparative chemical composition (per 100 g dry matter) of fresh cow, goat and sheep whole milks is also reported in Table 7.3.

Table 7.2 Chemical compositions of cow, goat, and sheep raw whole milks.

Properties	Cow	Goat	Sheep
Dry matter (g per 100 g)	13.1 ± 0.13^{b}	11.23 ± 0.13^{c}	17.60 ± 0.13^{a}
Protein (g per 100 g)	3.62 ± 0.05^{b}	3.17 ± 0.05^{c}	6.27 ± 0.05^{a}
Fat (g per 100 g)	4.06 ± 0.11^{b}	3.23 ± 0.11^{c}	6.31 ± 0.11^{a}
Lactose (g per 100 mL)	4.56 ± 0.04^{a}	3.92 ± 0.04^{c}	4.17 ± 0.04^{b}
Ash (g per 100 g)	0.77 ± 0.02	0.80 ± 0.01	0.86 ± 0.08
Protein to fat ratio	0.89 ± 0.02	0.98 ± 0.02	0.99 ± 0.02
Calcium (mg per 100 mL)	125.68 ± 3.4^{b}	112.40 ± 3.4^{c}	188.16 ± 3.4^{a}
Inorganic phosphorus (mg per 100 mL)	88.13 ± 0.39^{b}	80.84 ± 3.56^{b}	139.21 ± 6.39^{a}
Gross energy (kcal per 100 g)	75.81 ± 0.74^{b}	61.11 ± 0.74^{c}	107.50 ± 0.74^{a}

$^{a–c}$Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SEM.

Table 7.3 Chemical compositions of cow, goat, and sheep raw whole milks (per 100 g dry matter).

Properties	Cow	Goat	Sheep
Protein (g)	27.63 ± 0.36^{b}	28.18 ± 0.36^{b}	35.66 ± 0.36^{a}
Fat (g)	30.98 ± 0.66^{b}	28.71 ± 0.66^{b}	35.86 ± 0.66^{a}
Lactose (g)	34.86 ± 0.66^{a}	34.91 ± 0.66^{a}	23.67 ± 0.66^{b}
Ash (g)	5.87 ± 0.30^{b}	7.18 ± 0.30^{a}	4.87 ± 0.30^{b}
Protein to fat ratio	0.89 ± 0.02^{a}	0.98 ± 0.02^{a}	0.99 ± 0.02^{a}
Calcium (mg)	959.42 ± 20.25^{b}	1000.73 ± 20.25^{ab}	1068.94 ± 20.25^{a}
Inorganic phosphorus (mg)	672.81 ± 27.98^{a}	719.92 ± 27.98^{a}	790.87 ± 27.98^{a}
Gross energy (kcal)	578.65 ± 3.53^{b}	544.14 ± 3.53^{c}	610.81 ± 3.53^{a}

$^{a–c}$Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SEM.

7.4.2 Fat globule and casein micelle sizes of different milks

The volume weighted (D_{43}) and surface weighted (D_{32}) mean diameter of fat globules of the different whole milks is reported in Table 7.4. The volume weighted (D_{43}) mean diameter of fat globules of goat milk (~3.6 μm) was smaller than that of cow milk (~4.4

μm) and sheep milk (~4.3 μm), both of which had similar D_{43} mean diameter. Similarly, the surface weighted (D_{32}) mean diameter of fat globules of goat milk (~2.9 μm) was smaller than that of cow milk (~3.8 μm) and sheep milk (~3.5 μm). The results are in line with previous studies which also reported that goat milk has smaller fat globule sizes than cow milk (El-Zeini, 2006; Gantner *et al.*, 2015; Park *et al.*, 2007).

The Z-average diameter (nm) of casein micelles (Table 7.4) of cow milk was found to be significantly ($P < 0.05$) lower (~158 nm) than that of sheep (~180 nm) and goat milk (~190 nm). The results are in accordance with previous studies which reported that goat and sheep milk have larger casein micelle sizes than cow milk (Nguyen *et al.*, 2018; Park *et al.*, 2007).

Overall, the differences observed in the chemical composition, fat globule diameter and casein micelle diameter of cow, goat, and sheep milk used in this study were similar to those reported in the previous chapters (Chapters 4, 5, and 6).

Table 7.4 Fat globule and casein micelle sizes of cow, goat, and sheep milk.

Properties	Cow	Goat	Sheep
Fat globule size			
D_{43} (μm)	4.38 ± 0.06^{a}	3.61 ± 0.02^{b}	4.29 ± 0.03^{a}
D_{32} (μm)	3.77 ± 0.06^{a}	2.86 ± 0.02^{c}	3.49 ± 0.02^{b}
Casein micelle size			
Z-average diameter (nm)	158.41 ± 1.68^{c}	189.98 ± 3.5^{a}	179.55 ± 0.73^{b}

$^{a–c}$Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SEM.

7.4.3 Gastric pH changes during digestion

The changes in the pH profile of the gastric chyme (both the liquid phase and the curd) of the piglets at different times are shown in Figures 7.3A and 7.3B.

There were no significant differences ($P > 0.05$) in the changes in gastric pH with time among milk from different species. The pH of the gastric liquid and the curd obtained from all of the piglets at time '0' (which represents the empty stomach) was ~3.0 (Figure. 7.3A) and ~3.3 (Figure 7.3B), respectively. Only a small amount of liquid and (or) curd was present at the '0' timepoint. 30 min after ingestion of the meal, the pH of the gastric liquid increased to ~5.9 (Figure. 7.3A). This was expected due to the high initial pH of the milk (~6.7) as well as the high buffering capacity of the different milks. As digestion progressed, the pH of the gastric liquid gradually decreased again to ~3.0 after 210 min of digestion (Figure. 7.3A). The pH of the gastric curds followed a similar trend to the gastric liquid (Figure. 7.3B). However, the pH value of the curds was higher and showed a slower decrease in pH with time compared to the pH of the gastric liquid phase, especially after 30 min of digestion (Figure. 7.3A and 7.3B). This was likely due to the higher buffering capacity of the aggregated protein phase. This observation is similar to that found for pH during dynamic *in vitro* gastric digestion (Chapter 6), wherein it was found that the pH at the centre of the curd (or clot) from raw whole milk of different species remained relatively higher (~3) compared to that of the liquid phase (~2) at the end of 240 min of gastric digestion.

The results indicate that the gastric pH of the bottle-fed piglets on a fresh milk diet does not drop to as low as pH 2. This is due to the lower acid secretion capacity of the piglet's stomach in comparison with adults (Moughan *et al.*, 1992). The trends in the gastric pH

observed in this *in vivo* study were similar to those reported by Mason (1962) in human infants fed breast milk. Mason (1962) reported a pre-feed mean pH of 3.5, which at 30 min post-feeding increased to 6.4 and gradually decreased to 3.2 at 210 min in infants who were feeding on mother's breast milk. The extent of the pH decrease is likely dependent on the type (composition) and quantity of diet provided as well as on the frequency of feeding. Moughan *et al.* (1991) also reported a gradual decrease in the gastric pH of bottle-fed piglets (gastric-cannulated) to ~2.5 after 240 min of ingestion of a cow milk-based formula. It has also been reported that along with hydrochloric acid (HCl), the presence of lactic acid (due to the gastric fermentation of lactose) may also contribute to the acidity of the stomach content of piglets (Cranwell *et al.*, 1976).

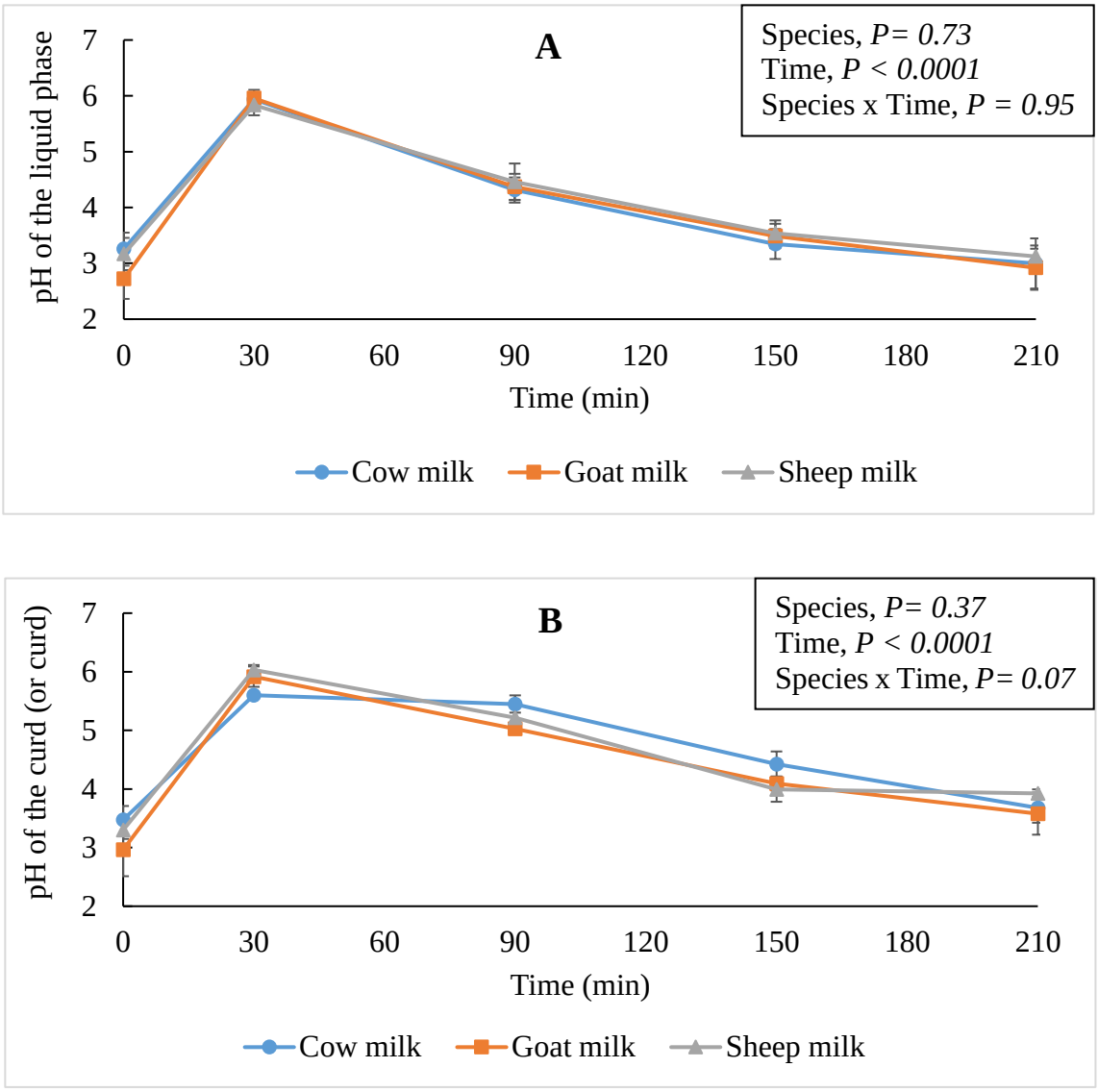


Figure 7.3 Changes in the pH of different whole milks during gastric digestion in the piglet. (A) pH of the strained liquid obtained at different timepoints from the piglet's stomach after sieving the gastric contents through a 1mm sieve; (B) pH of the curd obtained at different timepoints from the piglet's stomach; Values are mean ± SEM.

7.4.4 Milk coagulation in the stomach

Representative photographs of the gastric chyme collected from the stomach of piglets are shown in Figure 7.4. The gastric chyme consisted of coagulated curd and a liquid phase which are largely removed by sieving the gastric contents through a 1 mm sieve as shown in Figure 7.4A. The quantity of the liquid and the coagulated phases differed at different digestion times.

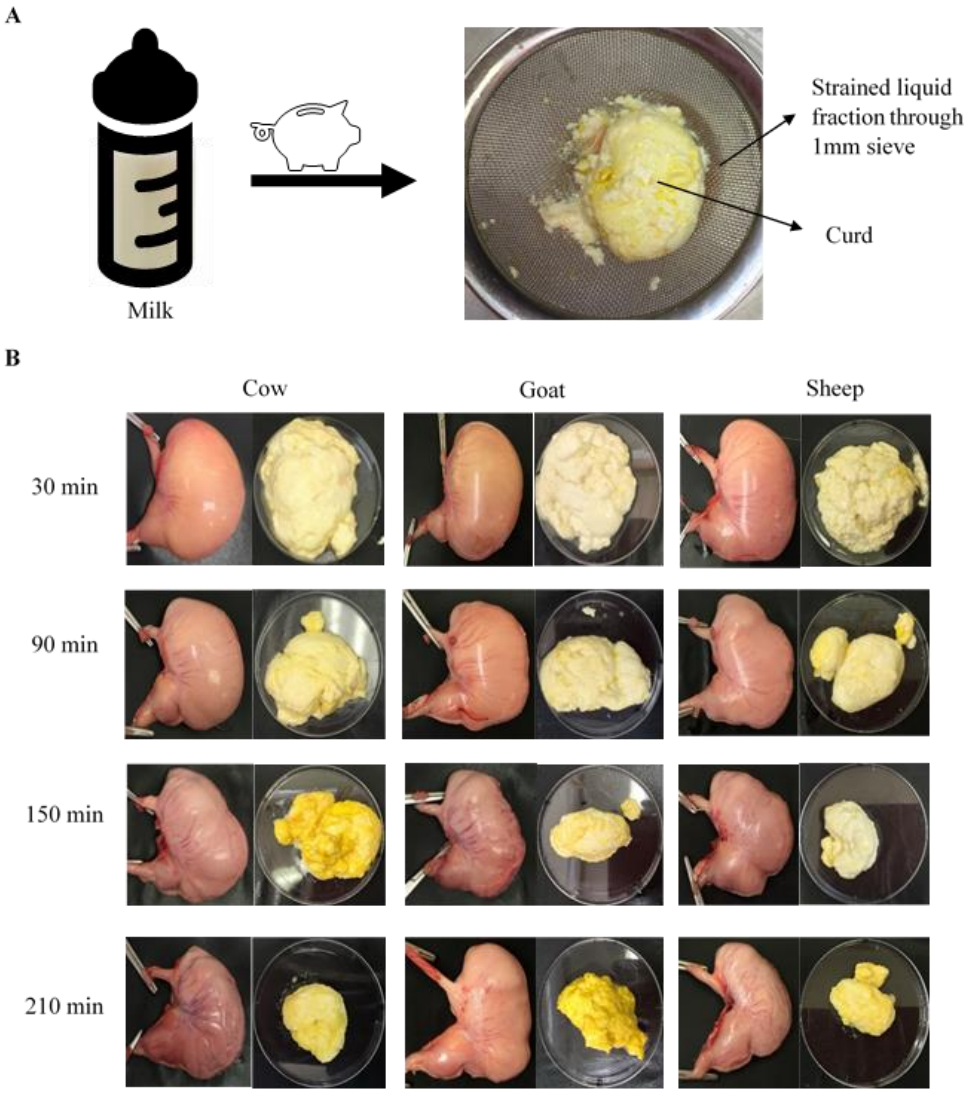


Figure 7.4 Photographs of the gastric chyme collected from the piglet's stomach. (A) an example of the stomach contents. (B) piglet's stomach (before dissection) and the curds obtained at different digestion timepoints. Note: only a representative photograph from one piglet at each timepoint is shown.

The stomach was extended at 30 min, while at 210 min it appeared to be nearly empty (Figure 7.4B). The whole milk of all the species formed a coagulum within 30 min of feeding at which time the pH was ~5.9 (for both the separated gastric curd as well as the liquid). The curd formed at 30 min appeared to be fragile and soft. With the increase in digestion time accompanied by a decrease in gastric pH, the curds became more compact, tighter, and smaller in size. This was due to the loss of curd material as well as the entrapped liquid. There was a similar trend in this respect for the milk curd of all of the species when observed visually. Similar observations were also noted during the *in vitro* gastric digestion of different milks using the HGS (Chapter 6).

The curds remaining in the piglet's stomach during the later stages of digestion appeared to be slimy and yellower in colour. This might be because of the coating of curd by gastric mucus and the presence of bile pigments due to the reflux of intestinal contents. Cranwell *et al.* (1976), while studying the gastric secretions and fermentation of pig's milk in 1-33-day-old piglets, reported that no physical changes to pig milk were observed when the stomach contents were collected 2-3 min post-suckling. However, soft and gelatinous curds were observed at 5 min post-suckling, and they also reported that the curds became granular in appearance, although still remaining soft during the subsequent 30 min digestion. During the next 30 min (i.e. ~60 min) the pig milk curds became drier, crumbled (pig's milk composition is different to cow's milk), and were frequently stained with bile pigments due to regurgitation of intestinal contents. Similar observations have been reported by Braude *et al.* (1970) who observed curd formation in the stomach contents of 28-day-old pigs after 15 min of feeding homogenized cow milk, and complete separation of curds and soluble or 'whey' fraction after 30 min post-feeding. They also

reported the presence of bile pigments in the stomach contents due to reflux of intestinal contents, which led to an increase in stomach pH after 1 h of digestion.

The curd formation in the raw whole milk from different species is due to the aggregation of casein micelles alone, as reported previously in Chapter 5; whey proteins remain soluble in the liquid phase of the gastric chyme and are not actively involved in curd formation (Chapters 5 and 6). The three-week-old suckling piglet is considered to have significant chymosin activity along with some pepsin activity and relatively lower gastric acid secretion capacity (Moughan *et al.*, 1992). Chymosin and pepsin belong to the same group of aspartic proteinases that uses aspartic acid residues in their active centre (Moschopoulou, 2011), and both are able to preferentially hydrolyse the Phe105–Met106 bond of κ-casein, except that pepsin also exhibits unspecific proteolytic activity towards bonds with Trp, Tyr, Leu or Val residues, and thus have a higher proteolytic activity than chymosin (Guinee & Wilkinson, 1992; Júnior *et al.*, 2015; Moschopoulou, 2011). The mechanism of action of chymosin and pepsin is considered to be similar in relation to milk clotting. Thus, the formation of the casein curd at around pH 6.0 (as observed in this study) would be due to the specific cleavage of the Phe105-Met106 bond of κ-casein by chymosin as well as pepsin. This resulted in the destabilization of casein micelles and their aggregation after a sufficient amount of κ-casein was hydrolysed (Jollès, 1966; Tam & Whitaker, 1972). Chymosin is most stable in the pH range of 5.3–6.3, but loses its activity rapidly under acidic conditions (i.e., below pH 3–4), as well as at high alkaline pH values, (i.e., above pH 9.8) (Crabbe, 2004). However, pepsin has maximum proteolytic activity at pH 2, with an optimum pH range of 2–5, and has some activity in the pH range pH 5.5–7.5. Pepsin is irreversibly inactivated at pHs above 7.5 (Piper & Fenton, 1965). This suggests that although the complete phase separation of milk was

observed in piglets' stomach at 30 min (pH ~5.9), the process of milk coagulation might have been initiated much earlier, i.e. when the pH was greater than 5.9.

