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Title	Biochemistry, physicochemical properties, and processing of human milk and comparison to bovine milk
Authors	Meng, Fanyu
Publication date	2021-06
Original Citation	Meng, F. 2021. Biochemistry, physicochemical properties, and processing of human milk and comparison to bovine milk. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2026-09-14 13:46:59
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UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Biochemistry, Physicochemical Properties, and Processing of Human Milk and Comparison to Bovine Milk

A Thesis Presented to the National University of Ireland for the Degree of

Doctor of Philosophy

By

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June 2021

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Prof. Mairead Kiely

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signature: Date: _____

致我的爷爷周其国、奶奶於玉芳及家人

世界，非世界，是名世界

To my dearest grandparents and family

Acknowledgement

My both tortuous and exciting PhD journey is finally coming to an end. Looking back, I would like to say, it is all of you that make me here.

First, I would like to show my biggest appreciation to my supervisor, Prof. **Alan Kelly**. It is you gave me the opportunity for this position, you guided me, supported me, and trusted me along the way. I cannot imagine a better supervisor than you. You always replied my email without hesitation and you accepted the heavy work of revising my manuscripts, version by version, even during weekends.

Dr. **Therese Uniacke-Lowe**, I would like to give my special thanks to you as I cannot finish my PhD without you. Because of you, I can have idea on creaming chapter, I can obtain the frozen milk samples to conduct the experiments. You also taught me for most lab techniques, Mastersizer, Urea-PAGE, Turbiscan, Lumisizer… Same as Alan, you helped me to revise manuscripts so many times. I hope you and Alan could forgive my habit about misplacing the definite article "the" and regard this as a special experience.

Dr. **Elisa Lanfranchi**, how lucky I meet a post-doc like you. You infected me by your enthusiasm and cautious attitude. I still remember you encouraged me by talking about your PhD experience, helping me contact the human milk analyser in Nutritional department, and attend the enzyme talking together in UCC. Thanks for your help during my PhD project.

My most sincere appreciation also goes to everyone in INFAMILK group. Brainstorming with everyone in INFAMILK is a fancy growing experience to me. Professor **Tony Ryan**, a real doctor retired from Cork university hospital, who gave me suggestions from clinical point of view. You care about students' opinion and I will always remember the question you asked every time at the end of our group meeting "Sonia, what did you learn from this meeting?". Professor **Catherine Stanton,** an outstanding researcher, from whom I learnt how to make good plans and push a project going on. **Katriona Lyons**, my co-partner in Teagasc, thanks for helping me with samples collection and I will always remember the time we talking about how stressful we were at the final stage of our PhD, which makes me feel I am not only myself on the PhD road. **Ruth Patricia Conboy**, I feel very glad to working with you for my final two chapters. Also, **Grainne Meehan** and **Carol-Anne O'Shea**, thanks for your hardworking on recruiting mothers and collecting human milk samples. I appreciate Department of Agriculture, Food and the Marine, Ireland, who provides funding and supports to the INFAMILK project.

I would like to say thank you to everyone in MiST group, my warmest family in UCC. **Patrycja Zakrys**, you are just like my elder sister. You cared about my feelings and taught me how to do project management and how to develop my career during my PhD, which really helps me a lot. Thanks to **Nikki** (盖楠) and **Bozhao** (李博照) for helping on my experiments and having dinner party with me together. You are really like my younger sister and bothers! Special thanks to Nikki for your superb series of cakes during my thesis writing period. Thanks to **Megan Ross**, you are warm and cool. I still

remember we were locked outside the food science building during the open day and I am so happy that I invited you to my home for some Chinese food. **Naomi Helen**, you are my best neighbour in the office. You are such an energetic person that I feel you make my PhD life brighter and I like your decoration during Halloween and Christmas. Thanks to **Jasper Van Der Schaaf**, you always had good ideas and be willing to share (both the knowledge and coffee capsules). Thanks for **Aisling Crotty**, you are considerate and I love cakes you baked! Thanks for **Costanza Bortolan**, **Costas Theodosiadisi**, **Nivedita Datta**, **Hiba Howsawi**, **Angela Di Conza**, and **Gaur Sawant**, I enjoyed the time when working with you. Last but not least, I would say thank you to **David Waldron**, our best technician. Thanks for your patient to help me ordering and looking for chemicals and organizing everything in our lab!

Many thanks to the technicians who have kindly helped me during my PhD study. **Jim McNamara**, your big smile shines my tough lab work and thanks for the helping in high-pressure processing. Thanks to **Avril McCord**, you are so helpful and thanks for showing me how to use colorimeter and spectrometer. Thanks to **Theresa Dennehy** for helping me with GC work and freeze-drying and **Johann Scollard** for helping me with freeze-drying as well. Thanks to **Yvonne O'Callaghan** for helping with the plate reader and **Karen Galvin** for helping with the fatty acids extraction methods.

I would also like to say thank you to all PhD students, postdoctoral researchers, and lecturers in Department of Food Science and Nutrition in UCC for your kindness and help. Special thanks to **Angeliki Angelopoulou** and **Elaine McCarthy** for helping me with the somatic cells counts and the human milk analyser. I would also like to thank all colleagues in Department of food science and Nutrition in UCC, who help me dealing with receipts, registration matters, and posting packages, etc.

I want to convey my thanks to **Maria** and **Pat**, you are not only my landlords, but my Irish parents. Meanwhile, I would also like to show my heartiest thanks to my dearest family and friends:

在此,我最感激的是我亲爱的的老爸孟庆民,老妈周秀萍,还有已故的爷爷周其国和奶奶於玉芳。谢谢你们对我的养育之恩，以及无条件的信任、支持和付出。同时也要感谢我的未婚夫曹江，谢谢你在我低谷时期的温暖陪伴,相信我们未来会更好。你们的存在让我感到幸福，我会永远爱你们。

感谢在爱尔兰的小伙伴们,马乔、曹曦、欧阳兴、韦丽妮、林莹琛……谢谢你们没事叫我吃吃喝喝，有你们在我感觉并不孤单。当然也要感谢已经回国的朋友们，然姐、莹莹、李煜、张逸群、李海洋……谢谢你们陪我在爱尔兰度过的美好时光。谢谢一直在国内的小伙伴们，子叶、佳博……你们是我早日回国的动力！谢谢博士同期的小伙伴们，谢谢学长学姐……你们是我学习的榜样和前进的动力！

Finally, I would like to thank my PhD! It's one of the most important periods in my life. **人生之路亦如博士之路，道阻且长，唯有坚定信念、持之以恒方能实现目标,到达终点！**

孟繁宇
2021.06

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Thesis Abstract

Human milk is a valuable biofluid that provides essential nutrients and immunological protection to newborns. In the thesis, levels and properties of macronutrients in human milk, their influencing factors, and physico-chemical properties were studied, and in some cases compared to those of bovine milk. A longitudinal analysis of human milk composition showed a decrease in protein level and plasmin activities in both term and preterm human milk over six months of lactation after the due date. Infant gender and pre-pregnancy maternal body mass index (BMI) influenced total fat, energy density, and fatty acid profiles in human milk. Concerning the physical stability of human milk, this is the first study to show that creaming rates of human milk increased with creaming temperatures in the range 5 to 40°C. There was no significant impact of freezing at -20°C for 1 week on creaming rates, while pre-heating at 70°C for 10 min slowed the creaming of human milk; the results may help to improve human milk handling methods. Compared to human milk creaming, bovine milk exhibited a lag phase at the beginning of creaming, followed by a much higher creaming rate at 5°C, suggesting the clustering of bovine milk fat globules through cold agglutination, which could occur in a greater extent than in human milk. Casein stability in human milk, in relation to ethanol stability, heat stability and heat-induced changes, chymosin-/pepsin-induced coagulation, chaotropic agent induced-dissociation, and cooling-induced dissociation were also examined in this thesis. Human milk showed higher resistance to heat-induced coagulation compared with bovine milk. Proteolysis occurred during incubation at 37°C; in human milk with low plasmin activity, proteolysis still occurred due to the action of aspartyl peptidases such as cathepsin D. In separate studies, novel processing methods that could be applied to human milk as alternatives to holder pasteurization were evaluated. High-pressure treatment at 400 MPa for 10 min was shown to be a promising preservation method to compromise between maintaining nutritional quality and ensuring microbial safety. Other non-thermal treatments showed better performance in preserving lysozyme and lactoperoxidase activities compared to holder pasteurization. However, addition of nisin A to human milk could not prevent the proteolysis by bacterial proteases and addition of lactose oxidase decreased the pH of human milk during storage at 4°C for 1 week. These preserving methods required further investigation. Overall, this thesis provides valuable data on nutrients in human milk provided by Irish mothers and expands

the knowledge of stability of milk from different species. This study will help improve the handling and storage methods in homes, hospitals, or human milk banks, therefore, helping infants to obtain better human milk and benefit their growth in the future.

List of Publications

Peer-Reviewed Journal Publications

Meng, F., Uniacke-Lowe, T., Lanfranchi, E., Meehan, G., O'Shea, C. A., Fox, P. F., Huppertz, T., Ryan, C. A., & Kelly, A. L. (2020). Factors affecting the creaming of human milk. *International Dairy Journal*, 104726.

Meng, F., Uniacke-Lowe, T., Ryan, C. A., & Kelly, A. L. (2021). The composition and physico-chemical properties of human milk: A review. *Trends in Food Science & Technology*. 112, 608-621.

Several further papers are in preparation for submission, delayed in part because of ongoing data analysis and in some cases additional trials.

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Introduction and Objectives

For infant feeding, there are three major strategies: mother's own milk, donor milk and infant formula feeding. Mother's own milk is the gold standard compared to donor milk and infant formula feeding as it provides more individualised and suitable nutritional compounds, as well as immunological protection, to neonates. Mother's own milk shows short-term protective effects against morbidity and mortality, particularly from necrotising enterocolitis (NEC) (Miller et al., 2018), as well as long-term benefits, including protection against child infections and malocclusion, increases in intelligence, and probable reductions in overweight and diabetes (Victora et al., 2016). Breastfeeding has been, therefore, considered as a public health priority and exclusive breastfeeding for about 6 months has been recommended (Eidelman & Schanler, 2012). The exploration on human milk is ongoing as knowledge on important nutrients, cells, and microbiome presented in human milk is still limited. For this reason, the INFAMILK project was funded by the Department of Agriculture, Food and the Marine (DAFM) Ireland to help improve breastfeeding, women's health and infants' growth. This work is part of this project and contributed to the project by measurement of macronutrients, fatty acids, and plasmin activity in human milk, as well as a deeper study of the properties of selected human milk samples.

Meanwhile, donor breast milk from human milk banks or infant formula are substitutes for breastfeeding when mother's own milk is not available (Haiden & Ziegler, 2016; Prell & Koletzko, 2016). As the number of human milk banks has increased (Arslanoglu et al., 2013), donor milk is more widely processed and used for infants who cannot receive their mother's own milk, although the knowledge of stability of such milk during processing and storage is insufficient. No milk bank in the Republic of Ireland has been established at the moment and the nearest human milk bank is in Northern Ireland, where donor milk is processed, transported, and stored. The practise of these processes may need to be standardised based on scientific studies. Also, in infant formula manufacturing plants, new ingredients are added to infant formulae in an attempt to mimic the composition or performance of human milk (Gallier et al., 2015; Martin, Ling, & Blackburn, 2016). However, some information on the structure of key components in human milk, such as casein micelles, as well as some physicochemical performance of human milk, remain unresolved.

Based on the above background, key questions come up (**Fig. 1**).

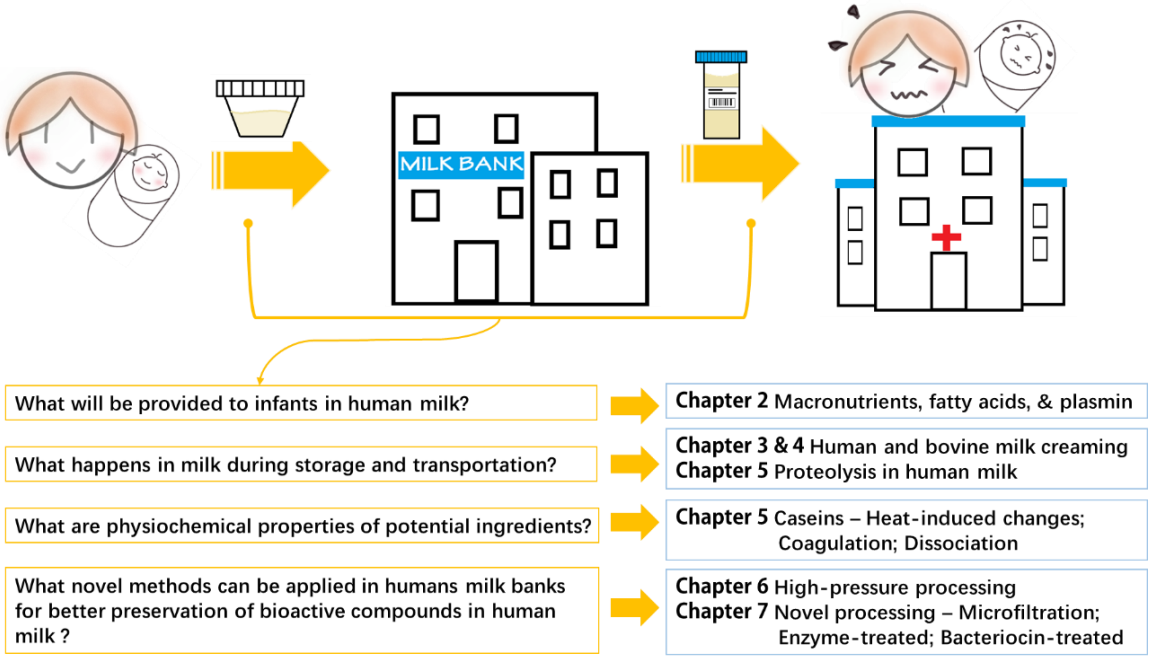

Fig. 1 Aims of the thesis and the guide for each chapter.

Our work aims to answer these questions and the objectives were as follows:

1. As a task of the INFAMILK project, to establish macronutrient composition of human milk over lactation, from mothers who delivered full term from birth to up to six months, as well as from mothers who delivered preterm from birth to the full-term due date and subsequently up to six months of lactation. The results are being further interpreted with microbial data and metabolomic data to elucidate their relationship.

2. To study the stability of human milk during storage, especially the creaming properties, which has not been well studied in human milk. Meanwhile, the method for studying the creaming properties based on bovine milk was compared to facilitate the study. Human milk creaming was then compared to bovine milk creaming to study the mechanisms involved.

3. To investigate the ultrastructure and stability of human casein micelles, which could be a model to inform next generation infant formulae.

4. To explore emerging technologies in preserving valuable components, such as lactoperoxidase and lysozyme, in donor milk without compromising microbial safety.

The information generated in this thesis will provide mothers, paediatric professionals, and infant formula manufacturers, as well as their ingredients suppliers, with essential knowledge.

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CHAPTER 1

The Composition and Physico-Chemical Properties of Human Milk: A Review

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Part of this chapter was published in:
Meng, F., Uniacke-Lowe, T., Ryan, C. A., & Kelly, A. L. (2021). The composition and physico-chemical properties of human milk: A review. *Trends in Food Science & Technology.* 112, 608-621.

Authors Contributions:
Fanyu Meng have drafted the manuscript.
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Abstract

***Background**:* Human milk is recommended as the optimal source of nutrition for babies up to six months of age, as it provides individualized, specific and abundant nutritional compounds, as well as immunological protection. Currently, there is wide interest in studying human milk because of this key role, as well as identification of biologically active compounds. In addition, human milk banks play an important role in supplying donor human milk to infants, especially premature infants, in hospitals, which involves processing and storage of such milk.

Scope and Approach: This review summarises recent studies on macronutrients in human milk, including their structure, levels, influencing factors, and contributions to the health benefits of infants. Physico-chemical properties of human milk are also reviewed, including pH, colour, stability of casein micelles, size and zeta-potential of fat globules, heat stability, freeze-thaw effect, dehydration and phase separation, and the impact of preservation processes on these.

Key Findings and Conclusions: The levels of human milk macronutrients and factors that influence these, such as lactation and gestation age, are ongoing research topics. Studies on physicochemical properties of some human milk components, such as caseins, are comparatively few, as research has mainly focused on bioactive peptides, milk fat globule membrane and human milk oligosaccharides. Processing and preservation are applied to human milk and the effects of these methods on the retention of human milk nutrients for use in human milk banks and hospitals must be considered. Some physico-chemical properties, such as milk pH, colour, and physical stability, may be possible indicators of milk quality.

1.1 Introduction

Human milk from the infant's own mother is the optimal source of nutrition for infants because: (1) it provides core required nutrients, including proteins, lipids, carbohydrates and minerals; (2) it provides bioactive factors, especially immunological factors, to protect infants from invading microorganisms (Ballard & Morrow, 2013; Lee et al., 2018); (3) it helps the development of the infant microbiome and thus the newborns' immune system (Kulinich & Liu, 2016; Lyons et al., 2020); and (4) some components in human milk are considered to influence the expression of genes in neonates, therefore influencing their health outcomes (Verduci et al., 2014). Breast-feeding also provides many other short-term and long-term benefits to infants, such as reducing the risk of necrotizing enterocolitis (in preterm infants) and obesity later in life (Mosca & Giannì, 2017; Boix-Amorós et al., 2019). Human milk from an infant's own mother is therefore recommended as the first-choice source of nutrition by the World Health Organization (WHO) (WHO, 2003)

When an infant's own mother's milk is unavailable or in limited supply, for example for vulnerable preterm infants, donor human milk (DHM) from milk banks is fed as a substitute because it can significantly reduce morbidity and mortality among vulnerable infants (WHO, 2003). DHM usually consists of pooled milk samples provided by lactating mothers who have been medically screened. It is then typically pasteurized, frozen and stored until required (DeMarchis, Israel-Ballard, Mansen, & Engmann, 2017; Weaver et al., 2019). As a result, some beneficial compounds, such as secretory immunoglobulin A (sIgA), lactoferrin, lysozyme, long-chain polyunsaturated fatty acids, cytokines and growth factors, may be present at lower levels than in fresh human milk (Malinowska-Pańczyk, 2020). Studies on how to minimize the nutrient gap between DHM and mothers' own milk, for example, finding alternatives to the major human milk banking preservation step, holder pasteurization (HoP, typically at 62.5°C x 30 min), are ongoing.

There is also an increasing interest in innovative applications of human milk, for instance, the possibility that the human milk component sIgA can be used to treat the newly identified illness severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Fox et al., 2020). Furthermore, human milk compounds should guide strategies for infant feeding, including identifying new critical ingredients for infant formula, such as oligosaccharides, components of the milk fat globule membrane (MFGM) and bioactive peptides (Koletzko, 2016; Sun et al., 2017; Wang et al., 2020). To achieve better preservation of bioactive compounds, in order to utilize the human milk more effectively,

it is important to study how best to process and preserve human milk, as well as evaluating the underlying physico-chemical properties of human milk.

The physical and chemical properties of milk refer to attributes such as pH, colour, and density; these properties depend on both intrinsic compositional and structural factors in the milk and extrinsic factors such as temperature and treatments (McCarthy & Singh, 2009). To the best of our knowledge, there is no systemic review on the physico-chemical properties of human milk and their relationship to milk composition, which are important in understanding milk quality and the impact of processing and preservation. The purpose of this review is to compile existing knowledge and new findings on human milk composition and its physico-chemical properties, with the goal of identifying new insights into human milk processing and storage and, ultimately, enhancing feeding strategies for vulnerable infants. It should be noted that the extensive topic of the microbiome of human milk was not included in this review, but recent reviews in this area include those of Lyons et al. (2020), Togo et al. (2019), and Moossavi et al. (2019).

1.2 Synthesis and Secretion of Human Milk in the Mammary Gland

Breast milk is the secretory fluid produced from the human mammary gland, a mechanism which is regulated by hormones and growth factors (Monks and McManaman, 2013; Montalbetti et al., 2014; Rezaei et al., 2016). As illustrated in **Fig. 1.1**, the basic elements of this glandular structure are alveoli and ducts; most components of the milk are synthesized and secreted from the specialized mammary epithelial cells (MEC) lining the lumen of alveoli, and then it is transported to the nipple by ducts (Monks and McManaman, 2013).

Most components (**Fig. 1.1**C), including casein, lactose, human milk oligosaccharide (HMO), calcium, phosphate, and citrate are secreted in the MEC through exocytic secretion processes, whereby the Golgi complex encapsulates these nutrients into secretory vesicles and carries them to the apical membrane. Synthesized in the MEC, lipids are transported through a different pathway because they are packaged into cytoplasmic lipid droplets (CLD) by enzymes in the endoplasmic reticulum without using the Golgi system (McManaman, 2014). Also, some amino acids and immunoglobulins that present in maternal blood circulation may be transported intact across the MEC (Rezaei et al., 2016).

Rather than being secreted from the MEC, immune cells, serum proteins, immunoglobulins and micronutrients can be taken up from mother’s blood directly and

enter the milk directly through a paracellular transport pathway (Monks and McManaman, 2013). This pathway is regulated by hormones, growth factors and mechanical constraints, while can be prevented by intact tight junction (Stelwagen and Singh, 2014). However, tight junctions are not fully closed during the first days of lactation and can reopen during weaning, which is consistent with the high proportions of immunoglobulins and serum proteins in colostrum and during weaning (Verd et al., 2018). Moreover, the integrity of tight junctions is influenced by mastitis and by preterm birth (Stelwagen and Singh, 2014). It has been hypothesized that high levels of immune cells, immune proteins and protease activity in preterm or mastitic milk can result from increased barrier permeability (Dallas et al., 2015).

Although general information about the synthesis and transport of the nutrients from mammary gland into human milk is known, details about these mechanisms remain unclear (Monks and McManaman, 2013; Montalbetti et al., 2014).

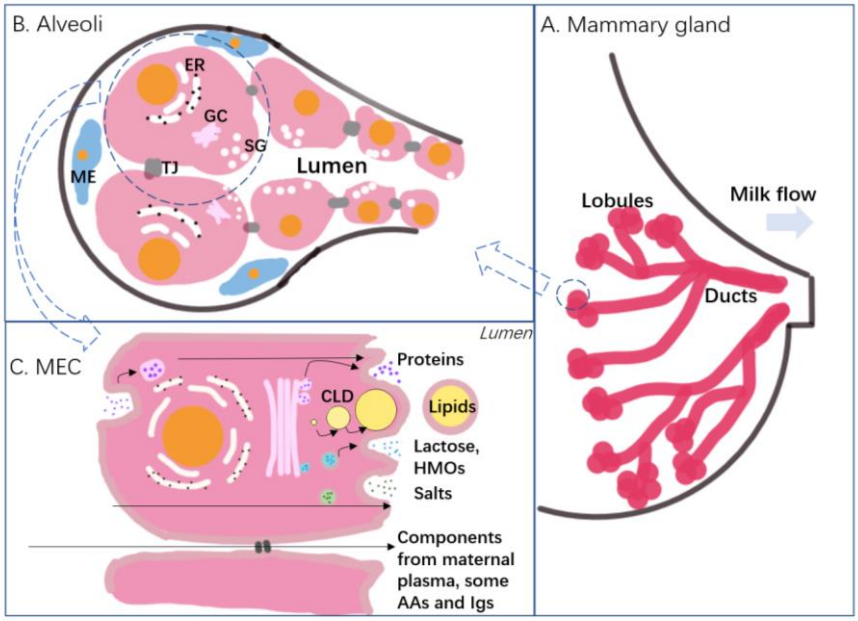

Fig. 1.1 Illustration of the structure of (A) mammary gland, (B) alveoli, and (C) specialized milk epithelial cell (MEC). ER: endoplasmic reticulum, GC: Golgi complex, SG: secretory granules.TJ: Tight junctions ME: myoepithelial cells (help milk expression). CLD: Cytoplasmic lipid droplets.

1.3 Macronutrients in Human Milk and Factors Influencing their Levels

Human milk is a complex mixture of water, carbohydrates, lipids, proteins and ash (Andreas, Kampmann & Mehring Le-Doare, 2015). The milk from well-nourished mothers who have a balanced diet should provide all the important nutrients to the infants during their first 6 months' of life (Eidelman et al., 2012; Prell and Koletzko, 2016). In addition to essential nutrients, human milk contains also bioactive molecules, such as cytokines, growth factors, and HMOs, which also contribute to the proper development of infants. Bioactivity and immunomodulatory activities of these molecules are of great research interest, and reviews in this area include those of Ballard et al. (2013), Gomez-Gallego et al. (2016), and Toscano et al. (2017).

As shown in **Table 1.1**, human milk contains 1-2% protein, 3-5% fat, and 6-8% carbohydrate. The individual differences and *intra*-analysis variations in content are influenced by several factors, including gestational age, stage of lactation, maternal characterization (BMI, age, parity, diet), birth weight of infants, diurnal variation, and the stage of expression or feeding, e.g., whether the milk is foremilk or hindmilk (**Table 1.2**). Moreover, methods of sampling and measurement also lead to different results among studies (Ballard and Morrow, 2013).

1.3.1 Gestational age

Milk composition is influenced by gestational age (GA), i.e., the period of time between conception and birth. According to the gestational age, human milk can be categorized as preterm milk (less than 37 weeks' GA) and term milk (37 to 42 weeks' GA) and preterm milk can be further grouped into extreme preterm (less than 28 weeks' GA), very preterm (28 to 32 weeks' GA) and moderate to late preterm (32 to 37 weeks' GA). The impact of gestational age on macronutrients levels, especially in terms of total fat and protein, is not conclusively understood (**Table 1.2**). The levels of macronutrients in milk from mothers of extremely preterm infants are of particular significance, due to the vulnerability of these babies, and there have been a number of studies comparing milk from extremely preterm, preterm, and term milk groups to understand best strategies for individualised nutrition for infants (Bauer & Gerss, 2011; Léké et al., 2019; Maly et al., 2019; Caldeo et al., 2020). As shown in **Table 1.1**, Maly et al. (2019) reported no difference of levels of milk macronutrients between very preterm (24 – 30 GA) and

preterm (31- 35 GA; average protein levels: 1.6 % during the first week of lactation) milk, whereas Bauer & Gerss (2011) reported that extreme preterm milk (GA<28 weeks) has higher proteins concentrations than preterm milk (32-33 weeks).

Recent studies provided longitudinal data on changing protein levels among term, preterm and very preterm groups (Caldeo et al., 2020); significantly higher protein levels in preterm or very preterm milk compared with term milk were found during days 0 – 7 and months 1 – 2 of the lactation. Fischer Fumeaux et al. (2019) reported a decrease in average protein concentrations from birth to four months after birth, from 2.2 to 1.5 % in preterm milk and from 2.5 to 1.7 % in term milk; they also reported that preterm human milk contains a higher mean fat content in the first week of lactation, but a lower mean fat content during 3 -8 weeks of lactation, compared with term milk. The carbohydrate level has been variously reported to be higher in term milk than in preterm milk (Léké et al., 2019), to not differ significantly (Caldeo et al., 2020), or to be higher (although not significantly) in preterm milk compared with term milk (Fischer Fumeaux et al., 2019).

The reason for the difference in composition between the term and pre-term milk is arguable. Some researchers consider that milk may be tailored for preterm babies, because of the higher amount of total protein and higher protease activity as well as more hydrolysed protein products, in preterm milk than in term milk; these may beneficial to pre-term neonates, who have immature digestive systems and require more nutrients to 'catch up' with the growth of full-term infants (Armaforte et al., 2010). In contrast, others have proposed that the difference may only be as a result of the immaturity of the mammary gland, e.g., leaky tight junctions of mothers of preterm neonates (Anderson, 1984; Molinari et al., 2012; Dallas et al., 2015). This hypothesis is supported by a lack of difference in the expression of immunological proteins and digestive enzymes between term and preterm milk. In addition, pre-term human milk feeding at routine enteral volume often fails to meet the recommendations for enteral nutrient intake for stable-growing pre-term infants, and thus fortification is required (Adamkin and Radmacher, 2014; Boyce et al., 2016; Kim, 2016; Radmacher and Adamkin, 2017).

Table 1.1 Studies on macronutrient (g dL^{-1}) and energy (kcal dL^{-1}) levels of human milk (modified from Ballard and Morrow, 2013).

Samples	Protein	Fat	Carbohydrate	Energy	References, n
Term infants, mature milk	1.2 (0.15) [a]	3.6 (0.7)	7.4 (0.15)	70 (6.7)	Nommsen et al. (1991), n=58
Term infants, mature milk	1.6 (0.4)	4.1 (0.7)	6.2 (0.9)	67.7 (3.9)	Bauer and Gerss (2011), n=10
Term infants, weight>2500g	1.2 (0.26)	3.5 (1.49)	7.6 (0.77)	66.3 (13.3)	Yamawaki et al. (2005), n=1197
Donor human milk samples	1.2 (0.25)	3.2 (1.0)	7.8 (0.88)	65 (11)	Wojcik et al. (2009), n=415
	0.9 (0.6, 1.4) [b]	3.6 (1.8, 8.9)	7.2 (6.4, 7.6)	67 (50,115)	Michaelsen et al. (1990), n=2553
Extremely Preterm, GA<28 weeks	2.3 (0.5)	4.4 (0.9)	7.6 (0.6)	78 (8.4)	Bauer and Gerss (2011), n=52
Preterm, Born 32-33 weeks	1.9 (0.3)	4.8 (1.0)	7.5 (0.5)	77 (6.5)	Bauer and Gerss (2011), n=20
Preterm, 24-30 GA, first week of lactation (4-6)	1.7 (1-5)	3.6 (0.9-6.1)	6.3 (3.5-7.2)	69 (45-91)	Maly et al. (2019), n=85
Preterm, 31-35GA, first week of lactation (4-6)	1.6 (1.1-3.6)	3.4 (1.6-7)	6.4 (0.6-7.1)	67 (42-104)	Maly et al. (2019), n=140

Data are presented as [a] mean (SD) or [b] median values and interquartile range or [c] median values with range. GA: gestational age.

Table 1.2 Factors affecting the gross composition of human milk.

Factors[1]	Impact					
	Protein		Fat		Carbohydrate	
	Findings	References	Findings	References	Findings	References
Ethnicity/ Geographic locations	Impact	Feng et al. (2016)	Consistent	Andreas et al. (2015)	Consistent	Andreas et al. (2015)
			Affects lipid profiles	Kumar et al. (2016)	Affects HMO level	Erney et al. (2000)
Age of mother	Highest in mothers aged 20-30	Kader et al. (1972)	Higher in older mothers	Hausman Kedem et al. (2013)	Higher in older mothers	Lubetzky et al. (2015)
Parity	Affects protein profile in preterm milk	Montagne et al. (1999)	Decreases	Jensen (1999)	No impact	Nommsen et al., (1991)
Maternal diet	High in milk from well-nourished mothers	Ballard & Morrow (2013)	Affects by protein intake	Ballard & Morrow (2013)	No significant relations	Andreas et al. (2015)
	Positive correlated with carbohydrate intake	Hascoët et al. (2019)	Correlated with carbohydrate intake and smoking	Hascoët et al. (2019)		
Maternal BMI	Positive correlated	Michaelsen et al. (1990); Mangel et al. (2017)	Slightly increased	Michaelsen et al. (1990)	No impact	Mangel et al. (2017)
			NS	Mangel et al. (2017)		
Infant birth weight	Inverse correlation	Andreas et al. (2015).	High in the low-birth-weight infants	Dritsakou et al., (2017)	Inverse correlation	Andreas et al. (2015)

Table 1.2 *(Continued)*

Factors	Impact					
	Protein		Fat		Carbohydrate	
	Findings	**References**	**Findings**	**References**	**Findings**	**References**
Gestational age	Higher in preterm milk	Gidrewicz & Fenton, (2014)	Higher in preterm milk	Bauer & Gerss, (2011); Guerra et al. (2016)	Higher in term milk	Guerra et al. (2016); Léké et al. (2019)
	Not conclusive	Bauer & Gerss, (2011); Maly et al. (2019)	Higher in term milk	Narang (2006)		
			NS	Granot et al. (2016)		
Lactational stage	Decreases	Gidrewicz & Fenton (2014)	Increases	Gidrewicz & Fenton (2014)	Consistent	Gidrewicz & Fenton (2014)
Diurnal variation	Higher Igs in the diurnal period	Franca et al. (2010)	Peak at midmorning	Jensen (1999)	NS	Paulaviciene et al. (2020)
	Higher at 12pm & 6pm compared with 12am & 6am	Paulaviciene et al. (2020)	Higher at 12pm & 6pm compared with 12am & 6am	Paulaviciene et al. (2020)		
Nursing process	-	-	Higher in hindmilk	Mizuno et al. (2009)	Higher in foremilk	Martin et al. (2016)
Mastitis	NS	Say et al. (2016)	Low in mastitic milk	Say et al. (2016)	Low in mastitic milk	Say et al. (2016)

1. Adapted from Jensen, (1999), and Andreas et al. (2015). HMO: Human milk oligosaccharides. BMI: Body mass index. NS.: No significant difference.

1.3.2 Stage of lactation

Human milk produced during the first 24 hours (or 2 to 4 days broadly) *post-partum*, i.e., colostrum, contains a higher level of immunological components and lower lactose concentration than mature milk produced by 28 to 40 days' *post-partum*, due to the loose structure of tight junctions in the mammary gland during the first few days after birth (Monks and McManaman, 2013). The milk produced between colostrum and mature milk, typically from day 4 to 10 *post-partum,* has been defined as transitional milk; this has a similar profile of macronutrients to colostrum, but in higher volume and a higher amount of nutrients than colostrum to ensure the rapid growth of infants. When milk is fully mature, the composition shows much smaller subsequent changes compared to colostrum or transitional milk (Monks and McManaman, 2013).

The total protein content decreases from colostrum and remains stable in mature milk (Gidrewicz and Fenton, 2014). Lönnerdal et al., (2017) reviewed studies from 1953 to 2011 on lactational effects on proteins, and found that the sharpest decrease in protein level occurs during the first month after delivery, and protein content in colostrum can be nearly twice as high as that in milk collected 3 to 4 weeks after delivery, no matter whether in pre-term or term milk. According to the systematic review of Gidrewicz & Fenton (2014), the total fat content increases from approximately 2.2% in colostrum and reaches a plateau at the second week of lactation of 34 g L^{-1} in mature term milk. Lactose is present at a level of 64-76 g L^{-1} of milk, which remains constant during lactation.

1.3.3 Maternal diet

Maternal diet has been suggested as an influencing factor of milk composition because: (1) some components are directly derived from the maternal intake through the maternal plasma, especially fatty acids and vitamins; and (2) it can influence the metabolism pathway indirectly, as has been proven in mice studies (Bravi et al., 2016; Kim et al., 2018). Two strong pieces of evidence are the correlation between the high docosahexaenoic acid (DHA) content of milk and maternal fish consumption, and the relationship between milk vitamin C level and its dietary intake (Bravi et al., 2016). Limited information on the relationship between energy, protein and carbohydrate levels in milk and maternal diet have been reported. A high energy content in milk may be

related to a low maternal intake of carbohydrate and high intake of fat (Mohammad et al., 2009), while a high total breast milk protein content has been linked to a high maternal protein intake (Forsum and Lönnerdal, 1980). No relationship has been found between mothers' energy intake and total carbohydrate content in milk (Quinn et al., 2012).

1.4 Proteins

1.4.1 Classification of human milk proteins

Based on proteomic studies, human milk contains more than 400 proteins, with functions in immune response, cellular metabolism and protein metabolism (Molinari et al., 2012; Zhu & Dingess, 2019). In general, human milk proteins can be classified into three groups: whey proteins, caseins, and MFGM proteins, accounting for 60 - 80%, 20 - 40%, and 1 - 4% of total proteins, respectively (Lönnerdal et al., 2017). Caseins and whey proteins can be separated due to their different solubilities; caseins are acid-insoluble, whereas whey proteins are acid-soluble. Unlike bovine milk, it is not easy to separate whey proteins and casein in human milk through the addition of acid alone and/or addition of calcium. A combination of adjustment of pH to 4.3, addition of calcium and ultracentrifugation has been used for whey protein and casein separation in human milk (Kunz & Lönnerdal, 1992). Furthermore, a method for the fractionation of caseins from human milk by addition of calcium and secondary phosphate has been recently developed; the precipitation of caseins accompanied the precipitation of colloidal calcium phosphate (CCP), which results from the reaction of added calcium and soluble phosphate in the milk (Ten-Doménech, Simó-Alfonso, & Herrero-Martínez, 2018).

As shown in **Table 1.3**, The whey protein to casein ratio changes progressively during lactation, from 90:10 in early lactation, to 60:40 in mature milk and 50:50 in late lactation, reflecting the changing requirements of the growing infant (Kunz & Lönnerdal, 1992). However, Liao et al. (2017) showed a high inter-individual difference of whey protein to casein ratio during lactation. In contrast to these changes in levels of whey proteins and caseins, the concentrations of MFGM proteins, i.e., proteins distributed on the membrane surrounding fat globules, are more stable during lactation (Lönnerdal et al., 2017).

In addition, **Table 1.3** demonstrates that both the concentrations and the diversity of human whey proteins are high, whereas casein levels are low in human milk compared

with well-studied bovine milk (Guo, 2021). Furthermore, human milk does not contain β-lactoglobulin and α_{s2}-casein, which are present at relatively high concentrations in bovine milk.

Table 1.3 Protein profile of term human milk at different lactational stages compared with that of mature bovine milk.