The dynamic macrostructural changes observed in milk from the three species in the piglet's stomach are expected to be mainly due to gastric shearing (contraction) action along with the proteolytic enzyme (chymosin as well as pepsin) activity and the acidic environment of the stomach. However, it should be noted that the gastric enzyme activities in piglets (mimicking human infants) are significantly lower than those in growing pigs (mimicking human adults), due to the lower pepsin secretion, acid output and higher gastric pH (higher than the optimum pH for maximum pepsin activity) (Bourlieu *et al.*, 2014; Moughan *et al.*, 1992). Despite differences in the enzyme concentration (and activity), acidity, and contraction frequency (or forces) between different models (*in vivo* piglet versus HGS) for mimicking gastric digestion, the mechanism of milk coagulation was observed to be similar in all the systems.

It has been reported that during the first few hours after birth, some human infants may have a chymosin like enzyme (which is not pepsin) along with pepsin (Henschel *et al.*, 1987), but this is considered to disappear from the gastric fluid 10 days after birth and is not found in adult gastric fluid (Dallas *et al.*, 2012). It is considered that a full term infant has 13% of the adult pepsin activity at birth, which increases to 30% at 1 month of age (Bourlieu *et al.*, 2014). In addition, the extent, frequency and speed of contractions in infants/toddlers are considered to be lower than the adults, while the transit time is quite similar to that of adults (Bourlieu *et al.*, 2014).

7.4.5 Microstructure of the gastric chyme (curd and liquid) observed under confocal scanning laser microscopy (CSLM)

The microstructures of the gastric curds and the liquid chyme obtained from milk of the three species at 30, 90, 150 and 210 min as observed by CSLM are depicted in Figures 7.5 and 7.6.

The gastric curd microstructures of cow milk showed a compact protein matrix (in green), while a significant proportion of fat globules (in red) remained entrapped (or embedded) within the protein matrix of the curd. Spherical, coalesced or aggregated (but globular) as well as non-spherical forms of fat globules (larger in size than those present in fresh milk) were observed in the gastric curds at all digestion timepoints (Figure 7.5). Similar observations were noted for goat and sheep whole milk curds at different digestion times (Figure 7.5). These results are similar to those observed in the previous chapter (Chapter 6) while studying the *in vitro* gastric digestion of raw whole milk from the different species. As reported in Chapter 6, pepsin can hydrolyse the MFGM proteins and increase the susceptibility of the fat globules to changes in structure by the shearing action of the stomach contraction. However, the extent of coalescence and the amount of non-spherical fat appeared to be much higher in the *in vivo* samples of the curd to that seen *in vitro*. This may potentially be due to the gastric lipase present in the piglet stomach along with pepsin.

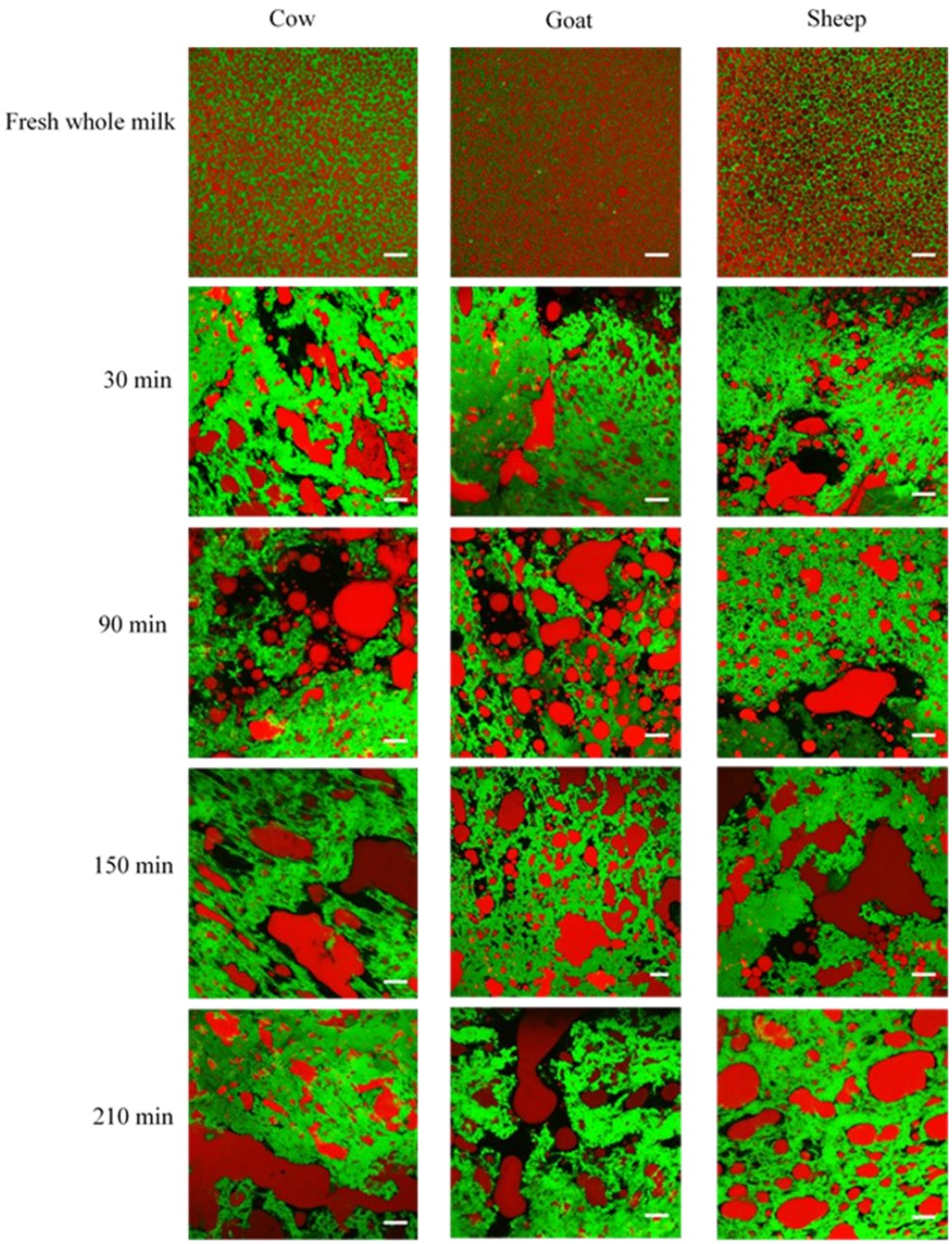


Figure 7.5 Confocal micrographs of the cow, goat, and sheep whole milk curds remaining in the piglet's stomach at different digestion times. Scale bars represent 25 µm. Note: only one representative micrograph from one piglet at each timepoint is shown.

Gastric lipase can diffuse into the triglyceride core of the milk fat globules through the altered MFGM (generated from hydrolysis of MFGM proteins to peptides by pepsin) and the lipolytic products generated can accumulate at the fat-water interface or even replace the MFGM phospholipids present at the interfacial layer of the fat globules. These products of lipolysis and proteolysis along with some intact or partially hydrolysed MFGM material may provide structural stability as well as a steric barrier or electrostatic repulsion to prevent the flocculation and coalescence of fat globules (Gallier *et al.*, 2013; Gallier *et al.*, 2012; Ye *et al.*, 2011). However, this modified interfacial layer is not as stable as the native MFGM and may be more susceptible to coalescence under the gastric digestion conditions.

The contraction forces and the frequency for the HGS used during *in vitro* gastric digestion studies are based on those occurring in the human stomach and those patterns may differ to that of a piglet/pig stomach (Ehrlein & Schemann, 2005) and may also be a reason for the observed differences. In addition, the piglet's body temperature was generally in the range of 38-40°C during the trial (usual body temperature), which is higher than a normal human body temperature or that followed in the HGS (36.5-37.5°C), which may also have contributed to the increased coalescence observed in the *in vivo* (versus *in vitro*) curd samples, considering that the milk fat globule is very sensitive to changes in temperature (Et-thakafy *et. al.*, 2017). It has to be also noted that this variation in body temperature may have increased the curd strength and contraction (a similar phenomenon happens during cheese making at higher temperature) (Patel *et. al.*, 1971), which may also have pushed the fat globules in closer contact, enhancing coalescence.

The microstructure of the liquid chyme obtained from whole milk of the three species at 30, 90, 150 and 210 min is depicted in Figure 7.6.

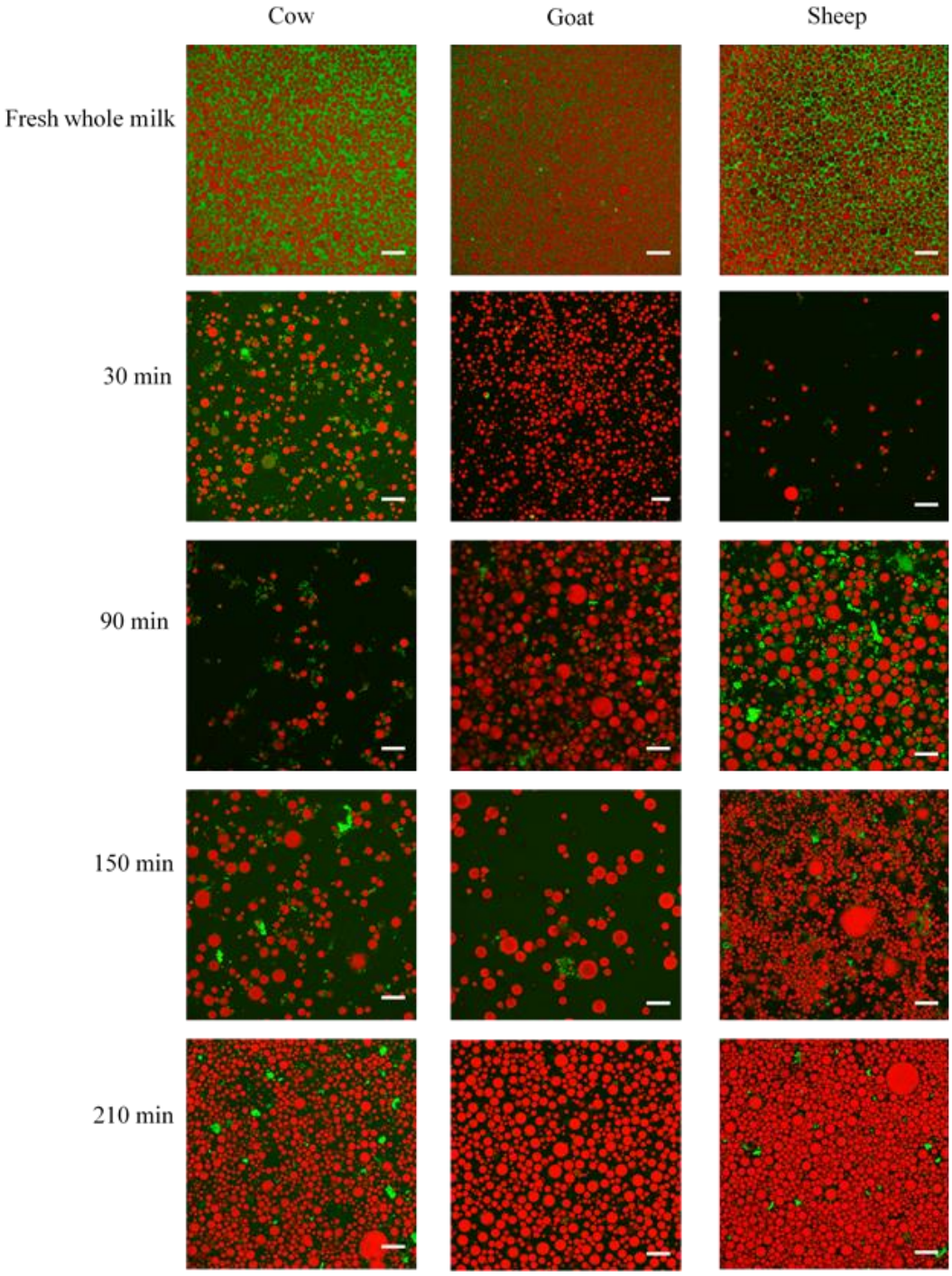


Figure 7.6 Confocal micrographs of the gastric liquid from cow, goat, and sheep whole milk remaining in the piglet's stomach at different digestion times. Scale bars represent 25 µm. Note: only one representative micrograph from one piglet at each timepoint is shown.

Compared to the fat globules in the gastric curds, the fat globules in the liquid chyme appeared to be less coalesced and maintained their spherical shape. The degree of coalescence appeared to be more prevalent as the digestion progressed (i.e. from 90 min onwards) (Figure 7.6). In addition, some small casein aggregates were also observed in the liquid chyme (Figure 7.6). These observations were similar for the liquid chyme of milk from all of the species. It was evident that the extensive coalescence of fat globules and non-globular forms of fat observed in the protein matrix of the curd was not observed in the liquid chyme. The presence of a dense protein matrix around the fat globules in the curd and the higher entrapped fat phase volume concentration in the gastric curd may have increased the chances of frequency of contact between the fat globule surfaces, leading to breakdown of the native or altered (stabilized by peptides, monoglycerides, diglycerides and/or phospholipids) surface membrane, enhancing coalescence, as discussed in the previous chapter (Chapter 6). In addition, the liquid chyme is readily emptied from the stomach as well as diluted (slightly) with gastric secretions, rendering it less prone to the shearing action of the stomach and the action of the gastric enzymes present (compared to the fat globules entrapped within the curd).

To the best of my knowledge, no *in vivo* studies on cow milk or non-cow milks have reported the microstructural changes occurring during the gastric digestion of fresh (raw) whole milks using confocal microscopy. However, the microstructural results observed in this study for different milks are in agreement with the previous *in vitro* adult gastric digestion observations (Chapter 6). Gallier *et al.* (2013) also reported an increase in size of the fat globules from raw (non-homogenized) cream in the rat's stomach. Overall, the microstructural results confirm that a combination of mechanisms appear to be responsible for the structural changes occurring in the protein and fat globules during

gastric digestion of milk from different species, but the changes appear similar for cow, goat, and sheep milk.

7.4.6 Fat globule size distribution of the liquid chyme

In raw whole milk from the cow, goat, and sheep, the average fat globule size distribution ranged from 1.45-15.14 µm, 0.55-13.18 µm, and 1.10 – 15.14 µm, respectively, before digestion (fresh milk) (Figure 7.7). During gastric digestion (30 to 210 min), the average fat globule size distribution of the liquid chyme from cow, goat, and sheep milk ranged from 0.06-724.44 µm, 0.03-724.44 µm, 0.03-630.96 µm, respectively. This indicates that there was an increase in larger particles (in the range of ~15 – 725 µm) and some smaller particles in the liquid chyme (Figure 7.7). However, there were no particular trends observed in the changes in the average D_{43} and D_{32} diameter of the milk fat globules during gastric digestion between and within species (Table 7.5A and 7.5B). EDTA-SDS solution was used to dissociate any flocs present as well as casein micelles/protein aggregates before particle size analysis of the liquid chyme (Wang *et al.*, 2020; Ye *et al.*, 2002). It cannot be discounted that the presence of other fine or slimy material such as mucins or mucus material from the piglet stomach may have interfered with the determination of the true particle size of the milk fat globules.

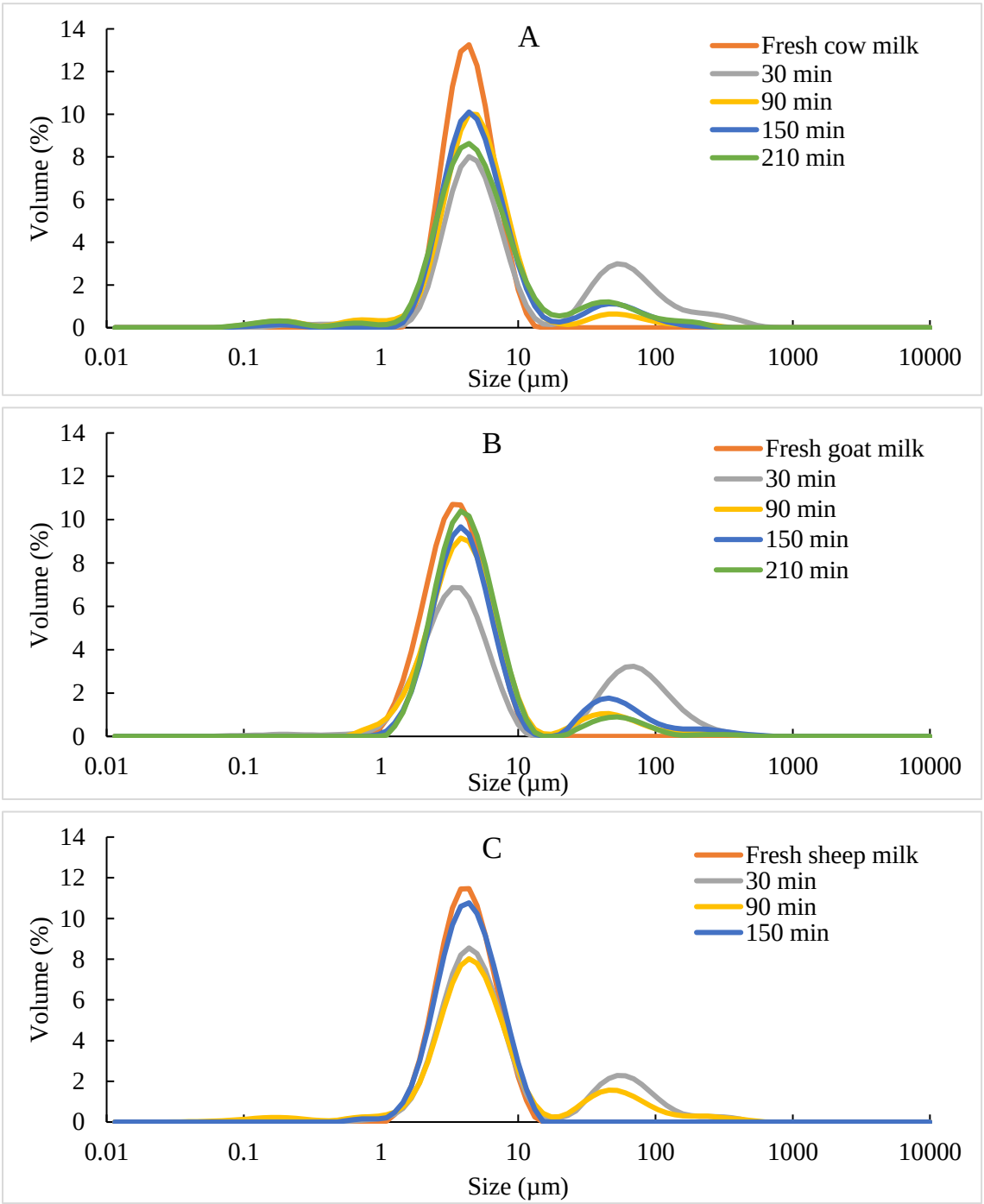


Figure 7.7 Particle size distribution of the gastric liquid of whole milk from different species at different digestion timepoints. (A) cow whole milk, (B) goat whole milk, and (C) sheep whole milk. Mean values for two to four piglets are reported. Note: insufficient sample for 210 min digestion timepoint of sheep milk for analysis.