Protein (g L^{-1})	**Human**			**Bovine**
	Lactational Stages			Lactational Stages
	Colostrum	Transitional	Mature	Mature
Total proteins	20	19	9-12 (10)	32 - 37 (35)
Whey protein: Casein Ratio	89:11	65:35	60:40	20:80
Casein[1]				
β-Casein	2.6	4.4	2.7	9.9
κ-Casein	1.2	1.3	0.9	3.1
α_{s1}-Casein	-	-	Trace	10.4
α_{s2}-Casein	-	-	-	2.1
Whey proteins[2]				
α-lactalbumin	4.3	4.2	3.0	1.4
β- lactoglobulin	-	-	-	3.4
Lactoferrin	5.1	3.3	1.9	Trace
Serum albumin	0.4	0.6	0.5	0.4
Lysozyme	0.3	0.3	0.98	Trace
Immunoglobulins	16% of total whey proteins			10% of total whey proteins
IgG	-	0.1	0.04	0.64[2]
IgM	-	0.1	0.04	0.09[2]
slgA	5.5	1.5	1.10	0.08[2,3]

[1] Adapted from Cuilliere et al. (1999), Lönnerdal et al. (2017) and Guo, (2021). [2] Adapted from Wheeler et al. (2008). [3] IgA level.

1.4.2 Caseins and casein micelles in human milk

1.4.2.1 Classifications of caseins

In human milk, 70% of total casein is β-casein (Carroll et al., 1985), a proline-rich amphipathic casein; the phosphorylation sites of human β-casein, at specific seryl and threonyl residues, were identified by Greenberg, Groves, & Dower, (1984). Human β-casein has six isoforms depending on the phosphorylation degree, from 0 to 5 (Sood & Slattery, 2002), which can be the major difference compared with the bovine β-casein (**Table 1.4**). These isoforms differ in solubility, polymerization manner, and calcium-binding capacity (Azuma, Kaminogawa, & Yamauchi, 1985).

From liquid chromatography analysis, human κ-casein has been shown to exist as its dimeric form (37 kDa), in which disulphide bonds join the single cysteine residue of each monomer, and the molecule contains 6 to 7 phosphate groups (Ferranti et al., 2004). κ-casein is important as a heavily glycosylated protein in human milk. It can inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa and trigger the growth of *Bifidobacterium infantis* and *Lactobacillus bifidus*, thus protecting breast-fed infants against gastrointestinal disease (Wada & Lönnerdal, 2014).

α_{s1}-casein has been characterised in mature human milk, and exists naturally as a multimer in complex with κ-casein (Rasmussen, Due, & Petersen, 1995). Martin et al. (2003) reported that human α_{s1}-casein contains cysteine, which is usually found in α_{s2}-casein instead of α_{s1}-casein in the milk of other species, which suggests that, even though α_{s2}-casein is absent from human milk, α_{s1}-casein plays its role, such as forming disulphide links with κ-casein (Rasmussen et al., 1999). In addition, α_{S1}-casein is present in human milk in an unphosphorylated or partially phosphorylated form with a lower level of phosphorylation than other species, which may benefit the infant immune system (Saenger et al., 2019). Unphosphorylated α_{S1}-casein can induce secretion of proinflammatory cytokines *via* Toll-like receptor 4 and its co-factors, whereas phosphorylated α_{S1}-casein cannot do this *in vitro* (Saenger et al., 2019).

1.4.2.2 The structure, size distribution, and stability of casein micelles

β-casein and κ-casein, with a small amount of α_{s1}-casein, can interact together with calcium ions creating a special dynamic and ordered structure, i.e., the casein micelle (CM) (Azuma et al., 1985; Martin et al., 2003). Human CMs can be observed from transmission electron microscopy as a cloud-like loose broadly spherical tangle (**Fig. 1.2** A). Based on electron microscopy studies, the size distribution of CMs ranges between

20-104 nm in mature human milk, with an average of 42 nm (Carroll et al., 1985). Human CMs are thus smaller than those of bovine, caprine and equine CMs (Inglingstad et al., 2010). The size of human CMs is positively correlated with β- to κ-casein ratio; when the κ-casein fraction increases, the number and the surface area of the micelles increase, and thus the size of CMs decreases (Dev et al., 1994). Although there has been no direct investigation of changes in the size of human CMs during lactation, the ratio of β- to κ-casein increases during lactation, suggesting that the size of CMs may increase during lactation (Cuilliere et al., 1999). The high level of κ-casein and small size of CMs in colostrum may compensate for the low digestive function of neonates (Cuilliere et al., 1999). To our knowledge, recent studies about human CMs, and a model for their structure, are lacking, and some key properties such the surface charge (zeta potential, an indicator of the stability of colloidal dispersions) of human CMs have not been reported.

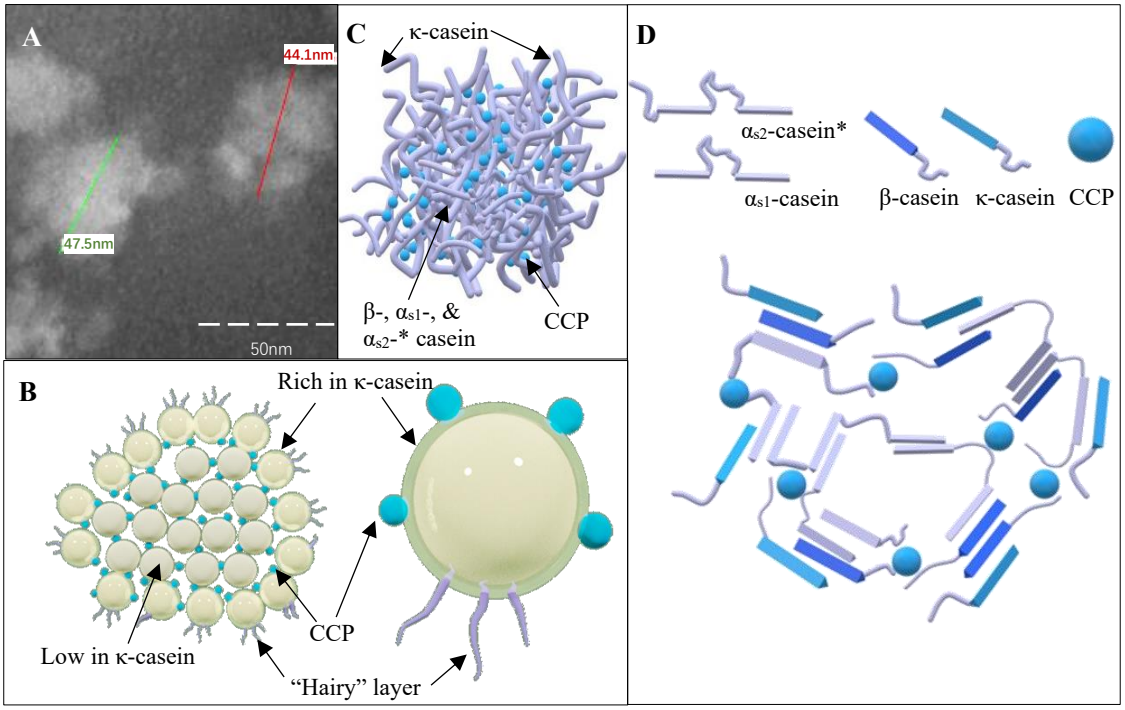

Fig. 1.2 Human casein micelles (A) observed by transmission electron microscopy of fresh skimmed human milk diluted 1:50 with 0.01M $CaCl_2$ stained with 20 g L^{-1} PTA (negative staining; see details in **Chapter 5**), and illustration of casein models from bovine milk studies: (B) the sub-micelle model (Slattery and Evard, 1973), (C) the nanocluster model of Holt (Holt, 1992), and (D) the dual-binding model (Horne, 1998; Horne, 2020). *α_{s2}-caseins present in bovine milk, but not in human milk.

Although the casein profile of human milk differs from that of bovine milk (Table 1.4), the ultra-structure appears similar, with β-caseins constituting the core of the micelle

through hydrophobic interactions and phosphoryl groups, while κ-casein is positioned on the surface, contributing to the stabilization of the micelle against precipitation by Ca^{2+} ions (Horne, 2020). Various models of internal bovine CM structure have been proposed from findings in bovine milk and they have been refined progressively. Among these, there are three detailed structural models of CMs (**Fig. 1.2** B-D): (1) the sub-micelle model (Slattery & Evard, 1973); (2) the nanocluster model of Holt (Holt, 1992); and (3) the dual-binding model (Horne, 1998, 2020).

In terms of bovine CMs, the 'dual-binding' model suggests that micelle structure is governed by a balance of hydrophobic interactions and CCP-mediated cross-linking of hydrophilic regions. The nanocluster model fits well with all changes of milk during processing, suggesting that not only the hydrophobic interactions, but also main-chain and side-chain H-bonds and other interactions play roles in the formation of the internal structure. All these models still have limitations in explaining the behaviour of caseins in reality (Li & Zhao, 2019).

From human milk studies, the 0P and 1P forms of β-casein require a much lower level of κ-casein to be suspended compared with the 4P and 5P forms, when Ca^{2+} is present, which reflects the fact that high phosphorylated forms are major β-caseins involving in forming intermolecular cross-links *via* calcium ions in micelles (Sood & Slattery, 2002). The extent to which κ-casein molecules in the human micelle shield the other proteins is less than that in bovine micelles, and so β-casein molecules may have greater potential mobility from the core to the surface of human CMs (Dev et al., 1994). Such an open structure may provide accessibility to digestive enzymes, and consequently, a large number of peptides could perhaps be released during proteolysis or digestion.

The formation of CMs can be influenced by many factors *in vitro*, such as ion concentration and the degree of phosphorylation of caseins. A lower level of calcium is required for human CM formation than for bovine CMs, which partly corresponds to the lower content of calcium in human milk than in bovine milk (Azuma et al., 1985). Sood and Slattery (2003) studied the nature of protein-protein interactions in human CMs through use of a mild chaotropic agent, LiCl, which can extract nondenaturing proteins from complexes. Their results suggest that the 0P form of β-casein may participate in interactions that are not available to the phosphorylated forms and that human CMs are formed and maintained through a combination of hydrophobic interactions and salt bridges involving protein phosphate esters and CCP.

As shown in **Table 1.4**, the effects of other chaotropic agents, cooling, and pH (from pH 5 to 10) on the dissociation of human CMs have also been studied (Sood, Herbert, &

Slattery, 1997). Similar to bovine milk, human β-caseins can be released during cooling with even higher extents (Sood et al., 1997). However, CMs are quite resistant to dissociation by chaotropic agents at 4°C due to their stabilizing salt bridges (Sood & Slattery, 2003). Bovine CMs are stable at high temperatures, but can be destabilized by freezing due to a decrease in pH and increasing level of ionic calcium in the unfrozen phase of milk, according to studies (Chen & Yamauchi, 1969); the corresponding information for human CMs, including the effects of freeze-thawing and heating, is currently lacking in the literature.

1.4.3 Whey proteins

1.4.3.1 Concentrations of whey proteins in human milk

Human milk is unique in that the percentage of whey protein in total protein (60-80%) is much higher than in milk of other species. The proteome of the human whey fraction has been extensively characterised and described by proteome mapping reports (Molinari et al., 2012; Zhang et al., 2016). The most abundant proteins present in human whey include α-lactalbumin, lactoferrin, lysozyme, immunoglobulins, and serum albumin, represent 26%, 10%, 16%, 10% of whey proteins respectively (Guo, 2021). Compared with bovine milk, human milk contains higher levels of lactoferrin, lysozyme and sIgA than bovine milk (**Table 1.3**). β-lactoglobulin, which is the dominant whey protein in bovine milk, is not detected in human milk (Rudloff and Kunz, 1997), except as an allergen (Axelsson et al., 1986; Dekker et al., 2020).

As shown in **Table 1.3**, the levels of whey proteins change during lactation. Human colostrum contains high levels of lactoferrin and sIgA, whereas mature milk contains much lower levels, which may relate to a higher requirement of immunologic components of new-borns to develop their weak immune system (Montagne et al., 2001; Rai et al., 2014; Yang et al., 2018). Although the content of α-lactalbumin decreases from colostrum (4.3 g L^{-1}) to mature milk (3 g L^{-1}), it remains high at all lactation stages compared with other proteins in human milk or compared to α-lactalbumin levels in bovine milk (1.4 g L^{-1}) (Affolter et al., 2016). In terms of gestational age, the levels of total IgA and lysozyme are higher, whereas the concentration of lactoferrin is lower, in preterm milk than in term milk has been reported (Mehta and Petrova, 2011). In contrast, no significant difference in lactoferrin level between term and preterm milk has been reported (Hsu et al., 2014).

Table 1.4 Summary of differences in structures, and stability between caseins in human and bovine milk.

	Human[1]			**Bovine**[2]			
Caseins	β-Casein	κ-Casein	α_{s1}-Casein	β-Casein	κ-Casein	α_{s1}-Casein	α_{s2}-Casein
Molar Mass (kDa)[4]	23.9 – 24.2	19.0	21.0	23.9 – 24.1	19.0	22.1 - 23.7	25.2 – 25.4
Structure	Multi-phosphorylation (0-5P)	High glycosylation (carbohydrate weight:40%)	9 potential phosphorylation sites; -0P or partially phosphorylated form	Major -5P, occasionally -4P form	Carbohydrate weight:10%	Major -8P and -9P form	10 - 13P form
	Human CM (mean size: 64 -80 nm)			Bovine CM (mean size:150-182 nm)			
Influencing Factors							
pH	Fractionation at pH 4.3 with Ca^{2+} addition; β-casein (especially -0P and -1P form) dissociates from CM with increased pH (pH 7.6 to pH 9.9)			Fractionation at pH 4.6; CM is unstable at pH < 5.2; the size of CM decreases at pH < 5.5; CM dissociates at pH > 9			
Heating	NS			Partial disruption at 50°C - 90°C			
Cooling /Refrigeration	Cooling: Reversable solubilization; 0P β-casein increases; mean size of CM increases; Refrigeration: β-casein dissociates			Reversable solubilization during cooling; β-casein dissociates during refrigeration			
Freezing (-20°C)	NS			Destabilization during freezing			
HPP	NS			Disruption at 400 MPa due to solubilization of CCP			

[1] Adapted from Liao et al. (2017), Azuma, Kaminogawa, and Yamauchi (1984), Ferranti et al. (2004), Carroll, Basch, Phillips, and Farrell Jr (1985), König et al. (2017), Kunz and Lönnerdal (1992), Sood, Herbert, and Slattery (1998), Sood, Herbert, and Slatter (1997), and Zhang et al. (2020). [2] Adapted from Swaisgood (2003), Schulte, Stöckermann, and Gebhardt (2020), Vaia, Smiddy, Kelly, and Huppertz (2006), Horne (2020), Fox and Brodkorb (2008), and Huppertz, Fox, and Kelly (2004b). [3]Levels of caseins in human milk were presented as average (range). [4] Molecular Mass is calculated from its amino acid sequence (carbohydrate free form). **Note**: CM: casein micelle; CCP: Colloidal calcium phosphate; NS: Not studied/ Report not found.

The content of lysozyme is changeable during lactation; however, no clear trends for lysozyme concentration were shown during the first 90 days after delivery (Lönnerdal et al., 2017). Some studies have shown an increase in the concentration of lysozyme during lactation, which is a contrast from other whey proteins (Montagne et al., 2001). Lönnerdal et al. (2017) concluded that the concentrations of lysozyme were highest at 31 to 60 days *post-partum* (approximately 1.1 mg mL^{-1}). However, only four articles were reviewed in that analysis and no data on lysozyme concentrations after day 90 were available (Lönnerdal et al., 2017). There is no consensus about the effect of malnutrition on levels of lysozyme in milk (Hamosh, 1998).

Immunoglobulins (Ig) in human milk include IgA, Immunoglobulin G (IgG) and Immunoglobulin M (IgM). The concentration of sIgA is high in colostrum, at approximately 2.0 g L^{-1}, and this level drops to 0.5 g L^{-1} and remains constant in mature milk (Goldman et al., 1982). The content of IgG, at approximately 2% of total human Ig (Hurley and Theil, 2011), has been reported to be low in colostrum, and it could not always be detected (Goldsmith et al., 1983; Lönnerdal et al., 2017). Interestingly, the dominant immunoglobulin in bovine milk is IgG (96 % of total bovine Ig) rather than IgA. Neither the changes in concentration of IgG or IgM during lactation has been clearly studied. However, the level of IgG in human milk has been detected to be higher in mature milk than in the transitional milk (Gao et al., 2012), which was supposed to compensate the immunity of infants, especially for opsonization and antibody-mediated cytotoxicity. In contrast, the level of IgM, which is also low in colostrum, decreases during lactation, because the newborn has the ability to produce enough IgM for an immune response (Gao et al., 2012; Lönnerdal et al., 2017).

In addition, some levels of bioactive proteins with low abundance have been reported. For example, haptocorrin (also known as transcobalamin) is a vitamin B12-binding protein that has been found has a higher content than that of vitamin B12 in milk (Lönnerdal, 2013). Human colostrum contains approximately 5 mg L^{-1} haptocorrin (4-5 days *post-partum*) and the level of this protein drops to approximately 3 mg L^{-1} in mature milk (4weeks *post-partum*) (Aoki et al., 1992). A longitudinal study on the level of this protein was also conducted by Greibe et al. (2013); the concentrations were approximately 25, 22, and 180 nmol L^{-1} (1.125, 0.99, and 8.1 mg L^{-1}) at 2 weeks, and 4 and 9 months of lactation, respectively.

Approximately 2000 nmol kg^{-1} of folate-binding protein (FBP) is found in human milk; FBP binds all naturally occurring folate in breast milk, preventing the oxidation of folate in the digestive tract (Haschke, Haiden, & Thakkar, 2016). It has also been reported that mature human milk contains approximately 140 mg L^{-1} osteopontin,

which has been demonstrated to help with immune activation, inhibition of ectopic calcification, cellular adhesion and migration, angiogenesis, and bone remodelling (Schack et al., 2009; Haschke et al., 2016).

Although a range of enrichment, separation, and detection techniques have been developed, knowledge on the detection of low-abundance proteins in human milk is still limited (Molinari et al., 2012). These minor proteins are involved in metabolism and other changes in mammary gland, such as mastitis, and thus could be used as biomarkers to detect inflammation or track the development of the mammary gland in the future. Moreover, they may provide nutritional, protective and developmental advantages to the infants (Molinari et al., 2012). Therefore, it is important to further explore the properties of minor proteins and to understand the mechanisms of activities in which they are involved.

1.4.3.2 α-lactalbumin

Human α-lactalbumin is a 14-kDa protein which has been reported to have bactericidal and anti-tumoral effects (Pellegrini et al. 1999; Svensson et al., 1999; Zhang et al., 2009). During lactose synthesis, α-lactalbumin acts as a regulatory subunit of galactosyltransferase, a lactose synthase in MECs (Turkington and Hill, 1969). With the completion of lactose synthesis, α-lactalbumin dissociates from galactosyltransferase and is transported with other components into the milk.

The primary structure of α-lactalbumin is the same in many species, including humans, consisting of 123 amino acid residues (Permyakov, 2020). As summarized in **Table 1.5**, α-lactalbumin contains a single strong calcium-binding site, as well as several zinc-binding sites, which can influence the stability of this protein. Compared to bovine α-lactalbumin, human α-lactalbumin can be more heat-stable (**Table 1.6**), especially when Ca^{2+} cations are bound to the calcium-binding site, which also increases protein stability against heat and many denaturing agents (Permyakov, 2020). In contrast, the binding of Zn^{2+} to the Ca^{2+}-loaded protein decreases its stability (Heine et al., 1991). Nevertheless, the role of binding of Ca^{2+}, Zn^{2+} and other divalent metal ions, such as cobalt and manganese, to α-lactalbumin in lactose synthesis is still unclear. It has also been reported that α-lactalbumin and lysozyme are similar in both amino acid sequence and three-dimensional structure. However, α-lactalbumin exhibits much lower cell lytic activity than lysozyme (Sava et al., 1989; Ho, Nadeem, & Svanborg, 2017).

Table 1.5 Differences in the structure and stability of α-lactalbumin and lactoferrin in human and bovine milk..

	α-Lactalbumin[1]		**Lactoferrin[2]**	
Milk Type	Human	Bovine	Human	Bovine
Molar Mass (kDa)	14.1	14.2	75 - 82.4	80 - 84
Structure	Contains a secondary Ca^{2+} binding site	Less Ca^{2+} bound	Glycoprotein; low degree of iron saturation (~10%)	Different N-glycan profiles; higher degree of iron saturation
Influencing Factors				
pH	Ca^{2+} can release and bind to oleic acid at low pH	Stable at low pH	Iron-binding capacity and antimicrobial activity remain at pH 2.0	Maximum exposure of hydrophobic regions at pH 3.0
Heating	Heat stable when Ca^{2+} bound	The presence of β-lg or α-CN decreases the stability	HoP affects proteolysis during gastric digestion; more heat-resistant than bovine lactoferrin	No significant effect after HTST; iron-binding ability decreases after UHT
Refrigeration	NS	NS	Levels remain for 5 d	NS
Freezing (-20°C)	NS	Stable	Decreased level	NS
Pressurization	NS	Stable up to 400 MPa	Decreased level	The structure is stable and the activity remains at 400 MPa

[1]Adapted from Lönnerdal and Lien (2003), Permyakov (2020), Wehbi et al. (2005), Huppertz, Fox, and Kelly (2004a) and Rodiles-López et al. (2010); [2]Adapted from Furmanski et al. (1989), Karav, German, Rouquié, Le Parc, and Barile (2017), Sreedhara et al. (2010), Rollo, Radmacher, Turcu, Myers, and Adamkin (2014), Suzuki et al. (2003), and Franco, Pérez, Castillo, Calvo, and Sánchez (2013). **Note:** HoP: Holder pasteurization; HTST: High temperature short time pasteurization; β-lg: β-lactoglobulin; UHT: Ultra-high temperature pasteurization; NS: Not studied/ Report not found.

Heine et al. (1991) considered that α-lactalbumin in human milk and β-lactoglobulin in bovine milk serve similar functions in the protein nutrition of the human infants and calves, as both of them provide a high ratio of essential amino acids

to total amino acids in each protein, helping with rapid growth of newborns. Furthermore, bovine α-lactalbumin shares similar amino acid compositions (89%) with human α-lactalbumin, but with lower levels of tryptophan and cysteine (Layman, Lönnerdal, & Fernstrom, 2018). In a clinical trial, term infants fed with low-protein formula fortified with bovine α-lactalbumin showed similar growth outcomes to human milk-fed infants (Trabulsi et al., 2011).

1.4.3.3 Lactoferrin

Lactoferrin is not only secreted by the mammary gland, but is sourced from the lachrymal, bronchial and salivary glands, the kidney and the mucosae of the endometrium, and seminal vesicles (Masson and Heremans, 1971). The synthesis of lactoferrin in the mammary gland depends on hormone, dominantly prolactin (Green and Pastewka, 1978).

Structure, properties and biological functions of lactoferrin were reviewed by Adlerova et al. (2008), Gonzalez-Chavez et al. (2009), and Nazir et al. (2017). Human lactoferrin is a 80-kDa glycoprotein belonging to the transferrin family, which is a single polypeptide chain folded into two globular lobes (Bloxam et al., 1987; Furmanski et al., 1989). Although lactoferrin has significant iron-binding capacity, the iron-free form is the most common form in human milk. There are two iron-binding sites located at the N- and C- terminus of lactoferrin, respectively. Similar to transferrin, iron binding causes conformational changes and leads to a decrease of the molecular mass (Nazir et al., 2017).

Human lactoferrin is involved in iron metabolism, exhibits antimicrobial activities, and plays roles in cellular proliferation and immunomodulatory effects (Nazir et al., 2017; Lönnerdal, 2013; Kirkpatrick et al., 1971). It has been shown that its bacteriostatic effect is due to it binding iron and making it inaccessible to pathogens, which require iron to grow (Brock, 1980). However, quantitative analysis of the specific levels of lactoferrin in human milk required to achieve biological effects still need further study.

Compared to bovine lactoferrin, lactoferrin in human milk has a lower degree of iron saturation, higher heat stability, and can remain antimicrobial activity at lower pH (**Table 1.5**). Recombined human lactoferrin (rhLF) has been produced from the milk of

transgenic goats and cows due to the high cost of lactoferrin isolation from human milk (Wang, Sun et al., 2017). Although some studies have shown that rhLF has similar physicochemical and biological properties to human lactoferrin, the glycosylation patterns of rhLF were suggested to be more similar to bovine lactoferrin than human lactoferrin, which may influence the function of lactoferrin (Parc et al., 2017). Despite some clinical studies demonstrating the protective effect of lactoferrin against infections of preterm infants, recent large trials showed no clear relationships between supplementation with bovine lactoferrin and reducing the risk of mortality/morbidity or the late-onset infection in preterm infants (Asztalos, Barrington, Lodha, Tarnow-Mordi, & Martin, 2020; Griffiths et al., 2019).

1.4.3.4 Lysozyme

Lysozyme in human milk is an acid-resistant glycoprotein containing 130 amino acids, with a molecular weight of approximately 15 kDa (Hamosh, 1998). Compared with well-studied chicken egg white lysozyme, human lysozyme has considerable homology, but a much greater hydrophobicity due to the difference in tertiary structure from chicken egg sources (Hamosh, 1998). As shown in **Table 1.6**, lysozyme in human milk is relatively stable during heating, cold storage and high-pressure treatment.

Lysozyme is critical in the human immune system as it is capable of breaking down the outer cell wall of Gram-positive bacteria by hydrolysing β-1, 4 linkages of N-acetylmuramic acid and 2-acetylamino-2-deoxy-D-glucose residues (Chipman and Sharon, 1969). Working together with lactoferrin, lysozyme has also been shown to kill Gram-negative bacteria *in vitro* (Ellison and Giehl, 1991). This is because lysozyme can access and degrade the inner proteoglycan matrix of the membrane of bacteria after removal of liposaccharide from the outer cell membrane of bacteria by lactoferrin, thereby killing the bacteria (Ellison and Giehl, 1991).

Zavaleta et al. (2007) showed that an oral rehydration solution fortified with recombinant human lysozyme and human lactoferrin decreased the duration of diarrhoea of children hospitalized with acute diarrhoea. Supplementing rice powder with rhLY and bovine lactoferrin improved the gut health of 2-year-old children (Cheng et al., 2019). Lysozyme has also been shown to inhibit the growth of HIV in vitro, but

in human milk it may only act upon the free virus and not on cell-associated virus (Lönnerdal et al., 2017).

1.4.3.5 Immunoglobulins

The most abundant and effective immunoglobulin in terms of anti-inflammatory effect is sIgA (Hettinga et al., 2011; Haschke et al., 2016). sIgA can recognize receptor-binding domains (e.g., reovirus type 1 Lang) as well as possibly affecting the virulence of bacteria directly (Andreas et al., 2015). This immunological inhibition of the microbial colonization and restriction of antigen uptake is commonly known as "immune exclusion" (Brandtzaeg, 2013). Immune exclusion by sIgA is activated by a broad-spectrum of commensal bacteria as well as potentially allergenic antigens, and it is thus important to the human immune system. It has been found that both sIgA and IgG can form complexes with antigens in human milk (Verhasselt, 2010). However, sIgA can trap antigens and prevent them from transferring to the child, whereas the IgG-antigen complex can easily cross the barrier of the infant gut (Verhasselt, 2010). A recent study reported that an immune response involving sIgA was found in human milk against the COVID-19 virus (Fox et al., 2020). This finding suggests that sIgA could be used as a novel therapeutic antibody; however, the efficacy and durability of the protein requires further studies.

sIgA is specific because of an epithelial glycoprotein called secretory component (SC) attaches to its dimeric structure (Kerr, 1990). SC helps sIgA to block toxins adhering to intestinal epithelium (Brandtzaeg, 2013). Also, this structure contributes significantly to the high resistance of sIgA to digestion (Lindh, 1975), which has been shown to be stable even at low pH *in vitro* (Chatterton et al., 2004) and it remains intact in infant faeces (Donovan et al., 1987). However, sIgA is sensitive to heat, and thus could be inactive after holder pasteurization (**Table 1.6**).

Other immunoglobulins, such as IgM and IgG, are also present in human milk. However, compared with sIgA, the concentrations of these immunoglobulins are much lower and they do not survive as intact proteins in the small intestine (Lönnerdal et al., 2017).

1.4.3.6 Other whey proteins

Functional whey proteins in human milk are still being explored and several of them have been relatively well described, including haptocorrin, a vitamin B12-binding protein (Jensen et al. 2011), folate-binding protein (Said et al., 1986; Haschke et al.,

2016), and osteopontin (Lönnerdal, 2013; Haschke et al., 2016). In common, these proteins are all glycosylated, which contributes to their biological functions, such as antimicrobial activities and surviving in the small intestine (Lönnerdal, 2013).

Table 1.6 Differences in the structure and stability of lysozyme and immunoglobulin A in human and bovine milk.

	Lysozyme[1]		**IgA**[2]	
Milk Type	Human	Bovine	Human	Bovine
Molar Mass (kDa)	15	16.5 - 18	162 (415 ± 60) [3]	160 (385 - 410) [3]
Structure	Both are c-type lysozymes; the enzymatic activity of human lysozyme is 10 times higher than bovine lysozyme		sIgA (effective IgA in human milk): SC attaches to dimeric structure to prevent adhesion of bacteria; free SC was isolated from bovine milk	
Influencing Factors				
pH	pH optimum: 6.35; more thermolabile at pH 7.2 than at pH 4.2	pH optimum: 7.9	Stable at low pH	NS
Heating	Heat stable; Decreased activity after HoP	Heat stable as c- type lysozyme	Decreased level after HoP;	No structural change after HTST; 80°C x 25 min inactivates IgA
Refrigeration	Stable for 96 h (PHM)	NS	Stable for 48 h	NS
Freezing (-20°C)	Decreased level	NS	No change	NS
Pressurization	Maintained level up to 600MPa	NS	Decreased level	NS

[1]Adapted from Castillo, Franco, Pérez, Calvo, and Sánchez (2011), Chandan, Parry Jr, and Shahani (1965), Parry Jr, Chandan, and Shahani (1969), Malinowska-Pańczyk (2020); [2]Adapted from Cakebread, Humphrey, and Hodgkinson (2015), Mainer, Sanchez, Ena, and Calvo (1997), Ustunol and Sypien (1997), Ramírez-Santana et al. (2012), and Malinowska-Pańczyk (2020); [3]Molar mass of sIgA, modified from Kerr (1990) and Korhonen and Marnila (2009). **Note:** sIgA: Secretory immunoglobulin A; SC: Secretory component; HoP: Holder pasteurization; HTST: High temperature short time pasteurization; PDM: Pasteurized donor milk; NS: Not studied/ Report not found.

1.4.4 Milk fat globule membrane (MFGM) proteins

As illustrated in **Fig. 1.3**, there is significant interest in human MFGM proteins, which represent 1% of the total fat globule mass (Liao et al., 2011). Compared with milk from other species, more than 100 types of MFGM proteins are uniquely present in human milk, including bile salt-stimulated lipase, lactotransferrin and clusterin. The dominant molecular function of MFGM proteins has been described as GTP-binding and lipoprotein binding (Lu et al., 2016). They are also crucial for lipid synthesis and secretion, and many of them have been described as bioactive proteins (Demmelmair et al., 2017). Well-described MFGM proteins in human milk, including Mucin 1 (MUC1), clusterin, lactotransferrin, and the enzyme complex, xanthine oxidoreductase (XOR), are discussed here briefly.

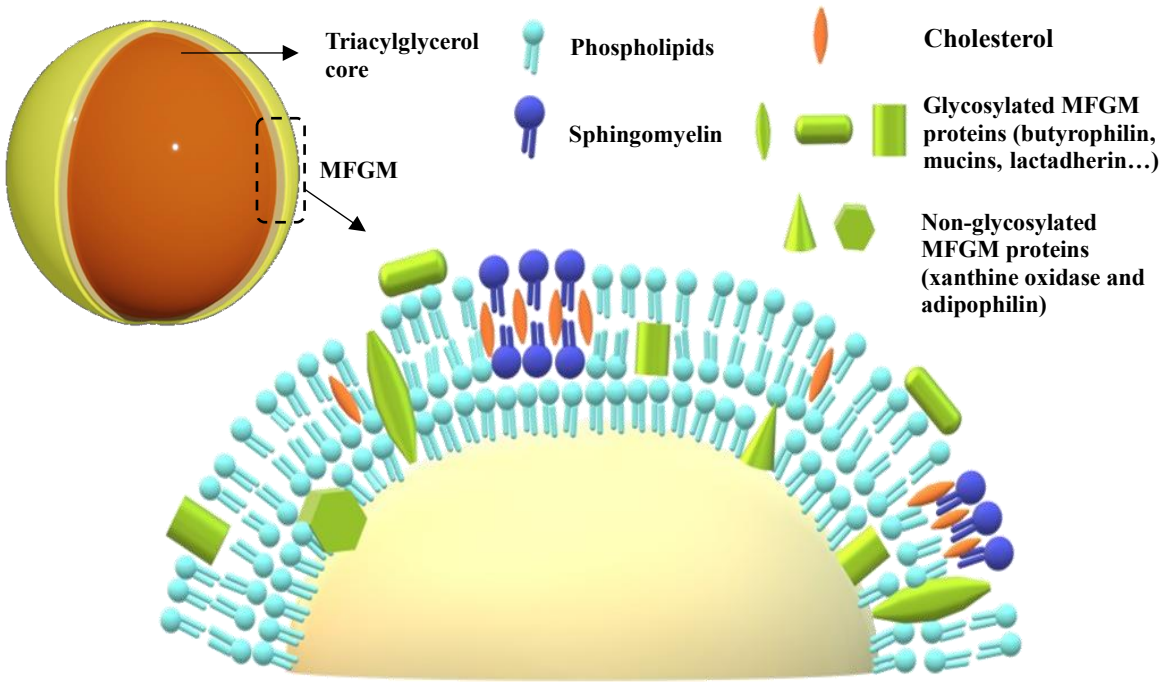

Fig. 1.3 Human milk fat globules and human milk fat globule membrane. Modified from Gallier et al. (2015).

Glycoproteins such as MUC1 and lactadherin exhibit antiviral ability *in vitro* (Lönnerdal, 2013; Yolken et al., 1992; Demmelmair et al., 2017). MUC1 may inhibit transmission of HIV to T cells through block the interaction of pathogens with dendritic cells (i.e., Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN)) (Saeland et al., 2009). Specifically, the interaction of MUC1 with

DC-SIGN was only found in human milk, but has not been found in infant formula, bovine or camel milk (Koning et al., 2015).

Clusterin, another highly glycosylated protein, has been observed to act as an inhibitor to suppress complement-mediated cell lysis (Jenne & Tschopp, 1992; Jones & Jomary, 2002). Although it has been known that clusterin is likely to provide a chaperone-like activity to protect other proteins against damage (Charlwood et al., 2002), the exact function of this protein is still being explored (Hettinga et al., 2011).

MFGM lactotransferrin has shown bacteriostatic activity because it is only partly saturated with iron in its native state, which is different from serum transferrin, and it can thus compete with bacteria for iron (Charlwood et al., 2002). Also, XOR, an enzyme as well as an endogenous metallo-flavoprotein in human milk, has been suggested to generate hydrogen peroxide and reactive nitrogen species, therefore contributing to antimicrobial activity (Harrison, 2006).

1.4.5 Proteolysis in human milk

Proteolysis occurs in human milk, before gastric digestion and further digestion in the small intestine of infants. Endogenous peptides present in human milk before infant consumption are mainly produced by indigenous or microbial proteases in the mammary gland or during storage (Armaforte et al., 2010; Guerrero et al., 2014). After consumption, indigenous proteases in human milk, together with infant proteases, produce peptides throughout the whole gastrointestinal tract, which can be considered as compensating for the low gastric acid level and protease activity of neonates (Dallas & German, 2017). The presence of peptides supporting the immune system derived from human milk can represent a major gap between infant formula and breast-feeding, according to an *in vitro* digestion study (Su et al., 2017).

The peptide profile in preterm milk is more complex compared to that in term milk (Ferranti et al., 2004). More low-molecular-weight hydrolysis products of caseins in preterm milk than term milk, linked to high proteolytic enzyme activity, have been reported (Armaforte et al., 2010). In contrast, larger-size peptides have been identified in preterm milk and it has been hypothesised that the immature mammary gland or a system present in preterm milk maintains the larger peptides (Ferranti et al., 2004, Dallas et al., 2015).

1.4.5.1 Protease systems in human milk

Human milk arguably has a more active and complex protease system than bovine milk, which is supported by the evidence of much higher and variable enzyme activity, and many bioactive hydrolysed casein products in human milk (Storrs and Hull, 1956; Heyndrickx, 1963; Armaforte et al., 2010; Dallas, Murray, & Gan, 2015). This system is a major reason for the efficiency of milk protein digestion in neonates. It has been proven that proteases can survive and digest milk protein in the stomach and intestine of infants, which can be considered as a compensation for low gastric acid level and protease activity of neonate (Dallas and German, 2017).

Plasmin (PL) is the predominant native milk enzyme that is capable of hydrolysis of proteins, including α_{s1}-casein, β-casein, osteopontin, and polymeric immunoglobulin receptor in human milk, with preferable cleavage at Lys-X and Arg-X bonds (Christensen et al., 2010; Dallas et al., 2015). The optimum pH and temperature of this serine proteinase are pH 7.5 and 37 °C, respectively (Korycha-Dahl et al., 1983).