Table 7.5 Changes in the particle size distribution of the gastric liquid of whole milk from different species at different digestion timepoints. (A) volume weighted mean diameter, D_{43} (μm), and (B) surface weighted mean diameter, D_{32} (μm). Values are mean ± SEM.

A. Volume weighted mean diameter, D_{43} (μm)*			
Time (min)	**Cow**	**Goat**	**Sheep**
Milk (before beginning digestion)	$4.38 \pm 0.06^{c,x}$	$3.61 \pm 0.06^{c,y}$	$4.29 \pm 0.07^{b,x}$
Liquid chyme			
30 min	26.82 ± 3.70^{a}	21.67 ± 3.70^{a}	20.57 ± 3.20^{a}
90 min	8.97 ± 3.13^{bc}	10.66 ± 3.62^{bc}	16.35 ± 3.13^{a}
150 min	$10.20 \pm 1.54^{b,y}$	$16.98 \pm 2.18^{ab,x}$	$4.45 \pm 2.18^{b,y}$
210 min	11.86 ± 3.39^{bc}	$9.40\ 3.39^{bc}$	_

B. Surface weighted mean diameter, D_{32} (μm)*			
Time (min)	**Cow**	**Goat**	**Sheep**
Milk (before beginning digestion)	3.77 ± 0.10^{xy}	$2.86 \pm 0.04^{b,z}$	$3.49 \pm 0.03^{b,y}$
Liquid chyme			
30 min	5.12 ± 0.45^{x}	$3.44 \pm 0.12^{a,y}$	$4.63 \pm 0.38^{a,xy}$
90 min	3.36 ± 0.88	3.38 ± 0.26^{ab}	3.52 ± 0.76^{ab}
150 min	3.79 ± 0.47	3.82 ± 0.16^{a}	3.47 ± 0.10^{b}
210 min	3.34 ± 1.19	3.60 ± 0.05^{a}	_

*Letters (a–c) in a column represent significant differences ($P < 0.05$) within a certain treatment across different digestion times. Letters (x-z) in a row represent significant differences ($P < 0.05$) across different treatments at a certain digestion time. If no letter is listed, there were no significant differences. Note: insufficient sample for 210 min digestion timepoint of sheep milk for analysis.

7.4.7 Changes in the gastric chyme (curd and liquid) observed under transmission electron microscopy (TEM)

The changes in the state of the protein network and fat globules within the gastric curds observed using TEM are shown in Figure 7.8. The light-greyish structures in the transmission represent fat, whereas the dark electron dense areas represent protein. In fresh milk from all of the species, the protein and fat globules were uniformly dispersed.

The casein micelles in goat and sheep milk appeared to have larger proportions of large casein micelles (Figure 7.8). At 30 min of digestion, it was clearly observed that the casein micelles aggregated to form a loose network with fat globules entrapped within this network (Figure 7.8). As the digestion progressed, i.e. with the increase in digestion time as well as decrease in stomach pH, the casein aggregate appeared to fuse together and become denser (Figure 7.8). In addition, the milk fat globules appeared to be disrupted with some coalescence. The fat globules located inside the protein matrix were not stained properly; this might be due to the hydrolysis of the fat globules by gastric lipase. These observations were similar for milk curd from all of the species. Interestingly, Berendsen (1982) and Berendsen and Blanchette-Mackie (1979) reported similar observations for the structural state of protein and fat during rat milk digestion in the 10-day old suckling rat.

There were obvious differences in the appearance of the protein network during digestion among the three milks (Figure 7.8). The extent of fusion, and compaction of the protein network with digestion time were less obvious in goat and sheep milk curds. However, the protein network of the sheep milk curds at 150 min also appeared to be more fused compared to the protein networks of sheep milk curds at other timepoints. Some remnants of milk fat globule membrane (MFGM) material were apparent, particularly in goat and sheep milk.

In the liquid chyme of the milk from all of the species, fractions of milk fat globule membrane (MFGM), milk fat globules, and some protein aggregates were observed (Figure 7.9). This indicates that although most of the fat was entrapped within the curds, some of the detached or broken fragments of the MFGM were released into the liquid

phase of the chyme, during the shearing action of the stomach contraction. Thus, it is expected that the MFGM fragments would rapidly transit the gastric phase into the small intestine; this may be an important factor in delivering some of the key bioactive nutrients associated with MFGM to the body.

It must be pointed out that the TEM analysis was conducted on samples from 1-2 piglets for each timepoint only due to the limits on the number of fresh samples that can be processed.

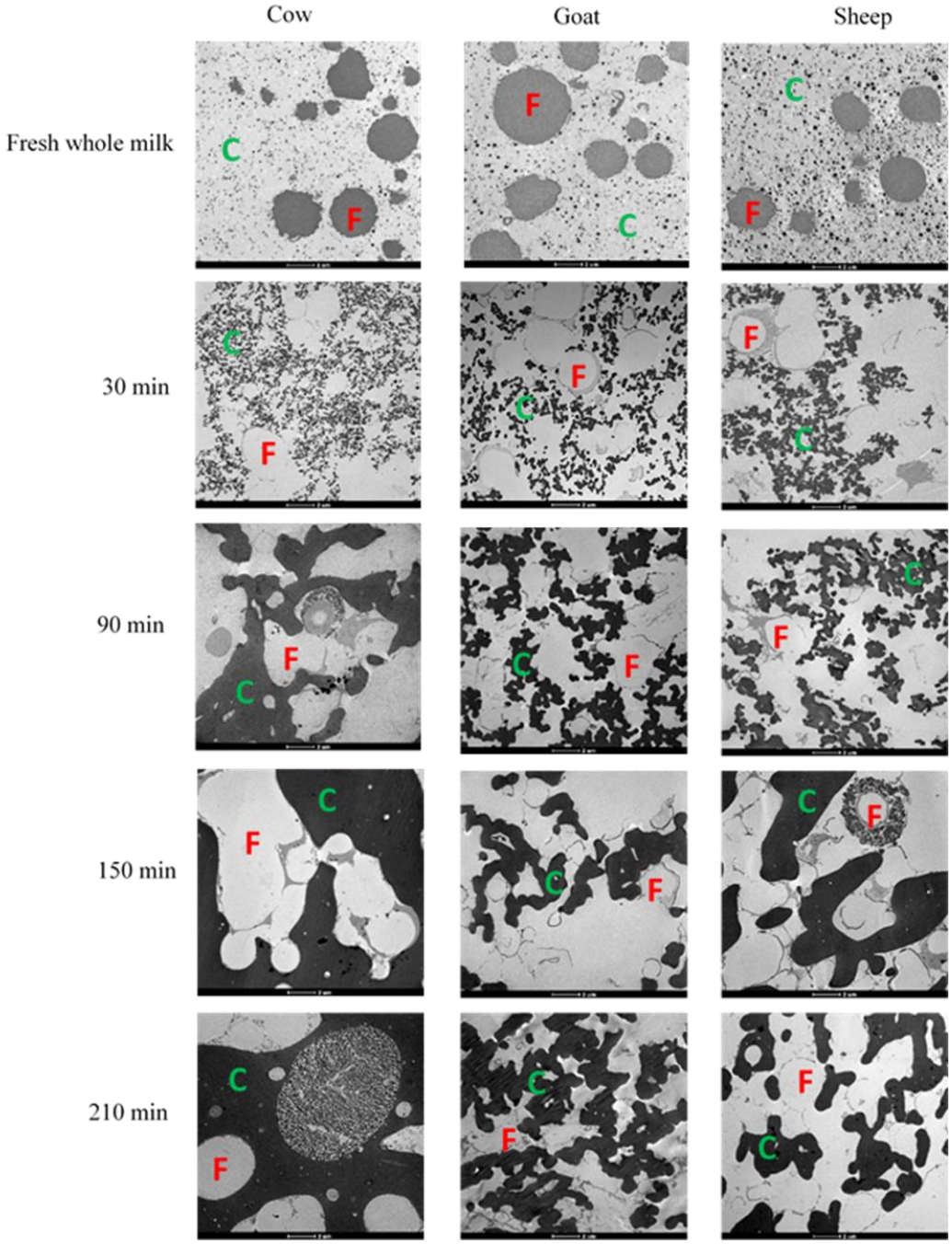


Figure 7.8 Transmission electron micrographs of the gastric curds from cow, goat, and sheep whole milk remaining in the piglet's stomach at different digestion times. Scale bars represent 2 µm. C represents casein, F represents fat. Note: only one representative micrograph from one piglet at each timepoint is shown.

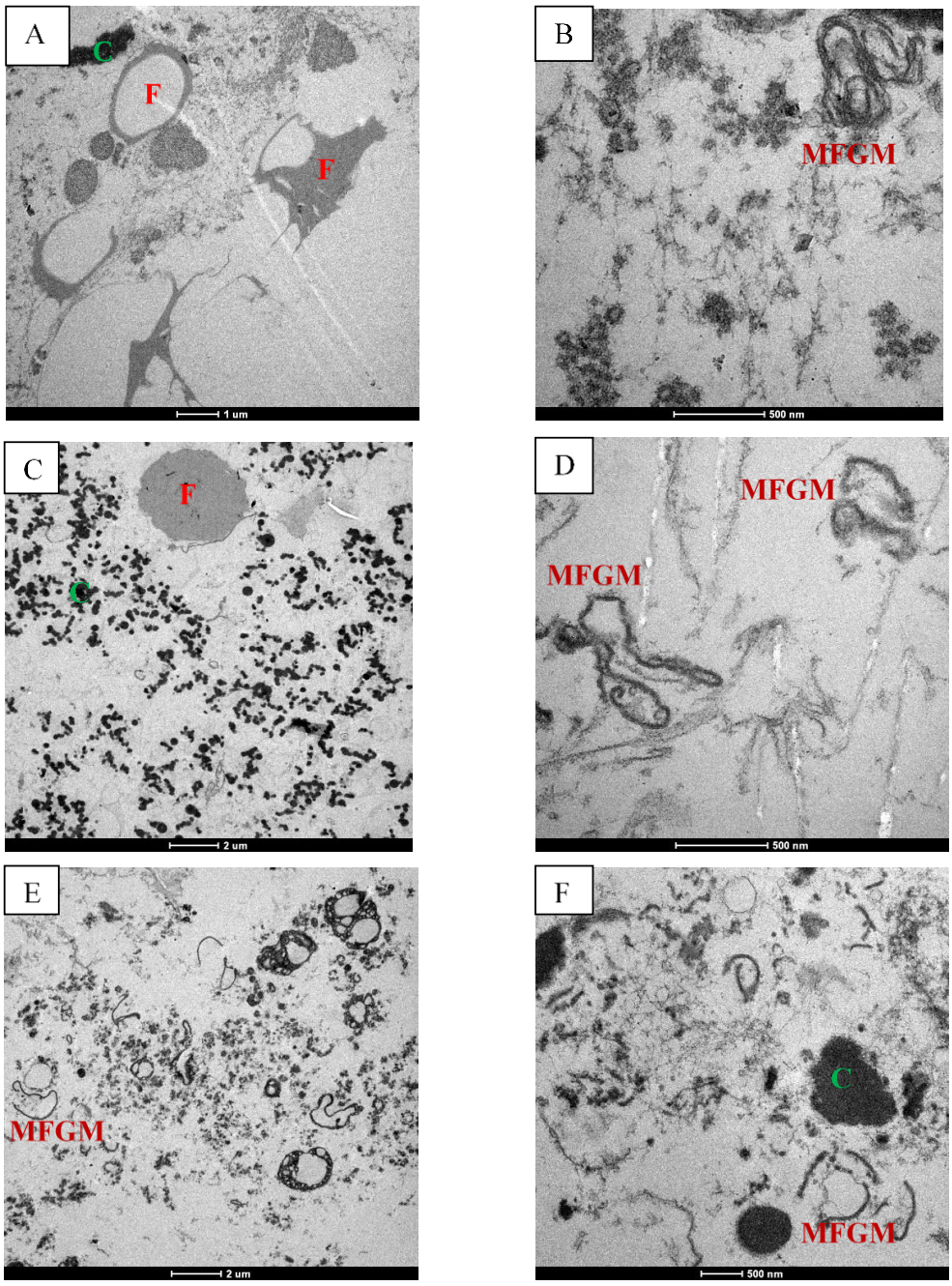


Figure 7.9 Transmission electron micrographs of the gastric liquid of different milks in the piglet's stomach. A and B represents 150 min gastric liquid of cow milk; C and D represents 90 min and 150 min gastric liquid of goat milk, respectively; E and F represents 90 min and 150 min gastric liquid of sheep milk, respectively. F, fat; C, casein aggregates; MFGM, milk fat globule membrane. Note: only one representative micrograph from one piglet at each timepoint is shown.

7.4.8 Tricine-SDS-PAGE of the gastric chyme (curd and liquid)

The protein composition of the gastric curds and liquid at different digestion times, determined using tricine-SDS-PAGE under reducing conditions is shown in Figure 7.10 and Figure 7.11, respectively.

In the SDS-PAGE of the gastric curds from cow whole milk (Figure 7.10A), it was evident that higher amounts of caseins were present at all timepoints in comparison to the whey proteins. The band intensities of the caseins appeared to be similar at all digestion times indicating slow digestion of caseins. A para-κ-casein band (above the α-lactalbumin band), was observed at 30 min as well as at other digestion timepoints. This band is derived from hydrolysis of κ-casein to para-κ-casein (~15 kDa) by chymosin (as well as pepsin). Similar results have also been reported by Miranda and Pelissier (1983) and Ye *et al.* (2019b) while studying *in vivo* digestion of unheated cow skim milk proteins and unheated cow whole milks proteins, respectively, in the male rat stomach. A faint β-lactoglobulin and α-lactalbumin band was also observed in all of the curds, the band intensity of which decreased with digestion time. The smaller amounts of β-lactoglobulin and α-lactalbumin observed in the curds was due to the entrapped liquid chyme in the curds which was expelled out slowly during digestion, leading to a decrease in β-lactoglobulin and α-lactalbumin band intensity. Also, traces of higher molecular weight whey proteins were found in the curds at initial digestion times, which disappeared with further digestion. The protein hydrolysis profile of the goat (Figure 7.10B) and sheep (Figure 7.10C) whole milk curds followed a similar trend to that observed for the cow whole milk curds. These results also indicate that caseins from the aggregated curd would have left the stomach mainly in the form of peptides and fine aggregates (comprising intact casein).

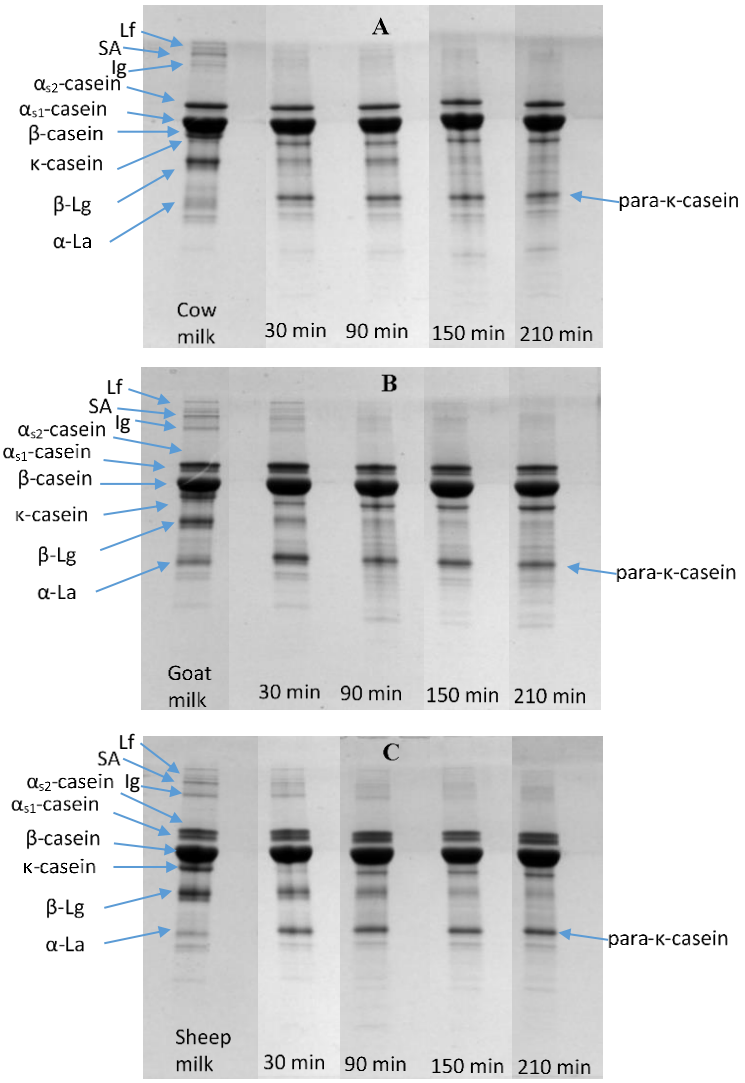


Figure 7.10 SDS-PAGE pattern of the freeze-dried curds (20 µg protein in each lane) obtained during the gastric digestion of whole milk from the piglet's stomach at different digestion times (30, 90, 150, and 210 min). (A) cow whole milk; (B) goat whole milk; (C) sheep whole milk. SA, serum albumin; Lf, lactoferrin; Ig, immunoglobulin; β-Lg, β-lactoglobulin; α-La, α-lactalbumin. Note: only one representative SDS-PAGE from one piglet at each timepoint is shown.

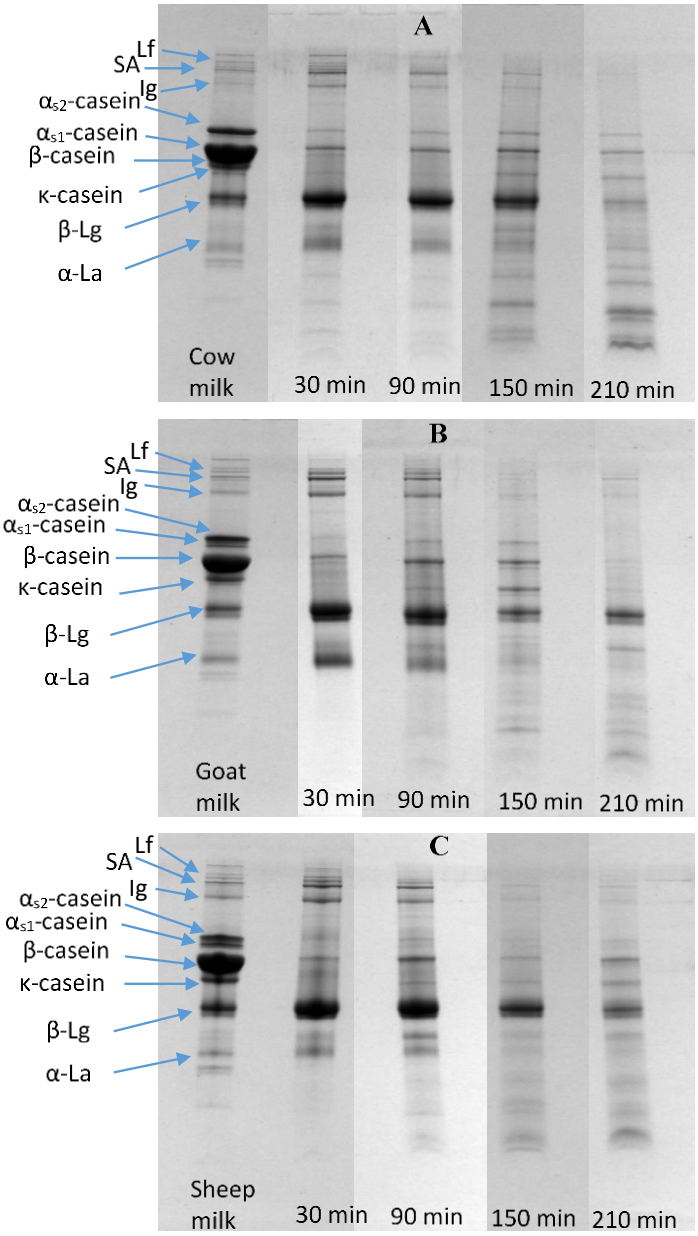


Figure 7.11 SDS-PAGE pattern of the freeze-dried liquid fraction (20 µg protein in each lane) obtained during the gastric digestion of whole milk from the piglet's stomach at different digestion times (30, 90, 150, and 210 min). (A) cow whole milk; (B) goat whole milk; (C) sheep whole milk. SA, serum albumin; Lf, lactoferrin; Ig, immunoglobulin; β-Lg, β-lactoglobulin; α-La, α-lactalbumin. Note: only one representative SDS-PAGE from one piglet at each timepoint is shown.

In the tricine-SDS-PAGE of the cow whole milk liquid chyme (Figure 7.11A), β-lactoglobulin and α-lactalbumin were present with intense bands at 30 min of digestion. The intensity of both the intact β- lactoglobulin and α-lactalbumin bands decreased gradually as digestion progressed. The α-lactalbumin band intensity decreased rapidly (especially from 150 min onwards) compared to β- lactoglobulin. Small amounts of intact casein were also found to be present at all times in the liquid chyme. Other whey proteins (such as lactoferrin, serum albumin, and immunoglobulins) were also present with intense bands in the 30 min samples. These higher molecular weight whey proteins were almost completely hydrolysed by 210 min of digestion and only traces were left in the stomach. Some peptides (<10 kDa) were also seen in the liquid chyme especially from 90 min onwards. These peptides might have been generated from the slow hydrolysis of clotted caseins during digestion along with hydrolysis of the intact whey proteins.

It should be noted that as the liquid chyme emptied rapidly from the stomach, most of the whey proteins would likely have left the stomach in intact form and only limited hydrolysis of intact whey proteins would have occurred. The results indicate that smaller and intact casein aggregates are also delivered to the small intestine along with soluble components of milk through the liquid phase; these results are in accordance with the microstructural observations of the liquid chyme (Figure 7.6 and Figure 7.9). Similar observations were noted for the protein profile of goat (Figure 7.11B) and sheep (Figure 7.11C) whole milk liquid chyme, but the extent of protein hydrolysis and the number of peptides generated may be different for milk from different species and this needs to be investigated further.

The changes in the protein composition of the curd and liquid phase observed *in vivo* were similar to those reported in previous chapters (Chapters 5 and 6) during dynamic *in vitro* gastric digestion of different milks, though there may be differences in the extent of protein hydrolysis, as the pH was dropped to as low as 2 during *in vitro* gastric digestion (Chapters 5 and 6).

7.4.9 Rigidity (or firmness) of the gastric curds

The complex modulus (G*) values of the gastric curds of milk from the three species remaining in the stomach at the different digestion times were determined using small deformation rheology (Figure 7.12). The G* value is a representation of firmness or stiffness of the gastric curds; the higher the G* value, the higher the rigidity of the curd.

For G*, there was a statistically significant interaction between species and time, meaning that overall G* differed over time but the effect was also different between the three species. The G* values of the curds from different species was similar at 30 min of digestion, and as the digestion progressed, the G* values of the curd increased to a different extent for the different species as depicted in Figure 7.12. Though the average G* value of the milk curds from goat and sheep milk remained lower than that of cow milk at all digestion timepoints, there were no statistically significant differences between them, except at 210 min of digestion, at which time the G* value of cow milk was the highest followed by goat and sheep milk (Figure 7.12). It appeared that the firmness of the sheep milk curds reached a plateau at around 150 min of digestion, however, cow and goat milk curds increased further in firmness beyond 150 min of digestion (Figure 7.12).

It should be noted that although careful uniformity was maintained during sample collection and analysis throughout the study, there may be variations in the different sections of the curd considering the heterogeneity of the curd in terms of hydration, microstructure as well as enzyme activities (Mulet Cabero, 2018; Ye *et al.*, 2016b). A small amount of curd remaining from the pre-sampling day diet was also found to be present along with the fresh milk curd formed from the final sampling day milk diet in the piglet's stomach. Only the fresh milk curd sections were sampled for the rheological analysis. It was also found that the curds remaining in the stomach at zero timepoint (fasted piglets) were hard and could not be pressed to achieve the required gap for analysis under the parallel plate geometry of the rheometer. Therefore, the rheological measurements couldn't be done on these curds. Moreover, it should be noted that the separated liquid chyme fraction in the stomach was not considered during the rheological measurements, which in the actual stomach environment would have an impact on the curd texture and properties. However, it was not possible to measure the rheological changes on the entire stomach contents (considering both the curd and the liquid chyme).

The rheological changes observed in the gastric curds of milk from the different species during digestion complemented the visual observations (Figure 7.4) and the findings observed in the electron micrographs (Figure 7.8) of the curds. The increase in the firmness (G*) of the milk curds over time was mainly due to the compaction of the aggregated caseins of the curd as digestion progressed, due to the loss of entrapped liquid from the curds. This is mainly because of the stomach contraction forces and decrease in pH to below the isoelectric point of caseins (pH 4.6) in the stomach. The differences observed in the consistency of the curds of milk from different species may be explained by the changes in the protein network of the curd observed using electron micrographs

(Figure 7.8). The less fused protein network of goat and sheep milk curds may be a key reason for the lower G* values and relatively soft curds formed from these milks at 210 min compared to cow milk (Figures 7.8 and 7.12).

It was observed in Chapters 5 and 6 (with the *in vitro* work) that initially the curd formed hold more water and had a soft texture, and as digestion progressed the curd became denser and stronger. Similar observations were found during this *in vivo* study. However, in the previous studies (Chapters 5 and 6), it was found, through texture analysis, that at the end of 240 min of *in vitro* gastric digestion that sheep milk curds had higher hardness compared to that of cow and goat whole milk, both of which had a similar degree of hardness.

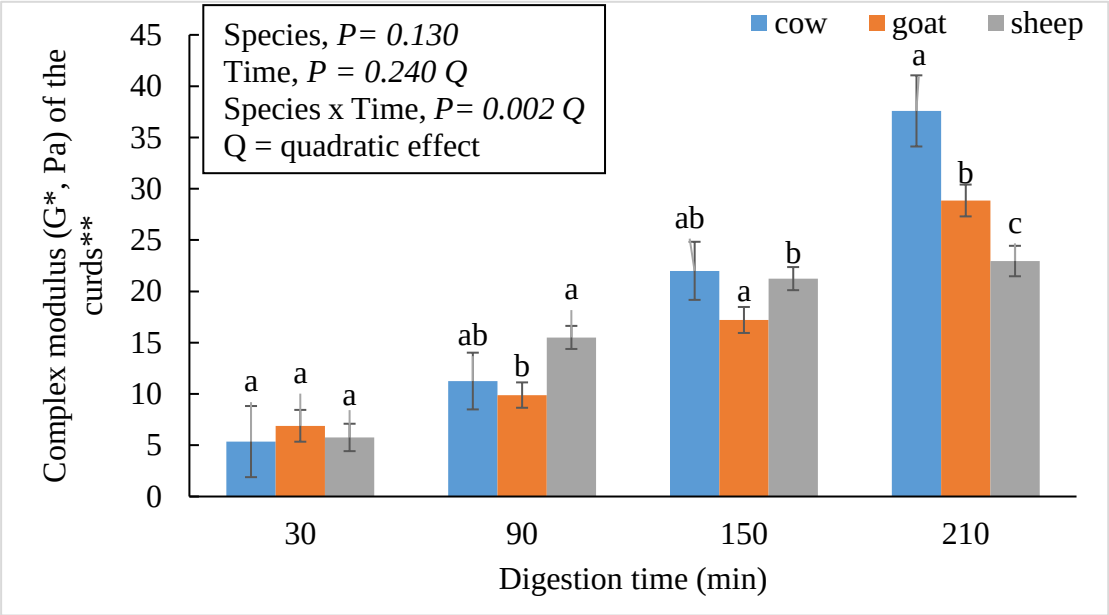


Figure 7.12 Changes in the complex modulus (G*, Pa) of the remaining gastric curd in the piglet's stomach at the different digestion times. Values are reported as mean ± SEM. Different letters (a–c) above the bars represent significant differences among the curds at a given digestion timepoint (P < 0.05); the statistical significance of differences for a particular milk sample across different digestion times are not represented.**square root transformation of raw data was required to fulfil model assumptions.

7.4.10 Gastric emptying of nutrients

The gastric emptying curves for the total stomach chyme of milk (given per 100g milk ingested) from the different species are shown in Figure 7.13. The time '0' in the graph represents the amount of milk in the piglet's stomach at the completion of suckling.

In general, as digestion progressed, the total dry matter content of the gastric chyme decreased. Compared to cow milk, goat and sheep milk had a faster rate of decrease in the dry matter content during digestion compared to cow milk Figure 7.13A. Thus, the T1/2 (time required to empty half the total dry matter of milk) followed the order: sheep milk (38 min) < goat milk (67 min) ≤ cow milk (81 min) (Figure 7.13A). Similarly, the rates of decrease in the amount of protein (Figure 7.13B) and fat (Figure 7.13C) retained in the stomach varied significantly for the total gastric chyme during digestion and generally followed the order: cow milk ≤ goat milk < sheep milk.

As the milk from the different species varied in nutrient content and the piglets received their meal based on an equal protein basis (and thus, different volumes), the data were also analysed and are plotted as g per 100 g of a particular nutrient (dry matter or protein or fat) as opposed to 100 g milk (Figure 7.13) to equate for differences in nutrient composition and observe the relative nutrient retention behaviour (Figure 7.14). These results also indicated that the rates of decrease in the nutrient content of goat and sheep milk varied significantly from that of cow milk and generally followed the order: cow milk < goat milk < sheep milk, and accordingly the T1/2 varied similarly (Figure 7.14).

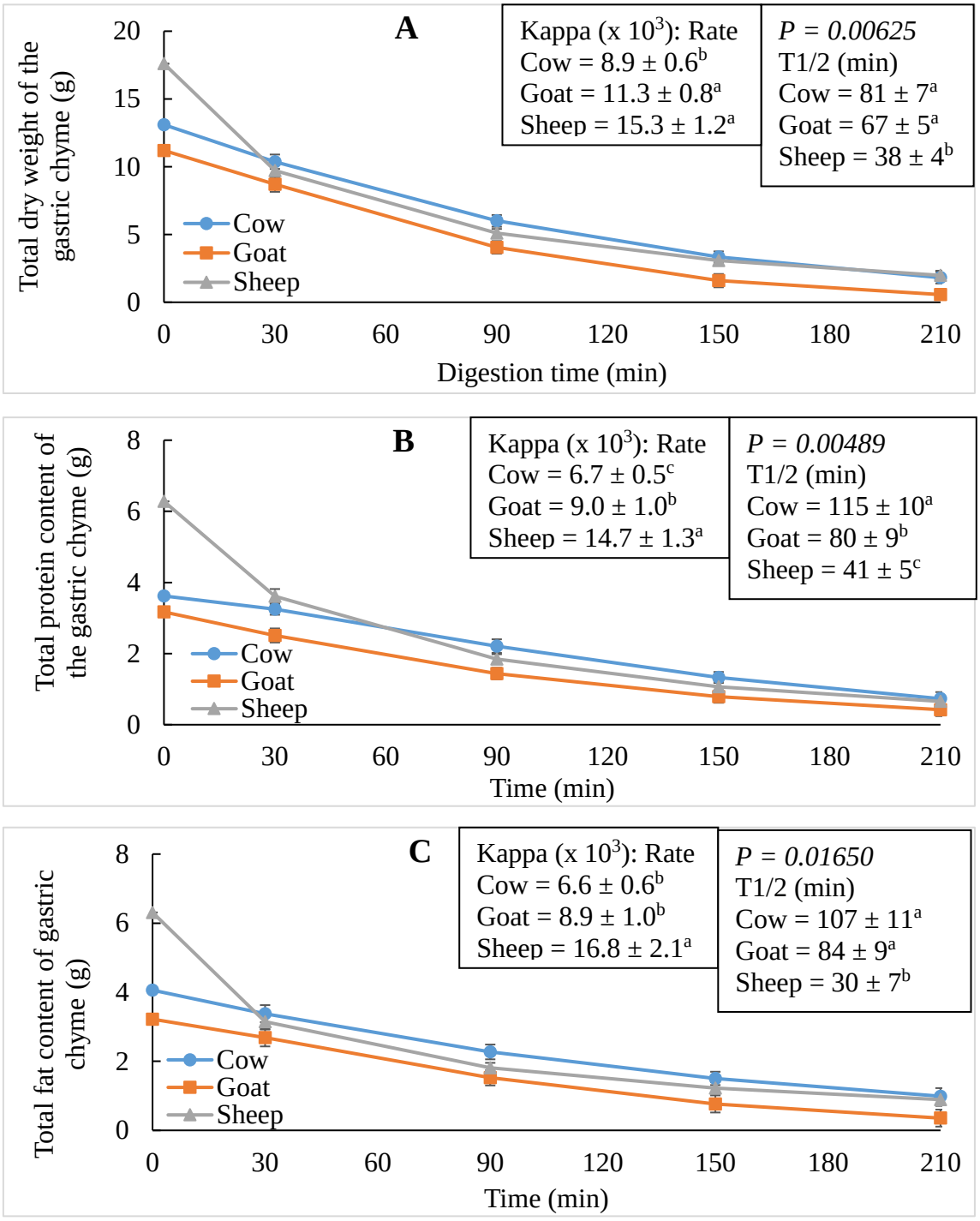


Figure 7.13 Changes in (A) dry weight, (B) protein, and (C) fat of the total chyme retained in the stomach during the gastric digestion of different milks. Values are reported as mean ± SEM. All values are related to the ingestion of 100 g milk.

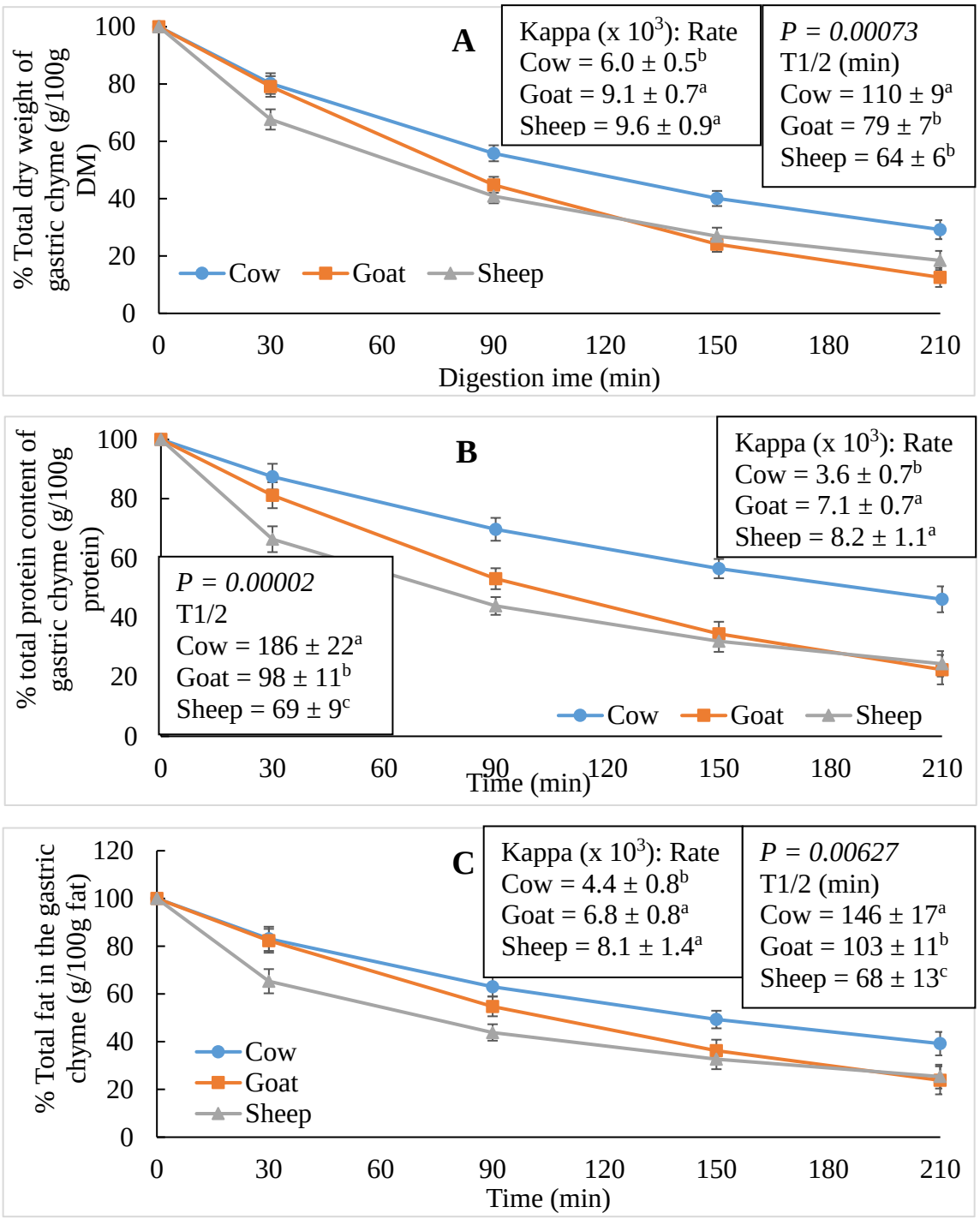


Figure 7.14 Relative retention of the total chyme (A) dry weight (g per 100 g dry matter), (B) protein (g per 100 g protein), and (C) fat (g per 100 g fat) during the gastric digestion of different milks in the piglet's stomach. Values are reported as mean ± SEM.