PL can be activated from its zymogen, plasminogen (PG), by plasminogen activators (PAs) in milk (Lu and Nielsen, 1993a). In human milk, PAs include streptokinase, urokinase (u-PA), and tissue-type PA (t-PA), all of which can cleave the Arg^{560}-$Va1^{561}$ bond in human PG to produce PL (Schaller and Gerber, 2011). Most PL, PG and PAs are associated with casein micelles in milk, whilst a small amount of them can be found on MFGM (Korycha-Dahl et al., 1983). Meanwhile, plasmin inhibitors (PIs, e.g., α_1-antitrypsin) and plasminogen activator inhibitors (PAIs, e.g., Type 1 plasminogen activator inhibitor) are present in the serum phase of milk, and can inhibit the activity of PL and PAs, respectively (Gans & Tan, 1967; Heegaard et al., 1994; Precetti et al., 1997). Interestingly, some of the PAIs are bound to t-PA in bovine casein micelles, which can thus prevent expensive degradation of casein micelles (Heegaard et al., 1994). Studies of bovine milk also showed that PL and PAs are heat-stable, and they can even survive in UHT milk (Metwalli, de Jongh, & van Boekel, 1998; Lu & Nielsen, 1993b), whereas PIs and PAIs are less heat-stable, especially PAI (Prado et al., 2006).

PL and PG levels are known to vary with stage of lactation, lactation number, and somatic cell count in bovine milk studies (Korycha-Dahl et al., 1983). However, there are no reports of longitudinal plasmin activity in human milk. The level of PI, α_1-antitrypsin was measured as 1.4-5.2 g/l in colostrum, dropping to 0.05 g/l 26-52 weeks *post-partum* (McGilligan et al., 1987). This decrease has been explained by the fact that

immunological milk proteins need protection by these inhibitors in the colostrum, which therefore facilitates the infant immune system (Dallas et al., 2015).

Korycha-Dahl et al. (1983) reported that PL and PG-derived activities in human milk were 4.8±4.2 U mL^{-1} and 12.4±9.9 U mL^{-1}, respectively at 6-7 days *post-partum.* These results showed no difference in PL activity, but much lower PG-derived activity, than in bovine milk. In contrast, a higher plasmin activity in human milk than in bovine milk has been reported (Storrs and Hull, 1956; Heyndrickx, 1963; Dallas et al., 2015), but overlapping of specificities with thrombin and trypsin could occur during the measurement (Dallas et al., 2015); however, it has been argued that activities of trypsin and thrombin have never been demonstrated in human milk (Dallas et al., 2015).

The plasmin activity of term milk samples is lower than that in pre-term milk samples (Armaforte et al., 2010). It has been hypothesized that incomplete closure of tight junctions in the immature mammary gland of preterm mothers leads to high leakage of proteases and protease activators into the milk (Dallas et al., 2015). This is consistent with higher peptide counts, ion abundance, and concentrations in preterm milk than in term milk (Armaforte et al., 2010; Dallas et al., 2015).

Although cathepsins, including cathepsin D (Větvicka et al., 1993), B (Lemay et al., 2013), H and S (D'Alessandro et al., 2010) are present in human milk, cathepsin D is particularly significant as it can act at neutral pH rather than acid pH (Lkhider et al., 2004). The zymogen of cathepsin D, procathepsin D, is the major form found in human milk. It has been predicted that activation of cathepsin D from procathepsin D by cysteine proteases happens in both bovine and human milk (Větvicka et al., 1993). Little is known about the activators and inhibitors of this enzyme (Wittlin et al., 1999; Dallas et al., 2015). The preference for cleavage of cathepsin D is for hydrophobic target sites and regions (e.g., Leu-Leu, Leu-Asn, Glu-Leu in β-casein), which are similar to elastase, pepsin, and chymotrypsin (Khaldi et al., 2014). However, this protease was found to hydrolyse a higher number of human milk proteins than other enzymes, especially β-casein and polymeric immunoglobulin receptors (Khaldi et al., 2014). To our best knowledge, the concentration of cathepsin D has been measured only in preterm human milk, and reported as 0.8 ± 0.2 ng mL^{-1} (Demers-Mathieu et al., 2017). In bovine milk, the level of cathepsin D was also low, and it has similar specificity to that of bovine chymosin (McSweeney, Fox, & Olson, 1995). Furthermore, cathepsin D activity in bovine milk is positively correlated with somatic cell count (O'Driscoll, 1999). A recent study showed that only cathepsin D and plasmin have the potential to continue degrading milk proteins within the preterm infant stomach (Demers-Mathieu et al., 2018).

The activity of elastase, thrombin, and kallikrein has been reported in preterm human milk (Demers-Mathieu et al., 2017). Elastase has similar cleavage sites (e.g., Leu-Leu in β-casein) on human milk protein to cathepsin D, and it has also been predicted as an active protease in polymorphonuclear neutrophils, a group of anti-infection cells in human milk (Prin-Mathieu et al., 2002). No elastase activators have been detected in milk yet, whereas inhibitors of elastase have been found (Dallas et al., 2015); inhibitors of elastase in human milk include antielastase and α_1-antitrypsin (Lindberg, 1979; McGilligan et al., 1987).

Thrombin is an enzyme that converts fibrinogen into fibrin; thrombin, its zymogen prothrombin, and its inhibitors, such as antithrombin III and α_1-antitrypsin, are present in human milk (Molinari et al., 2012). However, no thrombin activators have been identified in human milk.

Kallikrein is a serine protease originated from blood that has been identified in human milk in its active form; however, it has very little proteolytic activity during gastric digestion of infants (Demers-Mathieu et al., 2018). An inhibitor of kallikrein, kallistatin has been identified in human milk (Zhou et al, 1992). The function of kallikrein in formation of pre-digested peptides in human milk requires further study. In addition, trypsin has been detected in human milk by radioimmunoassay; however, the activity was not detected using spectrophotometric substrate assays (Monti et al., 1986; Demers-Mathieu et al., 2018).

1.4.5.2 Product of Proteolysis - Peptides and Amino acids

In human milk, most peptides in milk originate from β-casein, α_{s1}-casein, osteopontin, and polymeric immunoglobulin receptor, due to their unfolded structure or the presence of regions for protease access (Ferranti et al., 2004; Guerrero et al., 2014). As seen in **Fig. 1.4**, the major cleavage site for PL on β-casein is Lys^{160} (C-terminal), while others include Lys^{18}, Lys^{23}, Ala^{73}, Ala^{180} and Glu^{186} in human milk (Ferranti et al., 2004). The bioactive peptides characterised from human β-casein include: casein phosphopeptides (CPP) that facilitating calcium absorption (Hansen et al., 1996); opioid peptides affecting the sleep-wake cycle of babies, such as caseinomorphins (Taira et al., 1990); and peptides involved in immunomodulation, such as hexapeptide 54–59 and the tripeptide Gly-Leu-Phe (Parker et al., 1984; Sahai et al., 1996; Migliore-Samour et al, 1989). In recent studies, antioxidant peptides such as CAMP 211-215 (Wang et al., 2020) and PDC213 (Sun et al., 2017) from β-casein were also identified. However, bioactivities of these peptides have only been confirmed in synthesised peptides (Ferranti et al., 2004).

κ-casein is stable to some proteases, such as elastase and cathepsin D (Khaldi et al., 2014), but can be digested by chymosin or pepsin. It has been deduced from the cleavage sites and κ-casein-derived peptides that plasmin also digests κ-casein, but this is less extensive than digestion of β-casein (Ferranti et al., 2004; Khaldi et al., 2014). The proteolytic cleavage of κ-casein generates two peptides; one is a glycomacropeptide (GMP), a peptide containing 98-162 residues for human casein (106-165 residues for bovine casein), and the second fragment is the hydrophobic *para*-κ-casein. GMP is more bioactive than intact κ-casein, which can be effective as a Bifidus growth-promoting factor even at low concentrations (50 ppm) (Azuma, Yamauchi, & Mitsuoka, 1984). Human GMP has a higher bioactive potency than bovine GMP due to a highly glycosylated structure and a wide spectrum of carbohydrates of human GMP (Yamauchi et al., 1981; Fiat and Jollès, 1989; Wada and Lönnerdal, 2014). Another important bioactive peptide released from human κ-casein is human κ-caseinoglycopeptide (hCGP), which has antithrombotic activity (Chabance et al., 1995). Peptides from α_{s1}-casein were found to be the most abundant in human milk, although β-casein released a greater number of peptides (Dallas et al., 2015). Casoxin D, an opioid-agonist peptide, has been isolated from human α_{sl}-casein as a proteolysis product of pepsin-chymotrypsin (Yoshikawa et al., 1994).

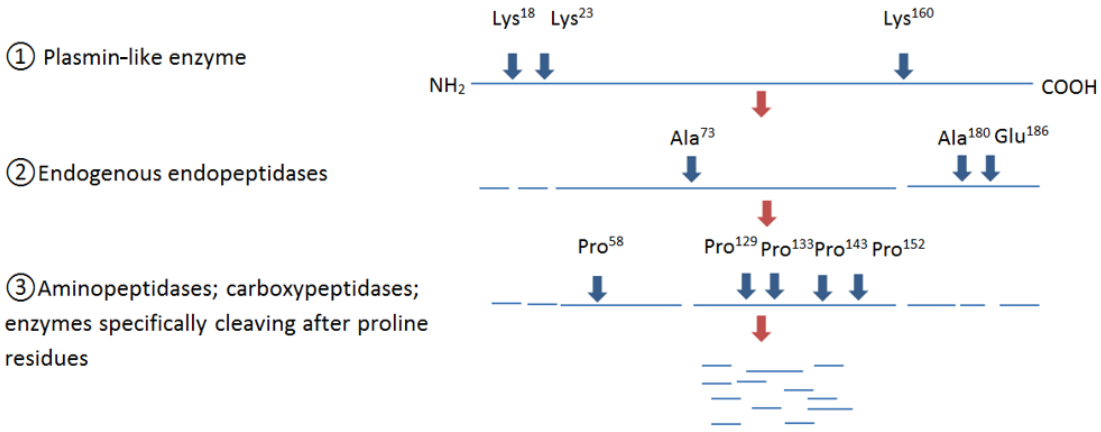

Fig. 1.4 Simplified pattern of proteolysis of human β-casein to peptides and amino acids (modified from Ferranti et al., 2004).

In terms of whey proteins, α-lactalbumin in human milk has been recently reported to be degraded by cathepsin D at pH 3-7 *in vitro* (Gan et al., 2019). Lactoferricin, a fragment of lactoferrin (corresponding to amino acid sequence: 17-41) showed an even stronger bactericidal activity than lactoferrin itself (Kirkpatrick et al., 1971). Although some bioactive peptides produced from sIgA and lactoferrin have been found, they are quite robust proteins to proteolysis, and can survive after the small intestinal digestion

(Lönnerdal, 2013). Some other whey proteins, for example, osteopontin, can be digested by plasmin and cathepsin D (Christensen et al., 2010).

As shown in **Fig. 1.4**, proteins and peptides can be digested by aminopeptidase and carboxypeptidase, which cut amino acids from the N- of C-terminals of proteins, respectively. These amino acids contribute to free amino acids, account for 5%–10% of total amino acids (Zhang et al., 2013), which has been speculated to be a rapid source for supplying amino acids to neonates without protein digestion by infants (van Sadelhoff et al., 2018). During the first 6 months of lactation, although absolute levels of most free AAs remained stable, relative levels of almost all FAAs increased and the ratio of total free AAs to total AAs almost doubled (van Sadelhoff et al., 2018). The levels of free AAs in term and preterm milk are similar except glutamine, which is the most abundant free AA and is present at significant lower levels in preterm milk than in term milk (Zhang et al., 2013; De Luca et al., 2016; Feng et al., 2016; van Sadelhoff et al., 2018).

1.5 Lipids

1.5.1 Major lipids in human milk

By supplying 50-60% of total energy, human milk lipids (4.1 ± 0.7 % in term mature milk; Bauer & Gerss, 2011) are essential to infant growth, as well as brain development, nerve function and vitamin absorption (Koletzko, 2016). Summarized in **Table 1.7**, human milk lipids are classified as triacylglycerols (TG, 98%), phospholipids (PL, 0.8%), and cholesterol (0.5%) (Koletzko, 2016). The majority of these are found in human milk fat globules (87%) (MFG) (Jensen, 1999). As shown in **Fig. 1.3**, TG form the core of MFG, while it is surround by a triple-layer membrane, i.e., MFGM, comprising of PL, cholesterol and MFGM proteins.

TGs are the major type of apolar lipid in human milk, comprising a glycerol backbone with three linked fatty acids (Jensen, 1999). The structure of TG in human milk is unique partly due to positionally distribution of fatty acids. For example, short-chain fatty acids and medium-chain fatty acids are mainly esterified at sn-3 position (Chen et al., 2020), while most palmitic acid (16:0) is esterified to the sn-2 position of TG in human milk (Carnielli et al., 1995). This preferential distribution protects long chain palmitic acid from being hydrolysed to free fatty acids, the form indigestible for

infants, as they tend to form insoluble soaps in the presence of calcium or magnesium (Carnielli et al., 1995). Therefore, the difference in positionally distribution of fatty acids in infant formula leads to impaired absorption of calcium, which may negatively influence early bone accretion of infants (Carnielli et al., 1995). Furthermore, faecal calcium-palmitate soap is associated with hard stools, which partly explains the greater stool softness in breast-fed infants than formula-fed infants (Béghin et al., 2019). Recent application of formulae enriched in sn-2 palmitate proved its benefits to stool consistency and bone mineral content in early infancy (Béghin et al., 2019).

Table 1.7 Differences between major lipids in human and bovine milk.

	Human (Total: 22 - 50 g L^{-1})			**Bovine (Total: 30-50 g L^{-1})**		
Major lipids	TG	PL	Cholesterol	TG	PL	Cholesterol
Level (g L^{-1})	21.5 - 49	0.4 - 0.7	0.1 - 0.2	29.4 - 49	0.24 - 0.4	0.09 – 0.15
Structure	TG at the core of MFG; MFGM comprises of polar lipids, such as PLs and cholesterol and MFGM proteins					
	Human MFG (3-5μm); zeta-potential: -5.6 to -8.2 mV; TG: positional distribution of FAs; PLs: SM dominate; high level of saturated FAs; higher levels and types of MFGM proteins			Bovine MFG (3-5μm); zeta-potential: -13.5 to -20 mV; SM: sphingoid base and FAs are different from human SM		
Influencing factors						
pH	NS			The interaction of MFG with caseins is higher at acid pH		
Heat treatment	No impact/ decrease after HoP			No impact on the level of total fat and fatty acids profiles		
Refrigeration	Crystallization of high-melting point TG in MFG			Lipid auto-oxidation; hydrolysis of MFG by indigenous and bacterial lipases		
Freezing	Activation of lipolysis			Freezing point: −0.564 to −0.516°C; unaffected level		
Pressurization	Unaffected structure up to 600 MPa (10min)			100–600 MPa for 0–60 min has little effect on MFG size		
Dehydration	NS			Spray- and freeze-drying affect the physical, chemical and microstructure characteristics of MFGs		

Adapted from Koletzko (2016), Ma et al. (2019), Lopez and Ménard (2011), Zou et al. (2012), Michalski (2013), Lima et al. (2018), Lopez et al. (2013), Wesolowska et al. (2019), Obeid et al. (2019), O'Connor and O'Brien (2006), Prajapati et al. (2017), Huppertz, Fox, and Kelly (2003), and Yao et al. (2016). Note: TG: Triglyceride; PL: Phospholipids; MFG: Milk fat globule; FAs: Fatty acids; SM: Sphingomyelin; MFGM: Milk fat globule membrane; HoP: Holder pasteurization; NS: Not studied/ Report not found.

The crystallization and melting properties of human milk TG, which occurs across a wide range of temperatures, were studied by Lopez, Briard-Bion, Bourgaux, and Pérez (2013). Lopez et al. (2013) reported that refrigerated storage of human milk induced the solidification of TG and that, after such storage of human milk, the final melting temperature of TG was reported to be 39.8 ± 1.5 °C, depending on the exact fatty acid composition.

PLs and other complex lipids are the key components of MFGM, including sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) (Koletzko, 2016). Interestingly, PLs are not evenly distributed on the human MFGM (**Fig. 1.3**); liquid-ordered domains that are rich in SM and cholesterol were present in the outer bilayer of the MFGM and surrounded by a liquid-disordered phase (PC, PI, PE, and PS) (Lopez and Ménard, 2011). The concentration of PLs is inversely correlated with MFG diameter (Zou et al., 2012). As well as a structural role, PLs have an impact on membrane functions and metabolism (Koletzko, 2016).

The principal sterol in human milk is cholesterol, the level of which (100 - 200 mg L^{-1} at 3 weeks *post-partum*) is much higher than that in bovine or caprine milk (Yao et al., 2016). Cholesterol is essential for all cell membrane structures, especially the myelin membrane in the nervous system, which is critical for brain growth (Saher et al., 2005). It also provides substrates for important modulators of infant growth, including bile acids, lipoproteins, vitamin D, hormones, and oxysterols (Koletzko, 2016).

1.5.2 Human milk fat globules

The size and available surface area of MFGs in milk influence the efficiency of lipolysis, and therefore digestion by infants (Berton et al., 2012; Garcia et al., 2014). Smaller micelle size and large available surface area of MFGs facilitate the digestion of human milk by infants (Armand et al., 1999). The average size of MFGs in human milk is approximately 3 - 5 μm. Compared with bovine MFG (D4,3: 4.2 μm), human MFG (D4,3: 5.0 μm) have been reported to be larger (Lopez, Cauty, & Guyomarc'h, 2015; Ma et al., 2019) or to not be significantly different in size (Meng et al., 2020).

Studies agree that the size of human MFG increases from transitional milk to mature milk (Jiang et al., 2020; Michalski et al., 2005; Rüegg & Blanc, 1981); however, the size of MFGs at the beginning of lactation is controversial. Michalski et al. (2005) reported a decrease at 0-4 day *post-partum* and Zou et al. (2012) described a decrease from colostrum (1-5 days *post-partum*) to transitional milk (6 – 15 days *post-partum*).

However, Rüegg and Blanc (1981) suggested an increase from colostrum (4 days *post-partum*) to transitional milk (9 days *post-partum*) and Jiang et al. (2020) noticed an increase from 3 to 12 days *post-partum*.

The zeta potential of MFG, i.e., the surface charge of the globules, which can influence its interactions with other milk components, such as proteins and lipases, was measured by Lopez and Ménard (2011), Zou et al. (2012), Michalski (2013), and Jiang et al. (2020); the absolute values (-5.6 to -8.2 mV) reported are lower than those reported for bovine milk (-13.5 to -20 mV; Michalski, 2013). This could be related to the presence of a glycoprotein 'coat', the sphingomyelin content of human MFGM, other compositional attributes of the MFGM, and the surrounding environmental mineral composition of the aqueous phase (Michalski et al., 2005).

1.5.3 Lipolysis

1.5.3.1 Lipase

Lipases are a subclass of esterases, which catalyse the hydrolysis of lipids. Human milk mainly contains two types of lipases, bile salt-stimulated lipase (BSSL) and lipoprotein lipase (LPL). BSSL is a carboxyl ester lipase (EC 3.1.1) secreted from the exocrine pancreas (Baba et al., 1991) that catalyses the hydrolysis of a broad range of fats: glycerides, cholesterol, retinyl-esters, fat-soluble vitamin esters, phospholipids and ceramides (Hernell & Bläckberg, 1994; Wang, Ding et al., 2017). It has been found in the milk of many species including humans, but, interestingly, it is not present in milk of domesticated milk-producing animals, i.e., cows or goats (Hernell et al., 1988; Olivecrona et al., 2003).

BSSL has attracted high attention because it not only catalyses hydrolysis of triglycerides but also breaks the bond of palmitic acid and glycerol in the sn-2 position that many other lipases cannot, which partly resolves the question as to why breast-fed infants have higher efficiency of fat absorption than formula-fed infants (Pollakis et al., 2013; Lu et al., 2016). Meanwhile, human BSSL shows a highly polymorphic behaviour, high acid tolerance and high resistance to proteolysis by pepsin or pancreatic proteases *in vitro* (Pollakis et al., 2013). This has been explained by the unique primary structure of human BSSL compared with that of other species, due to a 16 O-glycosylated repeat of 11 amino-acid (proline-rich) residues each at C-terminal parts of the protein (Lindquist et al., 2002). Furthermore, BSSL has been found to be an HIV-1 inhibitory factor by potently binding DC-SIGN (Pollakis et al., 2013).

In human milk, BSSL represents 1-2% of total protein and the concentration increases with lactation, whilst the activity remains almost constant in term milk (Mehta et al., 1982). The content of BSSL can be higher in pre-term milk, perhaps to facilitate the digestion of lipids by preterm infants (Liao et al, 2011; Molinari et al., 2012). However, some studies did not find a significant difference in either the content or activity of BSSL between term and preterm milk as a function of lactation (Mehta et al., 1982; Freed et al., 1989). The lipolysis of human milk lipids by BSSL is limited during storage at 4 °C for up to 96 h (Bertino et al., 2013). However, BSSL retains activity during storage at - 20 °C and the level of lipolysis increased after several freeze-thaw cycles (Penn et al., 2014).

Recombinant human BSSL (rhBSSL) can readily be purified from transgenic cattle and support large-scale production (Wang, Ding et al., 2017). In addition, recombinant human BSSL (rhBSSL) expressed in hamster ovary cells has been supplied to preterm infants in clinical trials; however, whether there is an improvement in the growth of preterm infants after feeding with rhBSSL is still inconclusive, according to a phase III clinical trial (Casper et al., 2016).

The other lipase present in human milk, LPL, shows much lower activity than BSSL (0.03%-5% of the activity of BSSL). LPL is distributed on the capillary walls of most tissues and is commonly known as a regulator of lipid and lipoprotein metabolism. It hydrolyses chylomicrons and very low-density lipoproteins, to monoglycerides, free fatty acids, and intermediate-density lipoproteins. It can also slowly hydrolyse very small amounts of monoglycerides and phospholipids. Lack of LPL or disorders of LPL metabolism can lead to a clinical symptom called hyperchylomicronaemia (Santer et al., 2005; Molinari et al., 2012).

1.5.3.2 Fatty acids

The fatty acid profile of human milk is highly dependent on the mother's diet and gestational age (Guerra et al., 2016). In general, human milk is rich in saturated palmitic and stearic acids, polyunsaturated fatty acids (PUFA) of the n-3 series (e.g., docosahexaenoic acid (DHA)) and n-6 series (e.g., arachidonic acid (ARA)), and minor bioactive fatty acids. It has been reported that the level of DHA and ARA can be 1.5-2-fold higher in pre-term milk than in term milk after 6 months of lactation (Luukkainen, Salo, & Nikkari, 1994). However, some studies have found no difference between these profiles in term and pre-term milk (Granot et al., 2016). In addition, oxidation of fatty acids in human milk is much less than in infant formula because of protection by antioxidants, such as vitamin E, as well as the MFGM structure in human milk

(Michalski et., 2008). The free fatty acid content is also low in human milk, unless stored frozen (-20°C) (Slutzah et al., 2010; Penn et al., 2014; Ahrabi et al., 2016). The level of free fatty acids can be higher in mastitic human milk than in healthy human milk (Lu et al., 2016).

1.6 Carbohydrates

1.6.1 Lactose

Lactose is the principal sugar in human milk, which remains constant during lactation at levels measured as 6.2 ± 0.9 % in mature term milk (Bauer & Gerss, 2011; Gidrewicz & Fenton, 2014). Besides supplying energy, lactose also plays a role in osmotic pressure regulation during milk production, as well as increasing the absorption of minerals, especially calcium (Ziegler & Fomon, 1983). This can be due to the ability of microbiota in the gastrointestinal tract to convert lactose into lactic acid, thus leading to a pH drop and a shift in the solubility of calcium (Ziegler & Fomon, 1983). Lactose is not hydrolysed in the upper gastrointestinal tract, and only to a very small extent in the proximal intestinal tract (Nommsen et al., 1991).

1.6.2 Human milk oligosaccharides

Human milk contains a high level of human milk oligosaccharides (HMOs); free oligosaccharides are built of five monosaccharides: glucose, galactose, N-acetylglucosamine, fucose, and sialic acid, with high structural diversity (Morozov et al., 2018). The concentration of total HMOs in colostrum can be as high as 20 - 25 g L^{-1}, decreasing to 5 - 20 g L^{-1} in mature milk (Xu et al., 2017).

To date, over 200 types of HMOs have been identified, and more than 100 of them have been characterized and structurally resolved (Kulinich & Liu, 2016; Cheng et al., 2020). Different profiles of HMOs can be a result of specific transferase enzymes expressed in the lactocytes in the mammary gland (Ballard & Morrow, 2013). The production of HMOs is controlled by genes, especially Secretor and Lewis blood group genes (Totten et al., 2012).

HMOs are important bioactive factors, through directly altering gene expression in immune cells as immune modulators, or by modifying the microbiota in the gastrointestinal tract as prebiotics, thus indirectly affecting the immune system of

infants (Totten et al., 2012; Ray et al., 2019). HMOs are resistant to low pH in the stomach and pancreatic brush enzymes (Engfer et al., 2000). Therefore, they can reach the large intestine to serve as substrates for bacterial metabolism. The protective mechanism by which HMOs prevent gastrointestinal infection by acting as receptor decoys is also crucial (Morozov et al., 2018). HMO and their derivatives are similar to the binding sites on cells, for example, DC-SIGN; they can, therefore, block the interaction between pathogens or viruses and their receptors by acting as receptor decoys. Many studies have also indicated that the branched chains of HMOs also contribute to blocking viral infection (Gao et al., 2020). The relationship between structures and antiviral effects of HMOs still needs further research.

HMO molecules can be synthesized chemically, via fermentation, and by enzymatic synthesis, which are still both expensive and challenging options (Cheng et al., 2020). Two HMOs, 2'fucosyllactose (2'FL) and lacto-N-neotetraose (LNnT) have been considered as novel foods by European Union in 2015 and been added to infant formula in clinical trials. However, the difference in the clinical outcomes between HMO-fortified formula and non-fortified formula is still not conclusive (Vandenplas et al., 2018).

1.6.3 Amylase and glycosidases in human milk

Amylase is an enzyme that can digest starch, glycogen, and some oligosaccharides by cleavage of α-D-1,4-glucan linkages. There are five isoamylases in human milk, including three salivary-type isoamylases and two unknown (neither salivary-type nor pancreatic-type) isoamylases (Fridhandler et al., 1974). Amylase can survive in the stomach due to the sharply increased pH resulted from having a meal of human milk (Lindberg and Skude, 1982). Interestingly, human milk has a high concentration of α-amylase compared with low α-amylase activity in milk of other species, even though there is no substrate for amylase in human milk (Jones, Mehta, & Hamosh, 1982; Fox et al., 2015). A decrease in amylase activity during lactation has been shown, from approximately 7900 units L^{-1} during the first 5 days to 1300 units L^{-1} after 90 days *post-partum* (Lindberg and Skude, 1982). Accordingly, it has been proposed that α-amylase in human milk is provided to compensate for the low level of amylase in saliva and duodenal juice of new-borns, which are not fully developed, especially in the initial stages of lactation. Meanwhile, it may help in digestion of complex amylase carbohydrates when feeding complementary foods (Owino et al., 2007).

Human milk also contains a series of glycosidases that can catalyse the hydrolysis of glycosidic bonds between oligosaccharides and glycoconjugates (e.g., glycolipids,

glycoproteins, and mucins), which cannot be achieved by amylase. Among glycosidases, α-L-fucosidase and N-acetyl-β- D-hexosaminidase has been reported as 250 to 2,000 nmol $mL^{-1}h^{-1}$, and 800 to 2,000 nmol $mL^{-1}h^{-1}$, respectively, with high variability among individual samples (Wiederschain and Newberg, 2001).

1.7 Vitamins and Minerals

Vitamins and minerals are part of the micronutrient profile of human milk (**Table 1.8** and **Table 1.9**), the content of which is highly influenced by maternal diet (Bravi et al., 2016; Hampel & Allen, 2016), including vitamin A, B2, B3, B12, C, D, selenium and iodine (Daniels et al., 2019; Hoppu et al., 2005; Perrella et al., 2021).

Fat-soluble vitamins include retinol (A), cholecalciferol (D), tocopherol (E), and vitamin K. Vitamin E (α-tocopherol), acts as a free radical scavenger and protects against PUFA peroxidation in the cell membrane. Milk vitamin content during the first six weeks *post-partum* of vitamin E (also vitamin A) has been reported to parallel mammary gland lipid synthesis (Chappell, Francis, & Clandinin, 1985). Preterm infants require this micronutrient as they have low serum levels of α-tocopherol (Bell et al., 2013).

Vitamin A can be directly obtained from breast milk or can be converted from β-carotene in the liver of infants. It is known to be abundant in colostrum but it decreases during lactation. The concentrations of vitamin A in the term human milk has been reported as higher in the preterm milk (Souza et al., 2014). However, both concentrations are lower than that recommended (2.3 mmol/L in breast milk) by Stoltzfus and Underwood (1995). Infant vitamin A deficiency, which is related to clinical disorders, has been reported to be rare among breast-fed infants (Newman, 1994). Moreover, maternal supplementation with high doses of vitamin A has been reported as influencing the bioavailability of vitamin E in colostrum, whereas it has no effect on the vitamin E level in mature milk (Grilo et al., 2016).

Despite it being essential for several aspects of infant health, such as the immune system and bone metabolism, vitamin D level is low in human milk (við Streym et al., 2015), which was defined as 25-hydroxy-vitamin D level lower than 50 nmol L^{-1} (Hollis et al., 2005). This low level has been suggested to be because vitamin D can be synthesised by infants themselves through sunlight exposure, and therefore there is no

essential requirement from human milk (Lakdawala & Widdowson, 1977; Bae & Kratzsch, 2018). Moreover, vitamin K, which is essential to proteins involved in blood coagulation, and some plasma proteins and organs is present at low levels in both preterm and term human milk (Guo, 2021). Low vitamin K concentrations in breastmilk, and thus low plasma vitamin K levels in infants may result vitamin K deficiency bleeding (VKDB) in newborns. Therefore, the ESPGHAN committee recommended that all newborns should receive vitamin K prophylaxis (Mihatsch et al., 2016).

Table 1.8 Representative levels of vitamins in human milk compared with bovine milk. Modified from Allen, Donohue, & Dror (2018) and Guo (2021).

Vitamins (mg L^{-1})	**Human milk**		**Bovine milk**
Fat-soluble vitamins			
Vitamin A	0.49	0.53	0.37
Carotene	0.38 – 1.3	0.24	0.21
Cholecalciferol(D)	-	0.001	0.0008
Tocopherol (E)	4.9	5.4	1.1
Vitamin K	0.002	0.015	0.03
Water-soluble vitamins			
Thiamine (B1)	0.21	0.15	0.42
Riboflavin (B2)	0.35	0.37	1.72
Pyridoxine (B6)	0.13	0.1	0.48
Cobalamin (B12)	0.00042	0.0003	0.0045
Niacin	1.5	1.7	0.92
Folic acid	0.085	0.043	0.053
Ascorbic acid (C)	50	47	18
Biotin	0.006	0.007	0.036
Pantothenic acid	2.2	2.1	3.6
References	World Health Organization (2004)	Guo (2021)	

B vitamins are an important water-soluble class, the content of which has been reviewed by Allen (2012). The level of B vitamins in human milk has been reported to decrease during lactation, and prolonged exclusive breastfeeding may not provide

sufficient B vitamins to infants (Torsvik et al., 2015). Vitamin C level in human milk is much higher than it in the serum of mothers, and it is highly influenced by maternal intake (Ahmed et al., 2004; Bravi et al., 2016). It has been suggested that high vitamin intake may reduce the risk of atopy in the infant (Hoppu et al., 2005). Compared with bovine milk, human milk contains much higher levels of vitamin A, C, inositol and niacin (**Table 1.8**); however, vitamin B and pantothenic acid contents of human milk are lower than in bovine milk (Guo, 2021).

Table 1.9 Representative levels of minerals in term and preterm human milk, compared with bovine milk during lactation.

Minerals (mmol L^{-1})	**Type of Milk**	**Human Milk**			**Bovine Milk**
		Colostrum	**Transitional**	**Mature**	**Mature**
Sodium	Preterm	23.3	15.2	8.1	24.2
	Term	19.2	8.1	14.1	
Potassium	Preterm	18.2	12.2	10.2	34.7
	Term	18.2	8.1	9.2	
Calcium	Preterm	5.8	5.8	4.8	29.4
	Term	5.8	4.6	4.6	
Magnesium	Preterm	1.2	1.2	1.2	5.1
	Term	1.1	1.2	1.2	
Chloride	Preterm	24.2	9.2	9.1	30.2
	Term	23.3	9.1	8.1	
Phosphate	Preterm	-	2.3	1.2	11.2
	Term	-	2.3	1.2	
References		Bauer and Gerss (2011)			Neville, Zhang, & Allen (1995)

Mineral supply to infants is also essential for a variety of bodily functions. Changes in human milk electrolyte concentrations were observed in the first day after birth and are closely related to the duration of lactation. As shown in **Table 1.9**, high levels of sodium chloride are present in colostrum and decline during the first week of lactation, to reach a constant level during later lactation for both term and preterm

infants (Bauer and Gerss, 2011). Moreover, sodium concentrations decrease as the volume of milk produced increases in preterm infants. Notably, potassium concentration also decreases significantly over time (Yamawaki, 2005). Other minerals, such as phosphate, calcium, zinc, copper and magnesium, are quite stable during lactation, with slightly higher levels in colostrum (Yamawaki, 2005; Shi et al., 2011; Bauer and Gerss, 2011). As seen in **Table 1.9**, calcium and phosphate levels are similar between term and pre-term human milk, but this level is much lower compared with bovine milk (Guo, 2021; Hsu et al., 2014). In addition, ionized calcium comprises a larger fraction of the total calcium concentration in human milk, as the concentration of calcium-binding proteins (caseins) is low in human milk, which may facilitate the absorption of calcium (Allen and Neville, 1983).

1.8 Physico-chemical Properties of Human Milk

1.8.1 Acidity

The major contributors to acidity in human milk are caseins, minerals and organic acids. However, acidity can further increase, i.e., pH can decrease, during storage and processing, primarily due to lactic acids and other organic acids being produced from microbial degradation of lactose. Therefore, human milk pH has been recommended as an indicator of the milk quality in human milk banks (Escuder-Vieco et al., 2016).

The typical pH of mature human milk ranges between 7 to 7.5 at room temperature, with a decrease during the course of lactation due to changes in composition, such as casein concentration (Morriss et al., 1986). In contrast, an increase in milk pH from 6.99 to 7.24 at room temperature during the first 20 days of lactation has been reported (Sunaric et al., 2017). No significant difference of pH between term and preterm human milk has been reported by Caldeo et al., (2020). The pH of human milk decreases after freezing at -20°C; Ahrabi et al., (2016) reported that it decreased from above pH 7 to pH 6.3 after storage at -20°C for 9 months.

pH is an important factor in proteolysis and lipolysis as it influences the activity of enzymes such as lipase (Erickson, Gill, & Chan, 2013). Moreover, acidification of human milk to pH 4.5 is reported to cause a decrease in white cell count, possibly due to lysis of white cells during the acidification process (Erickson et al., 2013). However,

there is still no conclusion as to whether milk pH is just the result of maternal mammary metabolism and of little importance to newborns, or if it is an important factor influencing the properties of milk and the health of infants (Demers-Mathieu et al., 2018).

1.8.2 Colour of human milk

The colour of milk mainly depends on CMs and MFGs that scatter light, and therefore or homogenised milk had higher luminosity compared with raw milk, which was largely due to an increase of visible light scattering caused by smaller fat globules (Truong et al., 2016). Meanwhile, the colour of skimmed bovine milk reflects the size change of CMs (Fox et al., 2015); no report on the colour of skimmed human milk has been found. Human milk can also take on different colours because of the food and medication intake of mothers; however, no conclusive research on food- and medication- induced colouration of milk exists (Anderson, 2018). Beta-carotene and other carotenoids in milk are considered to be related to dietary intake of beta-carotene-rich foods such as carrots and sweet potatoes, and lead to an orange colour of colostrum and the pale white to cream colouration of mature human milk (Anderson, 2018). A green colour of milk has occasionally been reported and could be due to: (1) high multivitamin and mineral (especially iron) intake; (2) low lactose and casein content; and (3) propofol administration (Anderson, 2018). Other colours have also been observed in human milk, including black due to minocycline administration and pink pigments caused by *Serratia* bacteria (Yazgan et al., 2012).

1.8.3 Phase separation: creaming and sedimentation

Breast milk is an emulsion of milk fat globules in milk plasma, which separates naturally into cream and serum layers under gravity. The creaming properties of human milk during storage were studied by Meng et al. (2020); human milk showed a faster creaming rate at 40°C, but a slower creaming rate at 5°C, compared with bovine milk. To our knowledge, the sedimentation of human milk, which usually refers to protein-rich material accumulating as a layer at the bottom of the container following expression and during the storage, has not been studied yet. Future studies are required as the sedimentation or creaming may result in protein and fat loss in the container on decanting and thus impair the nutritional value of human milk.