7.4.11 Changes in the nutrient composition of the gastric chyme fractions (curd and liquid)

The total stomach chyme was composed of the curd and gastric liquid phase from 30 min onwards. Thus, how these two fractions changed within the stomach with digestion time and this impacted the overall gastric emptying rates was further analysed. The wet weight, dry weight, protein, and fat content of the curd and the liquid fractions (g per 100 g of milk) of the gastric chyme from 30-210 min of gastric digestion are reported in Figures 7.15 and 7.16.

The wet weight of the liquid (Figure 7.15A) and the curd (Figure 7.16A) fractions decreased as digestion progressed. The changes in the wet weight of the gastric liquid and gastric curd fractions of the chyme indicated that the gastric liquid emptied out relatively faster compared to the gastric curd during digestion, irrespective of the species. Thus, the gastric curd constituted the major fraction of the total stomach chyme remaining especially from 90 min onwards (Figure 7.15A and 7.16A). As expected, the dry matter content of the liquid phase and the curd also decreased as the digestion progressed. The dry matter content of the curd remained relatively high at all digestion points compared to the dry matter content of the liquid phase. These results indicate that majority of the dry matter content was retained or trapped in the gastric curds during digestion.

Similarly, the protein and fat content of the curd (Figure 7.16C and Figure 7.16D) was much higher than that of the liquid phase (Figure 7.15C and Figure 7.15D). The low protein content of the liquid phase was expected, because of the formation of the casein curd (leading to higher protein content of the curd) as well as rapid gastric emptying of the liquid phase. The higher fat content of the curd compared to that of the liquid phase

was due to the physical entrapment of fat within the curds. Some (~64-80%) of the milk fat (depending on species) was retained in the gastric curds at 30 min, the amount of which decreased as digestion progressed (Figure 7.17B). Generally, the protein or fat content of the curd remaining in the piglet's stomach from goat and sheep milk was lower than that of cow milk during digestion, although the effect of time of digestion for changes on the protein and fat content of the curd were not statistically significantly different for the three species (Figures 7.17A and 7.17B).

These observed decreases in the nutrient content of the curd fractions (dry matter or protein or fat) obtained from the piglet's stomach (Figures 7.16 and 7.17) were similar to those noted during the *in vitro* gastric digestion of whole milk (Chapter 6). It should be noted that due to the continuous gastric emptying of the liquid phase, it was not possible to relate the changes occurring in the protein and fat composition of the curd with the changes in nutrient content of the liquid phase.

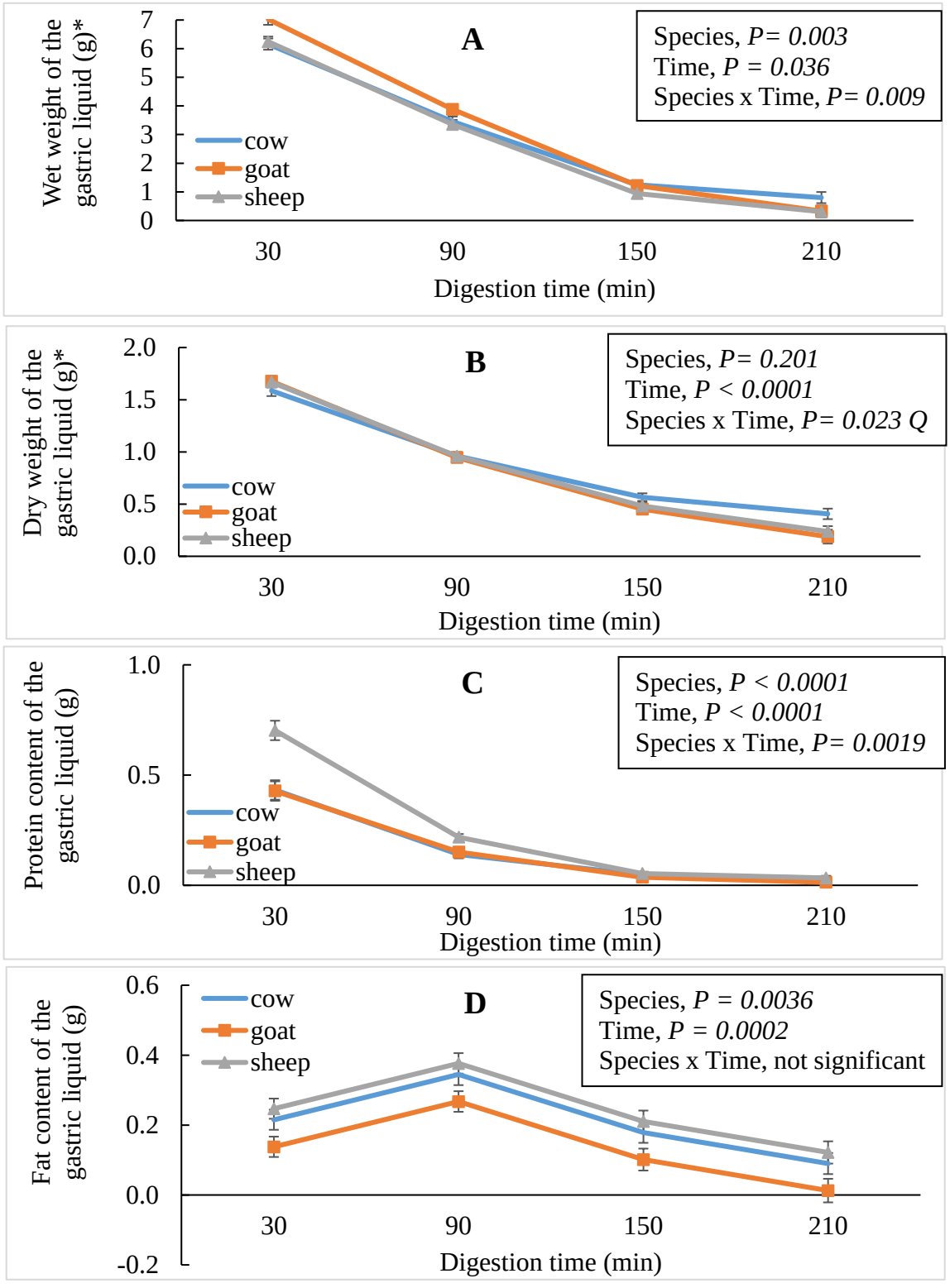


Figure 7.15 Changes in the liquid chyme retained in the piglet's stomach during the gastric digestion of different milks. (A) wet weight; (B) dry weight; (C) protein; (D) fat. Values are reported as mean ± SEM. All values are related to the ingestion of 100 g milk. *square root transformation of raw data was required to fulfil model assumptions.

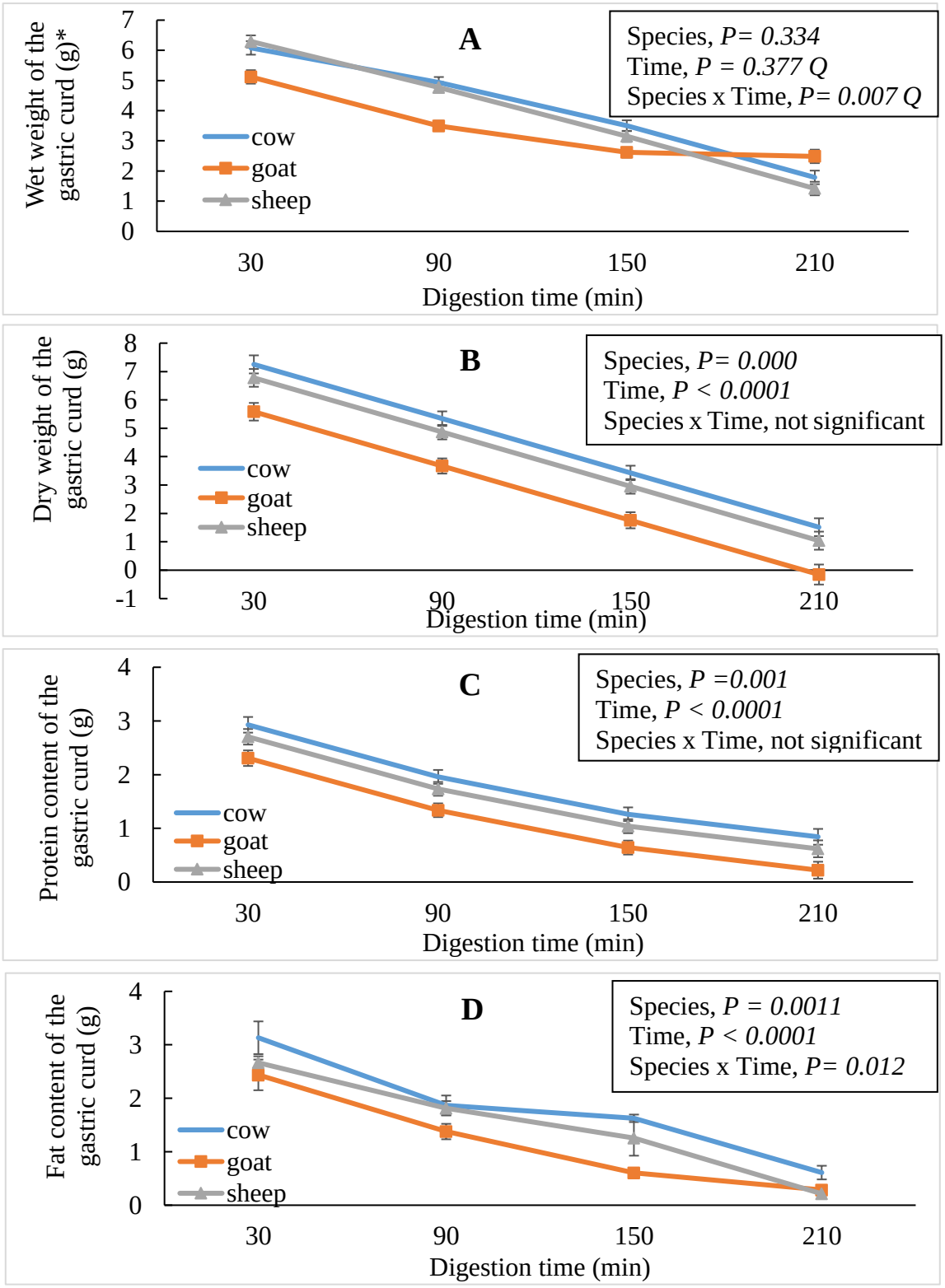


Figure 7.16 Changes in the curd retained in the piglet's stomach during the gastric digestion of different milks. (A) wet weight; (B) dry weight; (C) protein; (D) fat. Values are reported as mean ± SEM. All values are related to the ingestion of 100 g milk. *square root transformation of raw data was required to fulfil model assumptions.

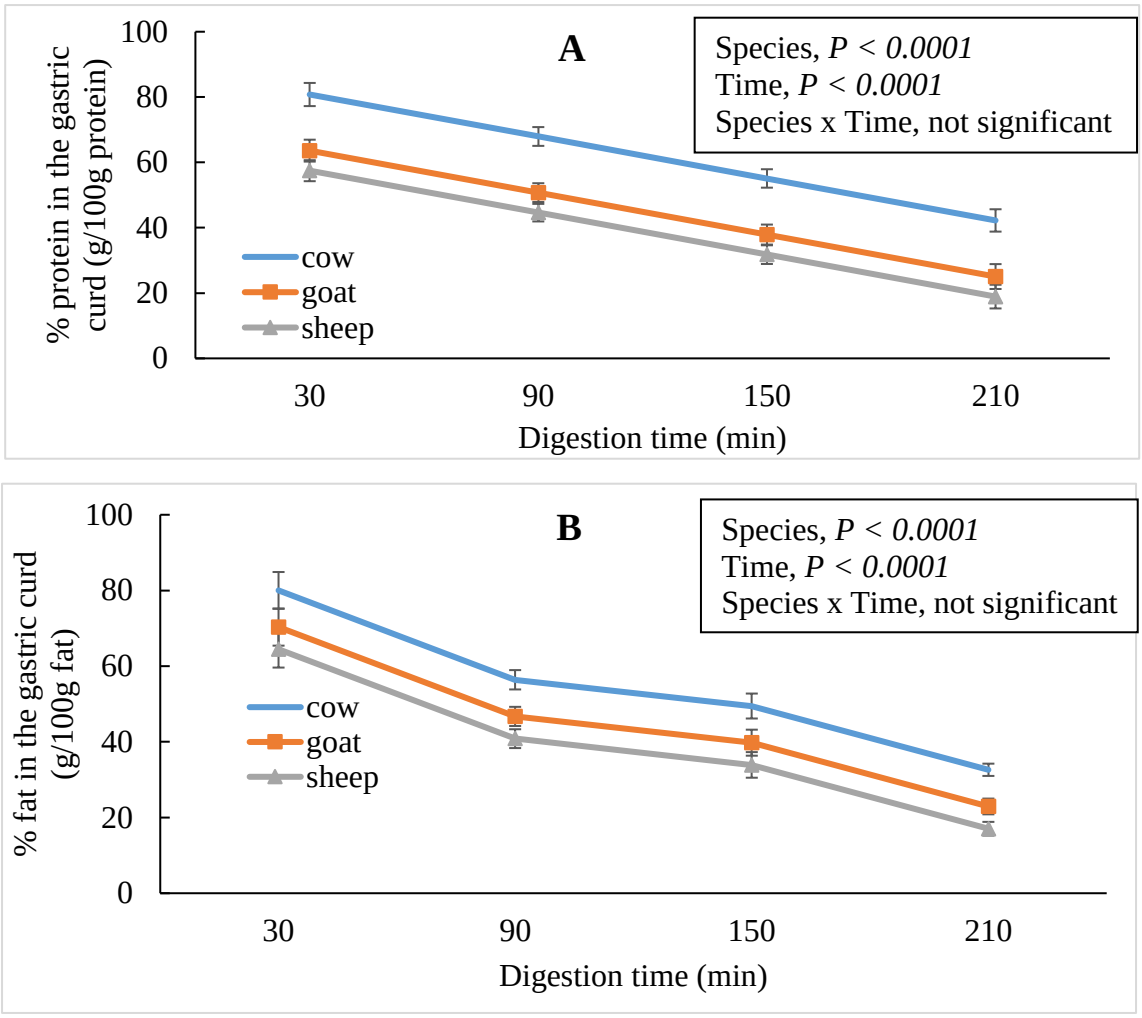


Figure 7.17 Changes in (A) % protein (g per 100 g protein) and (B) % fat (g per 100 g fat) retained in the curd during the gastric digestion of different milks in the piglet's stomach. Values are reported as mean ± SEM.

7.4.12 Relationship between protein and fat lost from the curds

During the *in vitro* gastric digestion study of whole milk from the different species (Chapter 6), it was reported that there was a strong correlation between the amounts of protein and fat lost from the curds. To confirm this finding *in vivo*, the amounts of protein and fat lost from the curd in the stomach of each piglet was plotted (Figure 7.18). The results obtained confirmed the positive linear correlations ($P < 0.001$) between the protein

and fat lost from the curd, irrespective of species (even though the changes in pH drop, gastric secretions, and digestion time were somewhat different for the *in vitro* system). This further emphasizes that the changes in curd structure (i.e. compaction or disintegration or hydrolysis) during gastric processing was the major factor responsible for the release of fat globules from the curd into the liquid phase.

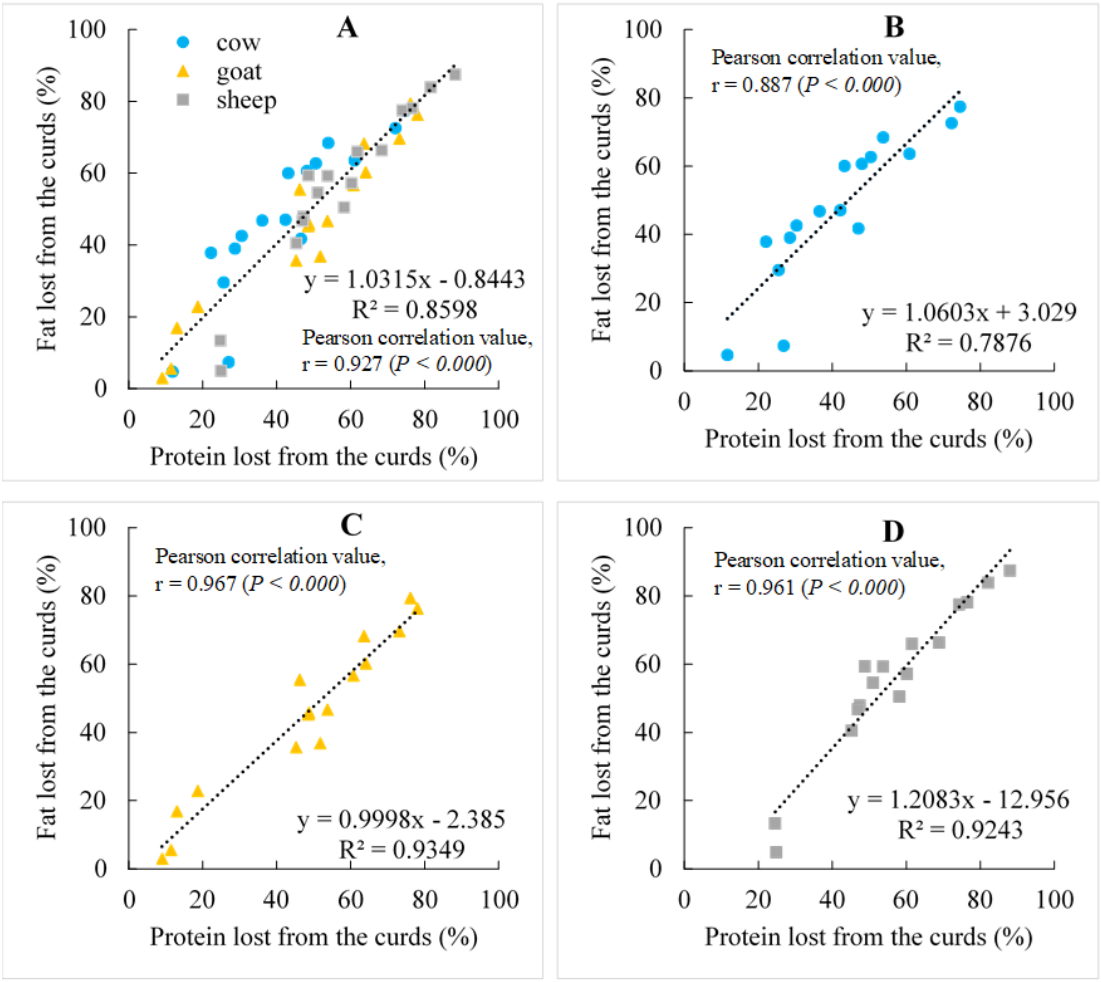


Figure 7.18 Relationships between the amounts of protein and fat lost from the curds (given as %) during gastric digestion from 30 to 210 min. (A) all whole milk curds from different species; (B) cow whole milk curds; (C) goat whole milk curds; (D) sheep whole milk curds. The Pearson correlation coefficient value (r), the regression line, and the linear regression equation are depicted. Each data point represents the results obtained from one piglet.