The milk emulsion can also be destabilised by the acidic pH of the stomach (Golding & Wooster, 2010). In an *in vitro* dynamic gastrointestinal system, aggregates formed during digestion, based on clusters of milk fat globules around which protein

aggregates form, are prone to creaming in the gastric compartment. This early gastric creaming could be a protective mechanism favouring emptying and compensating for the immature gastric motility in preterm new-borns (de Oliveira, Deglaire, et al., 2016).

1.9 The Effect of Preservation Methods on Nutritional and Biological Properties of Human Milk

1.9.1 Refrigeration, freezing and thawing

Freezing may bring benefits to human milk as it has been regarded as an effective method for reducing bacterial colony counts and viral load (Handa et al., 2014). Most studies agree that the level of total protein is stable during freezing at -20°C (Orbach et al., 2019). The level of carbohydrate has been reported to not significantly change, while the level of fat has been reported to decrease or not significantly change (up to 9 months) after freezing at -20°C (Ahrabi et al., 2016; Orbach et al., 2019). The major hypothesis for the reduction in fat due to freezing is the oxidation of both free fatty acids and those bound to glycerol which occurs due to a reduction in antioxidants during freezing at -20°C (García-Lara et al., 2013). Adhesion of human milk fat to container walls has also been considered as a possible reason for the loss of fat during freezing (Chang, Chen, & Lin, 2012; Lev et al., 2014). Others have proposed that slow freezing or long-term freezing can lead to the destruction of MFGM and allow access of lipases to the TG (Berkow et al., 1984). Deep freezing (-80°C) for 1 week has a similar impact on the level of macronutrients as freezing at -20°C (Handa et al., 2014); however, a higher level of fat and energy was maintained after 4, 12, and 24 weeks at -80°C than at -20°C (Orbach et al., 2019).

The level of IgA after freezing at -20°C has been studied but is still controversial. Some studies reported no significant change of its level after freezing at -20°C for 1, 3, and 9 months (Ahrabi et al., 2016). In contrast, a decrease in IgA level after 1 month at -20°C was reported (Akinbi et al., 2010). Ramírez-Santana et al. (2012) also reported that the concentration of IgA decreased in colostrum after freezing at either -20°C or -80°C after 12 months. Moreover, there could be a decrease in total antioxidant status and an increase of rancid flavour compounds during storage of human milk at -20°C (Hung et al., 2018; Păduraru et al., 2018).

In terms of thawing methods, Handa et al. (2014) reported no significant difference between thawing in tepid water (37°C, 10 min for thawing and 10 min for warming) and water-less thawing (in an enclosed chamber with warm air, no temperature indicated, time limit set automatically according to the volume of milk). In a recent study, room-temperature thawing, water-less thawing (in a bottle warmer) and microwave thawing showed different impacts on the levels of macronutrients (Kim et al., 2019). Compared with fresh milk, samples thawed at room temperature showed a significant decrease in fat content; in contrast, microwave thawing for 1 minute led to no significant difference in fat, but levels of protein and carbohydrate increased significantly compared with fresh milk. Samples treated with the water-less thawing method showed significant differences in all macronutrients compared with fresh milk (Kim et al., 2019). Studies on the impact of thawing methods on human milk composition are limited partly because the impact of thawing is hard to isolate from that of the freezing method used. More detailed and complete description of freeze-thawing cycle may be useful for future study.

Compared with freezing at -20°C (from 7 days to 2 months), short-term refrigeration (< 72 hours) retains more energy and macronutrients (Pramitasari, Sidiartha, & Pratiwi, 2019). However, a higher level of lipids was peroxidised in human milk during the storage at 4°C than freezing (Miranda et al., 2004). Refrigeration also leads to the crystallization of high-melting point TG within MFG, therefore decreasing the rate of TG hydrolysis and solubilisation, which could affect the absorption of milk fatty acids by infants (Lopez et al., 2013).

1.9.2 Heat treatment of human milk

For safety purposes, pooled donor human milk is frequently heated in human milk banks at 62.5°C for 30 minutes, i.e., HoP, which allows a good compromise between achieving microbiological safety and retaining the quality of human milk. Macronutrient content is relatively unaffected after HoP (Lima et al., 2018). It is well known that enzymes are sensitive to temperature and thus that heat treatment leads to decreased enzyme activity in human milk. Henderson, Fay, and Hamosh (1998) reported that LPL and BSSL were completely destroyed after HoP. The activities of glutathione peroxidase, a selenium-containing enzyme that plays roles in protecting cells against lipid oxidation-induced damage, also decreased after HoP (Guerra et al., 2018). Immunological profiles were also altered after HoP, and IgG showed the highest retention levels, followed by IgA and IgM (Escuder-Vieco et al., 2018).

The impact of HoP on the gastrointestinal digestion of human milk has been considered, as different bioactive peptides were released from caseins in pasteurized milk compared to raw milk during intestinal digestion (Deglaire et al., 2019). In addition, a clinical trial demonstrated that HoP of preterm milk did not affect lipolysis, but affected the proteolysis of lactoferrin and α-lactalbumin during gastric digestion by preterm infants (de Oliveira, Bourlieu, et al., 2016).

High temperature short time pasteurization (HTST, 72°C for 15 seconds), the principal dairy preservation technique, has been demonstrated to give a better retention of antiviral activities and antioxidant activity than HoP (Donalisio et al., 2018). The level of Ig retention in milk after HTST has been considered to be higher than in milk after HoP (Escuder-Vieco et al., 2018). Other thermal methods, such as ultrasonic heating and microwave-assisted heating, have also been investigated. Homogenisation of milk (at 45 or 50°C) occurred after 2 min of ultrasonic heating (20 kHz, 150 W) and temperature of milk increased 10.3 ± 0.4°C, with bioactive components, including sIgA, lysozyme, lactoferrin and BSSL, being retained (Czank, Simmer, & Hartmann, 2010).

1.9.3 Non-thermal alternatives to holder pasteurization

Because thermal methods influence some of the nutritional and biological properties of human milk, and also eliminate some of the beneficial microbiota present in the milk, non-thermal treatments for preserving human milk have been studied (Peila et al., 2016).

High pressure processing (HPP) is a novel non-thermal method which can effectively reduce the bacterial population while retaining higher levels of Ig, lysozyme activities, and BSSL activities in human milk compared to the HoP method (Mayayo et al., 2016; Sousa, Delgadillo, & Saraiva, 2014; Wesolowska et al., 2019). The structure of TGs is unaffected by HPP at 600 MPa for 10 min (Wesolowska et al., 2019). However, control of pressure and time for HPP is crucial and the optimal conditions that should be applied to human milk still need to be defined.

Ultraviolet (UV)-C (200 to 280 nm) irradiation is also an emerging non-thermal technology applied in human milk preservation, which can reduce levels of bacteria in breast milk without losing activities of some heat-sensitive enzymes (Christen et al., 2013). Another non-thermal treatment is ultrasonic processing, which provides better retention of BSSL, lactoferrin, and lysozyme compared with ultrasonic heating (40 or 50°C) method and HoP. However, application of ultrasonic processing alone is not as

effective as when combined with heat in terms of inactivation of bacteria (Czank et al., 2010).

1.9.4 Stability to dehydration

Drying of human milk can be an option for human milk storage as it saves storage space, prolongs storage time, and also reduces the cost of refrigeration. Spray-drying and freeze-drying of human milk have been investigated in several studies; the profile of Ig was evaluated because they are essential to infant immune system, especially IgA as discussed in Section 1.4.3.5; however, all Ig are sensitive to heat. Spray-drying was associated with good retention of IgG (> 88%) and IgM (> 67%) but retained only 38% of IgA (Castro-Albarrán et al., 2016). Freeze-drying of milk showed even better retention of Ig compared with spray drying, as the highest product temperature reached is 40°C (Castro-Albarrán et al., 2016). In addition, freeze-drying improved the retention of vitamins, fats, and antioxidant capacity compared with frozen unpasteurized milk after storage for 3 months (Lozano et al., 2014). Hahn, Kim, Song, Park, and Kang (2019) also reported that HMO profiles were not affected by freeze-drying. Therefore, applying freeze-drying in human milk banks is suggested as an option to extend the life of milk while maintaining immunological properties that benefit the health of the newborn, presuming the material can be readily reconstituted in sterile water before use.

1.10 Conclusion

Levels, structures and functions of macronutrients in human milk, including fat, carbohydrate, and protein have been extensively evaluated. Macronutrients not only supply energy, but also have significant biological and immunological roles and consequently, bring short-term and long-term benefits to infants. Proteins, consisting of casein, whey and MFGM proteins, were a particular focus of this review; caseins are major digestible proteins, while many whey proteins, e.g., Ig, are responsible for the immunity of infants, and both have biological roles depending on their structures. There are still gaps in knowledge of physiochemical properties of milk proteins, such as zeta potential and the effects of freeze-thawing on casein micelles. In addition, MFGM proteins provide health benefits and remain to be further explored. The unique composition and structures of human milk lipids is also significant, as it leads to a higher digestion efficiency of infants for human milk compared to infant formula. Finally, physico-chemical properties of human milk may affect its stability during handling and storage, such as many thermal and non-thermal strategies for milk preservation, which may differ in retention of bioactive and immunological compounds. Knowledge on some such properties of human milk, for example, sedimentation during milk storage, is limited and future studies are required. Questions also remain about whether pre-digestion proteolysis and some physiochemical properties are important factors influencing the properties of human milk and the health of neonates, or if they are just the outcome of maternal mammary gland with no function as indicators of milk quality. Overall, the knowledge of some of these properties requires updating and deeper exploration in many areas.

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CHAPTER 2

Longitudinal Analysis of Human Milk Macronutrients, Protease, and Fatty Acid Profiles

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Abstract

Human milk provides nutrients essential for infant growth and health, levels of which are dynamic during lactation. This work analysed changes in macronutrients, fatty acids, and plasmin activities over the first six months of lactation in term milk as well as up to 64 gestational weeks, i.e., six months after the term due date of preterm infants. pH and colour of human milk was also monitored. There was a significant influence of lactation on levels of protein and energy, but not on levels of fat and carbohydrate or pH in term milk. Significant effects ($p < 0.05$) of infant gender on fat and energy contents were found; milk for male infants had higher levels of fat and energy compared to milk for female infants. Levels of fat and energy were higher, whereas that of carbohydrate was lower, in milk from overweight mothers (pre-pregnancy body mass index (BMI) ≥ 25 kg m^{-2}) compared to mothers with normal weight ($18.5 \leq$ BMI ≤ 25 kg m^{-2}) at 4 weeks after birth in term milk. Concerning fatty acids, oleic acid, palmitic acid, linoleic acid, stearic acid, myristic acid, and lauric acid were the most abundant fatty acids in human milk. In term milk, levels of caproic acid and α-linolenic acid increased significantly ($p < 0.05$), whereas arachidonic acid and docosahexaenoic acid decreased, during six months after birth ($p < 0.05$). Term human milk expressed from overweight mothers had higher levels of some fatty acids, particularly saturated fatty acids, compared to that of normal-weight mothers. Human milk for female infants contained higher levels of monounsaturated fatty acids, including palmitoleic acid and nervonic acid. Plasmin activities decreased during lactation in both term and preterm human milk. Colour did not differ between term and preterm groups, but was correlated with levels of fat, protein, energy, and milk pH. The study provides information on human milk composition and physicochemical properties, which are meaningful for improving infants' nutrition.

2.1 Introduction

Human milk contains essential macro- and micro- nutrients and is rich in bioactive components such as microRNA and cells, which provide short-term and long-term benefits to infants (Perrella et al., 2021). The composition of human milk has been studied as a standard for infant formula manufacture, as well as for better understanding of infant nutrition. Despite human milk providing bioactive and anti-inflammatory effects, it may not fulfil the nutritional requirements of very preterm infants (Boyce et al., 2016). Therefore, the fortification of human milk has been recommended and the composition of human milk in term and preterm infants continues to be studied to elucidate the optimal nutrition for those infants.

As summarized in **Chapter 1, Table 1.2**, the levels of macronutrients in human milk have been reported to be influenced by many factors, including lactational stages, gestational age (GA), maternal diet, age of mothers, parity, maternal body mass index (BMI), infant birth weight, and diurnal variation. However, the impact of those factors on human milk composition is still controversial.

Concerning the lactational effect, it is well accepted that levels of proteins decrease during lactation (Allen et al., 1991; Anderson, Atkinson, & Bryan, 1981; Caldeo, Downey, O'Shea, et al., 2020; Fischer Fumeaux et al., 2019; Grote et al., 2016; Maly et al., 2019). The levels of carbohydrate have been reported to increase during the early stages of lactation (approximately one-week *post-partum*) and remain constant afterwards (Anderson et al., 1981; Bauer & Gerss, 2011; Maly et al., 2019), remain stable (Caldeo, Downey, O'Shea, et al., 2020; Fischer Fumeaux et al., 2019) or increase from 1 to 6 months (Allen et al., 1991). The content of lipids shows large individual difference among samples, and has been reported to increase in early lactation (Anderson et al., 1981; Sahin et al., 2020) and then remain stable (Fischer Fumeaux et al., 2019; Maly et al., 2019) or remain stable at all lactational stages (Caldeo, Downey, O'Shea, et al., 2020). One recent study reported a decrease in fat and a significant decrease in energy from 1 to 3 months (de Fluiter et al., 2020). Energy levels, calculated from total fat, protein, and carbohydrate levels, have been reported to follow a similar trend during lactation compared to total lipids (Gidrewicz & Fenton, 2014). Over prolonged lactation, up to 2 years, levels of fat, protein and energy in human milk are

reported to increase, whereas carbohydrate levels decrease (Czosnykowska-Łukacka, Królak-Olejnik, & Orczyk-Pawiłowicz, 2018).

Gestational effects on human milk composition are still controversial, as some studies have reported that preterm milk has higher fat, carbohydrate, protein and energy during lactation (Anderson, 1981; Bauer and Gerss, 2011). Some studies concluded there is no significant effect of GA on human milk macronutrients when comparing term and preterm groups (Caldeo and Downey et al., 2020), as well as when comparing preterm and very preterm groups (Maly et al., 2017; Caldeo and Downey et al., 2020). Fischer Fumeaux et al. (2020) reported higher fat and energy levels in preterm milk in the first two weeks, while term human milk had higher fat and energy contents during 3 to 8 weeks *post-partum*.

Of milk lipids, more than 98% (w/w) are neutral lipids, which are located at the core of milk fat globules. Fatty acids (FAs) in human milk are of significance because they contribute to 95% (w/w) of neutral lipids (Jensen, 1999). Human milk is a rich source of n-3- and n-6-polyunsaturated fatty acids (n-6 PUFA and n-3 PUFA) and their long-chain PUFA (LC PUFA) derivatives, such as arachidonic acid (AA) and docosahexaenoic acid (DHA), which have been demonstrated to promote neonatal growth and brain development (Demmelmair et al., 2017). Fatty acid profiles have been reported not to vary during lactation (Shi et al., 2011), but may be influenced by maternal diet, especially the intake of protein and carbohydrate (Ballard & Morrow, 2013; Hascoët et al., 2019). Guerra et al. (2018) reported higher LC PUFA levels in preterm milk than in term milk. Thakkar et al. (2019) did not find differences between DHA, AA and eicosapentaenoic acid (EPA) levels between term and preterm human milk. A higher level of saturated FAs (SFA), with a major contribution from medium-chain FAs and higher levels of C18:2 n-6 in preterm milk compared to term milk has been reported (Thakkar et al., 2019). Most studies on human milk FAs only reported some specific FAs, such as DHA and AA, which have been widely accepted to improve infants' health, in the lactational stages categorized as colostrum, transitional milk and mature milk, rather than specific lactational weeks.

Besides macronutrients, human milk also provides enzymes that break down macronutrients into small fragments to aid infant digestion. Proteases that hydrolyse

the peptide bonds in proteins and peptides present in human milk include plasmin, trypsin, kallikrein, cathepsins, elastase, thrombin, amino- and carboxypeptidases and matrix metalloproteinases (Chan, Beverly, & Dallas, 2021). Among these, plasmin has been reported to be the major protease, activity of which can be higher in preterm milk than term milk (Armaforte et al., 2010; Caldeo, Downey, O'Shea, et al., 2020), or be comparable in term and preterm milk (Namachivayam et al., 2013). A recent study reported that plasmin activity decreased during lactation (Caldeo, Downey, O'Shea, et al., 2020) and may be influenced by the degree of prematurity.

This study aims to report the changes in milk composition, including macronutrients, fatty acid profiles, plasmin activities, and some physico-chemical properties up to 6 months of term human milk lactation in a large sample size. Meanwhile, the changes of composition in a small cohort of preterm human milk samples up to 6 months after the proposed full-term due date are reported.

2.2 Materials & Methods

2.2.1 Human milk collection & storage

This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. All participants were recruited at Cork University Maternity Hospital, provided written consent, and all relevant guidelines and regulations were followed.

Healthy mothers, including 88 mothers who had given birth full term delivery (118 recruited, 30 withdrew) and 12 mothers who had given birth prematurely (13 recruited, 1 withdrew) were enrolled in this study. Term milk was collected at 1-, 4-, 8-, and 24-weeks post-partum, while preterm milk was collected every two weeks until the full-term due date of the infant and subsequently followed the term protocol. Questionnaires of a 24-hour dietary recall were collected with the milk samples. For milk sampling, mothers expressed 10-20 mL of milk into the sterile container provided and stored it at 4°C until delivery to the laboratory. Samples for macronutrients, plasmin activity and fatty acids analysis were stored at -80 °C until analysis (3 years maximum). All

chemicals were obtained from Merck KGaA, Darmstadt, Germany unless otherwise stated.

2.2.2 Determination of levels of macronutrients

Total fat, protein, carbohydrate and energy contents of human milk were determined with a human milk analyser (MIRIS, Uppsala, Sweden) based on mid-infrared (mid-IR) transmission spectroscopy principles. The levels of energy were calculated by MIRIS as: Energy (kcal L^{-1}) = protein (g L^{-1}) × 4.4 + fat (g L^{-1}) × 9.25 + carbohydrate (g L^{-1}) × 4. Samples collected for compositional analysis were thawed at 4°C overnight, warmed to 40°C and homogenized using a MIRIS sonicator (MIRIS, Uppsala, Sweden) before analysis. Approximately 1.5 mL of human milk was injected into the human milk analyser and the measurement of each sample was performed in duplicate.

2.2.3 Determination of fatty acids in human milk

2.2.3.1 Sample preparation

Prior to the analysis, human milk samples were thawed at 4°C overnight and inverted 20 times to achieve a good mixing of milk fats. Total fatty acids were extracted from human milk and fatty acid methyl esters (FAME) were prepared according to the method of Kelishadi et al. (2012) with some modifications. Briefly, 1 mL of internal standard (1 mg mL^{-1} of C11:0 in methanol) was added to 1 mL milk followed by 0.7 mL of 10 N KOH and 5.3 mL of methanol in a screw-threaded clear glass tube. The tube was incubated in a water bath at 55°C for 1.5 h and vortexed for 5 s at 20 min intervals. Samples were cooled to below room temperature with ice water and 0.58 mL of 24 N H_2SO_4 was added. Tubes were mixed by inversion and incubated at 55°C as before for 1.5 h, vortexing for 5 s every 20 min. Tubes were then cooled and 2 mL of hexane was added. Each sample was vortexed for 2 min followed by centrifugation (Beckman J-E Highspeed, Beckman Coulter, Brea, California) for 2 min at 5000 rpm at 4°C. The clear upper phase (FAME extract) was transferred to a screw-cap Pyrex tube with Na_2SO_4 (1 g) added to remove water, followed by transfer to the GC test vial. Vials were capped and stored at −80°C until gas chromatography (GC) analysis.

2.2.3.2 Calibration and standards preparation

A Supelco 37 Component FAME mix (product CRM47885) was used as stock solution to identify and quantify fatty acids in milk samples. Calibration curves were produced for each individual fatty acid from 5 working standards prepared by diluting the stock solution with hexane (10, 7, 5, 2 and 1 mg mL^{-1} standard mixes). Calibration curves consisted of a plot of peak area versus concentration. The response linearity of each fatty acid had r^2 values higher than 0.99. The calibration was validated and tested between batches of 20 samples. The limit of quantitation and limit of detection were established using the lowest concentration standard, where all FAME compounds assessed exhibited signal-to-noise (S/N) values of at least 11. Undecanoic acid (C11:0) was used as internal standard to monitor the efficiency of the FA extraction process and to calculate recovery rates. The latter was done by analysing and comparing the 5 mg mL^{-1} working standard (1) spiked with C11:0, at a concentration similar to that added to samples (2) and C11:0 analysed alone at this concentration (3). In all cases, the area counts from (2) and (3) were similar at > 95%.

2.2.3.3 Gas chromatography of FAMEs

GC analysis of FAMEs was carried out with a Shimadzu GC-2010 Plus Gas Chromatograph (Shimadzu Scientific Instruments, Kyoto, Japan), equipped with a split/splitless injector, an AOC-20i autoinjector, an AOC-20s autosampler, a flame ionization detector (FID) and a high polarity cyanopropyl siloxane phase-based column (Agilent J&W CP-Sil 88 for FAME), 50 m × 0.25 mm with 0.2-µm film thickness (Agilent Scientific Instruments, Santa Clara, California). Helium was used as carrier gas at a linear velocity of 0.5 mL min^{-1}. Air and hydrogen flow rates were set at 400 and 40 mL min^{-1}, respectively. The make-up flow rate was set at 30 mL min^{-1}, and the auto injector and the detector temperatures were set at 260°C and 270°C, respectively. Two µL of sample was injected with the split set at 1/100. The column oven temperature was maintained at 30°C for 2 min, then increased at 10°C min^{-1} to 175°C and held for 15 min, followed by increasing at 5°C min^{-1} to 205°C, and finally by increasing at 3°C min^{-1} to 220°C and holding at this temperature for 24.5 min. The total runtime was 75 min. LabSolutions software (Shimadzu Scientific Instruments) was

used for peak area analysis and quantification of fatty acids. Samples were analysed in duplicate and the results for each FA were expressed as both percentage of total fatty acids/peak area (%) and mg g^{-1} of total fat in a milk sample. A hexane blank was run between every 4 samples to ensure no carryover between samples.

2.2.4 Determination of plasmin activity

Plasmin activity in milk was measured in duplicate using the modified method of Richardson and Pearce (1981). Human milk samples were thawed at 4°C overnight and centrifuged at 14,000 rpm for 20 mins with 0.4 M tri-sodium citrate added (3:1 ratio) to obtain a clear serum phase. A reaction mix of the sample (100 μL), coumarin substrate (40 μL) and 50 mM tris-HCl buffer (pH 7.5, 60 μL) were added into a well of black 96-well microplates (VWR International Ltd, Luttervorth, UK). The fluorescence intensity was determined at 380 nm (excitation) and 460 nm (emission) using a plate reader (Spectrafluorplus, Tecan). One unit of plasmin activity is defined as the activity necessary to release 1 nmol 7-amino-4-methyl coumarin (AMC) per mL per min under the conditions of the assay.

2.2.5 Measurement of pH and colour

pH measurements of human milk were conducted on fresh milk samples using a FiveEasy Plus Benchtop FP20 pH/mV Standard Kit (Mettler Toledo, Leicester, UK) at 4°C. The colour of human milk was measured using a Minolta CR-400 microcolour tristimulus chromameter (Konica Minolta Sensing Inc., Milton Keynes, UK) and presented as L*, a* and b* values. L*, a*, b* represent the lightness from black (0) to white (100), colour from green (−) to red (+), and colour from blue (−) to yellow (+), respectively. All measurements were conducted in triplicate.

2.2.6 Statistical analysis

Results were evaluated with SPSS software version 17.0 (IBM, Armonk, New York). Descriptive analysis was done to determine the frequency and the mean ± SD of some variables. Independent t-tests were applied to compare major characteristics of infants and mothers in term and preterm groups. The level of significance, α, was set at 0.05 in the study.

A mixed linear model was applied with the postmenstrual age (as categories) to determine whether the macronutrients, pH, plasmin activity, and fatty acids changes over lactation time in the term group and to assess the impact of following factors on milk composition: maternal pre-pregnancy BMI and infant gender. No statistical analysis was performed to compare term and preterm groups due to the low number of subjects in the preterm group after the due date. To estimate the effect of infant gender during lactation, the postmenstrual age (as categories), infant gender and their interaction were considered as fixed effects and the subject identification was considered as a random effect. Similar method was used to estimate pre-pregnancy BMI (as categories: BMI < 25 kg m^{-2} and BMI ≥ 25 kg m^{-2}) and the interaction with age. The data for each nutrient was tested for normality according to Q-Q plots and Kolmogorov-Smirnova test. Logarithmic transformation was applied to each nutrient when the data were not normally distributed.

Pearson correlation tests were used to check the correlations between the milk colour and levels of macronutrients. Repeated measures data for each mother were averaged prior to performing the Pearson correlation. The level of significance, α, was set at 0.05 in the study.

2.3 Results

2.3.1 Participants

In total, 88 term mothers and 12 preterm mothers were enrolled in this study. As shown in **Table 2.1**, the average gestational age (mean ± SD) at birth was 39.5 ± 1.0 weeks and 31.3 ± 2.1 weeks for mothers of term and preterm infants, respectively, which was significantly different ($p < 0.05$), as expected. Preterm infant birth weight was significantly lower ($p < 0.05$) and c-section delivery rate was higher ($p > 0.05$) compared to term infants.

The total number of milk samples decreased during lactation as the number of mothers who stopped breast-feeding increased (**Table S2.1**). In mothers who continued breast-feeding, exclusive breast-feeding decreased from 82% to 56 % during lactation

in the term group. The collected diet information showed that the proportions of mothers who had oily fish (< 20%), white fish (< 9%), or beans and pulses (< 19%) were low in both term and preterm group compared to the consumption of other types of foods. Supplements, especially supplements providing multi-vitamins and minerals, were taken by more than 39% of mothers. No milk samples were provided from mothers who had alcohol within the 24 hr before the milk expression in preterm group, whereas a low proportion mothers (4 - 6 %) had beer or wine in the term group.

Table 2.1 Key demographic and infant data for participants in the study.

Parameter	Full-term	Pre-term	P-value
N	88	12	-
Mothers' BMI pre-pregnancy (kg/m^2)	26.5 (8.0)	26.6 (4.4)	NS
Infant gestational age (week)	39.5 (1.0)	31.0 (2.4)	<0.0001
Infant birth weight (g)	3629.8 (436.8)	1893.3 (646.5)	<0.0001
Infant gender (% Female)	46.6	58.3	NS
Mode of delivery (% C-section)	34.1	50	NS

Values are present as Mean (standard deviation); NS: Not significant.

2.3.2 Macronutrients levels changes during lactation

As shown in **Fig. 2.1**, levels of protein (Mean ± SD) in term milk decreased significantly ($p < 0,05$), from 1.7 ± 0.3 % to 1.0 ± 0.2 % from the first week to the six months after birth, i.e., postmenstrual age at 64 weeks (**Table S2.2**). Similarly, levels of protein in preterm milk decreased from 1.6 ± 0.6 % when postmenstrual age was lower than 36 weeks to 0.9 ± 0.1 % at 64 weeks of postmenstrual age. Levels of total fat increased slightly in preterm milk from postmenstrual age lower than 36 weeks (3.7 ± 1.2 %) to proposed full-term due date (4.2 ± 1.7 %). In contrast, the average total fat in term milk at each lactational stage did not show clear trends (ranged from 3.0 ± 1.8 % at 64 weeks' GA to 3.8 ±1.7 % at 44 weeks' GA). Carbohydrate content remained relatively unchanged during lactation (6.7 – 7.1 %) and comparable between term and preterm milk. Energy content showed a similar trend to the fat content during lactation, in that the highest energy content in term human milk was at 4 weeks *post-partum* (44

weeks' GA); however, there was a significant association with lactation stage ($p < 0.05$). In preterm milk, energy increased to the full-term due date followed by a decrease to 44 weeks' GA, whereas increased to 64 weeks' GA. Compared to term milk, preterm milk contained higher levels of energy and fat, but lower levels of proteins, at the same postmenstrual age.

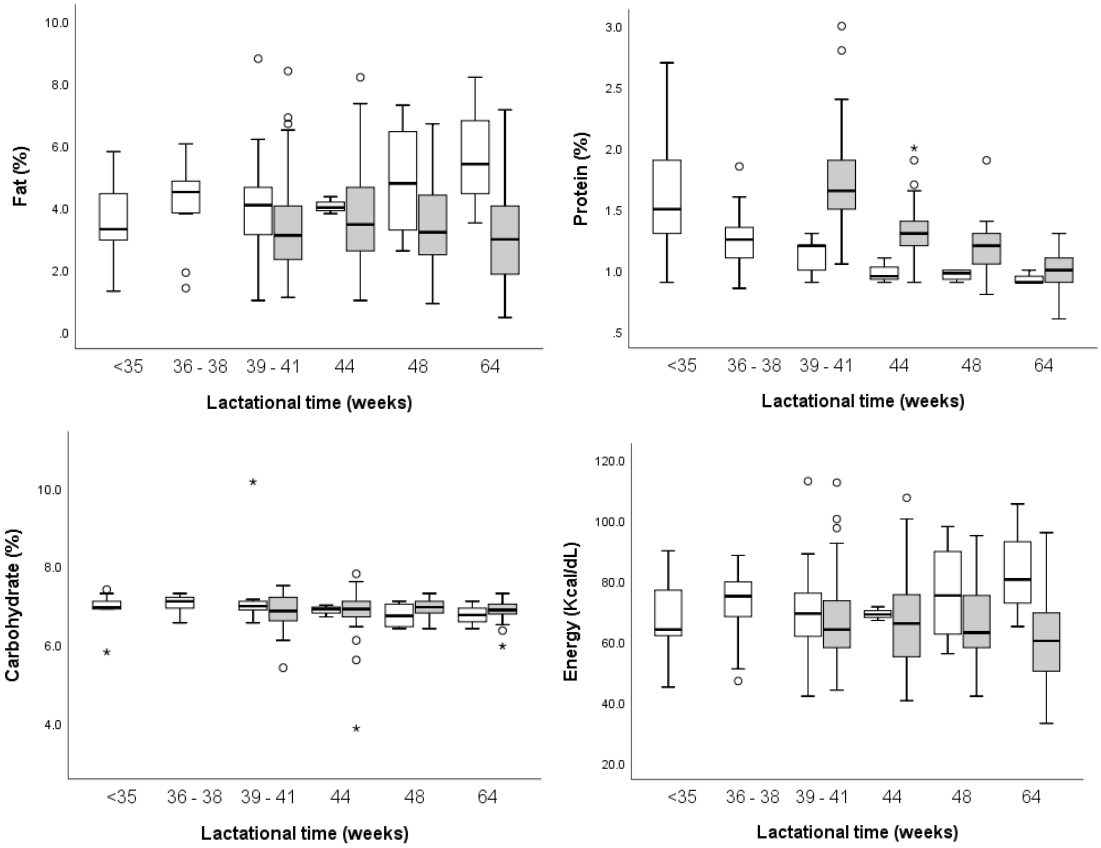

Fig. 2.1 Macronutrients changes of term human milk (grey) and preterm human milk (white) at same postmenstrual age. Horizontal lines inside boxes indicate the median value. Lines extending vertically from the boxes indicate variability outside the upper and lower quartiles. Mild outliers (○, 1.5-3 times outside the interquartile range) and extreme outliers (×, 3 times outside the interquartile range) are indicated.

In addition, the impact of maternal BMI and infant gender on macronutrient levels in term human milk was estimated. Infant gender had a significant impact ($p < 0.05$) on fat and energy content in term milk. When considering the interaction with lactation stages, milk for male infants had higher levels of fat and energy compared to milk for female infants at all lactational stages, especially at 4 weeks after birth; however, the

difference was not statistically significant ($p > 0.05$). While maternal BMI significantly ($p < 0.05$) influenced levels of fat, carbohydrate, and energy at week 44, i.e., 4 weeks after birth of term infants; fat and energy levels were lower, while the carbohydrate level was higher, in term milk expressed form mothers with normal weight than the milk expressed form high BMI mothers after 4 weeks *post-partum*.

2.3.3 Fatty acid profiles

GC chromatograms of the standard mix and an example of human milk FAs are shown in **Fig. 2.2,** and it can be seen that separation was achieved with good resolution**.** Concentrations of SFA, monounsaturated fatty acids (MUFA), n-3 LC PUFA, and n-6 LC PUFA are summarised in **Table 2.2**, which were all not significantly influenced by lactation ($p > 0.05$), i.e., they remained relatively constant during lactation in both term and preterm milk. Total SFA (mean ± SD) content was 160.4 ± 42 to 164 ± 23 mg g^{-1} total fat in term human milk, which was higher than total MUFA, which was measured as 104.4 ± 52 to 117.6 ± 43 mg g^{-1} total fat. In terms of LC PUFAs, levels of n-3 LCPUFAs were lower than n-6 LCPUFAs. Compared with the term group, preterm milk contained lower levels of SFA, MUFA, and n-3 LC PUFA, but contained similar levels of n-6 LC PUFA compared to term human milk at the same postmenstrual age.

Table 2.2 Concentrations (mg g^{-1} total fat) of the sum of saturated fatty acids, monounsaturated fatty acids, n-3 long-chain polyunsaturated fatty acids, and n-6 long-chain polyunsaturated fatty acids in term and preterm human milk during lactation.

	Term				**Preterm**			
Lactational time (weeks)	41	44	48	64	41	44	48	64
N	16	14	10	16	3	2	3	3
SUM SFA	162.1 (35.7)	162.6 (23.6)	160.4 (41.7)	164.0 (28.3)	141.6 (12.9)	150.9 (8.8)	120.8 (34.8)	148.6 (15.2)
SUM MUFA	117.6 (43.8)	113.6 (32.5)	109.7 (49.5)	104.4 (52.3)	55.7 (66.0)	78.2 (85.4)	57.9 (77.3)	60.2 (76.0)
SUM n-3 LCPUFAs	7.3 (1.7)	7.5 (1.1)	7.4 (2.3)	7.5 (1.7)	6.3 (0.6)	5.9 (1.3)	5.7 (2.0)	7.0 (1.6)
SUM n-6 LCPUFAs	36.9 (15.9)	36.0 (6.1)	37.4 (11.7)	38.1 (9.1)	38.3 (6.9)	35.4 (2.1)	33.3 (12.9)	30.9 (11.2)

SFA: Saturated Fatty Acids; MUFAs: Monounsaturated Fatty Acids; LCPUFAs: Long-Chain Polyunsaturated Fatty Acids.

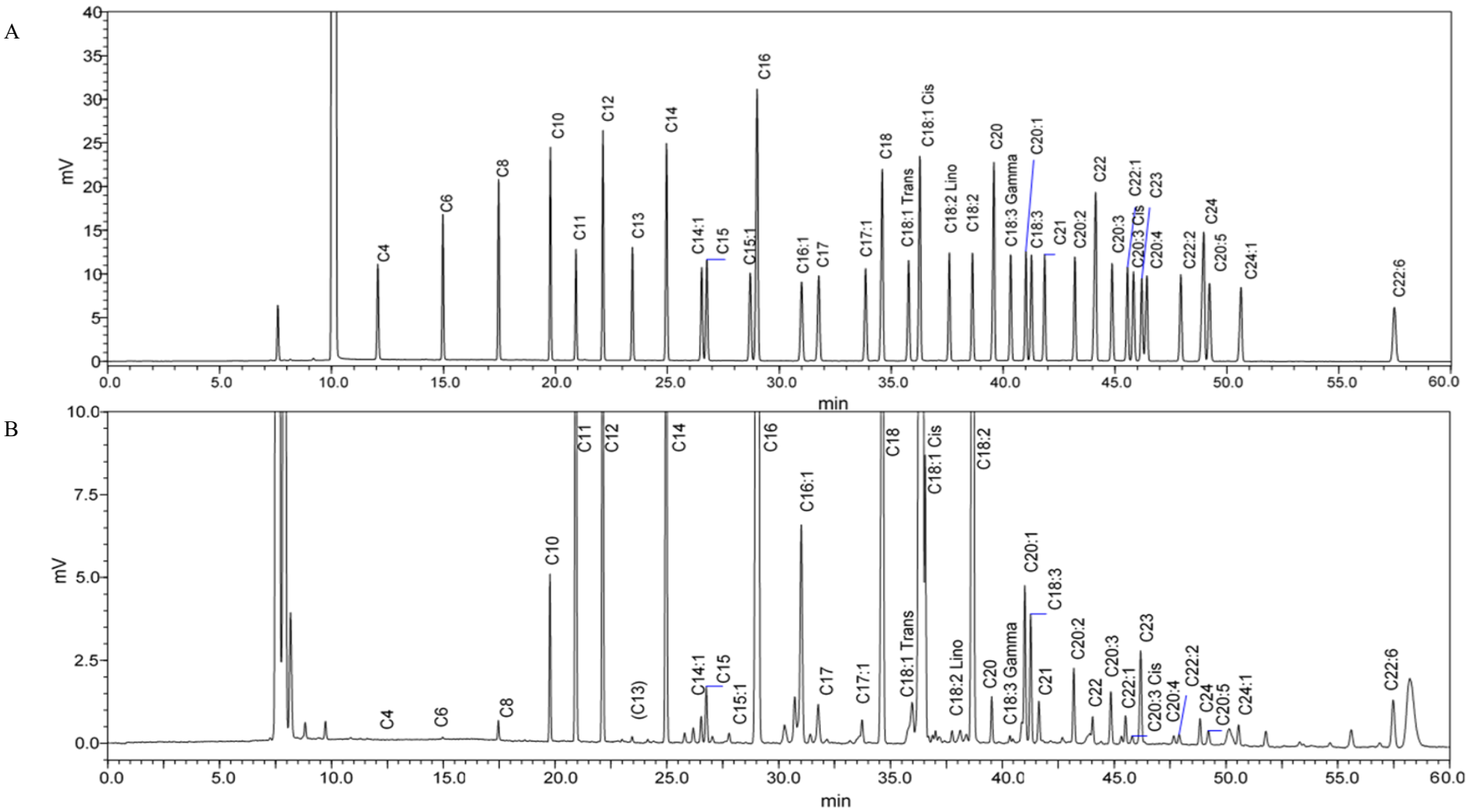

Fig. 2.2 GC-FID chromatograms of (A) Supelco 37-Component fatty acid methyl esters (FAME) standard mix and (B) FAME from a term human milk sample after one-week *post-partum*. Note: The chromatogram of the milk sample is scaled to show all significant peaks.

Average concentrations of individual fatty acids in term and preterm milk are shown in **Tables 2.3** & **2.4**, respectively. According to the percentage of total fatty acids/peak area in GC chromatogram (see the example in **Fig. 2.2** B), the most abundant FAs (% total FAs) in term human milk was oleic acid (C18:1 n-9; 28-31 %), out of 35 FAs measured in this study. Other abundant FAs (% total FAs) in term milk included palmitic acid (C16:0; 20-21 %), linoleic acid (C18:2 n-6; 10-11 %), stearic acid (C18:0; 6.4-6.9 %), myristic acid (C14:0; 5.8-6.4 %), and lauric acid (C12:0; 5-5.1 %). In preterm milk, although the types of abundant FAs were similar, the ranking according to the percentage of total fatty acids was different in terms of oleic acid (C18:1 n-9; 14-20 % total FAs) and palmitic acid (C16:0; 20-21 % total FAs). Although levels of oleic acids were lower compared to term milk on average, it is hard to draw conclusions as the sample size of preterm group was so small (n=3). The percentage of total FAs of linoleic acid (C18:2 n-6; 9.1-11 %), stearic acid (C18:0; 5.6-6.0 %), myristic acid (C14:0; 4.3-6.3 %), and lauric acid (C12:0; 3.6-5.3 %) in preterm milk were all slightly lower compared to those in term milk.

In term human milk, there was a significant increase in levels of caproic acid (C6:0) and α-linolenic acid (ALA; C18:3 n-3) during lactation (**Table 2.3 & Fig. 2.3**). Levels of long chain FAs in term human milk, including AA (C20:4 n-6), erucic acid (C22:1), docasadienoic acid (C22:2), DHA (C22:6), and lignoceric acid (C24:0) decreased significantly ($p < 0.05$) during lactation. Concentrations of *cis*-11-eicosenic acid (C20:1) and tricosanoic acid (C23:0) decreased significantly ($p < 0.05$) from postmenstrual age week 41 to week 44, i.e., during the first 4 weeks after birth, but thereafter remained consistent after 24 weeks after birth. Lactational stage also had a significant impact on γ-linolenic acid (C18:3 n-6) levels in term milk ($p < 0.05$), the concentrations of which increased during 1 to 4 weeks after birth, whereas it decreased from 4 weeks to 24 weeks after birth.

Table 2.3 Fatty acid profiles of term human milk during lactation. Results are presented as mean (standard deviation in brackets).

	Concentrations (mg g^{-1} fat)				**% Total fatty acids/Peak Area**			
LT (week)	41	44	48	64	41	44	48	64
N	16	14	10	16	16	14	10	16
Saturated Fatty Acids (SFAs)								
C6:0	0.00 (0.2)	0.17 (0.3)	0.28 (0.5)	0.52 (0.5)	0.00 (0.0)	0.00 (0.0)	0.01 (0.0)	0.02 (0.0)
C8:0	1.83 (0.6)	2.17 (0.8)	2.07 (0.8)	2.06 (1.1)	0.10 (0.0)	0.14 (0.0)	0.14 (0.0)	0.11 (0.0)
C10:0	5.51 (1.9)	6.70 (1.7)	6.44 (2.2)	6.21 (1.5)	0.95 (0.3)	1.22 (0.3)	1.17 (0.3)	1.00 (0.2)
C12:0	20.17 (8.6)	20.25 (6.3)	20.08 (7.7)	21.09 (6.0)	4.89 (1.7)	5.08 (1.5)	5.05 (1.6)	5.11 (1.5)
C13:0	0.64 (0.4)	0.67 (0.4)	0.57 (0.3)	0.50 (0.4)	0.03 (0.0)	0.03 (0.0)	0.03 (0.0)	0.02 (0.0)
C14:0	21.35 (6.7)	21.13 (4.5)	21.01 (5.5)	23.94 (6.6)	5.79 (1.7)	5.79 (1.1)	5.80 (1.0)	6.39 (1.7)
C15:0	1.68 (0.5)	1.79 (0.6)	1.84 (0.7)	1.91 (0.9)	0.30 (0.1)	0.32 (0.1)	0.34 (0.1)	0.29 (0.1)
C16:0	68.94 (13.1)	66.86 (10.8)	66.15 (18.0)	64.98 (12.8)	21.08 (2.6)	20.84 (2.4)	20.38 (2.9)	19.68 (2.5)
C17:0	1.63 (0.4)	1.68 (0.4)	1.67 (0.6)	1.79 (0.7)	0.33 (0.1)	0.32 (0.1)	0.33 (0.1)	0.30 (0.1)
C18:0	21.74 (7.0)	22.13 (4.9)	23.02 (9.3)	22.53 (4.0)	6.38 (1.4)	6.77 (1.4)	6.88 (2.2)	6.66 (0.8)
C20:0	1.78 (0.5)	1.88 (0.6)	1.78 (0.6)	2.07 (1.1)	0.20 (0.0)	0.20 (0.0)	0.19 (0.1)	0.17 (0.0)
C21:0	1.32 (0.5)	1.50 (0.5)	1.51 (0.7)	1.74 (0.7)	0.25 (0.1)	0.30 (0.1)	0.31 (0.1)	0.32 (0.1)
C22:0	1.46 (0.7)	1.68 (0.9)	2.02 (1.0)	1.16 (0.8)	0.16 (0.2)	0.24 (0.2)	0.33 (0.2)	0.14 (0.1)
C23:0	2.63 (0.9)	2.09 (0.4)	2.09 (0.6)	2.15 (0.5)	0.57 (0.1)	0.42 (0.1)	0.43 (0.1)	0.39 (0.1)
C24:0	1.08 (0.4)	0.68 (0.4)	0.64 (0.4)	0.36 (0.4)	0.08 (0.0)	0.04 (0.0)	0.03 (0.0)	0.02 (0.0)
Monounsaturated Fatty Acids (MUFAs)								
C14:1	1.29 (0.4)	1.52 (0.5)	1.53 (0.7)	1.43 (0.7)	0.17 (0.1)	0.21 (0.1)	0.23 (0.1)	0.18 (0.1)
C15:1	0.72 (0.4)	0.80 (0.4)	0.82 (0.5)	0.75 (0.5)	0.05 (0.0)	0.06 (0.0)	0.06 (0.0)	0.05 (0.0)
C16:1	6.95 (1.9)	7.38 (2.1)	8.14 (3.1)	8.16 (2.3)	1.90 (0.6)	2.02 (0.6)	2.31 (0.8)	2.13 (0.6)

Table 2.3 *(Continued)*

	Concentrations (mg g^{-1} fat)				% Total fatty acids/Peak Area			
LT (week)	41	44	48	64	41	44	48	64
N	16	14	10	16	16	14	10	16
Monounsaturated Fatty Acids (MUFAs)								
C17:1	1.23 (0.3)	1.27 (0.4)	1.27 (0.5)	1.16 (0.6)	0.18 (0.0)	0.17 (0.0)	0.18 (0.0)	0.16 (0.1)
C18:1 n-9	100.63 (42.8)	96.03 (30.6)	91.77 (46.3)	86.08 (50.6)	31.35 (11.5)	31.09 (9.5)	30.12 (13.4)	28.10 (15.7)
trans-C18:1 n-9	2.37 (1.4)	2.56 (1.1)	2.23 (1.5)	2.51 (1.9)	0.56 (0.4)	0.62 (0.3)	0.52 (0.4)	0.55 (0.5)
C20:1	2.52 (0.6)	2.06 (0.3)	2.02 (0.6)	2.11 (0.4)	0.62 (0.1)	0.47 (0.1)	0.46 (0.1)	0.44 (0.1)
C22:1	0.92 (0.3)	0.74 (0.3)	0.66 (0.3)	0.63 (0.3)	0.13 (0.0)	0.08 (0.0)	0.07 (0.0)	0.07 (0.0)
C24:1	1.00 (0.3)	1.24 (0.7)	1.22 (0.7)	1.54 (0.9)	0.19 (0.1)	0.25 (0.2)	0.25 (0.2)	0.33 (0.2)
n-3 Long-Chain Polyunsaturated Fatty Acids (n-3 LCPUFAs)								
C18:3 n-3	3.88 (1.4)	4.55 (0.7)	4.63 (1.7)	5.10 (1.1)	1.01 (0.3)	1.27 (0.3)	1.28 (0.4)	1.36 (0.3)
C20:3 n-3	0.81 (0.3)	0.55 (0.3)	0.46 (0.3)	0.37 (0.3)	0.08 (0.0)	0.04 (0.0)	0.04 (0.0)	0.03 (0.0)
C20:5 n-3	0.68 (0.3)	0.91 (0.4)	0.83 (0.3)	0.63 (0.4)	0.06 (0.0)	0.08 (0.0)	0.08 (0.0)	0.05 (0.0)
C22:6 n-3	1.91 (0.5)	1.51 (0.5)	1.50 (0.5)	1.40 (0.6)	0.41 (0.1)	0.28 (0.1)	0.29 (0.1)	0.21 (0.1)
n-6 Long-Chain Polyunsaturated Fatty Acids (n-6 LCPUFAs)								
C18:2 n-6	32.75 (14.8)	31.41 (6.6)	33.27 (11.1)	34.53 (8.7)	9.94 (2.4)	10.32 (2.3)	11.03 (3.4)	11.07 (2.7)
trans-C18:2 n-6	0.82 (0.4)	0.80 (0.4)	0.80 (0.5)	0.73 (0.5)	0.08 (0.0)	0.07 (0.0)	0.08 (0.0)	0.07 (0.0)
C18:3 n-6	0.72 (0.4)	1.05 (0.4)	0.97 (0.3)	0.75 (0.5)	0.06 (0.0)	0.11 (0.0)	0.10 (0.0)	0.08 (0.0)
C20:3 n-6	1.91 (0.7)	1.88 (0.6)	1.64 (0.6)	1.60 (0.5)	0.41 (0.1)	0.40 (0.1)	0.34 (0.1)	0.26 (0.1)
C20:4 n-6	0.69 (0.3)	0.85 (0.3)	0.76 (0.2)	0.52 (0.3)	0.10 (0.1)	0.12 (0.0)	0.10 (0.0)	0.06 (0.0)
Other Polyunsaturated Fatty Acids (PUFAs)								
C20:2	1.97 (0.8)	1.44 (0.4)	1.28 (0.4)	1.24 (0.5)	0.42 (0.1)	0.26 (0.1)	0.22 (0.1)	0.18 (0.1)
C22:2	0.71 (0.3)	0.32 (0.3)	0.35 (0.3)	0.28 (0.3)	0.07 (0.0)	0.03 (0.0)	0.03 (0.0)	0.02 (0.0)

LT: Lactational time (postmenstrual age).

In preterm human milk, concentrations of some FAs, including ALA (C18:3 n-3), eicosadienoic acid (C20:2), docasadienoic acid (C22:2), and tricosanoic acid (C23:0) showed a similar trend to that in term milk during lactation (**Table 2.4**). Comparison of selected important FAs in term and preterm milk are shown in **Fig. 2.3**. Concentrations of palmitic acid (C16:0), linoleic acid (C18:2 n-6), and ALA (C18:3 n-3) in preterm milk were comparable to those in term human milk during lactation, while concentrations of γ-linolenic acid (C18:3 n-6) at week 44, AA (C20:4 n-6) at week 44 and 48, stearic acid (C18:0) at week 48 and 64, nervonic acid (C24:1) at week 64, and DHA (C22:6 n-3) at all lactational stages in preterm milk was lower compared to those in term milk.

In addition, maternal BMI had a significant impact ($p < 0.05$) on some SFAs in term milk samples, including caprylic acid (C8:0), pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), heneicosanoic acid (C21:0), two MUFA (myristoleic acid (C14:1) and nervonic acid (C24:1)), and *trans*-linolelaidic acid (*trans* C18:2 n-6); higher levels of those FAs were present in overweight mothers (BMI $\geq$ 25 kg m^{-2}). Significant relationships were also seen between gender of infants and two MUFAs: palmitoleic acid (C16:1) and nervonic acid (C24:1); female infants obtained higher levels of those FAs from human milk. When considering the impact of gender at each lactation stage, there is no significant difference of each FA between milk for male or female infants after one week or four weeks after birth. Significantly higher levels ($p < 0.05$) of ALA (C18:3 n-3) and total n-3 LCPUFAs were present in milk for female infants than in milk for male infants after 8 weeks *post-partum*. In term milk after 24 weeks *post-partum*, concentrations of SFAs, including caprylic acid (C8:0), myristic acid (C14:0), and total SFA, as well as some MUFAs, including myristoleic acid (C14:1), palmitoleic acid (C16:1), nervonic acid (C24:1), and *trans*-9-elaidic acid (*trans* C18:1 n-9) were significant higher in milk for female infants than in milk for male infants.

Table 2.4 Fatty acid profiles of preterm human milk during lactation. Results are presented as mean (standard deviation in brackets).

	Concentrations (mg g^{-1} fat)				**% Total fatty acids/Peak Area**			
LT (week)	41	44	48	64	41	44	48	64
N	3	2	3	3	3	2	3	3
Saturated Fatty Acids (SFAs)								
C6:0	0.52 (0.5)	0.83 (0.2)	0.43 (0.4)	0.90 (0.3)	0.01 (0.0)	0.02 (0.0)	0.02 (0.0)	0.03 (0.0)
C8:0	1.52 (0.3)	1.38 (0.2)	1.35 (0.6)	1.46 (0.4)	0.11 (0.0)	0.11 (0.0)	0.11 (0.0)	0.13 (0.0)
C10:0	5.13 (0.6)	5.15 (0.1)	4.23 (1.5)	5.51 (1.1)	0.94 (0.2)	0.94 (0.1)	0.92 (0.3)	1.09 (0.1)
C12:0	14.89 (5.9)	15.89 (3.9)	13.14 (4.4)	20.52 (3.6)	3.62 (1.7)	3.79 (1.5)	4.09 (1.7)	5.26 (0.7)
C13:0	0.61 (0.2)	0.55 (0.1)	0.27 (0.3)	0.58 (0.2)	0.03 (0.0)	0.04 (0.0)	0.03 (0.0)	0.04 (0.0)
C14:0	16.16 (5.7)	18.34 (2.5)	14.98 (5.6)	22.51 (4.9)	4.31 (1.8)	4.75 (1.3)	5.04 (1.9)	6.27 (1.1)
C15:0	1.56 (0.3)	1.51 (0.4)	1.35 (0.5)	1.54 (0.3)	0.31 (0.1)	0.30 (0.0)	0.31 (0.0)	0.34 (0.0)
C16:0	69.46 (5.1)	73.72 (6.8)	57.53 (18.0)	65.33 (3.0)	21.03 (2.6)	21.12 (0.9)	21.10 (0.8)	20.34 (0.9)
C17:0	1.49 (0.3)	1.37 (0.4)	1.25 (0.5)	1.32 (0.2)	0.33 (0.1)	0.30 (0.0)	0.31 (0.0)	0.31 (0.0)
C18:0	19.15 (2.1)	21.51 (7.6)	15.72 (5.5)	18.45 (0.7)	5.72 (0.9)	6.01 (1.4)	5.60 (0.2)	5.69 (0.6)
C20:0	1.34 (0.3)	1.28 (0.3)	1.14 (0.6)	1.17 (0.3)	0.16 (0.0)	0.18 (0.0)	0.14 (0.0)	0.15 (0.0)
C21:0	1.42 (0.3)	1.08 (0.1)	1.07 (0.4)	1.18 (0.4)	0.32 (0.1)	0.22 (0.0)	0.26 (0.0)	0.27 (0.1)
C22:0	0.81 (0.1)	0.97 (0.5)	0.61 (0.2)	1.07 (0.6)	0.08 (0.0)	0.12 (0.1)	0.06 (0.0)	0.17 (0.2)
C23:0	2.21 (0.5)	1.80 (0.3)	1.77 (0.8)	1.74 (0.2)	0.50 (0.1)	0.38 (0.0)	0.45 (0.1)	0.40 (0.0)
C24:0	0.37 (0.3)	0.58 (0.1)	0.27 (0.3)	0.31 (0.3)	0.02 (0.0)	0.03 (0.0)	0.02 (0.0)	0.02 (0.0)
Monounsaturated Fatty Acids (MUFAs)								
C14:1	1.23 (0.3)	1.22 (0.1)	1.13 (0.4)	1.17 (0.2)	0.20 (0.1)	0.21 (0.0)	0.23 (0.0)	0.21 (0.0)
C15:1	0.69 (0.2)	0.62 (0.1)	0.47 (0.2)	0.63 (0.2)	0.06 (0.0)	0.06 (0.0)	0.05 (0.0)	0.07 (0.0)
C16:1	8.89 (0.5)	7.80 (0.9)	7.78 (3.4)	7.61 (2.8)	2.47 (0.3)	2.05 (0.1)	2.54 (0.4)	2.19 (1.0)

Table 2.4 *(Continued)*

	Concentrations (mg g^{-1} fat)				% Total fatty acids/Peak Area			
LT (week)	41	44	48	64	41	44	48	64
N	3	2	3	3	3	2	3	3
Monounsaturated Fatty Acids (MUFAs)								
C17:1	1.27 (0.2)	1.11 (0.2)	1.00 (0.5)	1.03 (0.1)	0.24 (0.0)	0.20 (0.0)	0.20 (0.0)	0.20 (0.1)
C18:1 n-9	39.21 (63.6)	62.88 (85.0)	43.79 (72.4)	45.34 (73.9)	12.56 (20.8)	20.10 (27.7)	14.45 (24.0)	13.52 (22.2)
trans-C18:1 n-9	0.91 (1.6)	0.83 (1.2)	0.73 (1.3)	1.03 (1.8)	0.22 (0.4)	0.22 (0.3)	0.16 (0.3)	0.25 (0.4)
C20:1	1.83 (0.2)	2.03 (0.0)	1.39 (0.4)	1.70 (0.1)	0.45 (0.1)	0.51 (0.1)	0.41 (0.1)	0.44 (0.1)
C22:1	0.63 (0.1)	0.73 (0.0)	0.55 (0.2)	0.64 (0.1)	0.08 (0.0)	0.13 (0.0)	0.10 (0.1)	0.11 (0.0)
C24:1	1.03 (0.5)	1.00 (0.6)	1.08 (0.3)	1.05 (0.8)	0.22 (0.2)	0.22 (0.2)	0.30 (0.1)	0.24 (0.2)
n-3 Long-Chain Polyunsaturated Fatty Acids (n-3 LCPUFAs)								
C18:3 n-3	3.62 (1.2)	3.99 (1.7)	3.62 (0.9)	4.85 (0.9)	1.00 (0.4)	1.10 (0.7)	1.27 (0.3)	1.44 (0.3)
C20:3 n-3	0.59 (0.2)	0.55 (0.1)	0.53 (0.2)	0.53 (0.1)	0.05 (0.0)	0.06 (0.1)	0.06 (0.0)	0.06 (0.0)
C20:5 n-3	0.67 (0.2)	0.52 (0.1)	0.59 (0.3)	0.58 (0.2)	0.07 (0.0)	0.05 (0.0)	0.07 (0.0)	0.07 (0.0)
C22:6 n-3	1.43 (0.7)	0.82 (0.4)	0.91 (0.6)	1.05 (0.5)	0.29 (0.2)	0.14 (0.1)	0.18 (0.1)	0.22 (0.2)
n-6 Long-Chain Polyunsaturated Fatty Acids (n-6 LCPUFAs)								
C18:2 n-6	34.87 (5.8)	32.51 (1.6)	30.19 (11.4)	28.13 (10.6)	10.99 (2.0)	9.71 (0.8)	11.35 (1.0)	9.13 (3.1)
trans-C18:2 n-6	0.64 (0.3)	0.59 (0.2)	0.62 (0.3)	0.55 (0.2)	0.06 (0.0)	0.07 (0.0)	0.08 (0.0)	0.06 (0.0)
C18:3 n-6	0.85 (0.4)	0.62 (0.2)	0.77 (0.4)	0.65 (0.2)	0.12 (0.1)	0.07 (0.0)	0.12 (0.1)	0.08 (0.0)
C20:3 n-6	1.47 (0.3)	1.23 (0.2)	1.27 (0.6)	1.10 (0.2)	0.33 (0.1)	0.26 (0.0)	0.32 (0.1)	0.24 (0.0)
C20:4 n-6	0.52 (0.1)	0.44 (0.0)	0.49 (0.2)	0.46 (0.1)	0.07 (0.0)	0.06 (0.0)	0.07 (0.0)	0.06 (0.0)
Other Polyunsaturated Fatty Acids (PUFAs)								
C20:2	1.19 (0.3)	1.08 (0.1)	0.93 (0.4)	0.91 (0.0)	0.24 (0.0)	0.22 (0.0)	0.20 (0.0)	0.18 (0.0)
C22:2	0.47 (0.1)	0.40 (0.1)	0.19 (0.2)	0.21 (0.2)	0.04 (0.0)	0.03 (0.0)	0.02 (0.0)	0.02 (0.0)

LT: Lactational time (postmenstrual age).

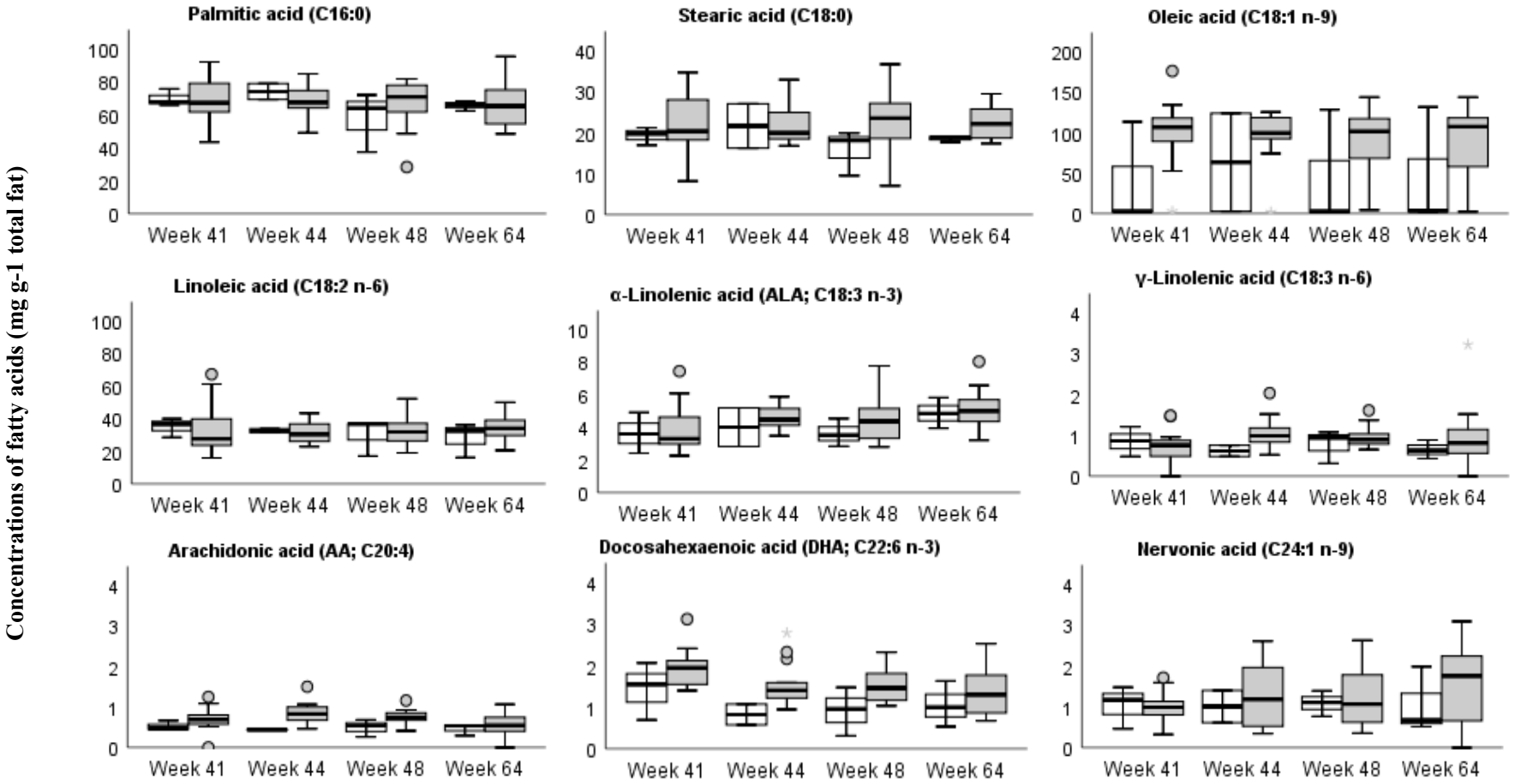

Fig. 2.3 Changes in selected fatty acids in term (grey) and preterm (white) milk during lactation. Horizontal lines inside boxes indicate the median value. Lines extending vertically from the boxes indicate variability outside the upper and lower quartiles. Mild outliers (○, 1.5-3 times outside the interquartile range) and extreme outliers (*, 3 times outside the interquartile range) are indicated.

2.3.4 Plasmin activities

As shown in **Fig. 2.4**, plasmin activity decreased during lactation in both term and preterm human milk with high inter-individual differences, especially at early stages after birth. In preterm milk, plasmin activities decreased from 0.116 ± 0.1 (<36 weeks' GA) to 0.006 ± 0.003 nmol AMC units min^{-1} mL^{-1} (64 weeks' GA) **(Table S2.2)**. In term milk, plasmin activities decreased from 0.069 ± 0.1 nmol AMC units min^{-1} mL^{-1} to 0.028 ± 0.04 nmol AMC units min^{-1} mL^{-1} at 44 weeks' GA and remained relatively constant after that.

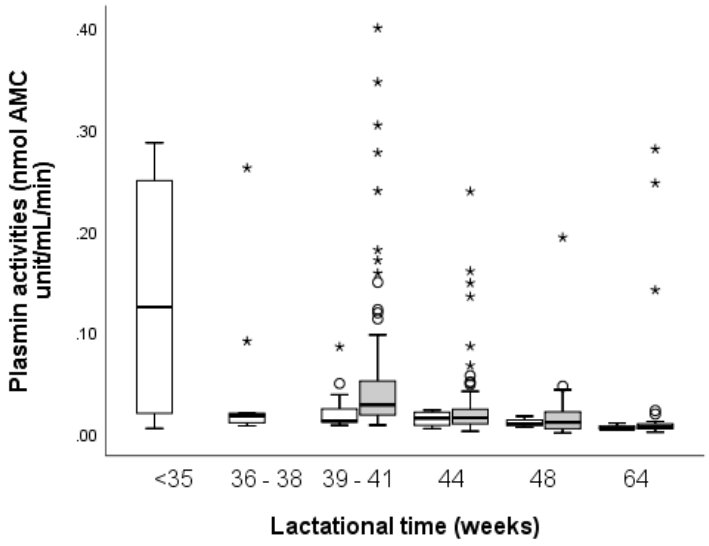

Fig. 2.4 Changes in plasmin activities in term (grey) and preterm (white) milk during lactation. Horizontal lines inside boxes indicate the median value. Lines extending vertically from the boxes indicate variability outside the upper and lower quartiles. Mild outliers (○, 1.5-3 times outside the interquartile range) and extreme outliers (×, 3 times outside the interquartile range) are indicated.

2.3.5 pH and colour changes

As shown in **Table S2.2**, average pH values remained relatively constant during lactation, with the maximum as pH 7.4 (preterm, 39 weeks' GA) and the minimum as pH 6.8 (term, 64 weeks' GA).

The average L*, a*, b* values (±SD) of term human milk (n=80) were 71.2 ± 7.7, -2.2 ± 0.6, and 7.7 ± 4.9, respectively, while the average L*, a*, b* values (±SD) of preterm human milk (n=8) were 77.3 ± 3.7, -2.4 ± 0.3, and 8.4 ± 2.0, respectively. L*, a*, b* values did not differ significantly between term and preterm groups ($p > 0.05$). The colour of milk was correlated with fat and protein levels in human milk (**Table 2.5**). Both L* and b* correlated with fat and energy significantly ($p < 0.05$), whereas b* was also correlated with the protein level (the Pearson's R^2 correlation coefficient of 0.304). In addition, L* value was negatively correlated with milk pH (R^2 of -0.217) and a* value was positively correlated with milk pH.

Table 2.5 Pearson correlations between colour (L*, a*, b*), macronutrients, and pH of human milk (n=88).

		Correlations		
		L*	a*	b*
L*	Pearson Correlation	1	-0.099	0.567**
	P values	-	0.36	< 0.001
a*	Pearson Correlation	-0.099	1	-0.294**
	P values	0.36	-	0.005
b*	Pearson Correlation	0.567**	-.294**	1
	P values	< 0.001	0.005	-
Protein	Pearson Correlation	-0.052	0.060	0.304**
	P values	0.63	0.58	0.004
Fat	Pearson Correlation	0.761**	0.051	0.477**
	P values	< 0.001	0.636	< 0.001
Carbohydrate	Pearson Correlation	0.016	-0.078	-0.083
	P values	0.88	0.47	0.44
Energy	Pearson Correlation	0.751**	0.059	0.493**
	P values	< 0.001	0.587	< 0.001
pH	Pearson Correlation	-0.217**	0.221**	-0.066
	P values	0.045	0.041	0.55

** Correlation is significant at the 0.05 level (2-tailed).

2.4 Discussion

A large cohort of mothers of term infants (n=88) with a smaller cohort of preterm mothers (n=12) was recruited in this study. Postmenstrual age rather than *post-partum* age was used in the study; in term milk, postmenstrual age from 41 to 64 weeks equals to *post-partum* age from 1 to 24 weeks, i.e., from 1 week to 6 months after birth whereas, when using postmenstrual age in preterm milk, for example, week 64, equals to sum of weeks to supposed due date (40 weeks) and weeks after supposed due date (24 weeks), i.e., nutrient content changes were observed over a longer time in human milk (up to 6 months after birth) for preterm infants.

Levels of proteins, fat, carbohydrate, and energy measured in the study were in accordance with many reports previously, which reported as 0.9-1.9 %, 3.2-4.1 %, 6.2-7.8 %, and 65-70 kcal dL^{-1}, respectively for term milk (Bauer & Gerss, 2011; Caldeo, Downey, O'Shea, et al., 2020; Michaelsen et al., 1990; Nommsen et al., 1991; Wojcik et al., 2009; Yamawaki et al., 2005) and as 1.6-2.3 %, 3.4-4.8 %, 6.3-7.6 %, and 67-78 kcal dL^{-1} for preterm human milk (Bauer & Gerss, 2011; Caldeo, Downey, O'Shea, et al., 2020; Maly et al., 2019). Fat and energy levels in preterm milk were slightly higher compared to their typical levels at week 64, which may result from the low number of preterm samples and no conclusion can be made. However, the results are consistent with increased fat levels after prolonged lactation time as reported by Czosnykowska-Łukacka et al. (2018).

Our results support reports that levels of protein decreased during lactation and that levels of carbohydrate are relatively static during lactation (Caldeo, Downey, O'Shea, et al., 2020). The increased fat and energy content from 1 week to 4 weeks after birth, has also been reported by previous studies (Bauer & Gerss, 2011; Saarela, Kokkonen, & Koivisto, 2005). There was a significant impact of infant gender on fat and energy content in term milk in agreement with the reports of Powe, Knott, and Conklin-Brittain (2010) and Fischer Fumeaux et al. (2019), who also reported that milk for male infants contains higher levels in fat and energy compared to milk for female infants. A sex-bias effect on milk composition has also been reported for some primate species, with richer fat and lower carbohydrate concentrations in milk for male than female offspring (Hinde, 2007). In addition, no statistically significant correlation was

found between maternal BMI and macronutrient contents in human milk. However, when considering the difference within the specific lactational stage, milk expressed from overweight mothers contained higher levels of fat and energy, but lower levels of carbohydrate levels than normal weight mothers at 4 weeks *post-partum*. Controversial results of the impact of maternal BMI on macronutrients were reported previously; some studies have reported lactose levels were lower in overweight/obesity mothers, especially in colostrum (Leghi et al., 2020; Young et al., 2017), and fat level was higher in milk from overweight/obese mothers (Bzikowska-Jura et al., 2018; Dritsakou et al., 2017; Kim et al., 2017), whereas others found no association between BMI and macronutrient levels in human milk (Leghi et al., 2020; Mäkelä et al., 2013).

Concerning fatty acids in milk, the major SFA presented in human milk was palmitic acid (C16:0; 20-21 % total FAs) and the major MUFA was oleic acid (C18:1 n-9; 28-31 % total FAs), and concentrations of these were in accordance with previous reports: 17-30 % of total FAs for palmitic acid (C16:0) and 22-42 % of total FAs for oleic acid (C18:1 n-9) (Floris et al., 2020; Krešić et al., 2013; Sánchez-Hernández et al., 2019).

AA (C20:4 n-6), EPA (C20:5 n-3), and DHA (C22:6 n-3) are widely reported LC PUFAs in human milk, because they are structural components of cellular membranes, and are incorporated in relatively large amounts during early growth of the brain and the retina (Koletzko, 2016). In our study, AA (C20:4 n-6), EPA (C20:5 n-3), and DHA (C22:6 n-3) accounted for 0.06-0.12 %, 0.05-0.08 %, and 0.14-0.41 % of total FAs in human milk fat, respectively. The levels of EPA (C20:5 n-3) and DHA (C22:6 n-3) were in the same range, whereas AA (C20:4 n-6) were lower compared to the literature, with reports of 0.04-0.15 % of total FAs for EPA (C20:5 n-3), 0.2-0.7 % of total FAs for DHA (C22:6 n-3), and 0.45-0.8 % of total FAs for AA (C20:4 n-6) (Genzel-Boroviczényy, Wahle, & Koletzko, 1997; Kelishadi et al., 2012; Kim et al., 2017; Krešić et al., 2013).

Linoleic acid (C18:2 n-6), which was measured as 9-11 % of total FAs, is the precursor of AA (C20:4 n-6), while ALA (C18:3 n-3), measured as 1-1.3 % of total FAs, is the precursor of EPA (C20:5 n-3) and DHA (C22:6 n-3) (Koletzko & Rodriguez-Palmero, 1999). The levels of linoleic acid (C18:2 n-6) were lower than

previous reports (17-18 % total FAs), while ALA (C18:3 n-3) levels were comparable to other studies (0.7-1.4 %) (Krešić et al., 2013; Romeu-Nadal, Castellote, & López-Sabater, 2008). Because of lower levels of AA (C20:4 n-6) and linoleic acid (C18:2 n-6), the total n-6 LCPUFAs (10.6-11.7 % total FAs) were lower compared to the literature (14-16 % total FAs); while levels of n-3 LCPUFAs (1.6-1.7 % total FAs) were higher as DHA (C22:6 n-3) levels were slightly higher in our studies compared to previous reports (0.5-1.0 % total FAs) (Genzel-Boroviczényy et al., 1997; Romeu-Nadal et al., 2008; Sánchez-Hernández et al., 2019).

The effect of lactation on FAs in term human milk was also examined. Significant increases in levels of caproic acid (C6:0) and ALA (C18:3 n-3) agreed with the increase of caproic acid from colostrum to mature milk reported in a meta-analysis by Floris et al. (2020). Decreased levels of AA (C20:4 n-6), DHA (C22:6 n-3), lignoceric acid (C24:0), erucic acid (C22:1 n-9), and docasadienoic acid (C22:2) during lactation found in our study have been reported previously (Floris et al., 2020; Genzel-Boroviczényy et al., 1997; Qi et al., 2018).

The impact of gestational age was also examined, but no statistical comparison was performed due to the small sample size of the preterm group. Lower levels of SFA, MUFA, and n-3 LC PUFA in preterm milk was observed compared to that in term milk. Berenhauser et al. (2012) reported preterm milk contains lower SFA and MUFA, but not n-3 LCPUFA compared to term milk, while Thakkar et al. (2019) reported lower MUFA, but not SFA and n-3 LCPUFA in preterm milk compared to term milk. As shown in **Fig. 2.2**, concentrations of DHA were lower in preterm milk than that in term milk across lactation, which agreed with the findings of Thakkar et al. (2019), whereas other studies have reported that DHA level was higher in preterm milk than term milk (Bokor, Koletzko, & Decsi, 2007) or found no difference between term and preterm milk (Floris et al., 2020).