7.5 Conclusions

This study enhances an understanding of gastric digestion of milk from different species and confirms some of the mechanisms of gastric digestion of milk observed during the *in vitro* studies described in the earlier chapters. The whole milk from all of the species disintegrated into a coagulum and liquid phase in the piglet's stomach within 30 min of milk ingestion at which time the pH was ~5.9. During digestion, the liquid phase emptied relatively faster, while the coagulated phase remained longer in the stomach. These results were similar to those observed during the *in vitro* studies. Microstructural results revealed that the extent of fusion and compaction of the protein network of cow milk curd was greater compared to goat and sheep milk curds. The less fused protein networks of goat and sheep milk is expected to be due to the differences in their protein composition and a possible reason for the overall lower protein or fat content of their curd in the piglet's stomach to that of cow milk, although no statistically significantly differences were observed for the changes on the protein and fat content of the curd of the three species over the time of digestion. As a major fraction of the nutrients (dry matter, protein, and fat) was entrapped in the curds, the changes in nutrient composition of the total gastric chyme (liquid + curd) was expected to be mainly due to the changes occurring in the coagulated curd phase. The results further emphasize that the dynamic digestion simulation models are able to mimic closely the key variables known to influence the digestion behaviour of complex structured foods such as milk.

Chapter 8.0. Concluding discussion and recommendations for future work

Milk is an integral part of a nutritionally balanced diet for humans. Non-cow milks are gaining the interest of people as well as industries, because of their perceived better nutritional properties than cow milk. However, most of these presumptions are based on anecdotal reports, and only little scientific research has been conducted to understand the nutritional and physicochemical properties of non-cow milks. Hence, the knowledge concerning the digestion behaviour of different milks is a subject of paramount importance. Considering the gastric digestion is a critical step in the overall digestion of milk, in this dissertation, the physiological behaviour cow, goat, and sheep milk has been investigated in detail using both *in vitro* and *in vivo* gastric digestion models.

The first objective of the thesis (Chapter 4) was to understand the differences in the gelation properties of the milk from different species in the presence of acid and enzymes as the gel-forming abilities of different milks have been related to their curdling properties in the stomach. This was achieved by studying the rheological and microstructural behaviour of acid (made with GDL only) or ‘combination' gels (made with both GDL and porcine pepsin) of different skim milks. The study revealed that milks with higher protein content (such as sheep, deer, and buffalo milk) resulted in stronger gel formation and relatively more compact protein network on acidification compared to the lower protein-containing milk (such as cow and goat milk). Goat milk had less protein than cow milk and thus, formed less firm acid gels and open protein networks compared to that of cow milk. Buffalo milk was an exception as, despite having lower protein content than deer milk and sheep milk, it formed stronger gels. Milk from different species also responds

differently to the combined action of GDL and pepsin. Goat milk showed a decrease in gel firmness with the addition of even the smallest amount of pepsin in contrast to other milks which showed an increase in gel strength with increase in pepsin concentrations. Overall, the findings of the Chapter 4 suggested that goat milk may form softer curd in the stomach compared to cow milk. This was tested under the dynamic conditions of the stomach. Thus, the second objective of the thesis (Chapter 5) was to investigate the comparative coagulation profile of cow, goat, and sheep skim milk under dynamic gastric digestion conditions. Deer and buffalo milk were not included as the intensive nature of the digestion experiments restricted the number of samples that can be tested. The dynamic gastric digestion behaviour of goat and sheep milk was investigated for the first time in this study.

A dynamic stomach model, i.e. a Human Gastric Simulator (HGS), was chosen to investigate the relative dynamic changes that occur in different milks during gastric digestion (Chapter 5). HGS is widely recognized as efficiently simulating the physiologically relevant mechanical forces that develop *in vivo*. The study revealed that milk from all the species showed signs of milk coagulation within the first 6-7 min of gastric digestion and separated into a clot and liquid phase within the next 4-6 min. The formation of the clot was due to the action of the pepsin on κ-casein to form para-κ-casein and glycomacropeptide, which results in destabilization of the casein micelles and their aggregation at ~pH 6.0. The amount of the clot formed at the beginning of the gastric digestion was directly proportional to the protein and total solids contents of the different milks, the gastric retention amounts of which decreased as the digestion progressed. The macro-and microstructures of the clots revealed that at the beginning of the digestion, the protein network was more open, and as the digestion progressed, the protein network

became more compact and dense. This was due to the loss of water from the clot protein network and the dynamic decrease in pH below the isoelectric pH of caseins along with the continuous action of the stomach contraction forces. The whey protein and other soluble components (minerals, lactose) of the milk remained in the liquid phase in the HGS and thus, were emptied much earlier and quickly during gastric digestion. A schematic diagram of the mechanism of milk protein coagulation is shown in Figure 8.1.

No differences in the disintegration behaviour of clots formed from different species were observed during the gastric digestion, except that lower amounts of goat milk clots remained in the HGS, while higher amounts of sheep milk clots remained in the HGS, compared to cow milk. These differences in the amount of clot retained were attributed to the differences in the initial protein (casein) content of the different milks, indicating that higher the casein content, higher is the weight of the clot and its retention during gastric digestion. The hydrolysis patterns of the different proteins from all raw skim milk were found to be similar despite the differences in protein composition as observed by SDS-PAGE. Texture analysis of the residual clots at the end of gastric digestion revealed that goat and cow milk clots had a statistically similar level of firmness, which was lower than the firmness of the sheep milk clots (the higher firmness of sheep milk clots was due to its less hydration). The pasteurized skim milk from different species showed similar observations with only slight differences in protein hydrolysis. These results from Chapter 5 indicated that although goat milk formed weak acid and combination skim milk gels (Chapter 4), under the complex conditions of the dynamic *in vitro* gastric digestion process (Chapter 5), no statistically significant differences in clot texture was observed between the goat and cow skim milk (the average firmness of goat milk clots was lower).

The *in vitro* dynamic gastric digestion study of different skim milks (Chapter 5) strengthened the fact that the physicochemical and structural changes that occur in casein micelles during gastric digestion play a crucial role in influencing the delivery state and rate of proteins.

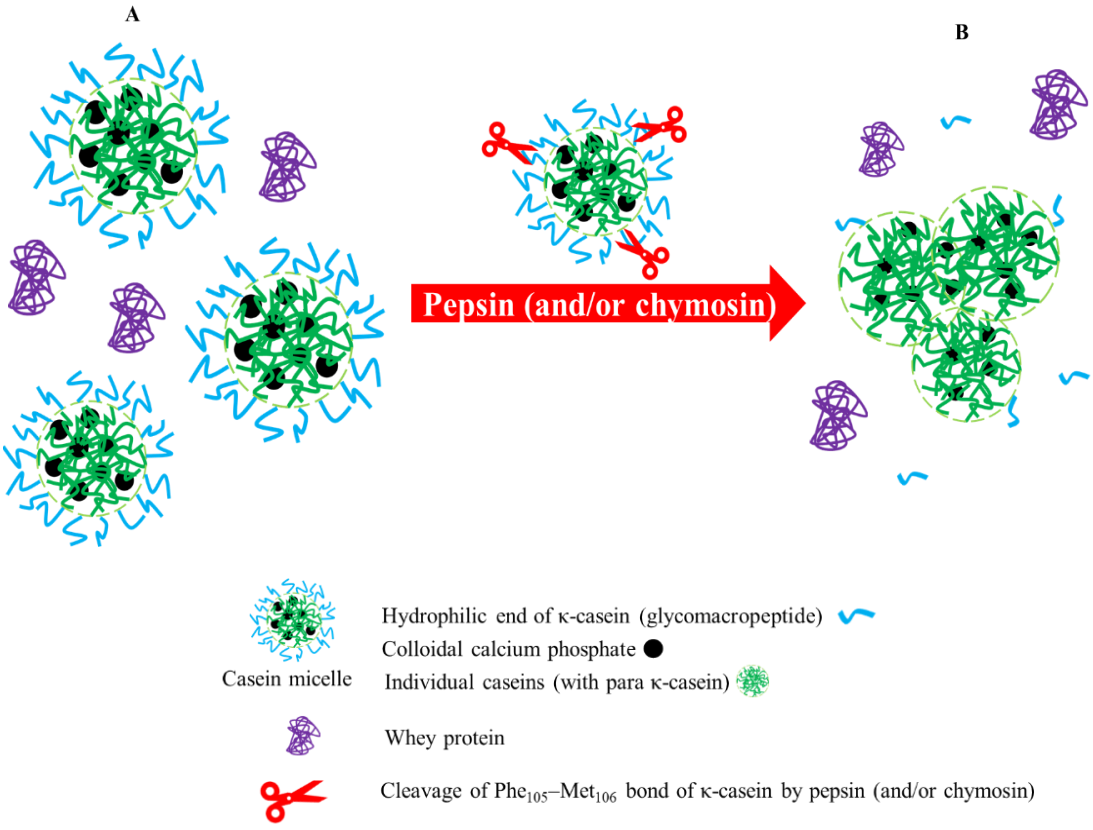


Figure 8.1 Schematic diagram illustrating the mechanism of milk protein coagulation: A, native casein micelles and native whey protein; B, aggregation of casein micelles (at pH ~6) because of the cleavage of the hydrophilic part of κ-casein by pepsin (and/or chymosin); whey proteins are not involved in the coagulation process of raw milk. Not to scale; highly schematic, and sizes are not proportional.

Fat is also a unique and complex component of the milk both in terms of its composition and structure. The coagulation of milk may have an impact on the distribution, state and release of fat globules in the stomach. Thus, to understand the impact of milk coagulation on the structure and liberation of fat globules, the previous *in vitro* gastric digestion study (Chapter 5) was further extended (the third objective of the thesis) to investigate the dynamic gastric digestion behaviour of cow, goat and sheep whole milk (Chapter 6). The dynamic gastric digestion behaviour of fat globules of goat and sheep whole milk was investigated for the first time through this study.

Similar to Chapter 5, the whole milk gastric digestion study (Chapter 6) revealed that both the raw and pasteurized (non-homogenized) whole milk from different species formed a curd in the HGS within 8-12 min of starting gastric digestion, at which time pH was ~6.0. It was found that the majority of the milk fat globules (~80-90%) were entrapped within its aggregated casein network (curd) at the beginning of the digestion. Thus, the gradual breakdown and hydrolysis of the surrounding casein network by pepsin and mechanical shearing of the HGS controlled the liberation of the fat globules from the structured curd throughout the gastric digestion. Coalescence was observed for the entrapped fat globules within the curd. This was due to a combination of factors, such as an increased collision between the adjacent fat globules (as higher volume fraction was retained in the curd), hydrolysis of native MFGM proteins by pepsin, and the rupture of the native or altered MFGM by the stomach shearing action. Interestingly, the fat globules in the liquid phase were less coalesced than those within the curd, indicating that only the smaller size fat globules were released from the curd into the liquid phase. These smaller fat globules are less susceptible to the changes in the microstructure due to the faster emptying of the liquid phase as well as their higher Laplace pressure (due to their smaller diameter), which

make them less susceptible to the shearing action of the stomach contraction forces. These mechanisms were found to be similar in milk from all the species. However, pasteurized whole milk from all the species formed relatively less structured coagula compared to their raw milk counterparts leading to a greater extent of protein hydrolysis and thus, lower proportions of fat remained in the pasteurized milk curds at the end of gastric digestion.

The *in vitro* gastric digestion studies (Chapters 5 and 6) suggested that beyond the individual nutrient contents (i.e. proteins or lipids or carbohydrates), the structural changes in a 'food matrix' plays a crucial role in influencing the digestion process. Little research has been carried out to understand in depth the intragastric changes in milk (such as phase separation, microstructural changes, textural attributes, and particle sizes) during digestion *in vivo*. Thus, the fourth objective of the thesis (Chapter 7) was to investigate the coagulation behaviour of cow, goat and sheep milk during gastric digestion under physiologically relevant conditions using bottle-fed piglet as an animal model. Bottle-fed piglets have been used previously to study the digestion of human milk and infant formulas. As goat and sheep milk are widely being used as a base in infant formulas and toddler foods, a piglet model was chosen. Besides, as the piglets consume only a milk-based diet, there will not be any interference from other food components for the physicochemical analysis of the gastric contents.

The *in vivo* study (Chapter 7) confirmed many of the structural and physicochemical changes observed during *in vitro* dynamic gastric digestion of milk using the HGS. Although the dynamic change in pH profile, enzymes, and other components (such as the presence of mucins, shearing force) was different between the *in vitro* and *in vivo* system,

the cow and non-cow milks (goat and sheep milk) separated into a curd (aggregated caseins) and liquid (soluble whey) phase in the piglet's stomach when the pH was ≥ 5.9. The three-week-old suckling piglet is considered to have significant chymosin activity along with some pepsin activity. Thus, it is expected that both chymosin and pepsin are involved in the hydrolysis of κ-casein, leading to aggregation of casein micelles. During digestion, the liquid phase emptied faster, while the slowly digested curds remained longer in the piglet's stomach.

The rheological and transmission electron micrograph analysis revealed that the compaction and firmness of the curd protein network increased as the digestion progressed. Compared to the cow milk curd, the curds formed by non-cow milks (goat and sheep milk) in piglet's stomach had relatively open protein microstructure and lower curd firmness at the end of gastric digestion. This may be the reason for the lower relative retention of protein and fat in the curds formed by goat and sheep milk. The protein hydrolysis profile of different milks was found to be similar. The microstructural and physicochemical analysis revealed that a significant fraction of the fat globules, i.e. ~64-80% (depending on milk) remained entrapped within the protein matrix of the curd. Coalesced fat globules (spherical as well as the non-spherical form) were observed at all digestion timepoints in the curds. Some extent of coalescence of fat globules was also observed in the liquid chyme. The coalescence observed during the *in vivo* study appeared to be more extensive than those observed during the *in vitro* study. This is expected to be due to the presence of lipase in the piglet's stomach which can easily access the triglyceride core of the milk fat globules through the altered MFGM (generated from hydrolysis of MFGM proteins to peptides by pepsin). The lipolytic products generated can accumulate at the fat-water interface or even replace the MFGM phospholipids

present at the interfacial layer of the fat globules. These products of lipolysis and proteolysis along with some intact or partially hydrolysed MFGM material may provide structural stability as well as steric barrier or electrostatic repulsion to prevent flocculation and coalescence of fat globules. However, this modified interfacial layer is not as stable as the native MFGM and may be more susceptible to coalescence under the gastric digestion conditions.

One of the key highlights of both the *in vitro* and *in vivo* studies was the strong positive linear correlation between the loss of protein and fat from the curds (irrespective of the species) (Figure 8.2). This emphasized that the disintegration of curd structure, due to protein hydrolysis (or breakdown) during gastric processing is responsible for the release of fat globules from the curd into the liquid phase.

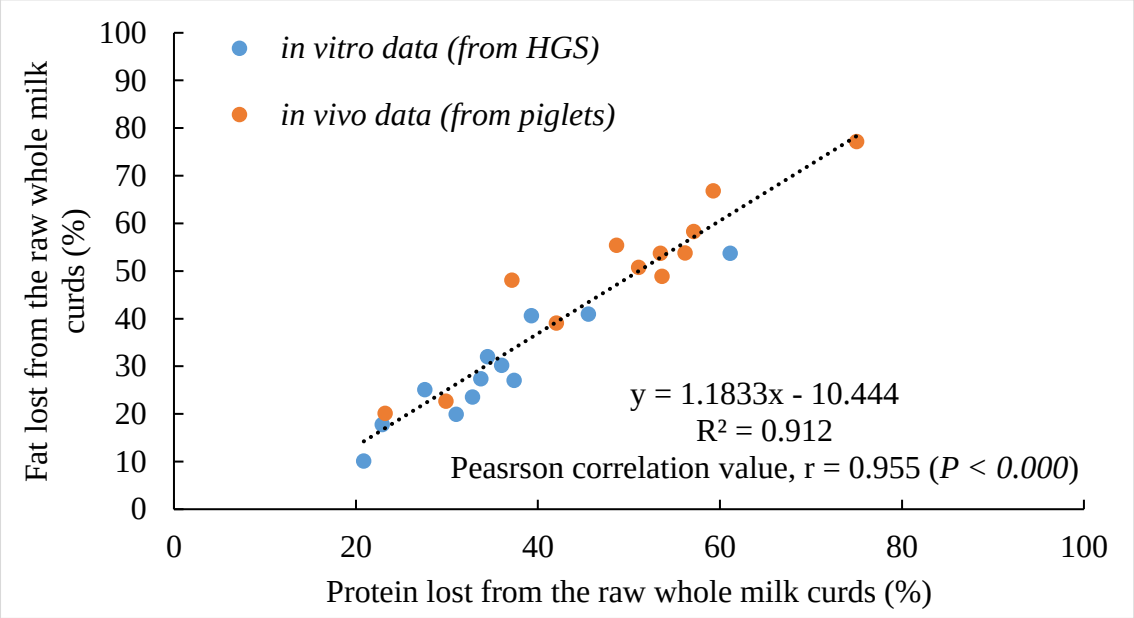


Figure 8.2 Relationship between the average amounts of protein and fat lost from the raw whole milk curds (given as %) from all the species (cow, goat, and sheep) during gastric digestion in vitro as well as in vivo from 30 to 210 min. The Pearson correlation coefficient value (r), the regression line, and the linear regression equation are depicted. Each data point represents the average results from all the species at different digestion timepoints.