Statistically significant ($p < 0.05$) associations between maternal BMI and FAs, including some SFAs, myristoleic acid (C14:1), nervonic acid (C24:1), and *trans*-linolelaidic acid (*trans* C18:2 n-6) were found in term milk. de la Garza Puentes et al. (2019) reported higher pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) levels in human milk from overweight/obese mothers, as shown here. They also

reported higher levels of caprylic acid (C8:0) in human milk from obese mothers (BMI ≥ 30 kg m^{-2}) compared to mothers with BMI between 25 and 30 kg m^{-2} (overweight mothers) in human milk. However, a statistically significant difference ($p < 0.05$) was only found in colostrum in their study, not in transitional or mature human milk. In terms of LC PUFA, higher levels of pentadecanoic acid (C15:0), heneicosanoic acid (C21:0) and *trans*-linolelaidic acid (*trans* C18:2 n-6) were found in overweight/obese mothers than mother with normal BMI, similar to our results; however, they did not find statistical significance ($p > 0.05$) (Ellsworth et al., 2020). Instead, these authors found significantly higher palmitic acid (C16:0) and significantly lower oleic acid (C18:1 n-9) levels in milk of overweight/obese mothers, which was not observed in our study.

To our knowledge, few reports have related levels of FAs to infant gender. Significant relationships were found between infants' gender and some FAs in this study. Milk from mothers who had female offspring contained higher levels of palmitoleic acid (C16:1) and nervonic acid (C24:1) compared to milk from mothers who had male offspring. The major impact of infant's gender was observed after 8- or 24-weeks *post-partum* in term milk. Higher levels of ALA (C18:3 n-3) and total n-3 LCPUFAs at week 8 *post-partum*, some SFAs and some MUFAs at week 24 *post-partum* were found in milk for female term infants compared to milk for male term infants. Thakkar et al. (2013) also reported higher myristic acid (C14:0), ALA (C18:3 n-3), and total SFA levels in milk for female offspring than for male offspring during a 120-day lactational observation; however, the difference was not significant ($p > 0.05$). They also reported significantly higher levels of linoleic acid (C18:2 n-6), 20:2 n-6 (eicosadienoic acid), and total PUFA in milk for female infants than milk for male infants, which was not observed in our study. No short chain SFA, capric acid (C8:0), no MUFA, and no trans FAs were studied by Thakkar et al. (2013). One recent study did not find significant relationships between infant gender and fatty acid profiles (Miliku et al., 2019). Interestingly, in terms of the sex-bias of nervonic acid (C24:1) levels found in our study, this FA has been reported to decrease during lactation (not found in this study) with higher abundance in preterm human milk (Ntoumani, Strandvik, & Sabel, 2013; Qi et al., 2018). Nervonic acid is important for white matter brain development and thus contributes to the central nervous system of infants

(Ntoumani et al., 2013), so the significance of variations in levels of this FA may need further investigation.

The changes in plasmin activities in a large cohort of human milk during lactation was measured in the study, because plasmin is the major active protease present in human milk (Dallas, Murray, & Gan, 2015; Ferranti et al., 2004). Notably, trypsin and thrombin, which have been detected in human milk, have similar specificities to plasmin (Dallas et al., 2015). However, thrombin does not interfere with the assay applied in our study (Richardson and Pearce, 1981) and levels of trypsin have been reported to be low in human milk (Monti, Mermoud, & Jolles, 1986). The levels of plasmin activities in term milk were in the range 0.045 - 0.14 nmol AMC units min^{-1} mL^{-1}, while the levels in preterm milk was slightly lower than previously reported by Armaforte et al. (2010) and Caldeo, Downey, O'Shea, et al. (2020) (0.27 – 0.43 nmol AMC units min^{-1} mL^{-1}), and the decreased trends of plasmin activities during lactation are in accordance with previous reports (Armaforte et al., 2010; Caldeo, Downey, O'Shea, et al., 2020). Although the longitudinal analysis of plasmin activities was similar to Caldeo, Downey, O'Shea, et al. (2020), a larger sample size was included in this study.

In addition, colour of human milk was measured in our study. L* correlated with fat and energy significantly, while b* correlated with fat, energy, and protein levels significantly ($p < 0.05$). Fat, present in human milk as milk fat globules and protein, partly present as casein micelles, both scatter light in the visible spectrum and thus are responsible for the whiteness, L*, of milk while carotenoids, the pigment present in milk fat, are responsible for yellowness, measured as b* value (Milovanovic et al., 2021; Owens, Brewer, & Rankin, 2001). From studies of bovine and ovine milk, levels of vitamins, for example, riboflavin (green pigment) have also been shown to contribute to the colour (Milovanovic et al., 2021; Solah et al., 2007). In human milk, levels of both carotenoids and riboflavin are diet-dependent, suggesting that the colour of milk is also influenced by diet (Lönnerdal, 1986; Zielinska, Hamulka, & Wesolowska, 2019). Measurement of milk colour may be a quick method to be applied in evaluation of human milk nutritional status and guide milk pooling in human milk bank in the future.

2.5 Conclusion

Changes in macronutrients, plasmin activities, and pH were monitored in a large cohort of term human milk over the first six months of lactation, as well as a small cohort of preterm human milk, up to six months after the term due date of preterm infants. A significant influence ($p < 0.05$) of lactation on levels of protein and plasmin activity was found. Infant gender and pre-pregnancy BMI influenced fat and energy contents of human milk. In addition, this study focused on the fatty acid profiles measured in a sub-group of term and preterm milk samples. There was significant influence ($p < 0.05$) of lactation, pre-pregnancy maternal BMI and infant gender on fatty acid profiles in term human milk. This study updates knowledge on the impact of human milk lactation, gestational age, pre-pregnancy maternal BMI, and infants' gender on human milk macronutrients, fatty acid profiles, protease activities, as well as milk pH and colour, providing an overall picture on human milk macronutrients from Irish mothers that can be useful in improving infant nutrition, for example, fortification or supplementation strategies for preterm infants in the future.

Supplementary Tables

Table S2.1 Diet information of mothers within 24 hours of each expression.

	Preterm		**Term**		
Lactational stage (week)	**-**	**1**	**4**	**8**	**24**
N	20	74	74	64	45
Pump/Manual (%Pump)	90	46	59	66	60
Express Time (%)					
0:00 - 6:00	5	7	9	6	0
6:00 - 12:00	20	38	27	39	49
12:00 - 18:00	35	24	32	23	20
18: 00 - 24:00	30	26	26	27	22
Unknown	5	5	5	5	9
Exclusive breast-feeding （% Exclusive)	75	82	74	73	56
Volume of formula per day if supply (mL)	80 -300	15-750	30 - 500	100-960	30-600
Oily fish (% Yes)	20	11	15	8	13
White fish (% Yes)	5	0	9	5	7
Red meat (% Yes)	4	66	49	56	47
Poultry (% Yes)	65	55	57	66	69
Vegetable oil (% Yes)	35	42	35	23	31
Olive Oil (% Yes)	70	46	46	48	49
Butter (% Yes)	85	80	91	80	67
Seeds (% Yes)	40	39	50	44	53
Nuts (% Yes)	48	35	39	42	29
Vegetables (% Yes)	100	96	93	94	91
Dairy Products (% Yes)	100	91	93	84	87
Bread (% Yes)	75	95	97	91	76
Beans, pulses (% Yes)	15	19	18	14	16
Supplements (% Yes)	40	55	57	59	39
Alcohol (% Yes)	0	5	4	6	5

Table S2.2 Summary of macronutrients, energy, pH and plasmin activity changes during the lactation.

		Levels of Macronutrients / Energy / pH / Plasmin Activities										***P*-value[a]**
Gestational/Postmenstrual Age (weeks)		<36	36-38	39-41		44		48		64		
Milk Type		PT	PT	PT	T	PT	T	PT	T	PT	T	T
Macronutrients												
N		8	12	16	69	3	65	4	51	3	40	
Mean (SD)	Protein (%)	1.6 (0.6)	1.3 (0.3)	1.1 (0.1)	1.7 (0.3)	1.0 (0.1)	1.3 (0.2)	1.0 (0.04)	1.2 (0.2)	0.9 (0.1)	1.0 (0.2)	<0.0001
	Fat (%)	3.7 (1.3)	3.9 (1.4)	4.2 (1.7)	3.3 (1.4)	4.1 (0.3)	3.8 (1.7)	4.9 (2.0)	3.4 (1.4)	5.7 (2.4)	3.0 (1.6)	0.067
	Carbohydrate (%)	6.9 (0.5)	7.1 (0.2)	7.1 (0.8)	6.8 (0.4)	6.9 (0.2)	6.9 (0.5)	6.7 (0.3)	7.0 (0.2)	6.8 (0.4)	6.9 (0.3)	0.547
	Energy (kcal dL^{-1})	69.1 (13.5)	70.2 (13.1)	71.6 (15.6)	66.2 (13.1)	69.2 (2.3)	68.0 (15.7)	76.1 (17.9)	65.4 (13.1)	83.7 (20.4)	60.0 (14.1)	0.022
pH												
N		7	13	11	38	4	37	3	38	3	32	
Mean (SD)		7.4 (0.2)	7.4 (0.2)	7.2 (0.4)	7.3 (0.2)	6.9 (0.6)	7.2 (0.4)	6.9 (0.3)	7.2 (0.5)	7.0 (0.4)	6.8 (1.3)	0.186
Plasmin activity (nmol AMC units min^{-1} mL^{-1})												
N		7	11	13	72	6	67	3	49	3	35	
Mean (SD)		0.116 (0.1)	0.066 (0.1)	0.019 (0.01)	0.069 (0.1)	0.015 (0.01)	0.028 (0.04)	0.034 (0.05)	0.017 (0.03)	0.006 (0.003)	0.026 (0.06)	0.001

Note: PT: Preterm milk; T: term milk. SD: standard deviation. [a]P-value for lactational effect in term milk.

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CHAPTER 3

Factors Affecting the Creaming of Human Milk

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This chapter was published in:

Meng, F., Uniacke-Lowe, T., Lanfranchi, E., Meehan, G., O'Shea, C. A., Fox, P. F., Huppertz, T., Ryan, C. A., & Kelly, A. L. (2020). Factors affecting the creaming of human milk. *International Dairy Journal, 108,* 104726.

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Fanyu Meng undertook all experimental work and drafted the manuscript.
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Abstract

The creaming properties of human milk have not been widely studied to date, and a mechanism for this phenomenon is not known. In this study, the natural creaming of human milk, as affected by temperature and pre-treatments, was studied using a dynamic light-scattering instrument. The creaming rate of human milk increased with temperature in the range 5°C to 40°C. Freezing human milk at -20°C and thawing at room temperature had little influence on creaming. Compared with bovine milk, human milk showed a faster creaming rate at 40°C, but a slower creaming rate at 5°C, suggesting a lack of cold agglutination. The mechanisms of creaming in milk from the two species were shown to differ also in response to heat treatment. This study expands the current knowledge on milk creaming in different species, and it may have potential application in storage and handling of human milk in hospitals or homes, therefore supporting optimal nutrition of infants.

3.1 Introduction

Human milk is essential as our first natural food, providing both nutrients and immunity to infants. Infants, especially preterm infants, who receive no or limited milk from their own mothers, can also be fed by human milk from donors as a substitute. Those milk donations are screened, received, pasteurized and stored in human milk banks (Hartmann et al., 2007). Moreover, working mothers may store their milk in the fridge (4°C) from 24 h to 8 d or in the freezer (-18°C) for even longer times (Hands, 2003; Weiss, 2005). Our initial observations showed rapid separation of human milk samples during the storage, however, little is known.

Natural creaming of milk occurs during storage because of the lower density of milk fat globules compared to milk serum, which leads to fat rising to the top under the influence of gravity. The most well studied subject of creaming has been bovine milk, since at least the work of Babcock (1889). Creaming properties of caprine (El-Ghannam, Attia, & Zeidanr 1986), buffalo (Abo-Elnaga, 1966), carabao (Gonzales-Janolino, 1968) and camel (Farah & Rüegg, 1991) milk have also been studied, and all of these showed much slower creaming rates than bovine milk at refrigeration temperature. Human milk has been reported to cream more slowly than bovine milk at body temperature (Whittlestone & Perrin, 1954), but overall the creaming of human milk and the factors affecting this process have not been extensively studied to date.

The creaming rate, $\boldsymbol{v}$, which is used to describe the creaming process in milk, is described by Stokes' Law (Walstra, 1995):

$$v = \frac{g(\rho_p - \rho_f)d^2}{18\eta_p} \qquad (1)$$

where g is acceleration due to gravity, d is the diameter of the fat globule, ρ_p and ρ_f are the densities of the plasma and fat, respectively, and η_p is the viscosity of the plasma.

However, applying Stokes' Law solely based on the size of individual milk fat globules cannot exactly predict the real creaming rate. For example, fresh bovine milk creams much faster than predicted by Stokes' Law at cold temperatures, which is explained by a phenomenon called cold agglutination, caused by the flocculation of fat globules (Sharp & Krukovsky, 1939). Cold agglutination is facilitated by agglutinins,

which attach to the milk fat globule membrane (MFGM) and cause fat globules to aggregate; these agents have been identified as Immunoglobulin M (IgM) (Euber & Brunner, 1984; Payens, Koops, & Mogot, 1965), and Immunoglobulin A (IgA) (D'Incecco et al., 2018; Honkanen-Buzalski & Sandholm, 1981) in bovine milk. A homogenisation-labile component, termed the skim milk membrane but now sometimes referred to as exosomes or extracellular vesicles (Benmoussa et al., 2017), was also reported to be involved in cold agglutination (Euber & Brunner, 1984). Other factors that have been reported to influence the rate of creaming of bovine milk include the presence of bacteria (Jenness, Shipe, & Sherbon, 1974) and somatic cells (Geer & Barbano, 2014), heating (>70 °C), and high-pressure treatment (≥400 MPa) (Huppertz, Fox, & Kelly, 2003).

Moreover, techniques for measuring creaming have improved. Traditionally, the creaming of milk was measured as the volume of cream produced from a specified volume of milk in a glass tube, of specific dimensions, at a stated temperature after certain time intervals, usually up to 24 h (Dunkley & Sommer, 1944; Euber & Brunner, 1984; Hammer, 1916; Kenyon, Jenness, & Anderson, 1966). More recently, the Turbiscan instrument has used to study the creaming behaviour of milk, based on multiple light-scattering principles; this gives information on creaming properties even when nothing is visible to the naked eye due to the opacity of milk samples, therefore removing the ambiguity of visual observation (Celia et al., 2009; Juliano et al., 2011).

In this study, the natural creaming properties of human milk under different temperature conditions were analysed using the Turbiscan stability analyser to fill the gap in knowledge in this area. The creaming properties of human milk were also compared to the creaming behaviour of bovine milk due to: (1) the widely studied bovine milk creaming; (2) the importance of ensuring homogenous milk for feeding; (3) the interest in the difference between human and bovine MFGs and (4) the mechanisms behind differences in physicochemical properties between them. In addition, the influence of different pre-treatments, such as freezing and pre-heating, which may be used in hospitals or milk banks to stabilise human milk, on the creaming were studied.

3.2 Materials & Methods

3.2.1 Human milk samples

Ethical approval for this study was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland, and fresh human milk samples (collected after the infant was satisfied, and hence being largely mid- to hind- milk) were collected from Cork University Maternity Hospital, Cork, Ireland. Full-term individual fresh milk samples from healthy mothers who were one week after birth (i.e., first week of lactation) were collected and used within 24 h. Fresh raw bovine milk was obtained from a local market. Both human and bovine milk were stored at refrigeration temperatures until the creaming analysis.

3.2.2 Compositional analysis

The fat, protein, lactose and total solids content of human milk was determined with a human milk analyser (MIRIS, Uppsala, Sweden) based on mid-infrared (mid-IR) transmission spectroscopy principles. Subsamples of fresh human milk (n=12) were collected for compositional analysis were frozen at -80°C, thawed at 4°C, warmed to 40°C and homogenized using a MIRIS sonicator (MIRIS, Uppsala, Sweden) before analysis. The fat, protein, lactose and total solids content of fresh bovine milk samples were measured using a Milkoscan FT 120 (Foss Electric, Hillerod, Denmark).

3.2.3 Size distribution of milk fat globules

The size distribution of milk fat globules (MFG) in human and bovine milk samples was determined with a Mastersizer 3000 laser particle size analyser (Malvern Instruments, Malvern, UK) equipped with a He–Ne laser (λ=633 nm). The refractive indices of the fat globule and of the dispersant (water) at 25°C were taken as 1.458 and 1.33, respectively, and the absorbance of fat globules was taken as 0.01 (Michalski, Briard, & Michel, 2001). The surface area mean diameter D[3,2], the volume-weighted mean diameter D[4,3], and the standard percentiles values, D_v10, D_v50 and D_v90 were measured in triplicate, as described earlier (Ménard et al., 2010).

3.2.4 Measurement of density of milk fat and viscosity of skimmed milk

Bovine milk fat was obtained by melting commercial butter and allowing to stand, followed by recovery of the upper fat layer. Human milk fat was obtained from a sample of donor milk which was agitated at high speed to destabilise the emulsion and recover a fat-rich phase, which was separated as for separation of bovine milk fat from butter to obtain a fat sample. The density of these human and bovine milk fat samples was determined using a pycnometer (SciLabware Ltd., Stoke-on-Trent, UK) at 20°C by following standard method ASTM D3505 (2018). To obtain skim milk, milk was centrifuged at 3000 g for 20 min at 5°C. Flow time of human skim milk, bovine skim milk, and pure water were measured by a U-shape glass viscometer (VWR International Ltd., Dublin, Ireland) at 5°C following standard method ASTM D445 (1997), and viscosities of human and bovine skim milk were calculated by:

$$\eta_{milk} = \frac{t_{milk}\,\rho_{milk}}{t_{H_2O}\,\rho_{H_2O}}\,\eta_{H_2O} \quad (2)$$

where t_{milk} and t_{H_2O} are the time (seconds) flow through the specific area of U-shape viscometer of milk and water, respectively; ρ_{milk} is the density of skimmed human (1.03×10^3 kg m^{-3}; Neville et al., 1988) or skimmed bovine milk (1.04×10^3 kg m^{-3}; Ma & Barbano, 2000) at 5°C; ρ_{H_2O} is the density of water at 5°C, which is 1.00×10^3 kg m^{-3} (Jones & Harris, 1992); η_{H_2O} is the viscosity of water at 5°C, which is 1.52 mPa s (Kestin, Sokolov, & Wakeham, 1978). Fresh milk samples were measured and each analysis was performed in triplicate.

3.2.5 Sample preparation for creaming analysis

For creaming analysis, five different temperatures, 5 (number of samples (n) =11), 20 (n=10), 37 (n=13), 40 (n=11) and 45°C (n=3) were selected in individual experiments for simulation of refrigeration, room temperature (RT), mammalian body temperature, and higher temperatures. Refrigerated milk samples were incubated in a water bath for 20 minutes to achieve the target temperature, and each sample (3 mL) was then inverted at least 10 times and placed in the Turbiscan immediately to make sure the back-scattering profile of the first scan was a flat line, and then scanned at each temperature for 10 hours. The time, 10 hours, was chosen as a compromise between the freshness and creaming

duration of the milk, and because, after this time, the peak thickness in cream layer of human milk reaches a plateau (as determined in preliminary work).

In the experiment with pre-heating, fresh milk samples were pre-heated in a water bath at 70 °C for 10 minutes. In a separate experiment, bovine whey protein isolate (WPI90, Carbery Group Ltd., Ballineen, Cork, Ireland) and immunoglobulins (Igs, isolated as described by McGrath, 2014), was added to human milk to achieve 1 g L^{-1} final Igs (average level of human and bovine Igs in milk). In a 'phase-reversal' experiment, human and bovine milk cream and serum were separated at 45°C, which can concentrate agglutinins from the whole milk to the milk serum layer, and recombined with the opposite fraction as described by Jennes & Parkash (1971).

3.2.6 Creaming analysis

The Turbiscan Lab^{TM} (Formulaction, Ramonville St. Agne, France) with an 800-nm near infrared (NIR) light source was used to study particle migration (creaming) in milk samples. The Turbiscan is based on multiple light scattering theory, where NIR photons are transmitted or back-scattered from the sample to transmission (T) or backscattering (BS) detectors (Carrentero et al., 2005).

Parameters quantified to describe creaming were the Turbiscan Stability Index (TSI), the Mean Value (of ΔBS, %), the peak thickness (ΔH), and the migration rate. The TSI was calculated by summing up the changes in BS at all measured positions, based on a scan-to-scan difference, over total sample height or a selected zone (Carrentero et al., 2005). In this study, the selected zone is the area where the cream layer forms; when the TSI value increases, the stability of the system decreases.

The Mean Value represents changes in the concentration and size of particles, i.e., signal variation of the ΔBS (%). It was calculated at the cream layer and for the bottom layer (lowest 20% of total height) of samples, and the ΔH measures the depth of the cream layer (represented by a peak in back-scatter data) which forms. A threshold value was set as 2% ΔBS. The slope of the curve of the ΔH as a function of time reflects the migration of the fat globules moving upwards within samples; the migration rate, calculated as the slope of the initial linear part of the ΔH plot, was taken to represent the creaming rate.

3.2.7 Statistical analysis

Data were analysed using Minitab® v18 (Minitab Inc., State College, PA, USA). Prior to analysis, data were tested for normality using the Anderson-Darling test. All TSI data, which were not normally distributed, were transformed according to a Box-Cox analysis. GLM ANOVA or one-way ANOVA followed by a paired multiple comparison test (Tukey's test) were used as appropriate. Human and bovine fat globule size distribution parameters were analysed using a 2-sample T-test. For all statistical analysis, the level of significance, α, was set at 0.05.

3.3 Results

3.3.1 Composition, size distribution and density of milk fat globules of human and bovine milk

The averaged composition of fresh human milk samples and fresh bovine milk samples tested in this study is reported in **Table 3.1** (details for the composition of milk samples from individual mothers are shown in **Table S3.1**). Compared with fresh bovine milk, human milk had lower levels of lipids and protein, but a higher level of carbohydrate and solids-not-fat content.

Table 3.1 Levels of macronutrients in human milk (n=12) and bovine milk (n=4) samples. Results are presented as mean and standard deviation (SD).

Type of milk	Fat ($g\ L^{-1}$)	Crude protein ($g\ L^{-1}$)	Carbohydrate ($g\ L^{-1}$)	Total solids ($g\ L^{-1}$)
Human	31 (1.04)	16 (0.18)	69 (0.25)	119 (1.16)
Bovine	40 (0.31)	35 (0.27)	45 (0.05)	130 (0.55)

Fat globules in human milk had a lower average D[3,2] (2.3 ± 0.3 μm) and a higher average D[4,3] (4.1 ± 0.9 μm) than those in bovine milk (2.4 ± 0.5 μm and 3.7 ± 0.3μm, respectively) (**Table 3.2**). In case of the size distribution of human MFGs, 10% of particles were below 1.1 ± 0.3 μm, and 50% of particles below 3.5 ± 0.7 μm, both of which are lower values than the values presented for bovine milk. However, the D_v90 of

human MFGs (7.1 ± 1.6 μm) was higher than that in bovine milk (6.6 ± 0.36 μm). Statistical analysis of human and bovine MFG size distribution parameters showed no significant difference ($p > 0.05$) between the parameters measured for the two types of milk.

The densities of human and bovine milk fat at 20°C were $0.88 \times 10^3 \pm 0.46$ kg m^{-3} and $0.88 \times 10^3 \pm 0.61$ kg m^{-3}, respectively. The viscosity of skimmed human milk at 5°C was measured as 2.53 ± 0.08 mPa s, which was lower than that of skimmed bovine milk at 5°C (3.45 ± 0.02 mPa s).

Table 3.2 Particle size distribution parameters of milk fat globules. Results are presented as mean (SD).

Size distribution parameters	Human milk (μm) (n=7)	Raw bovine milk (μm) (n=3)
D [3,2]	2.27 (0.33)	2.35 (0.47)
D [4,3]	4.11 (0.92)	3.69 (0.32)
Dv10	1.09 (0.32)	0.97 (0.12)
Dv50	3.53 (0.74)	3.63 (0.32)
Dv90	7.10 (1.56)	6.64 (0.36)

3.3.2 Creaming profiles of human and bovine milk at different temperatures

The formation of the layer of fat droplets (creaming) is illustrated in the back-scattering profiles of the samples (**Fig. S3.1**) as an increase in ΔBS at the top of samples over time, while a decrease of the back-scattering is indicative of clarification in the middle and lower phases of samples. As calculated from the backscattering profiles, the TSI values of cream layers (TSI_{cream}) in both types of milk increased with time (**Fig. 3.1**). In human milk, the TSI_{cream} increased with temperature from 5 to 40°C, and there were significant differences ($P < 0.05$) between TSI_{cream} at 5°C and TSI_{cream} at 40°C at each time point.

Bovine milk held at 5°C had a lower TSI_{cream}, i.e., a higher stability, than that at 40°C during the first hour; however, instability increased dramatically between 1 and 4 h of measurement. The average TSI_{cream} values of human milk were lower at 5°C but higher at 40°C, compared to those of bovine milk at each time point during 10 h. However, no significant difference ($P > 0.05$) of TSI_{cream} was found between bovine and human milk at 10 h at either temperature.

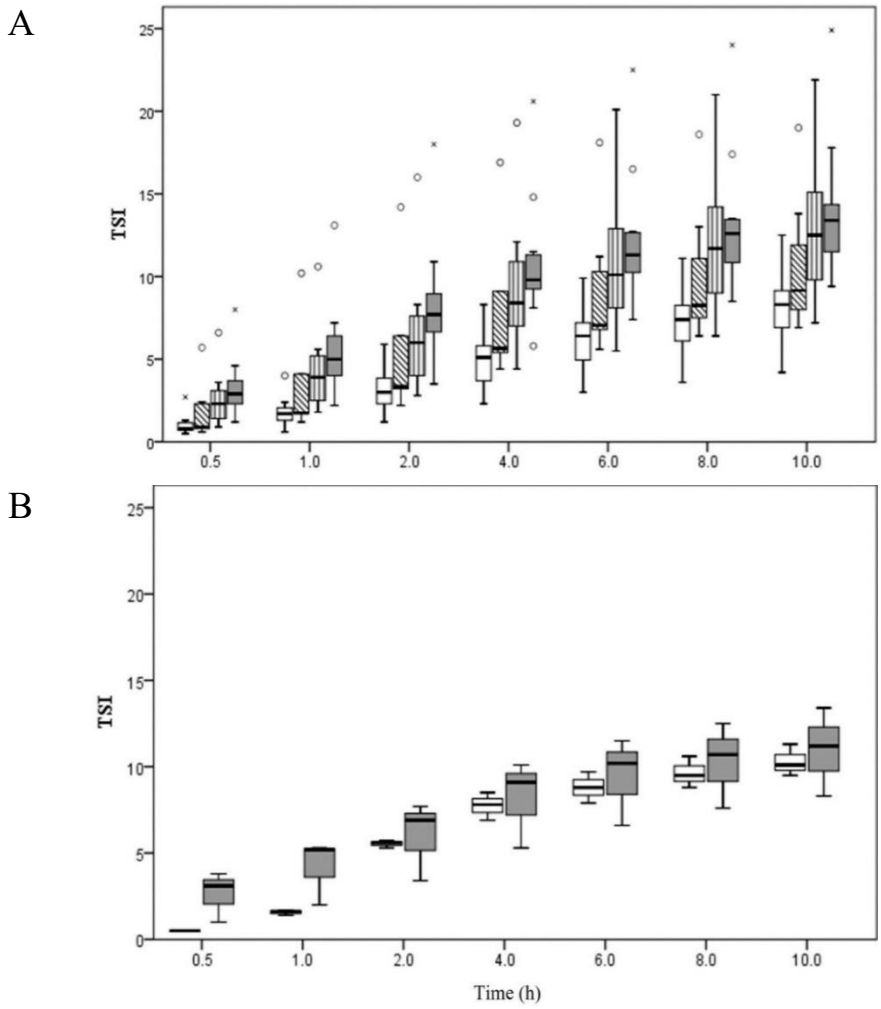

Fig. 3.1 Turbiscan stability index (TSI) distribution of the cream layer for fresh human milk (A) and fresh bovine milk (B) at 5°C (white), 20°C (backslashes), 37°C (vertical lines), and 40°C (grey) as a function of time. Horizontal lines inside boxes indicate the median value. Lines extending vertically from the boxes indicate variability outside the upper and lower quartiles. Mild outliers (○, values which lie between 1.5 - 3 times the interquartile range below the first quartile or above the third quartile) and extreme outliers (*, values which lie more than 3 times the interquartile range below the first quartile or above the third quartile) are indicated.

The Mean Value of ΔBS in cream layer (MV_{cream}), which can be estimated from ΔBS profiles (**Fig. S3.2**), increased with temperature for human milk (**Fig. 3.2** A), i.e.,

the higher the temperature, the more rapidly the fat globules concentrated in the human milk cream layer. The averages of MV_{cream} for human milk ranged from 9.5% to 16.3% after 10 hours from 5°C to 40°C. The average MV_{cream} for bovine milk after the same time at 5°C and 40°C were similar to each other (**Fig. 3.2** B). However, the time for bovine cream to reach the maximum ΔBS decreased from 6 h at 5°C to 3 h at 40°C. The rate of increase in ΔBS for milk from both species started to decrease after around 2 h creaming at all temperatures; however, this decrease in rate was more significant for bovine milk than for human milk.

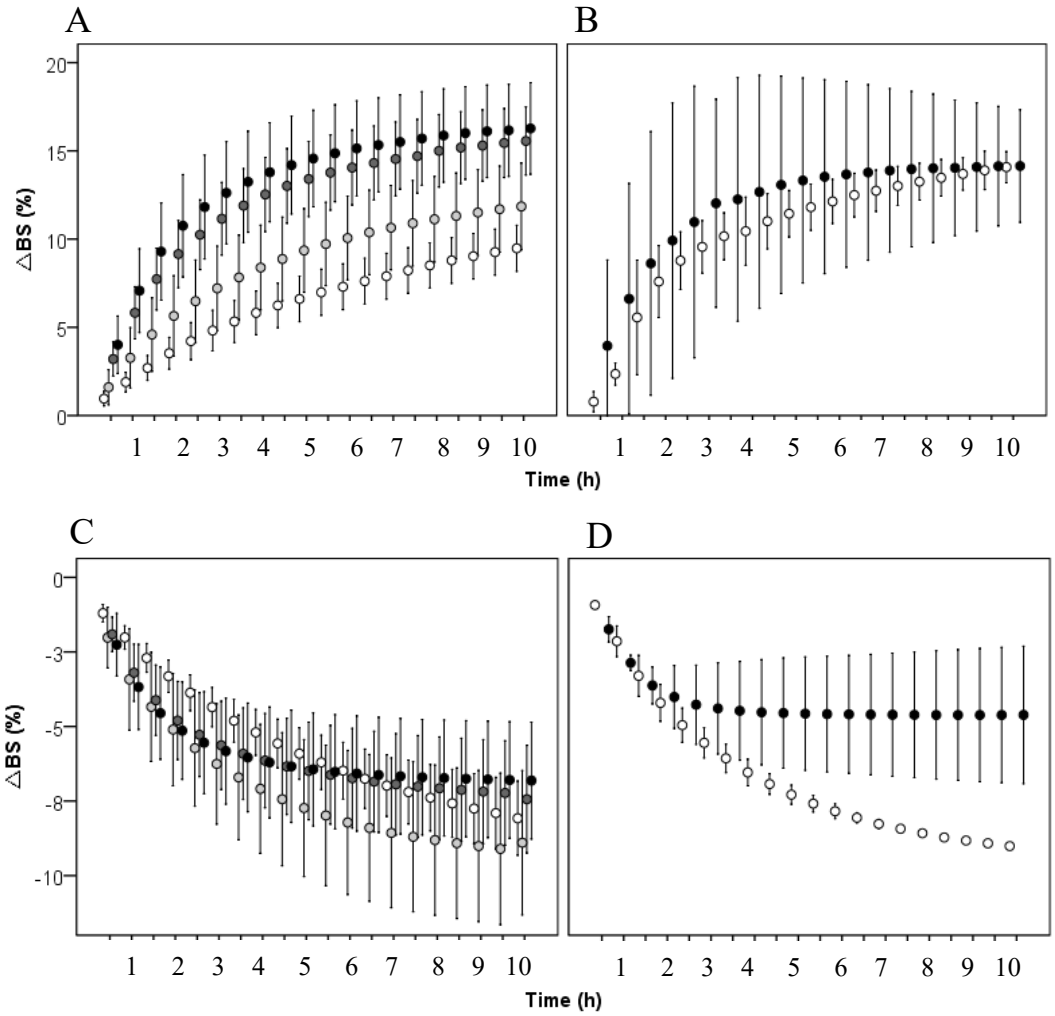

Fig. 3.2 Mean Value of delta backscattering (%) of (A, B) cream layer for fresh human milk and the bottom layer (C, D) and cream layer for fresh human milk (A, C) and fresh bovine milk (B, D) at different temperatures as a function of time. Temperature are 5°C (white), 20°C (light grey), 37°C (dark grey), and 40°C (black). Data points correspond to average Mean Value and error bars show the 95% confidence interval (CI).

The Mean Value in the bottom layers (MV_{bottom}) of the milk samples decreased from over time due to the fat globules moving upwards (**Fig. 3.2** C & D). In the bottom layer, human milk held at 20, 37, and 40°C showed similar initial behaviour, while milk held at

5°C had a lower initial rate of the MV_{bottom} curve. Interestingly, human milk held at 20°C had a higher decrease of MV_{bottom}, i.e., a more complete clarification, compared with that at 5°C. Moreover, in the bottom layer of both human and bovine milk, the clarification was more extensive at 5°C than at 40°C.

The peak thickness (ΔH) of human milk cream layer varies due to creaming temperature at different time points. As seen from **Table 3.3**, there were significant differences ($P < 0.05$) in ΔH among temperatures at 0.5 and 4 h, whereas there was no significant difference ($P > 0.05$) in ΔH among temperatures at 10 h creaming. Compared with bovine milk (**Fig. 3.3**), ΔH values of human milk cream increased steadily to a maximum at the end of 10 h, whereas it reached a plateau for bovine milk after 2 h at 5°C. There was a lag phase for bovine milk cream formation at 5°C, which was not seen 40°C, while human milk started to cream earlier than bovine milk at both 5°C and 40°C. The final ΔH value of the human milk cream layer at 5°C was lower than that at 40°C, whereas it was notably higher for bovine milk at 5°C than at 40°C.

Table 3.3 Peak thickness (ΔH; in mm) of human milk cream layer at different temperatures and time points. Results are presented as mean (SD); N is the number of samples at each temperature. Different letters (a, b, c) indicate statistically significant difference at $P < 0.05$ between temperatures within each column.

Temperature (°C)	**N**	**Creaming Time (h)**		
		0.5	4	10
5	11	0.50 (0.37)^{a}	1.73 (0.50)^{a}	0.84 (0.82) ^{a}
20	10	0.76 (0.63)^{b}	2.22 (0.90)^{ab}	1.65 (1.66) ^{a}
37	13	0.94 (1.61)^{bc}	2.36 (0.76)^{ab}	1.78 (1.38) ^{a}
40	11	1.17 (0.38)^{bc}	2.57 (0.53)^{ab}	1.97 (1.59) ^{a}
45	3	2.38 (0.65)^{c}	3.28 (1.10)^{b}	3.29 (0.98) ^{a}

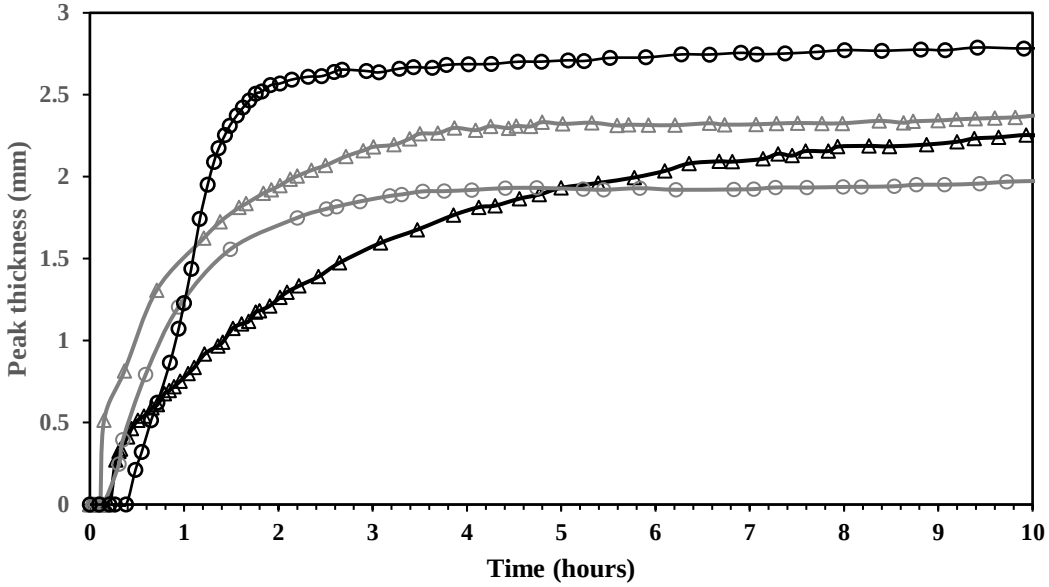

Fig. 3.3 Peak thickness of the cream layer for fresh human milk (△) and fresh bovine milk (○) at 5°C (black) and 40°C (grey), respectively. Peak thickness profiles are from individual samples but are representative of data from others.

The average creaming rate, i.e., the migration rate of MFGs, or clusters thereof, of human milk increased with temperature (**Table 3.4**); there was a significant difference ($P < 0.05$) between rates at 5°C and 40°C. The rate was lower than that of bovine milk at 5°C, whereas it was higher than that of bovine milk at 40°C, suggesting different mechanisms of creaming in the milk of the two species. However, there was no significant difference ($P > 0.05$) in creaming rate between bovine and human milk at 5 °C or at 40 °C.

Table 3.4 Creaming rates (mm h^{-1}) measured in the cream layer and the clarification layer (See **Fig. S 3.1** zone r_2) of human and bovine milk.

Milk Type	Temperature (°C)	N	Migration rates in creaming zones Mean (SD)	Migration rates in clarification zones Mean (SD)
Bovine	5	3	2.37 (1.45)	1.34 (0.58)
	40	3	2.38 (0.52)	2.69 (0.27)
Human	5	11	1.25 (0.95)	0.61 (0.14)
	20	10	1.81 (1.85)	1.46 (1.80)
	37	13	2.27 (1.61)	2.18 (1.31)
	40	11	3.34 (2.11)	3.33 (3.00)
	45	3	6.06 (1.69)	6.88 (3.82)

3.3.3 Influence of freezing-thawing and preheating on creaming

The TSI cream values of individual human milk samples after different pre-treatments as a function of time are shown in **Fig. 3.4**. Refrigeration for 1 week resulted in a lower TSI_{cream} at each time point, whereas a 9-month frozen and thawed milk had a higher TSI_{cream} at each time point. As seen in **Fig. 3.4**, short-term frozen storage (one week) has less impact on the physical stability of human milk than refrigerated storage and longer-term frozen storage. However, no significant difference ($P > 0.05$) was found among TSI_{cream} values for fresh milk, one-week refrigerated milk, one-week frozen milk, and 9-mo nth frozen milk at all time points.

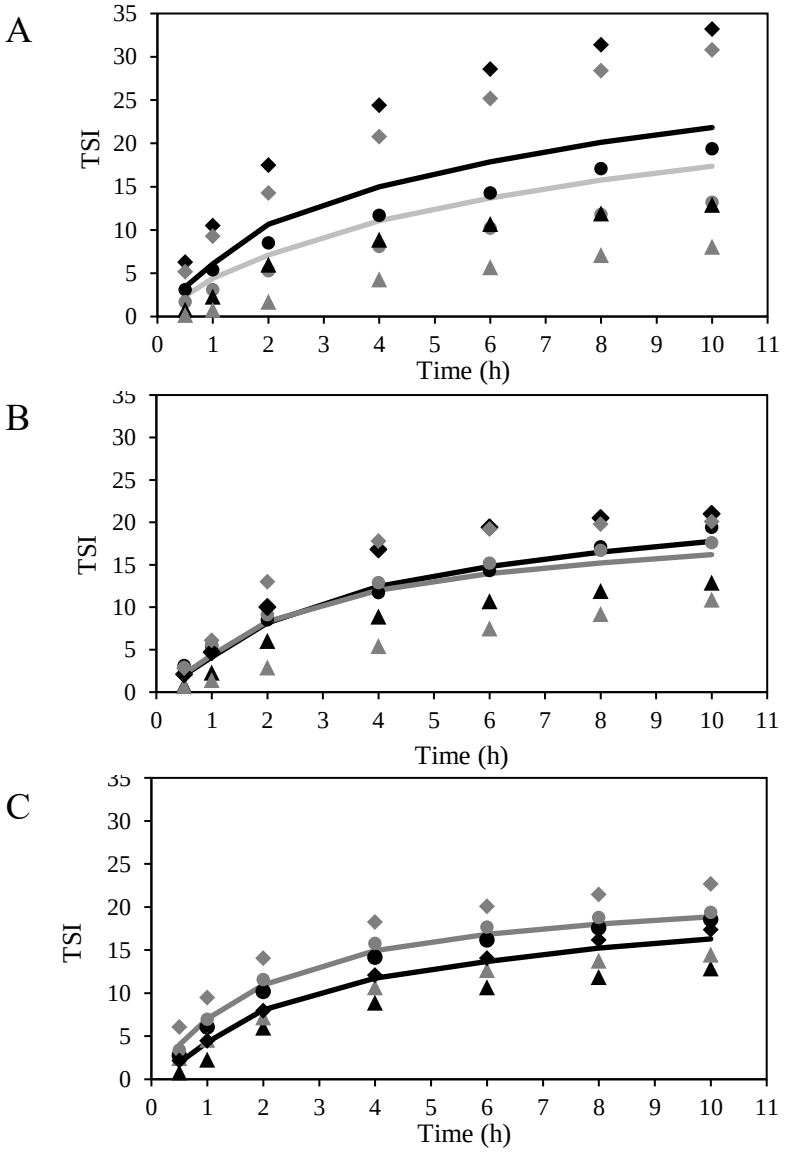

Fig. 3.4 TSI profiles of human milk cream layer for milk stored at (A) 4°C for 1 week, (B) milk frozen at -20°C for 1 week (thawed at RT), and (C) milk frozen at -20°C for 9 months (thawed at RT) compared to fresh human milk before treatments. The TSI_{cream} before treatments (black) and the TSI_{cream} after treatments (grey) are shown. The solid lines are averages of the TSI_{cream} before and after treatments, and ♦, ▲, ● represent three individual samples in each treatment.

The effect of preheating on human milk creaming was also studied. Compared to the control, the TSI of human milk cream layer decreased significantly ($P < 0.05$) after preheating **(Fig. S3.3)**. The final peak thickness of the cream layer was lower after preheating at 70°C for 10 min; combined with the fact that △BS also decreased significantly ($P < 0.05$) compared with the control, which suggests that preheating human milk led to impaired creaming capacity.

3.3.4 Mechanistic studies of creaming of human milk

When human and bovine milk were mixed in a ratio of 1:1 (v/v), the mixture behaved in a manner closer to bovine than human milk (**Fig. 3.5** A), with a lag in time before creaming commenced as observed above, but then the migration rate increased rapidly. When the cream from human milk was mixed with bovine skimmed milk, and *vice versa*, the recombined milk prepared by mixing human cream and bovine skim milk had higher TSI_{cream} (from 10 to 50 min, **Fig. 3.5** B), MV_{cream} (0 to 7 h, **Fig. 3.5** C), final △H of the cream layer, and initial creaming rate at each time point, compared to fresh bovine milk, fresh human milk, or milk recombined from skimmed human milk and bovine cream (**Fig. 3.5** D).

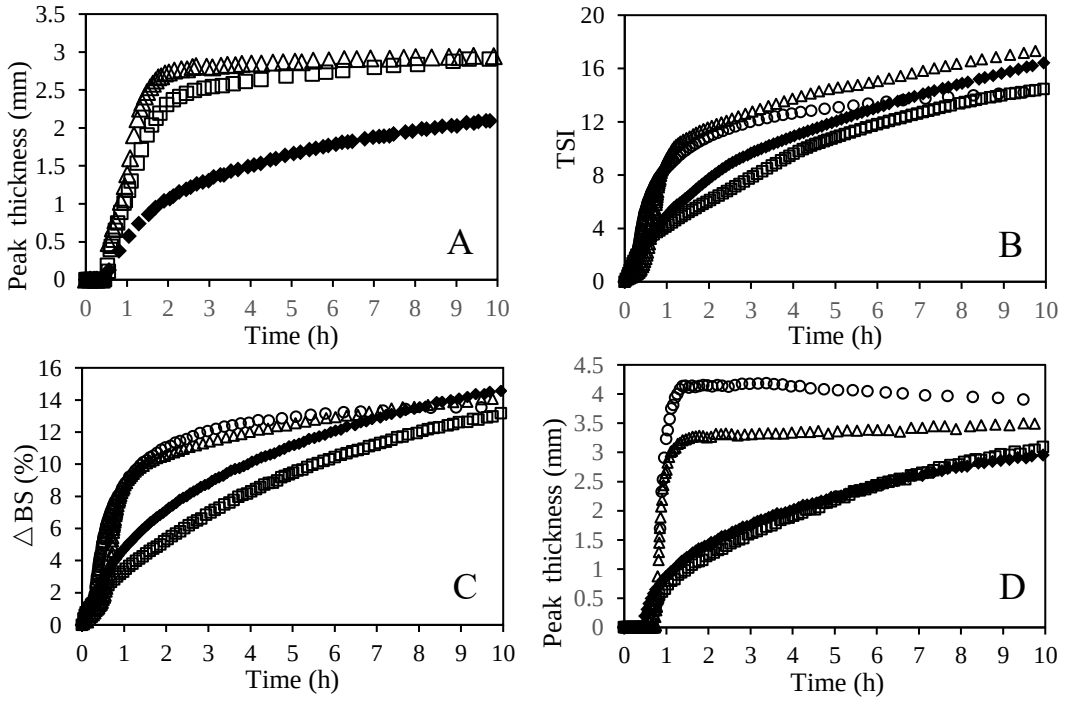

Fig. 3.5 The peak thickness profiles (A) of cream layer for fresh human milk (♦), fresh bovine milk (Δ), and mixed bovine and human milk (1:1 v/v; □) and the TSI (B), Mean Value (C), and peak thickness (D) profiles of cream layer for fresh human milk, fresh bovine milk, recombined milk of human cream and bovine serum (○), and recombined milk of bovine cream and human serum (□) held at 5℃, as a function of time. The profile is from an individual sample but is representative of data from others.

After the addition of bovine WPI (0.83% w/v) to human milk, both the peak thickness and the mean △BS increased slightly. The initial creaming rates of human milk also increased after addition of both bovine WPI and bovine Igs, and the addition of bovine Igs had a higher impact on human milk creaming than the addition of WPI. However, the increases due to addition of bovine WPI and Igs were not significant ($P > 0.05$) in the case of TSI_{cream} (**Fig. S3.4**) at each time point.

3.4 Discussion

To our knowledge, this is the first detailed study on creaming behaviour of human milk, which contributes to knowledge of physico-chemical properties of human milk, specifically its creaming and cold agglutination behaviour. A similar or even higher creaming rate of human milk than that of bovine milk at warm temperature (40°C) is inconsistent with the only report on human milk creaming, which suggested that the rate of creaming of human milk was lower than that in bovine milk at 37°C (Whittlestone & Perrin, 1954). This inconsistency may be due to the high individual variation of human milk samples, more advanced analytical methodologies, and data coming from a larger sample size in our study. In this study, fresh human milk samples were obtained in the early stages of lactation (one-week *post-partum*), when milk contains a lower fat content and a higher protein content than in mature human milk (Gidrewicz & Fenton, 2014). A lactational effect was noticed and samples at 8- and 24-weeks *post-partum* appear to have higher values of peak thickness than samples at 1-week *post-partum* (data not shown). Mid- to hind-milk, which was collected after the babies have been satisfied in this study, may contain a higher level of fat than foremilk (Mizuno et al., 2009); however, the milk fat globule size is not expected to be significantly different (Mizuno et al., 2009). The content of macronutrients of human and bovine milk measured was, however, consistent with the literature values (Anderson, Atkinson, & Bryan, 1981; Bauer & Gerss, 2011; Gidrewicz & Fenton, 2014; Guo & Hendricks, 2008). The lower average fat content and a higher average solids-not-fat content in human milk than in bovine milk samples may slow fat separation in the milk (Webb & Hall, 1935), and may partly explain the observed slower creaming rate of human milk than bovine milk at 5°C.

The range of MFG size of human and bovine milk was similar to those reported by Rüegg & Blanc (1981), although some studies reported a higher MFG size of human milk than bovine milk (Lopez, Cauty & Guyomarc'h. 2015; Ma et al., 2019). It has previously been noted that the human milk expressed from the fourth day to the first month of lactation, as for samples in this study, can have a lower average size than that of colostrum or mature milk from the first month until the end of lactation (Michalski et al., 2005).

According to Stokes' Law, the creaming rate rises when the diameter of MFG increases. A small extent of fat globule clustering, which increases the effective diameter of MFGs, was found in human milk at 5°C, as the average MFG size of human milk cream layer decreased after dilution with 1% SDS (**Table S3.2**), which has also been reported in bovine cream (Boode, 1992; D'Incecco et al., 2018).

Considering the average diameter D[3,2] of human MFGs during the first week of lactation (2.27 μm) and the average of bovine MFG diameter (2.35 μm), the creaming rate for individual MFGs can be predicted by Stokes' Law (Equation (1)). In this estimation, it is assumed that densities of human and bovine milk serum at 5°C are 1.03 × 10^3 kg m^{-3} and 1.04 × 10^3 kg m^{-3}, respectively (Neville et al., 1988; Ma & Barbano, 2000), and, based on measurements in this study, densities of human and bovine milk fat are both 0.88 × 10^3 kg m^{-3}, and viscosities of human and bovine milk at 5°C are 2.53 mPa s and 3.45 mPa s, respectively. On this basis, the calculated creaming rates of human and bovine milk are 0.60 mm h^{-1} and 0.51 mm h^{-1}, respectively. The predicted creaming rates for human and bovine milk are thus both lower than the measured averages (0.84 mm h^{-1} for human milk and 1.75 mm h^{-1} for bovine milk) at 5°C, especially for bovine milk, which may reflect the occurrence of cold agglutination in bovine milk.

IgM and IgA are reported as major agglutinins to accelerate bovine milk creaming during cold storage (D'Incecco et al., 2018; Hansen, Larsen, & Wiking, 2019), but remain in skim milk after separation at 45°C (Jennes & Parkash, 1971). Although the protein content of human milk was lower than that of bovine milk, human milk is reported to contain a higher percentage of Igs (approximately 16% of total whey proteins) than bovine milk (approximately 10% of total whey proteins; Guo & Hendricks, 2008). Also, human milk has IgA-dominated Igs, whereas bovine milk Igs are IgG-dominated (Hurley & Theil, 2011). The level of IgA has been reported to be 0.13 kg m^{-3} and 1 kg m^{-3} for bovine and human milk, respectively, whereas the level of IgM is 0.04 kg m^{-3} and 0.1 kg m^{-3} for bovine and human milk, respectively (Hurley, 2003), i.e., human milk contains higher levels of agglutinins (IgA and IgM). However, the cream volume and the creaming rate of human milk at 5°C were lower than that of bovine milk (**Fig. 3.3** and **Table 3.4**), which may result from a higher extent of agglutinins being associated with bovine milk fat globules than human milk. This is supported by the fact that the creaming ability of human milk was increased after mixing with bovine skim milk.

There are two possible reasons why human milk contains high levels of agglutinins, but has a lower creaming rate than bovine milk at 5°C. Firstly, although hydrophobic interactions will be weakened at low temperatures, bovine milk has been reported to have the most hydrophobic secretory IgA among species, including humans, which can result

in a higher level of bovine IgA associated with fat globules (Honkanen-Buzalski & Sandholm, 1981). Secondly, competition between soluble antigens and MFGM antigens to capture IgM may occur in human milk (Euber & Brunner, 1984). It is known that the interaction of IgM with MFG is specific and involves carbohydrate moieties (Euber & Brunner, 1984); therefore, milk oligosaccharides and κ-casein molecules containing carbohydrate moieties may be involved in the interaction of IgM. The oligosaccharides have not been considered as a factor in bovine milk due to their low concentration (Euber & Brunner, 1984), but should be considered in human milk due to their higher levels (Kunz & Rudloff, 1993). Moreover, the content of sialic acid, which has been demonstrated to inhibit milk creaming (Euber & Brunner, 1984), is much higher in human milk than in bovine milk (Wang & Brand-Miller, 2003; Wang et al., 2001). Further research on the reasons behind the difference in creaming behaviour between bovine and human milk appears warranted.

Human milk was stable following short-term freezing, which is consistent with an unchanged size distribution of human MFG before and after freezing (data not shown). It has been reported that no significant change in concentration occurs for IgA, the major agglutinin in mature human milk, at -20°C for 1 month (Evans et al., 1978) or 3 months (Reynolds et al., 1982). In contrast, a decrease in IgA level after 4 weeks at -20°C has also been reported (Akinbi et al., 2010), and Ramírez-Santana et al. (2012) reported a decrease in IgA concentration in human colostrum when the frozen storage period was increased to 12 months, indicating that IgA can be temperature liable. Moreover, although the MFG can be resistant to lipolysis as long as its structure remains intact, slow freezing or long-term freezing can lead to the destruction of MFGM and allow access of lipases to the core of MFG, i.e., to triglycerides (Berkow et al., 1984; Munkwitz, Berry, & Boyer, 1933).

Pre-heating human milk at 70°C for 10 min slowed cream separation and decreased the volume fraction of milk fat in cream layer, which has also been found for bovine milk (Caplan, Melilli, & Barbano, 2013). One possible reason could be that heat treatment increased the viscosity of the serum phase and thereby reduced the rate of creaming. Also, it has been hypothesized that heat-induced denaturation of proteins can result in interactions with milk fat globules and therefore increase their density (Caplan et al., 2013). A thinner cream layer can be thus formed after preheating (**Fig. S3.3**), shown as a lower $\triangle H$ of cream layer in preheated human milk than the untreated sample.

This human milk creaming study can be applied to remixing practices when milk is separated and to maintain the quality of milk by ensuring correct levels of fat during

human milk handling. Moreover, it may be applied to separate human milk skim layer and cream layer when they are specifically required as fortifiers or supplements for infants.

3.5 Conclusion

The creaming behaviour of human milk was affected by temperature and pre-treatments, including freezing-thawing and preheating. The creaming capacity of human milk was correlated with the temperature in the range 5 to 40°C. Short-term freezing-thawing had no effect on creaming while heating at 70°C for 10 min slowed the creaming of human milk. Similarities and differences of human and bovine milk creaming behaviour at low temperature are discussed, and may be caused by the fat content, the properties of MFGM, the size of MFG, the presence of agglutinins (IgA and IgM), and competition for attachment of agglutinins in milk serum. This study provides the information for both mothers and human milk banks in terms of handling and storing human milk and feeding infants with optimal methods in terms of ensuring proportionate levels of fat alongside other constituents.

Supplementary Tables and Figures

Table S3.1 Composition of human milk from individual mothers. Results presented are the averages of duplicate measurements.

Samples	Fat ($g\ L^{-1}$)	Protein ($g\ L^{-1}$)	Carbohydrate ($g\ L^{-1}$)	Total solids ($g\ L^{-1}$)
HM001	37	16	70	125
HM002	53	19	69	142
HM003	41	15	75	134
HM004	24	15	74	115
HM005	26	17	68	113
HM006	26	15	68	113
HM007	24	16	73	114
HM008	32	18	67	120
HM009	27	16	69	114
HM010	41	18	69	130
HM011	13	14	65	93
HM012	32	13	70	117

Table S3.2 The size distribution of fat globules in the human milk cream layer after 24 hours creaming at 5 °C. Results are presented as average (SD). The average size decreased after dilution with 1% SDS, indicating that flocculation occurred during human milk creaming.

	Dx (10)	Dx (50)	Dx (90)	D [4,3]	Dx (75)	Span	D [3,2]
Untreated	4.00 (0.02)	6.87 (0.12)	11.40 (0.36)	7.33 (0.16)	9.12 (0.22)	1.08 (0.03)	6.29 (0.09)
Diluted in 1% SDS	3.39 (0.03)	5.90 (0.07)	10.33 (0.06)	6.42 (0.04)	8.08 (0.08)	1.17 (0.01)	5.43 (0.05)

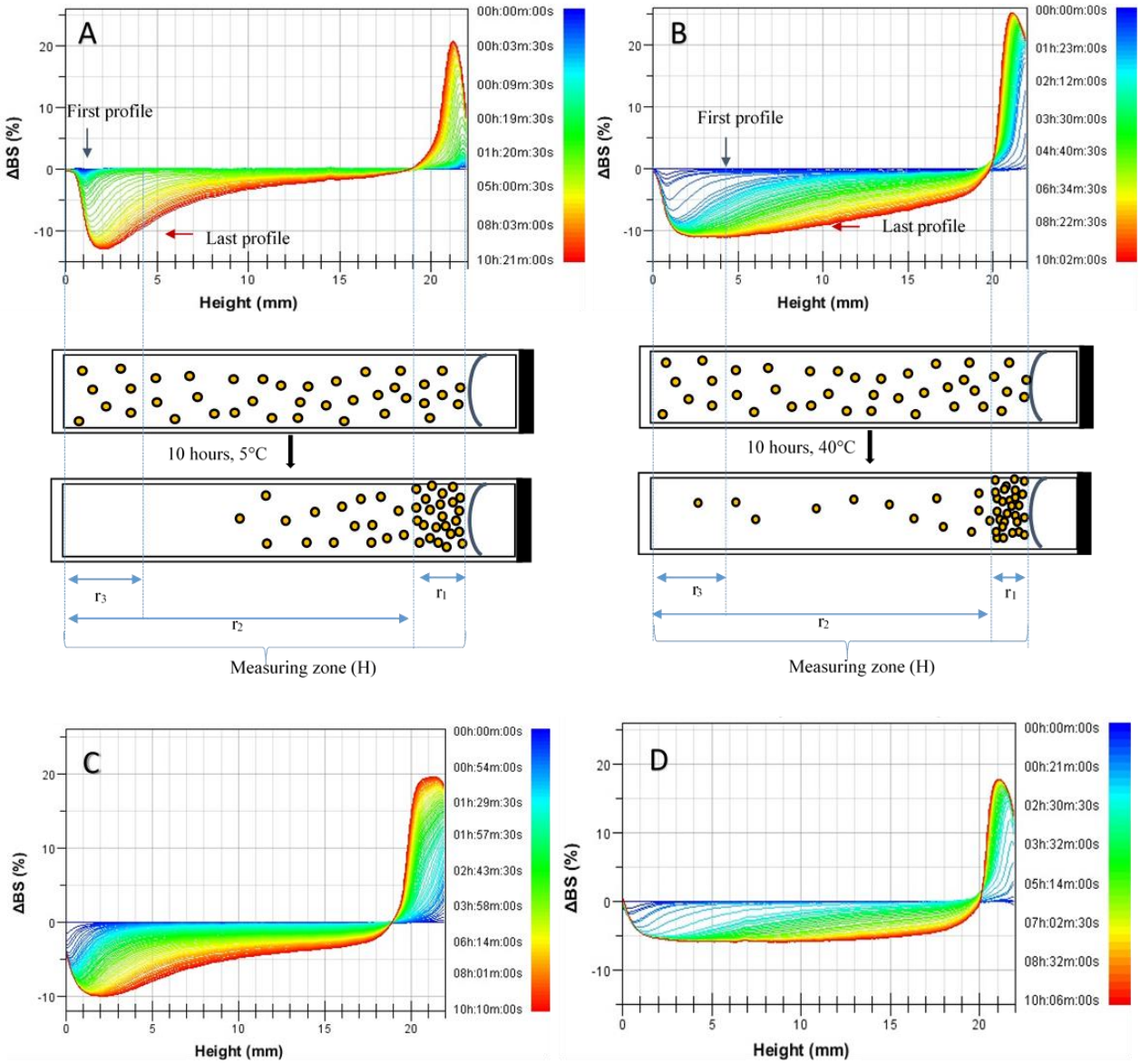

Fig. S3.1 Delta backscattering (△BS) profile (backscattering of near-infrared light through sample cell as a function of height using the first scan as reference scan) for (A) fresh human milk at 5°C, (B) fresh human milk at 40°C, (C) fresh bovine milk at 5°C, and (D) fresh bovine milk at 40°C. Samples were scanned every 30 seconds; however, only the most significant scans were kept by software after 10 hours scanning. The colours of the curves correspond to the time on the second Y axis. Calculation regions of creaming (r_1), clarification (r_2), and bottom (r_3, 1/5 H) are indicated with blue broken lines. Schematic illustrations of the influence of temperature on the profiles are shown for the first (0 s, stable dispersion) and the last (10 h, phase separation) profiles. Creaming profiles are from individual samples but are representative of data from multiple independent samples.

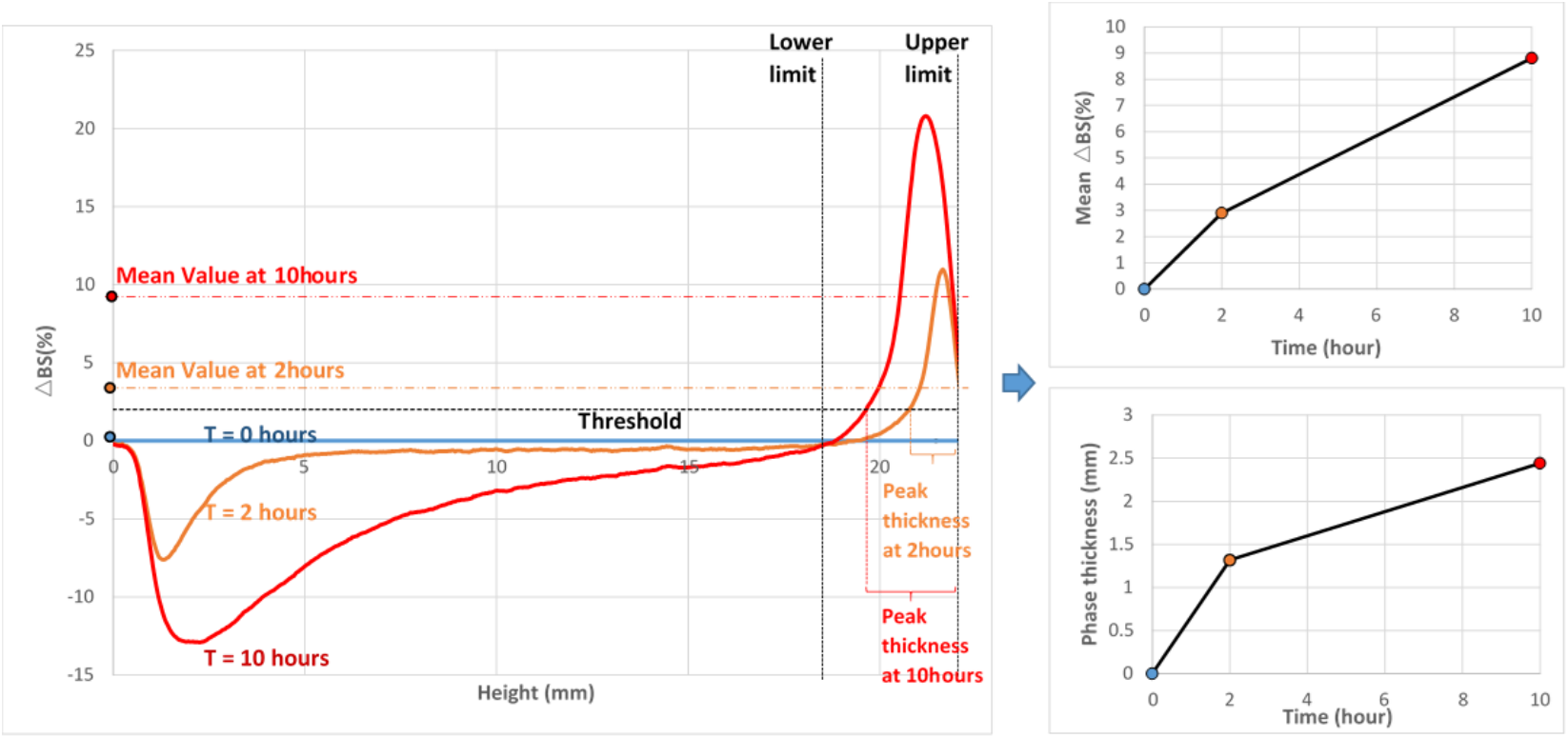

Fig. S3.2 Conversion of △BS profile (A) to Mean Value profile (B) and peak thickness profile (C) of cream layer as a function of time. △BS profile of human milk at time 0 (reference scan; blue line), 2 hours (orange line) and 10 hours (red line) are shown. To convert to Mean Value (of △BS) profiles, lower and upper limits were set to define the cream layer (or other zones). Mean Value of cream layer as a function of time was then computed by Turbiscan software (B). The thickness of the cream layer, starting where the backscattering value is greater than 2% (absolute) in comparison to the baseline, and the threshold are indicated with the broken black line. The peak thickness (cream layer) as a function of time (C) was computed by Turbiscan software automatically.

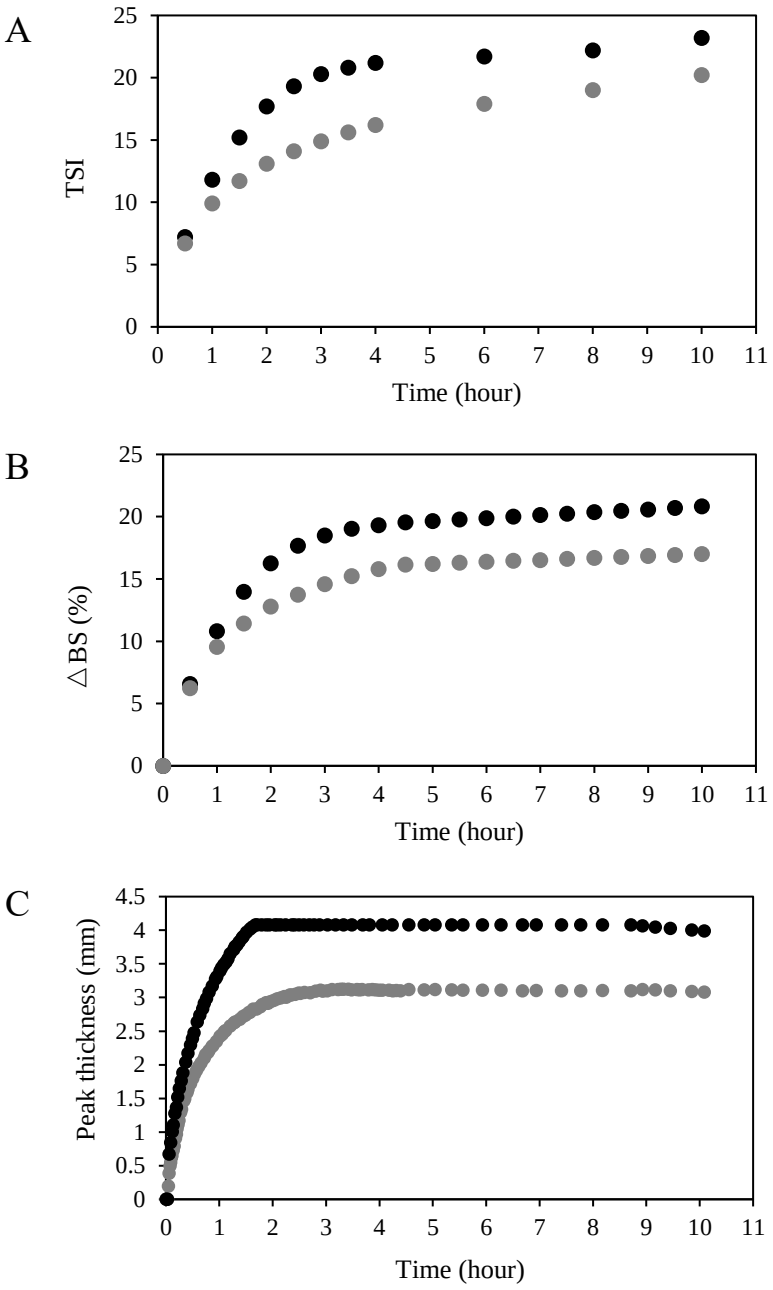

Fig. S3.3 The TSI (A), Mean Value (B), and peak thickness (C) profiles of human milk cream layer before (black) and after preheating at 70℃ for 10 minutes (grey) as a function of time. The profile is from an individual sample but is representative of data from others.

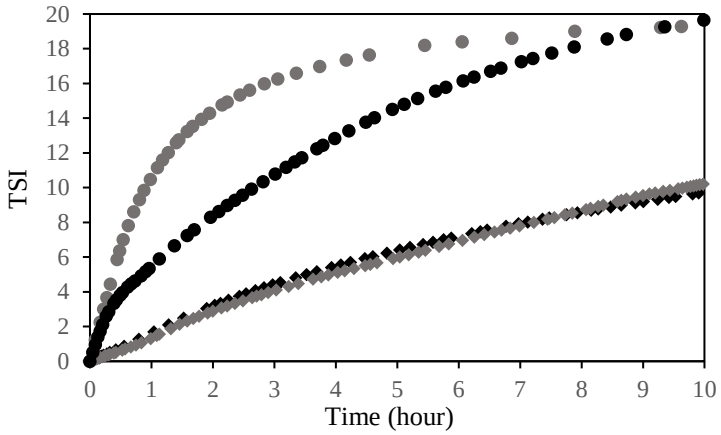

Fig. S3.4 TSI values of cream layer of fresh human milk (black) and treated human milk (grey) with added bovine WPI (♦) and human milk with added bovine Igs (●) held at 5°C. TSI profiles are from individual samples but are representative of data from multiple experiments.

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CHAPTER 4

Factors Affecting the Creaming of Raw Bovine milk: Comparison between Natural and Accelerated Methods

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Fanyu Meng undertook all experimental work and drafted the manuscript.
Therese Uniacke-Lowe and Alan Kelly critically reviewed the manuscript.

Abstract

The objective of this study was to study the effects of heating, addition of calcium-chelating agents, sonication, incubation, and freezing on the creaming of bovine milk. Both accelerated centrifugal and gravitational creaming methods were used to examine the creaming profile of bovine milk at 20°C; gravitational creaming was also examined at 5°C. The creaming rate of milk slightly decreased following heating at 70, 75, 80, or 85°C for 10 min, but the change was not significant ($p > 0.05$). Proteolysis during incubation or adding trisodium citrate enhanced creaming rate. The flocculation of fat globules occurred in the cream layer after 24-hour gravitational creaming, especially in sonicated milk and milk heated at 80 or 85°C. Measuring creaming using either accelerated or gravitational creaming techniques gave similar broad information on creaming rate; however, mechanistic insights provided differed.

4.1 Introduction

Raw milk, left to stand undisturbed, readily separates into a cream layer and a fat-depleted skimmed milk phase. This is routinely either accelerated or prevented during dairy processing, for different reasons, by centrifugal separation or homogenization, respectively, while other products exploit separation phenomena for specific reasons. For example, natural creaming of raw bovine milk by gravity separation is a traditional cheese making step for the manufacture of Grana and Parmigiano-Reggiano raw milk cheeses in the north of Italy (Fox et al., 2015). Gravity separation can produce a part-skim milk that has a larger proportion of small fat globules compared with the milk separated from a cream separator, which could influence the milk fat-derived flavor development in those cheese (Ma & Barbano, 2000). However, the work described in **Chapter 3** showed there are still unsolved questions regarding the creaming properties of bovine milk, and most studies were undertaken many years ago, while new sophisticated analytical methodologies are now available for studying separation phenomena of this type. Therefore, the objective of this study was to expand the body of knowledge in terms of bovine milk creaming.

The rate of cream separation, v, is usually described by Stokes' law (Walstra & Oortwijn, 1975):

$$v = \frac{g(\rho_p - \rho_f)d^2}{18\eta_p} \qquad (1)$$

where g is acceleration due to gravity, d is the diameter of the fat globule, ρ_p and ρ_f are the densities of the plasma and fat, respectively, and η_p is the viscosity of the plasma.

According to the equation (1), creaming will be accelerated for larger milk fat globules (MFG) and in cases of larger density differences between the particles and the continuous phase. Small particles will rise very slowly under gravity, because they have low mass and thus rise slowly and diffuse from regions with higher fat concentrations to regions with lower fat concentrations. Conversely, large fat particles will rise rapidly and diffuse slowly. Notably, in centrifugal separation, gravity in Stokes' law is replaced by centrifugal force, which depends on the speed of angular rotation and the distance of the particle from the axis of rotation.

Predictions from Stokes' law when applied to separation of bovine milk can be inaccurate, as other factors also influencing creaming rate, including temperature, immunoglobulins, the presence of bacteria and somatic cells (Geer & Barbano, 2014;

Jenness, Shipe, & Sherbon, 1974), pre-heating (Rowland, 1937), homogenisation, and high-pressure treatment (Huppertz, Fox, & Kelly, 2003). These factors all influence the parameters in Stokes' law; for example, immunoglobulin M could lead to flocculation of MFG, thereby increasing the particle size (D'Incecco et al., 2018), while homogenisation decreases creaming rate by reducing fat globule size (Ertugay, Şengul, & Şengul, 2004).

This study aims to update the knowledge of the influence of heating, sonication and freezing on bovine milk creaming by measuring creaming phenomena using accelerated and gravitational creaming techniques rather than by recording using the naked eye. Meanwhile, the effect of changes of casein micelles on creaming was studied by incubation of milk at 37°C, allowing proteolysis to occur, or adding trisodium citrate (TSC), a calcium-chelating agent that dissociates native casein micelles into smaller casein aggregates.

4.2 Materials & Methods

4.2.1 Samples and treatments

Fresh raw bovine milk was purchased from a local market (milk was produced on the day) and transported in a cool bag to the laboratory within one hour. All chemicals were obtained from Merck KGaA, Darmstadt, Germany unless otherwise stated. Sodium azide (0.05% w/v) was added to the milk to prevent microbial growth.

As shown in **Fig. 4.1**A, the milk was split into the fresh group, the frozen group, and the incubated group. In the fresh group, fresh raw milk was heated, had TSC added or was sonicated. In terms of heating, fresh milk samples at 4°C (15 mL each tube) were heated in a water bath to 65°C, 70°C, 75°C, 80°C, or 85°C and retained at each temperature for 10 min. The samples were then cooled down in ice-water to 20°C. TSC was added to fresh raw bovine milk sample (1:100 v/v) to make up a final concentration of 20mM and left at room temperature for 2 h before analysis. An aliquot of milk (50 mL) was sonicated using a sonicator (MIRIS, Uppsala, Sweden) with energy output at 20 J s^{-1} mL^{-1} for 75s. The "fresh group" samples were analysed immediately after treatments.