A summary of the different *in vitro* and *in vivo* conditions and changes during gastric digestion of whole milks is shown in Table 8.1.

Table 8.1 Summary of the different in vitro and in vivo conditions and changes during gastric digestion of whole milks.

Property	***In vivo* observations in piglet (Chapter 7)**	***In vitro* observations in HGS (Chapters 5 and 6)**
Targeted digestion time	210 min	240 min
Digestion temperature	38-40°C (piglet normal body temperature).	36.5-37.5°C (mimicking human body temperature).
pH changes	Dynamic decrease in pH after milk suckling; pH dropped to ~3 at the end of digestion (the 3-week piglet represents a 3-month old infant).	Dynamic decrease in pH after adding milk to the HGS; pH drop was adjusted to ~2 (simulated an adult digestion system). The pH drop can be adjusted to the desired profile in the HGS.
Enzymes	Chymosin, pepsin, and lipase are present. Chymosin and pepsin concentrations are lower than that in adults; lipase concentration is higher than that in adults.	Pepsin only, lipase was not used.
Stomach contraction	Stomach contraction occurs in the piglet, but not quantified. The no. of contractions in a human infant is considered to be same as an adult system.	Rollers belts mimic the contraction mechanism, three contractions per minute; a validated model for the contraction forces that occurs in a human adult.
Curd formation in the stomach	Observed at pH $\geq$ 5.9; separation of milk into a curd (aggregated casein micelles) and liquid phase occurs. The curd formation at this high pH was due to both chymosin and pepsin action.	Observed at pH $\geq$ 6.0; separation of milk into a curd (aggregated casein micelles) and liquid phase occurs. The curd formation at this high pH was due to the action of pepsin.

Property	***In vivo* observations in piglet (Chapter 7)**	***In vitro* observations in HGS (Chapters 5 and 6)**
Firmness of the curd	Curd firmness increased over time; monitored by rheological measurements.	Firmness of the curd increased over time (based on visual observations).
Curd macro/microstructure	Initially, casein micelles formed a network, and as the digestion progressed, the extent of fusion between the protein network increased. During the later time points, the curds appeared to be yellowish (due to bile reflux) and covered by gastric mucus.	Initially, when the curd is formed, the protein network was more open, and as the digestion progressed, the protein network became dense and compact.
Location of the fat globules	~64-80% of fat globules are entrapped within the curd; high volume fraction of fat globules in the curd which are closely packed compared to those present in the liquid phase.	Majority of the fat globules (~80-90%) were entrapped within the curd (high volume fraction, close packing) as soon as it is formed, and only little remained in the liquid phase.
Microstructure of the fat globules	Extensive coalescence of fat globules (spherical and non-spherical) is observed within the curd and to a lesser extent in the liquid phase. Broken MGFM fragments were observed both in the curd and the liquid phase.	Coalescence (spherical and non-spherical fat globules) is observed within the curd; coalescence is also observed in the liquid phase but to a lesser extent. The degree of coalescence was higher *in vivo* than *in vitro*.
Liberation of fat globules	Release of fat globules from the curd was directly dependent and proportional to the rate of breakdown of the curd protein network (strong correlation); Pearson correlation coefficient, r = 0.978.	Release of fat globules from the curd was directly dependent and proportional to the rate of breakdown of the curd protein network (strong correlation); Pearson correlation coefficient, r = 0.927.

Property	***In vivo* observations in piglet (Chapter 7)**	***In vitro* observations in HGS (Chapters 5 and 6)**
Protein hydrolysis	Caseins are slowly hydrolysed, released from the stomach as fine aggregates or peptides; whey proteins are also found to be hydrolysed but mostly released in intact form due to rapid emptying from the stomach.	Caseins are slowly hydrolysed, released from the stomach as fine aggregates or peptides; whey proteins were also found to be hydrolysed but mostly released in intact form due to rapid emptying from the stomach.
Gastric retention	Gastric retention of nutrients (dry matter, proteins and fats) in the curd phase as well as in the total gastric chyme decreased over time due to emptying of nutrients.	Gastric retention of nutrients (dry matter, proteins and fats) in the curd phase decreased over time.

A schematic diagram of the possible mechanisms occurring during gastric digestion of whole milk in the stomach is shown in Figure 8.3.

Overall, the findings presented in this thesis provided new insights about the physicochemical and structural changes that occur during digestion of milk from different species. The knowledge gained might have important application in designing bioinspired structures for controlled digestion and delivery of nutrients. In addition, the *in vivo* and *in vitro* study (Chapters 5, 6, and 7) also indicated the importance of dynamic *in vitro* gastric digestion models (such as HGS) in better mimicking or simulating the structural changes that occur in complex structured food systems (such as milk) in the gastric environment.

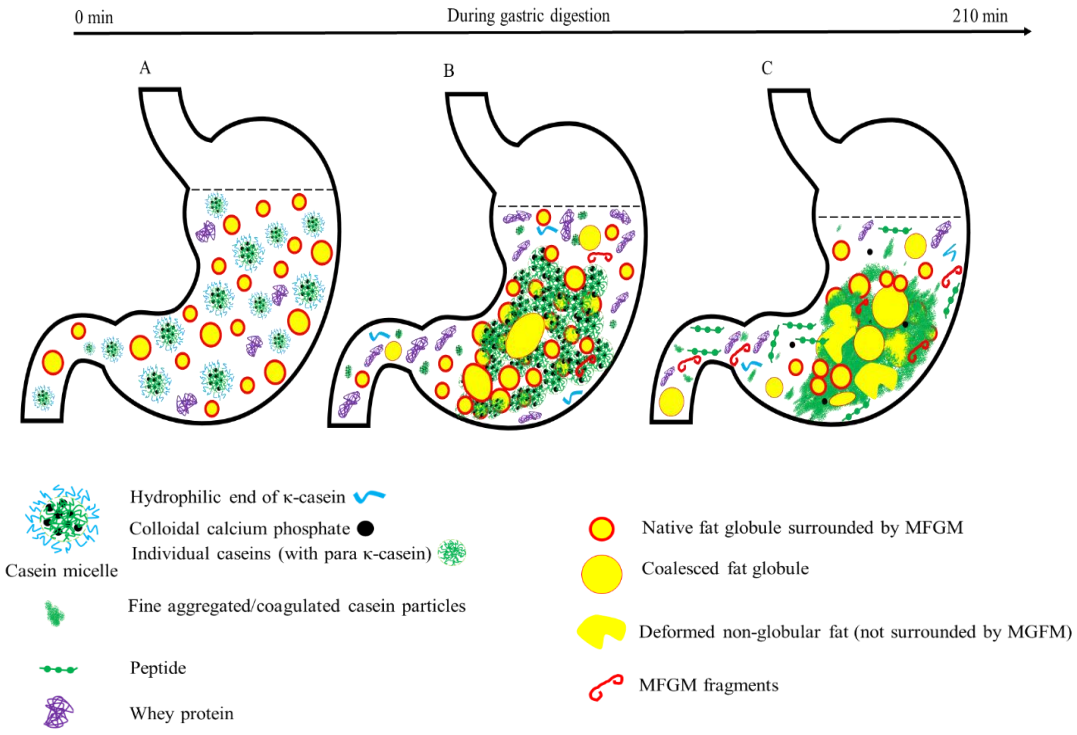


Figure 8.3 Schematic diagram illustrating the possible changes in the casein micelles and the fat globules during gastric digestion of whole milk in the stomach chamber of the HGS: A, whole milk before beginning digestion (pH ~6.7, proteins and fats are homogeneously dispersed in the milk matrix); B, intragastric coagulation of the casein micelles (at pH ~6) because of cleavage of the hydrophilic part of κ-casein by pepsin (and/or chymosin), with the majority of the fat globules being closely entrapped (or packed) in the aggregated casein network (curd); mostly smaller fat globules move to the liquid phase; C, increase in denseness of the coagulated casein network (loss of colloidal calcium phosphate around pH <4.6 and expulsion of the entrapped liquid); curd proteins are hydrolysed and broken down by pepsin (and shear); they then move into the liquid chyme, and the loss of fat from the curd is directly proportional to the loss of protein; coalescence of the globules increases and non-globular fat is observed; MFGM, milk fat globule membrane. Not to scale; the drawing is highly schematic, and sizes and quantities are not proportional.

Future directions

To bridge the important gaps that arose during this PhD research, future investigations have been recommended as follows-

- The acid and combination gel behaviour of milk from different species was studied in this thesis from the perspective of understanding the gastric digestion behaviour. In-depth fundamental studies need to be conducted in future to understand the observed unique rheological behaviour of different milk samples to lower pepsin concentrations (in comparison to cow milk). Understanding of such rheological properties (with rennet and bacterial cultures) is also important for developing high value products such as yoghurt and cheese, which should be investigated in detail in future.
- The thesis provided an understanding of the cow, goat, and sheep milk composition, structural assemblies (casein micelle and fat globules), and the changes that occur during gastric digestion, mainly in their natural state. However, the impact of commercial processing treatments (such as standardisation, homogenisation, pasteurisation (homogenisation), UHT (homogenisation), spray drying) on the digestion behaviour of different milks need to be studied to understand the process-induced changes in the structure that may affect the release of nutrients during digestion. Also, it has to be noted that the pasteurisation conditions (72°C, 15 sec) used in this study were kept same for all the milks for equal comparison. However, milk with higher total solids (>15%) or fat (>10%) may have different processing requirements which need to be considered in future.
- The thesis investigated in detail the structural changes in different milks during gastric digestion. However, the oral digestion step and gastric lipase was not included during the *in vitro* adult gastric digestion study. It is recommended that in future, these steps/enzymes are included during *in vitro* gastric digestion studies.

- The gastric digestion results suggested the differences in the delivery of proteins and fats due to the different structures formed in the stomach. Further intestinal digestion studies need to be conducted to understand in detail how the changes during gastric digestion impact the overall digestion, absorption, and metabolism of nutrients.
- During the gastric digestion study, the protein hydrolysis profiles using SDS-PAGE appeared to be similar for milk from different species. It is possible that the peptides/bioactive peptides formed are different. Thus, advanced quantification and identification of peptides, using proteomics and metabolomics, are recommended for future studies.
- The study showed exciting results regarding how the coagulation of milk protein provides controlled release of fat globules during gastric digestion and influence the structural changes in milk lipids. However, the changes occurring in the MFGM or fat globule interface during gastric digestion were not studied directly. In-depth structural and interfacial studies with advanced monitoring or staining tools are recommended in future. For example, confocal microscopy or other microscopic techniques can be used to study the distribution of neutral fats, phospholipids, glycolipids and glycoproteins. Small-angle X-ray scattering (SAXS) or ultra-small angle neutron scattering (USANS) can be used to understand the self-assembly of milk lipids (and casein micelles) during gastric digestion.
- Considering the focus of the thesis was around protein digestion, lipolysis was not studied as part of this study. Milk from different species varies in the proportion of different fatty acids, so, in future in-depth lipolysis studies such as lipid digestion and lipidomic need to be considered.

- The *in vivo* study in this thesis provided more in-depth insight about the structural changes that occur in milk from different species during gastric digestion and also confirmed many of the *in vitro* dynamic gastric digestion results observed in HGS. However, the piglet is a representative model for infant digestion. Though the results can be extrapolated to the other age groups, the use of growing pig model to better study the mechanisms of digestion, absorption and metabolism (representative of adult digestion conditions) is recommended.
- The gastric digestion studies revealed that the *in vitro* dynamic models with relevant physiological conditions of the stomach (such as enzyme concentrations, pH, as well as mechanical contraction) are instrumental in mimicking the gastric digestion conditions more realistically. Thus, the development of dynamic digestion models needs be emphasized and encouraged. Besides, the development of dynamic *in vitro* gastro-intestinal digestion model representative of different age groups (such as infants, elderly) needs to be considered.
- Further human digestion trials need to be conducted to validate the dynamic structural changes that occur during gastric digestion of milk (as observed from *in vitro* and animal studies) and understand the impact of gastric restructuring on milk digestive (appetite or fullness) and nutrient response (proteins, lipids, micronutrients).

A New Zealand Milk Means More (NZ3M) research programme has been initiated by the Riddet Institute/Massey University (funded by Ministry of Business, Innovation and Employment Endeavour Fund) which will be taking into consideration the above future directions. The programme is an integrated research work across multiple disciplines (food technologists, material scientists, animal scientists, nutritionists) to study using

advanced methodologies how both composition and structure plays a key role in providing better nutrition. The knowledge generated will help in designing value-added milk products with scientifically validated nutritional outcomes for people of different age groups. An overview of the programme is provided in Figure 8.4.

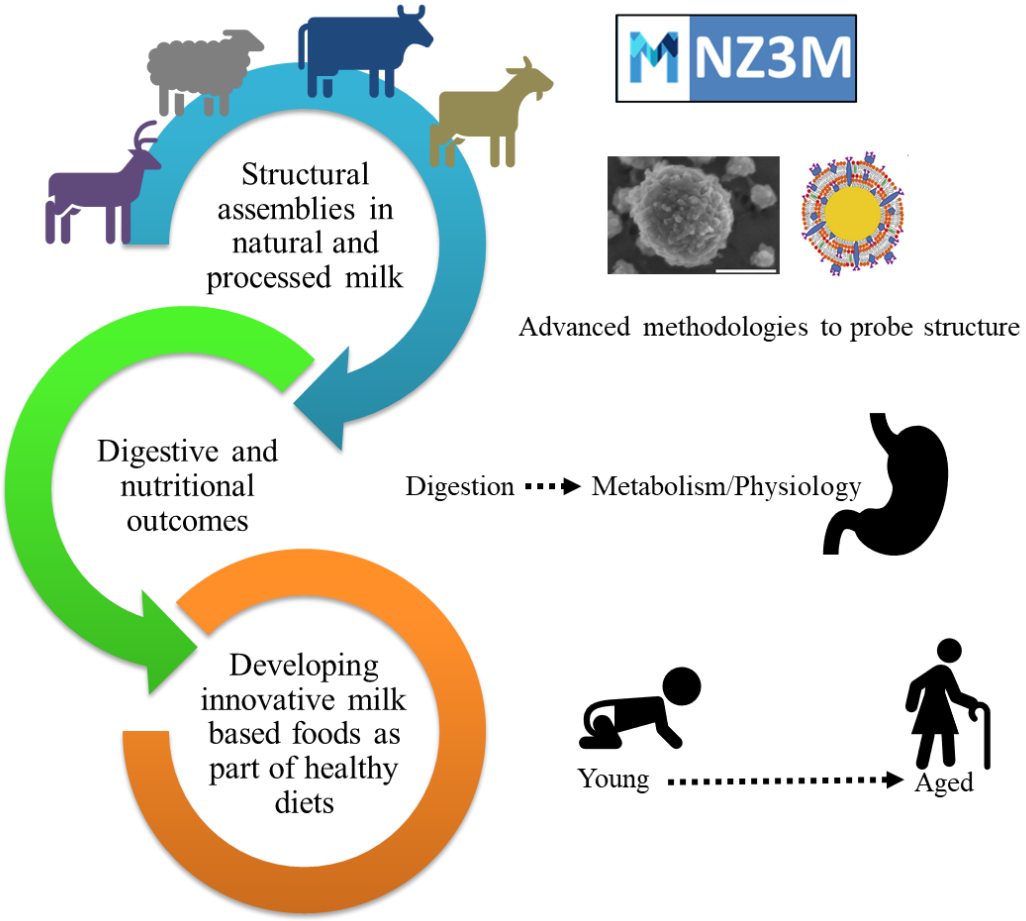


Figure 8.4 Overview of New Zealand Milk Means More (NZ3M) research programme.

Annexures

Annexure 1: Chemical composition and fat globule characteristics of different milks

Table A1.1 Chemical composition and fat globule size of cow, water buffalo, goat, sheep, and red deer whole milks.

Properties	Cow	Water buffalo	Goat	Sheep	Red deer
Dry matter (%)	14.67 ± 0.03^{c}	19.89 ± 0.01^{b}	11.47 ± 0.05^{d}	19.72 ± 0.13^{b}	27.02 ± 0.15^{a}
Protein (%)	4.07 ± 0.02^{d}	4.96 ± 0.02^{c}	3.09 ± 0.03^{e}	6.40 ± 0.02^{b}	8.16 ± 0.01^{a}
Fat (%)	5.07 ± 0.01^{d}	9.06 ± 0.06^{b}	3.47 ± 0.05^{e}	7.80 ± 0.04^{c}	13.49 ± 0.11^{a}
Carbohydrate (by difference, %)	4.83 ± 0.12^{a}	4.95 ± 0.00^{a}	4.09 ± 0.02^{c}	4.58 ± 0.04^{b}	4.17 ± 0.03^{c}
Ash (%)	0.70 ± 0.12^{b}	0.92 ± 0.03^{b}	0.83 ± 0.05^{b}	0.95 ± 0.07^{ab}	1.20 ± 0.02^{a}
Fat globule size[1]					
D_{43} (μm)	4.39 ± 0.07^{c}	7.12 ± 0.03^{a}	3.69 ± 0.07^{d}	4.45 ± 0.09^{c}	6.58 ± 0.07^{b}
D_{32} (μm)	3.60 ± 0.06^{b}	4.63 ± 0.99^{a}	2.96 ± 0.06^{b}	3.59 ± 0.08^{b}	2.67 ± 0.03^{b}

$^{a–e}$Values within each row with different superscripts are significantly different ($P < 0.05$). Values are reported as mean ± SD.

In the fat globule size distributions, a small peak ranging from 0.15 to 1 μm was observed for buffalo milk and deer milk, even after dissociation with EDTA-SDS buffer (Figure A1.1). This could have been due to the presence of casein micelles that were still associated with the fat globules or to the presence of very small fat globules in these milks, which needs to be further investigated and may be an interesting aspect to consider for future work with particle size measurements. The presence of small particles of size <1 μm has been reported by Ménard *et al.* (2010) in buffalo milk and cow milk and by El-Zeini (2006) in goat milk and camel milk. No scientific data on the fat globule size distribution of deer milk are available.

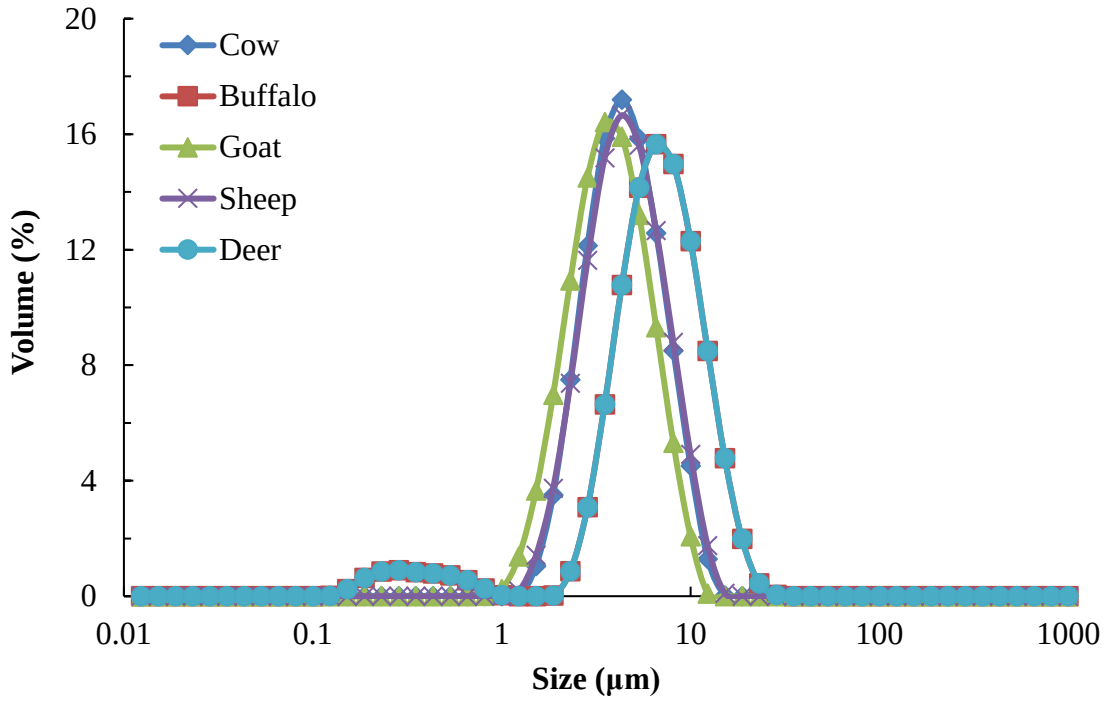


Figure A1.1 Fat globule size distributions of different whole milks dissociated with EDTA-SDS buffer. Mean values are depicted.

Confocal scanning laser microscopy of the different whole milks was performed as described in the research paper. A 1.0% (wt/vol) solution of Nile Red in acetone was used to stain the fat globules (argon laser with an excitation line at 488 nm). Confocal micrographs (Figure A1.2) of the fat globules of the whole milk from different species depicted qualitatively that those in buffalo milk and deer milk were comparatively larger than those in the milk from other species. Goat milk can be seen to have a larger number of smaller fat globules, whereas the fat globules in sheep milk and cow milk seem to be more or less similar. The whole milk from all species was found to have spherical fat globules; to a lesser extent, a few oval fat globules were found in the buffalo, sheep, and deer milk. Ménard *et al.* (2010) also reported a circular shape of the fat globules in cow milk and buffalo milk using optical microscopy. El-Zeini (2006) reported that the fat

globules of cow, buffalo, goat and sheep milk were spherical in nature 'with an oval shape in irregularity of the spatial arrangement' when viewed using a scanning electron microscope.

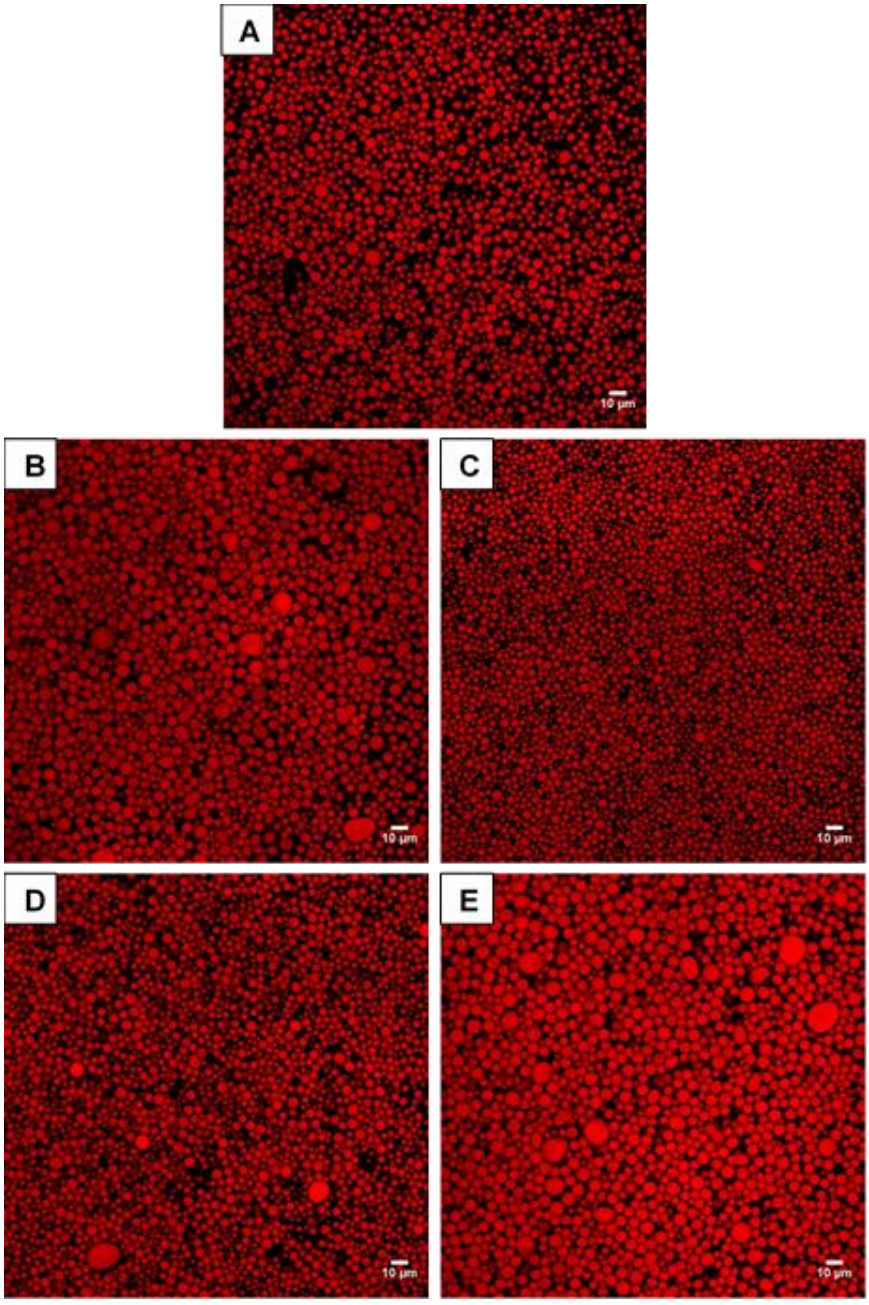


Figure A1.2 Confocal microscopy images of different whole milks showing the differences in the fat globule size distributions: A, cow milk; B, water buffalo milk; C, goat milk; D, sheep milk; E, red deer milk. Scale bar represents 10 µm.

Transmission electron microscopy of the different whole milks was performed as described in the research paper. The large light-brown particles in the transmission electron micrographs (Figure A1.3) are the fat globules, whereas the tiny dark circular to oval-shaped particles represent the protein component of the whole milks. The transmission electron micrographs of the fat globules in the whole milk from different species also depicted qualitatively that the fat globules in buffalo milk and deer milk were comparatively larger than those in the milk from other species. In general, the fat globules appeared to be circular in all milks, except for deer milk, in which the fat globules appeared to be oval in shape.

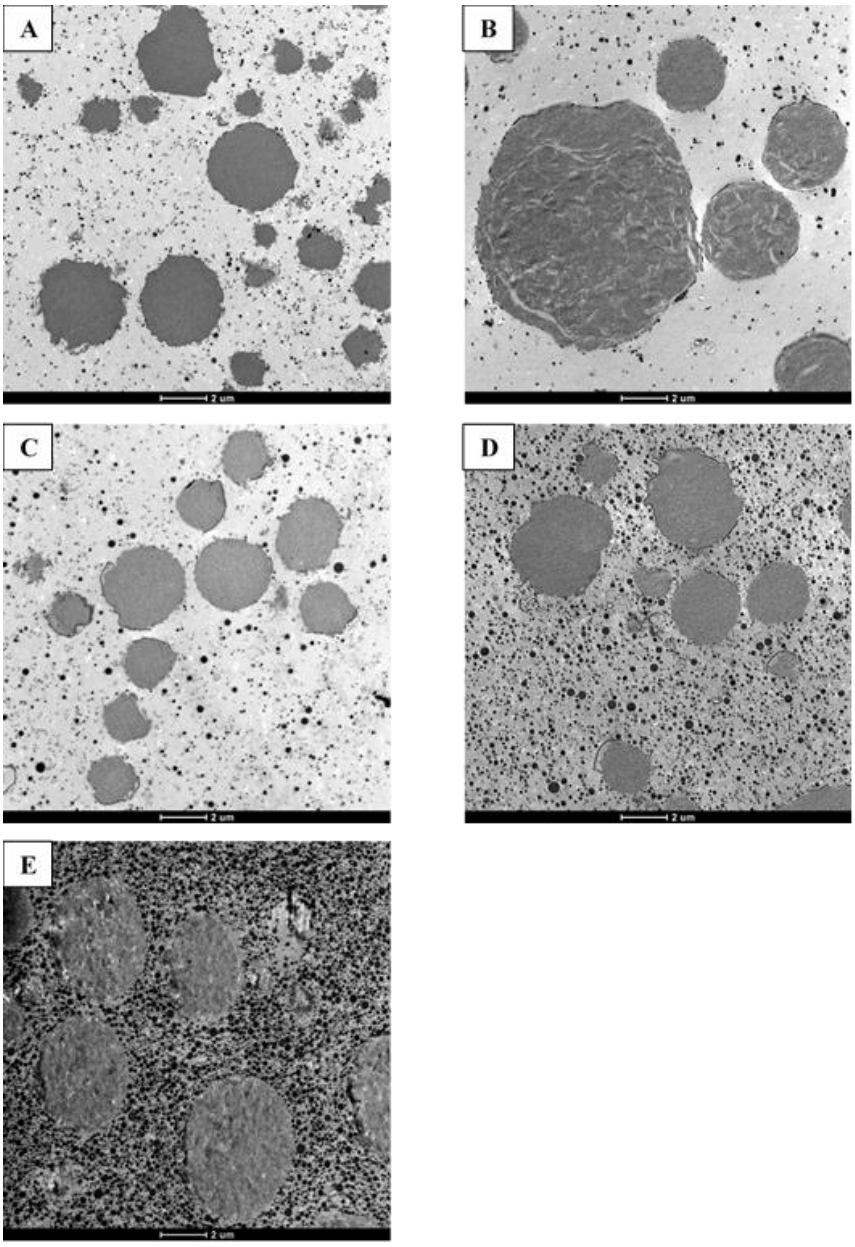


Figure A1.3 Transmission electron micrographs showing fat globules in whole milk from different species at SA6,000x magnification: A, cow milk; B, water buffalo milk; C, goat milk; D, sheep milk; E, red deer milk. Scale bar represents 2 µm.

Annexure 2: Composition and properties of the whole milk curds obtained after *in vitro* gastric digestion

The amounts of protein and fat retained (g/200 g milk) in the dried curds from the different milks with increasing digestion time are shown in Figures A2.1(A) and A2.1(B), respectively. In general, the total protein and fat contents of the dried curds decreased gradually as the digestion progressed for the milk of all species; however, the decreases in protein content and fat content were much higher for the pasteurized milk of all species than for their raw milk counterparts, indicating that the protein hydrolysis or breakdown was greater for the pasteurized whole milk curds.

The correlation between the dry matter and fat lost from the whole milk curds of all the species is shown in Figure A2.2.

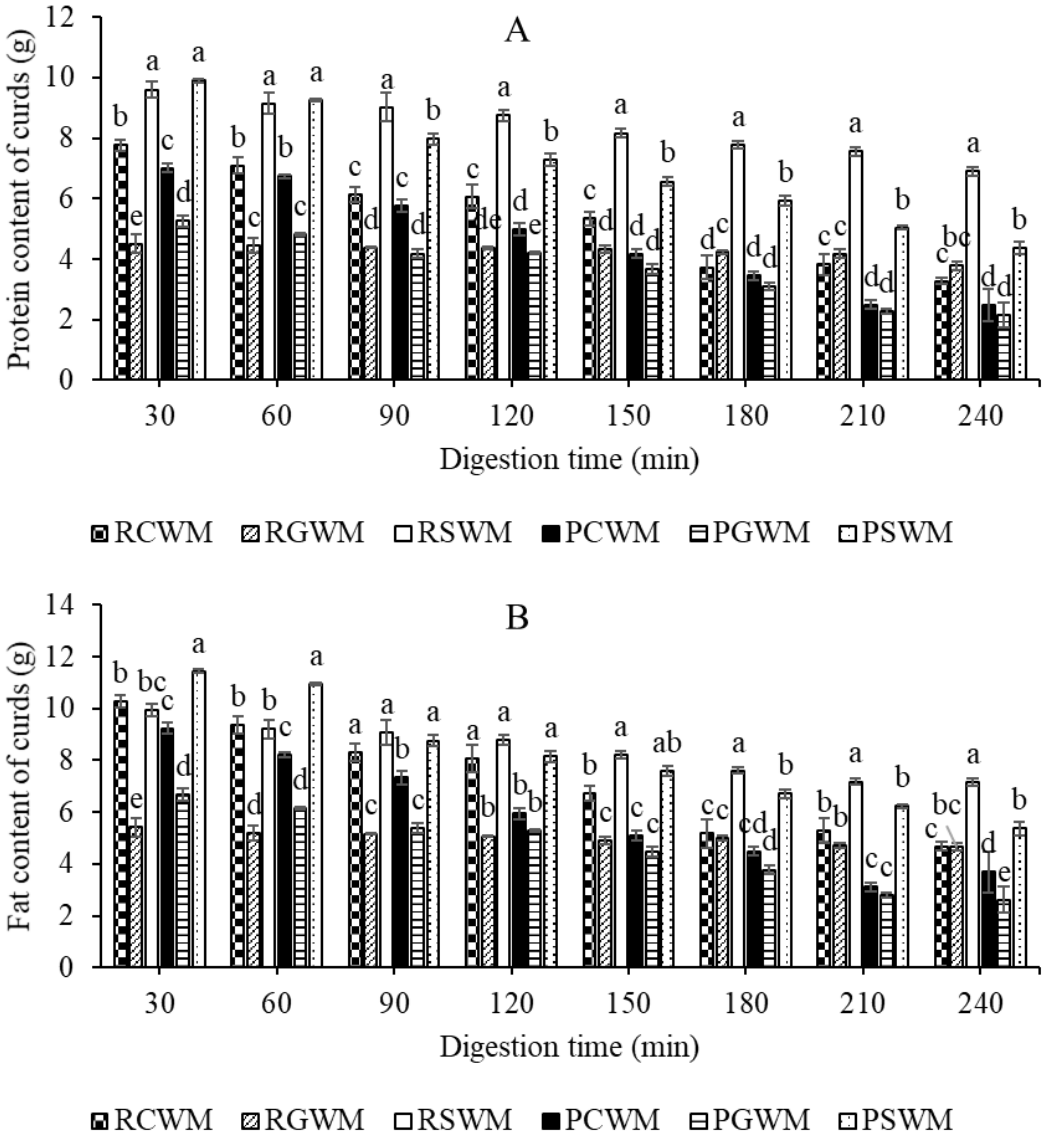


Figure A2.1 Changes in the protein and fat contents of the curds during the gastric digestion of 200 g of different milks in the HGS: (A) protein content of the curds; (B) fat content of the curds. RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk. Different letters (a–e) above the bars represent significant differences among the milk samples at a given digestion timepoint (P < 0.05); differences within a particular milk sample across different digestion times are not represented. Each data point represents the mean ± SD of results from at least two different batches of milk.

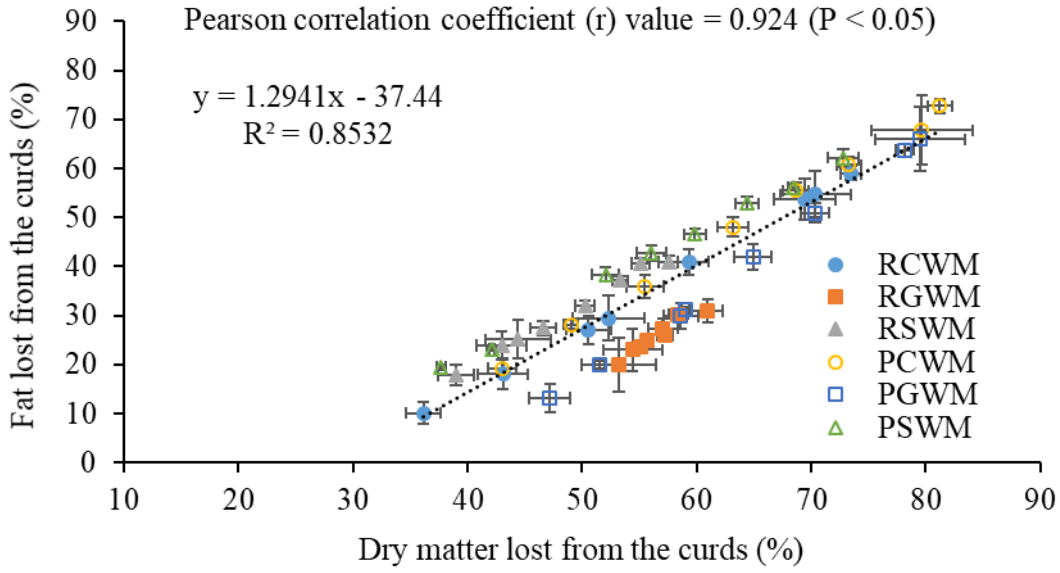


Figure A2.2 Relationship between the fat and dry matter contents lost from the curds of different whole milks during gastric digestion in the HGS from 30 to 240 min (RCWM, raw cow whole milk; RGWM, raw goat whole milk; RSWM, raw sheep whole milk; PCWM, pasteurized cow whole milk; PGWM, pasteurized goat whole milk; PSWM, pasteurized sheep whole milk). Each data point represents the mean ± SD of results from at least two different batches of milk. The Pearson coefficient value as well as the regression line and the equation for all points are depicted.

Statement of contribution (DRC 16 forms)

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Ye, A., Roy, D., & Singh, H. (2020). Structural changes to milk protein products during gastrointestinal digestion. In M. Boland & H. Singh (Eds.), Milk Proteins (Third Edition) (pp. 671-700): Academic Press. https://doi.org/10.1016/B978-0-12-815251-5.00019-0

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Roy, D., Ye, A., Moughan, P. J., and Singh, H. (under review). Impact of gastric coagulation on the kinetics of release of fat globules from milk of different species. Targeted Journal - Food and Function.

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