In the frozen group, samples of control (untreated milk) and milk heated at 70°C for 10 min were frozen at -20°C for 1 week and 1 month, respectively, and then thawed at 4°C overnight. Meanwhile, the control and heated milk (70°C x 10 min) were

incubated at 37°C for 1 day and 1 week. All treatments were performed in triplicate on separate raw milk samples.

4.2.2 Measurement of composition and size distribution of milk fat globules

The levels of macronutrients in bovine milk were measured using a Milkoscan^{TM} Mars (Foss, Hillerød, Denmark). Particle size distribution of bovine milk was determined using a Mastersizer 3000 laser particle size analyser (Malvern Instruments, Malvern, UK) equipped with a He–Ne laser (λ=633 nm). The refractive indices of the fat globule and of the dispersant (water) at 25°C were taken as 1.458 and 1.33, respectively, and the absorbance of fat globules was taken as 0.01(Michalski, Briard, & Michel, 2001). The surface area mean diameter D [3,2] and the volume-weighted mean diameter D [4,3] were measured in triplicate.

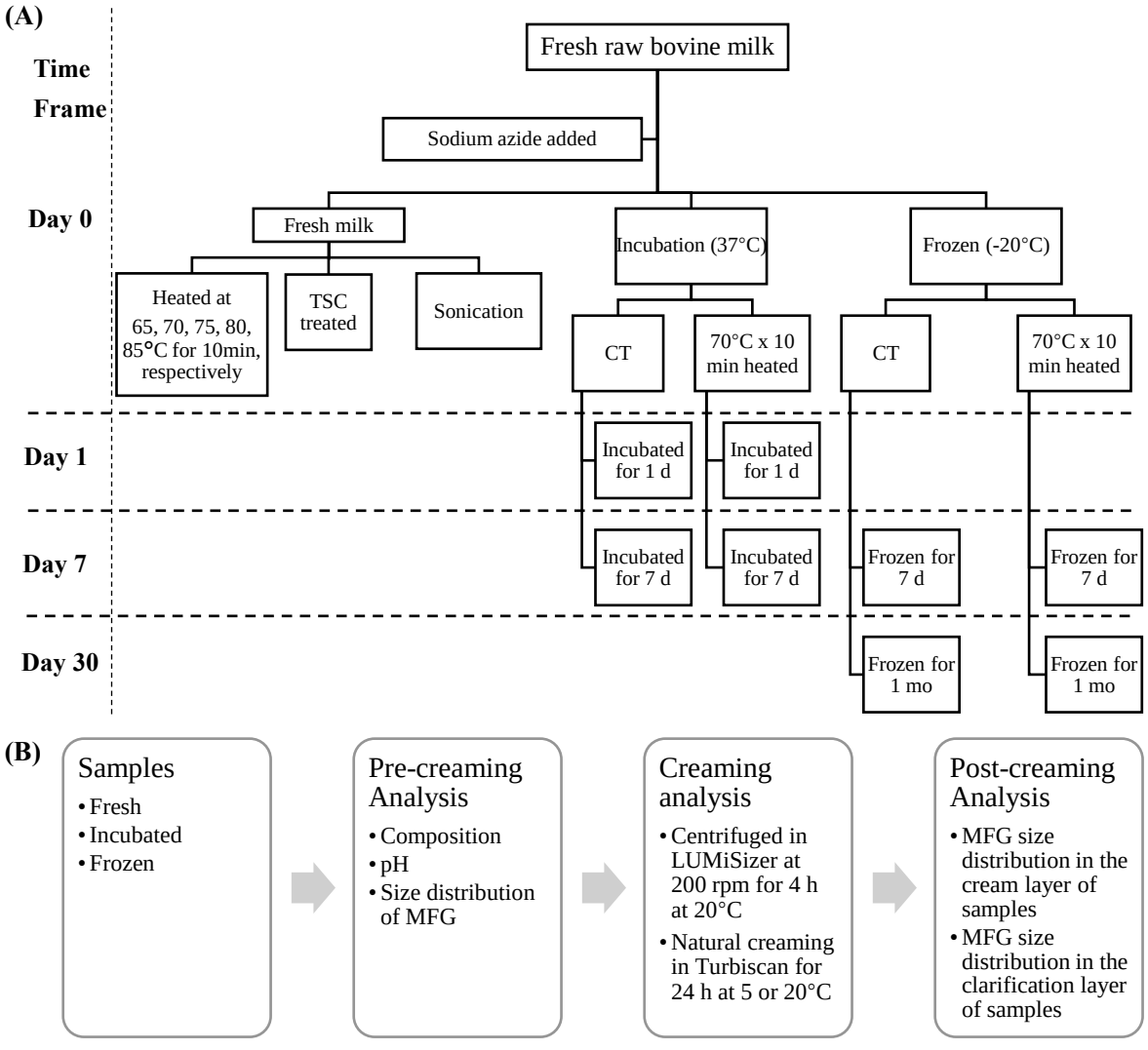


Fig. 4.1 Study design of (A) samples and treatments and (B) analysis.

As shown in **Fig. 4.1**B, the size distribution of milk MFG was determined in samples at different stages and position within the samples as follows: (i) bovine milk before creaming analysis; (ii) the cream layer of milk after creaming analysis (samples were carefully taken from the top of the sample using a syringe from the LUMiSizer cuvette (Lum GMBH, Berlin, Germany) or using a spatula from the Turbiscan cuvette (Formulaction, Ramonville St. Agne, France); (iii) the clarified layer of milk after creaming analysis (samples were taken from the bottom of the sample (30%) by a syringe); and (iv) the cream layer of milk samples after mixing with sodium dodecyl sulphate (SDS; final concentation:1%) after gravity creaming.

4.2.3 Creaming analysis

Creaming of milk samples was measured using a LUMiSizer (LUM GMBH) and Turbiscan Lab^{TM} (Formulaction). Each sample was gently inverted 20 times before measurement and each analysis was duplicated.

4.2.3.1 Measurement of accelerated creaming using a LUMiSizer

Accelerated creaming testis was performed using a LUMiSizer, an analytical centrifuge, and accompanying SEPVIEW v.6.4 software (LUM GMBH). The principle of operation of the LUMiSizer was described in detail by Lerche and Sobisch (2011). Approximately 400 µL aliquots of sample were transferred to a polycarbonate cuvette (PC 110-131XX; LUM GMBH) using a syringe and wide-bore needle. Profiles of phase separation (transmission of near-infrared light through sample cell as a function of position in centrifugal field) were then recorded during centrifugation at 200 g for 4 h at 20 °C.

Mean levels of clarification (i.e., delta transmission; ΔT_{clar}) and front tracking were integrated from phase separation profiles within a defined region (110 to 130 mm) and are recorded. In the clarification analysis, where initial transmission is subtracted from any later recorded transmission profiles. In this study, ‘‘front tracking’’ refers to the “creaming front” and the profile of front tracking indicates the movement of the phase boundary within the clarification region at a threshold of 5% change in transmission. Front tracking is used to calculate creaming/clarification rate (mm h^{-1} at 200 g). The instability index, a factor calculated by the software and whose derivation and calculations can be found elsewhere in detail (Detloff, Sobisch, & Lerche, 2013), is also presented to compare the overall stability of milk samples; this value is between 0 and 1, where 0 indicates no separation or change in stability, i.e., the highest stability and 1 indicates a complete separation between phases, i.e., the lowest stability.

4.2.3.2 Measurement of physical stability using a Turbiscan

The natural gravitational creaming of milk samples was measured using a Turbiscan Lab^{TM} (Formulaction) and accompanying TurbiSoft Lab v.2.2 software (Formulaction). Aliquots of milk (3 mL) were transferred into a glass tube and were scanned by an 800-nm near infrared (NIR) light source for 24 h at 20°C for comparison with the accelerated creaming method. The principle of the Turbiscan Lab^{TM} is based on the measurement of backscattering (BS) signals, resulting from fluctuations on particle volume and size, meaning migration or particle aggregation phenomena, respectively. A delta backscattering (△BS) profile (BS of NIR light through sample cell as a function of height, using the first scan as reference scan) was recorded during gravitational creaming for 24 h at 20 °C. Meanwhile, a subgroup of raw bovine milk samples was observed at 5°C for 24 h to compare rates of cold separation to those at 20°C.

In the study, (i) Turbiscan stability index of the whole samples (TSI_{global}), creaming region (TSI_{cream}), and clarification region (TSI_{clar}); (ii) mean △BS of the clarification region, calculated from △BS profile; and (iii) peak thickness of the creaming region (threshold value was set as 5% ΔBS) were presented, as described in **Chapter 3**. The profile of peak thickness of the clarification was used to calculate creaming/clarification rate (expressed in mm h^{-1} at 1 g). It should be noted that 24 h was chosen for the gravitational creaming as the TSI profiles reached a plateau in this time in preliminary trials.

4.2.4 Urea-polyacrylamide gel electrophoresis

Urea-polyacrylamide gel electrophoresis (Urea-PAGE) (12.5% total monomer, 4% crossing-linking agent as percentage of total monomer, pH 8.9) of the incubated bovine milk was performed using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, UK) according to the method of Andrews (1983) with the modifications of Shalabi and Fox (1987). The gels were stained directly with Coomassie Brilliant Blue G-250 by the method of Blakesley and Boezi (1977) and destained using DI water.

4.2.5 Statistical analysis

Data were analysed using IBM SPSS Statistics, version 26 (IBM Corp., Armonk, USA). One-way ANOVA followed by a paired multiple comparison test (Tukey's test) were used as appropriate. For all statistical analysis, the level of significance, α, was set at 0.05.

4.3 Results

4.3.1 Composition

Fresh bovine milk contained 4.3 ± 0.08 % fat, 3.4 ± 0.06 % protein and 4.3 ± 0.02 % lactose (**Table S4.1**). No significant difference of levels of fat, protein and lactose was found after incubation for 1 week or frozen storage for 1 month. After heating at 70°C for 10 min, the level of protein and carbohydrate remained unchanged, while the level of fat decreased to 4.2 ± 0.11 %; however, this decrease was not significant ($p > 0.05$).

4.3.2 Size distribution of milk fat globules

As shown in **Table 4.1**, the volume weighted mean diameter D [4,3] of raw bovine milk was 3.4 ± 0.1 μm, which did not change significantly after different treatments ($p > 0.05$). The D [3,2] value of sonicated milk decreased from 1.9 ± 0.1 μm to 1.3 ± 0.02 μm, indicating that sonication increased the volume percentage of smaller particles, as expected (**Table 4.1**). Particle size of control milk slightly increased after freezing (after 1 week: D [4,3] = 4.2 ± 1.3 μm; after 1 month: D [4,3] = 3.9 ± 0.4 μm); however, the increase was not significant ($p > 0.05$). Notably, frozen heated samples had a higher increase of average size after freezing compared with unheated frozen samples (**Table 4.2**).

Table 4.1 Changes of particle size distribution parameters of milk fat globules in milk before and after accelerated creaming (200 g) for 4 hours at 20°C. Milk samples were untreated (control), heated from 65 to 85°C for 10 min, added with 20 mM TSC, or sonicated. Results of D [4,3] (μm) and D [3,2] (μm) are presented as mean (SD).

Samples	Whole milk		Cream layer		Clarified layer	
	D [4,3]	D [3,2]	D [4,3]	D [3,2]	D [4,3]	D [3,2]
Control	3.35 (0.1)	1.85 (0.1)	4.81 (1.9)	2.74 (0.7)	1.75 (1.4)	0.47 (0.3)
65°C x 10 min	3.45 (0.3)	2.12 (0.4)	4.69 (1.9)	2.74 (0.1)	1.17 (1.4)	0.26 (0.1)
70°C x 10 min	3.28 (0.1)	1.83 (0.1)	3.95 (0.6)	2.46 (0.2)	1.35 (1.7)	0.26 (0.1)
75°C x 10 min	3.32 (0.4)	1.86 (0.6)	6.96 (2.8)	2.85 (0.2)	2.00 (1.7)	0.27 (0.1)
80°C x 10 min	3.34 (0.3)	1.91 (0.5)	14.37 (8.8)	3.85 (1.1)	1.11 (1.2)	0.21 (0.02)
85°C x 10 min	3.30 (0.4)	1.91 (0.5)	7.58 (2.6)	2.30 (0.6)	0.96 (1.1)	0.35 (0.2)
TSC 20 mM	3.39 (0.3)	2.14 (0.4)	7.63 (4.3)	2.38 (0.2)	2.23 (1.8)	0.73 (0.7)
Sonicated	3.41 (0.3)	1.31 (0.02)	12.70 (4.0)	3.38 (0.5)	1.80 (1.1)	0.27 (0.04)

SD: Standard deviation; TSC: Trisodium citrate.

Table 4.2 Changes of particle size distribution parameters of milk fat globules in milk before and after accelerated creaming (200 g) for 4 hours at 20°C. Milk samples were untreated (control) or heated at 70°C for 10 min combined with incubation at 37°C for 1 or 7 days or frozen storage at -20°C for 1 week or 1 month. Results of D [4,3] (μm) and D [3,2] (μm) are presented as mean (Standard deviation).

Samples	**Whole milk**		**Cream layer**		**Clarified layer**	
	D [4,3]	D [3,2]	D [4,3]	D [3,2]	D [4,3]	D [3,2]
Raw bovine milk (Control) before and after storage						
Before Storage	3.35 (0.1)	1.85 (0.1)	4.81 (1.9)	2.74 (0.7)	1.75 (1.4)	0.47 (0.3)
37°C x 1 d	3.53 (0.1)	2.32 (0.1)	3.67 (0.2)	2.58 (0.1)	2.24 (1.1)	0.29 (0.02)
37°C x 7 d	3.39 (0.4)	2.27 (0.4)	4.15 (0.6)	2.51 (0.2)	3.73 (3.1)	0.35 (0.2)
-20°C x 1 wk	4.23 (1.3)	1.96 (0.7)	7.86 (5.0)	2.50 (0.4)	0.24 (0.01)	0.20 (0.02)
-20°C x 1 mth	3.89 (0.4)	1.97 (0.1)	3.74 (0.1)	2.29 (0.1)	0.61 (0.4)	0.20 (0.01)
Raw bovine milk heated at 70°C for 10 min before and after storage						
Before Storage	3.28 (0.1)	1.83 (0.1)	3.95 (0.6)	2.46 (0.2)	1.35 (1.7)	0.26 (0.1)
37°C x 1 d	3.46 (0.3)	2.15 (0.5)	3.79 (0.4)	2.55 (0.1)	0.72 (0.4)	0.23 (0.04)
37°C x 7 d	3.23 (0.3)	1.85 (0.4)	3.71 (0.4)	2.31 (0.1)	2.68 (3.6)	0.21 (0.03)
-20°C x 1 wk	5.03 (2.9)	1.68 (0.5)	17.84 (6.6)	4.36 (0.9)	0.48 (0.1)	0.20 (0.01)
-20°C x 1 mth	6.71 (3.5)	1.99 (0.5)	10.67 (6.3)	3.14 (0.7)	2.93 (1.5)	0.27 (0.1)

After accelerated creaming, the mean diameter of fat globules increased in the cream layer (D [4,3]: 4.81 ± 1.9 μm), while it decreased in the clarified layer (D [4,3]: 1.75 ± 1.4 μm) of control (**Table 4.1**). Compared with accelerated creaming, the mean particle size in the cream layer of milk after gravitational creaming (**Table 4.3**) was slightly lower, while that in the clarified layer was higher; however, the difference was not significant ($p > 0.05$). Notably, it was observed that fat globules in the cream layer of sonicated milk covered a wider range of size classes compared with other samples (**Fig. 4.2**). Also, the peak volume density of fat globule size of sonicated milk and milk heated at 80°C moved to higher size classes, i.e., the majority of milk fat globule size increased.

Table 4.3 Particle size distribution parameters of milk fat globules after gravitational creaming for 24 hours at 20°C. Results of D [4,3] (μm) and D [3,2] (μm) are presented as mean (Standard deviation).

Samples	Cream layer after gravitational creaming		Clarification layer after gravitational creaming	
	D [4,3]	D [3,2]	D [4,3]	D [3,2]
Fresh bovine milk				
Control	4.66 (1.8)	2.72 (0.3)	1.85 (0.8)	0.53 (0.2)
65°C x 10 min	8.73 (4.6)	3.53 (0.9)	2.52 (2.1)	0.66 (0.6)
70°C x 10 min	6.73 (5.0)	3.35 (1.0)	1.25 (0.6)	0.35 (0.1)
75°C x 10 min	4.37 (0.9)	2.63 (0.01)	1.20 (0.5)	0.34 (0.1)
80°C x 10 min	25.6 (0.8)	7.05 (0.5)	1.17 (0.1)	0.30 (0.01)
85°C x 10 min	22.32 (18.8)	6.15 (3.7)	2.32 (1.6)	0.39 (0.1)
TSC 20 mM	6.57 (2.3)	2.69 (0.5)	3.75 (2.1)	1.01 (0.2)
Sonicated	11.10 (3.5)	4.01 (1.0)	1.66 (0.4)	0.47 (0.1)
Raw bovine milk (Control) after storage				
37°C x 1 d	3.20 (0.4)	1.81 (1.0)	0.94 (0.3)	0.32 (0.001)
37°C x 7 d	4.26 (0.3)	2.77 (0.0)	2.20 (0.1)	0.45 (0.004)
-20°C x 1 wk	9.19 (2.3)	2.84 (0.1)	1.71 (0.8)	0.33 (0.05)
-20°C x 1 mth	6.48 (0.8)	3.14 (0.2)	0.94 (0.1)	0.29 (0.01)
Raw bovine milk heated at 70°C for 10 min after storage				
37°C x 1 d	4.21 (0.7)	2.23 (0.4)	1.48 (0.2)	0.35 (0.1)
37°C x 7 d	12.49 (9.1)	4.16 (1.6)	1.54 (1.2)	0.45 (0.2)
-20°C x 1 wk	8.30 (3.4)	2.99 (0.4)	1.51 (1.0)	0.30 (0.02)
-20°C x 1 mth	20.07 (10.4)	5.72 (2.0)	1.24 (0.3)	0.31 (0.03)

TSC: Trisodium citrate.

After both gravitational and accelerated creaming, D [4,3] values for fat globules in the cream layer of milk heated at 80°C and 85°C, sonicated milk, and milk heated at 70°C followed by freezing for 1 week or 1 month were much higher compared with the control (**Table 4.3**). The fat globule size in those samples decreased after addition of 1% SDS (**Fig. S4.1**), for example, D [4,3] of the cream layer of milk heated at 80°C after gravitational creaming was 25.6 ± 0.8 μm (**Table 4.3**), which decreased to 7.3 ± 1.1 μm with the SDS addition (data not shown). In contrast, D [4,3] values of the cream layer in control milk only decreased from 4.66 ± 1.8 μm to 4.05 ± 0.05 μm after SDS addition. This indicated that aggregation during creaming occurred in samples, such as milk heated at 80 and 85°C, sonicated milk, and milk heated at 70°C and frozen for 1

week or 1 month to a greater extent compared with the control or samples heated at 65 or 70°C. In the clarified layer, milk with TSC addition showed higher mean fat globule size compared with others after both accelerated and gravitational creaming (**Table 4.1 & 4.3**).

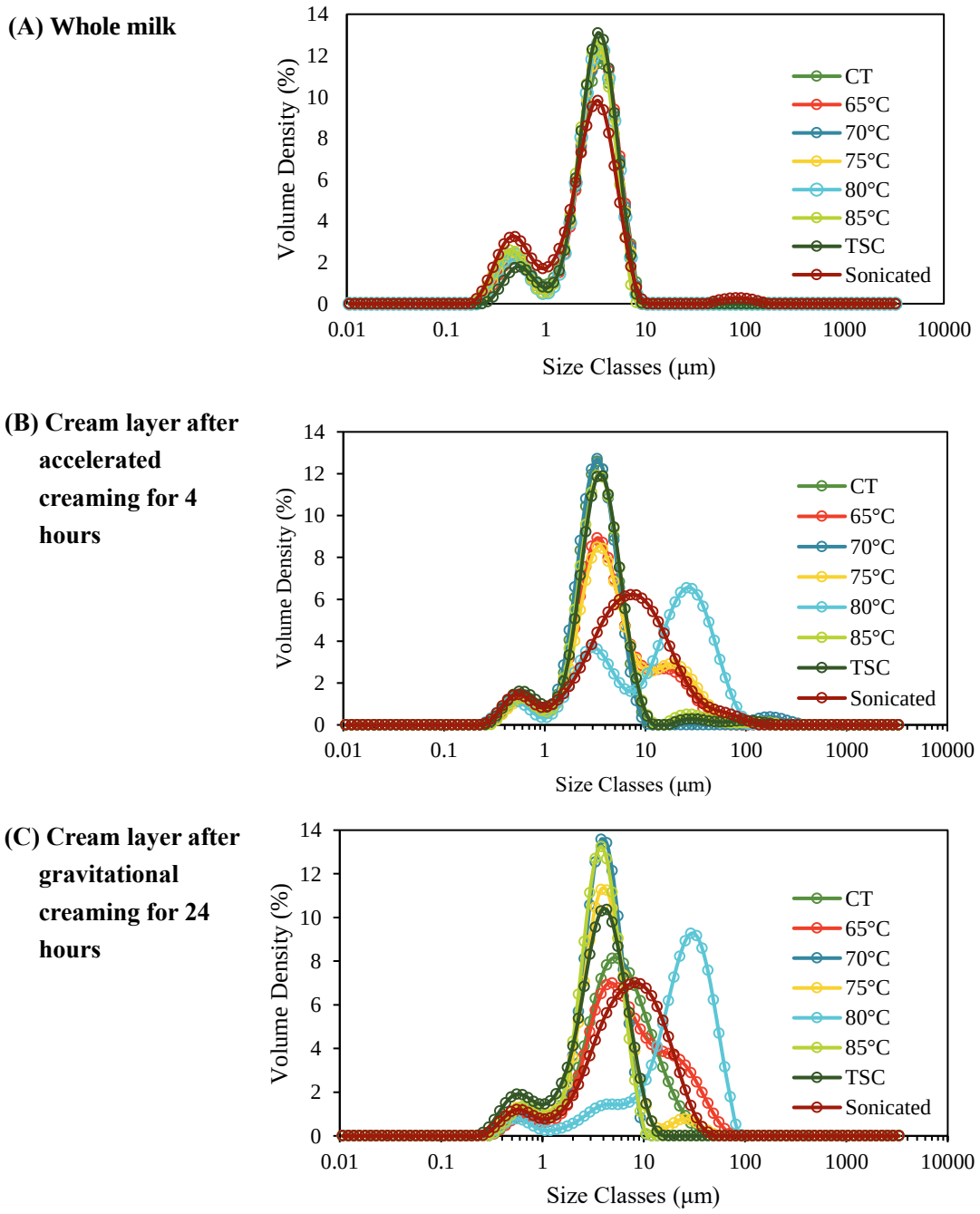


Fig. 4.2 Distribution of fat globule size in milk samples after preheating. Data are shown from one trial, but representative for others.

4.3.3 Clarification profiles from centrifugal separation

As shown in **Fig. 4.3**, the ΔT_{clar}, i.e., changes of transmission in the clarification layer increased during creaming of milk samples, indicating that particles moved from the bottom to the top of milk, as would be expected. Sonicated milk had significant lower mean $\triangle T_{clar}$ (0.11 ± 0.03 %) compared with control milk (0.42 ± 0.09 %) or milk heated at 65°C (0.43 ± 0.09 %) at 30 min ($p < 0.05$). During accelerated creaming, the difference between sonicated milk and others increased; mean $\triangle T$ of sonicated milk was significantly lower than others at each time point from 1 to 4 h ($p < 0.05$).

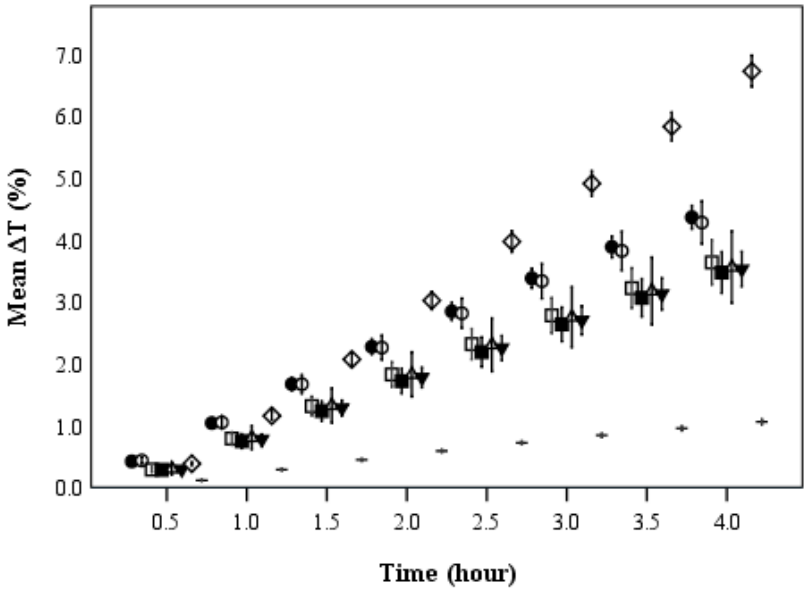


Fig. 4.3 Mean clarification levels of bovine milk samples: control (●); 65°C (○), 70°C (□), 75°C (■), 80°C (△), and 85°C (▼) preheated (10 min) samples; TSC added samples (◇); and sonicated samples (—) during centrifugal creaming at 20°C. Bars represent mean ± standard error.

Milk with added TSC had significant higher ΔT_{clar} values compared with other samples at each time point between 2 to 4 h during accelerated creaming ($p < 0.05$). Among heated samples, ΔT_{clar} ranged from 0.28 ± 0.12 % (milk heated at 75°C) to 0.42 ± 0.09 % (control) at 30 min of accelerated creaming, which increased to values from 3.5 ± 0.58 % (for milk heated at 75°C) to 4.4 ± 0.33 % (control) after 4 h of accelerated creaming. Heating at temperatures higher than 70°C resulted in lower ΔT_{clar} compared with the control during creaming; however, the difference between heated samples at each time point was not significant ($p > 0.05$).

The influence of incubation and freezing on ΔT_{clar} is shown in **Fig. 4.4** A & B, respectively. After incubation at 37°C for 1 day or 1 week, the mean $\triangle T$ increased

during subsequent creaming at each time point compared to samples without incubation; however, the increase was not statistically significant ($p > 0.05$). It was observed from **Fig. 4.4**B that the mean △T increased after 1 week freezing at -20°C, while the value decreased but was still higher than that of the control after freezing at -20°C for 1 month. However, there was no significant difference in terms of mean △T between the frozen samples (1 week or 1 month) and control ($p > 0.05$).

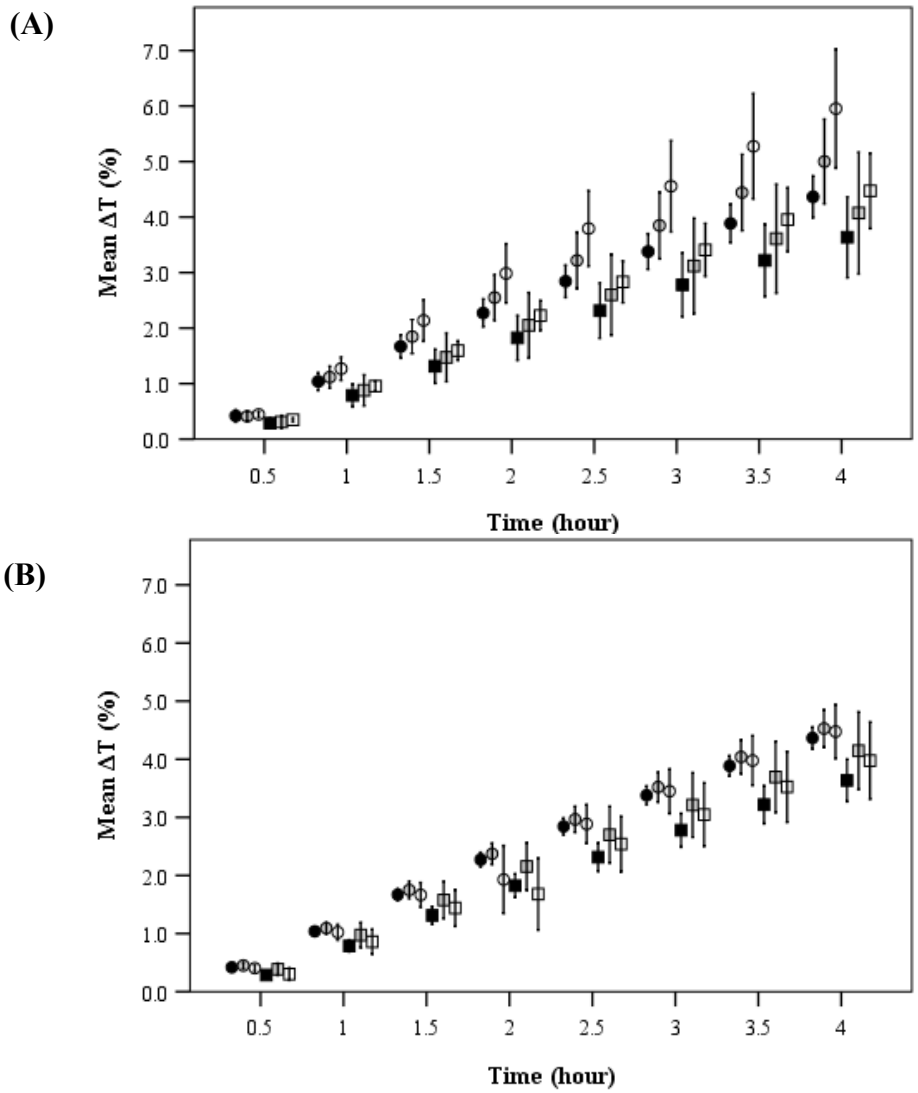


Fig. 4.4 Mean clarification of control (circle) and samples heated at 70°C for 10 min (square) after (A) incubation at 37°C for 0 (black), 1 (grey), and 7 days (white) and (B) freezing at -20°C for 0 (black), 7 (grey), and 30 days (white) as a function of creaming time. Data was obtained from LUMisizer and bars represent mean ± standard error.

The clarification rates at 200 g were calculated from front tracking profiles (**Fig. S4.2**). As shown in **Table 4.4**, sonicated milk had the lowest clarification rate compared

with others, which is significant at 30 min and 4 h of accelerated creaming ($p < 0.05$). Except for sonicated milk, there was no significant differences in clarification rate at 30 min or 4 h of creaming (at 200 g) between samples ($p > 0.05$). Samples heated at 85°C had the fastest creaming rate (8.30 mm h^{-1}) among samples at 30 min, while the creaming rate of untreated samples after incubation for 7 days at 37°C was the highest at 4 h after creaming.

Table 4.4 Clarification rates and instability index of bovine milk samples during accelerated creaming in LUMisizer for 0.5 hours or 4 hours. Results are presented as mean (standard deviation). Different letters (a, b, c, d) indicate statistically significant difference at $P < 0.05$ between samples with different treatments within each column.

Samples	**Clarification rates at 200g (mm h^{-1})**		**Instability index**
	0.5h	4h	4h
Fresh bovine milk			
Control	5.80 (0.13) ^{a}	2.63 (0.05) ^{a}	0.092 (0.006) ^{bc}
65°C x 10 min	5.92 (0.36) ^{a}	2.63 (0.24) ^{a}	0.088 (0.013) ^{bc}
70°C x 10 min	7.64 (1.61) ^{a}	2.38 (0.15) ^{a}	0.079 (0.011) ^{b}
75°C x 10 min	6.11 (0.88) ^{a}	2.32 (0.15) ^{a}	0.076 (0.010) ^{b}
80°C x 10 min	6.98 (1.84) ^{a}	2.32 (0.49) ^{a}	0.073 (0.016) ^{b}
85°C x 10 min	8.30 (1.86) ^{a}	2.41 (0.12) ^{a}	0.077 (0.008) ^{b}
TSC 20 mM	5.41 (0.30) ^{a}	2.89 (0.18) ^{a}	0.156 (0.011) ^{d}
Sonicated	0 (0) ^{b}	0.89 (0.20) ^{b}	0.022 (0.002) ^{a}
Raw bovine milk (Control) after storage			
37°C x 1 d	5.79 (0.35) ^{a}	2.90 (0.19) ^{a}	0.109 (0.014) ^{bc}
37°C x 7 d	5.69 (0.53) ^{a}	3.07 (0.30) ^{a}	0.127 (0.020) ^{cd}
-20°C x 1 wk	5.30 (0.32) ^{a}	2.69 (0.15) ^{a}	0.095 (0.012) ^{bc}
-20°C x 1 mth	5.62 (0.36) ^{a}	2.78 (0.24) ^{a}	0.098 (0.015) ^{bc}
Raw bovine milk heated at 70°C for 10 min after storage			
37°C x 1 d	5.97 (0.27) ^{a}	2.62 (0.36) ^{a}	0.090 (0.020) ^{bc}
37°C x 7 d	6.02 (2.37) ^{a}	2.76 (0.10) ^{a}	0.103 (0.015) ^{bc}
-20°C x 1 wk	6.86 (0.81) ^{a}	2.54 (0.50) ^{a}	0.085 (0.022) ^{bc}
-20°C x 1 mth	7.67 (1.90) ^{a}	2.60 (0.53) ^{a}	0.088 (0.022) ^{bc}

The highest instability index of samples at 4 h of accelerated creaming was seen for milk with added TSC (0.156 ± 0.01), while the lowest was for sonicated milk (0.022

± 0.002), i.e., the clarification of samples with TSC was the most complete that a higher amount of fat moved to the top of samples, while sonicated milk had the smallest change after accelerated creaming for 4 h. In addition, the instability index of samples incubated at 37°C after 7 days (0.127 ± 0.02 after creaming for 4 h) was significantly higher than that for samples heated at temperatures from 70 to 85°C for 10 min without incubation.

4.3.4 Creaming profiles from gravitational creaming

As shown in **Table 4.5**, TSI values was presented and used to rank the stability of selected regions (global region, i.e., from the bottom to the top of the sample; creaming region; and clarification region) of samples after gravitational creaming for 24 h at 20°C. In terms of the entire sample (global region), bovine milk with added TSC (TSI_{global} =13.6 ± 0.8) was the most unstable, followed by untreated milk after freezing at -20°C for 1 week (TSI_{global}: 13.6 ± 0.8), while heated milk and frozen heated milk (70°C x10 min combined with storage at -20°C for 1 month had relatively lower TSI_{global} values. The control after freezing for 1 week and samples with TSC added showed the highest TSI_{cream} and TSI_{clar}, respectively.

Heating and sonication of milk decreased the change in mean ΔBS in both cream and clarified layer (**Table 4.6**). In contrast, addition of TSC or incubation at 37°C increased the mean ΔBS of the cream layer. Freezing at -20°C for 1 week or 1 month did not impact on the mean ΔBS in either the cream or clarified layers. Peak thickness significantly ($p < 0.05$) increased after incubation at 37°C for 1 week (from 3.1 mm to 3.7 mm). In terms of creaming rate, the initial creaming rate decreased after freezing for 1 month (from 3.1 ± 0.4 mm h^{-1} to 2.0 ± 0.24 mm h^{-1}) when calculated from the profiles of peak thickness of cream layer. In contrast, no significant difference ($p > 0.05$) in creaming rate between the control and milk after freezing for 1 month was found when calculated from the profiles of peak thickness of clarification.

Table 4.5 Turbiscan stability index (TSI) of bovine milk during gravitational creaming over 24 hours. Results are presented as mean (Standard deviation). Different letters (a, b, c) indicate statistically significant difference at $P < 0.05$ between temperatures within each column. TSC: Trisodium citrate.

Samples	TSI		
	Global	**Cream**	**Clarification**
Fresh bovine milk			
Control	9.9 (0.6) ^{ab}	19.0 (1.2) ^{ac}	8.5 (0.9) ^{a}
65°C x 10 min	9.2 (0.4) ^{ab}	17.2 (0.6) ^{ac}	7.8 (0.5) ^{a}
70°C x 10 min	8.0 (0.5) ^{a}	14.7 (0.4) ^{a}	6.9(0.8) ^{a}
75°C x 10 min	7.9 (0.1) ^{a}	16.3 (0.4) ^{ac}	6.8 (0.2) ^{a}
80°C x 10 min	7.8 (0.4) ^{a}	14.7 (0.3) ^{a}	6.8 (0.2) ^{a}
85°C x 10 min	8.0 (0.8) ^{a}	14.9 (0.3) ^{a}	6.8 (0.8) ^{a}
TSC 20 mM	13.6 (0.8) ^{c}	19.7 (0.7) ^{bc}	12.5 (0.6) ^{b}
Sonicated	8.1 (0.2) ^{ab}	14.5 (1.6) ^{a}	7.1 (0.1) ^{a}
Raw bovine milk (Control) after storage			
37°C x 1 d	8.8 (0.3) ^{ab}	16.8 (2.5) ^{ac}	7.3 (0.1) ^{a}
37°C x 7 d	9.4 (0.7) ^{ab}	17.4 (2.1) ^{ac}	7.3 (0.9) ^{a}
-20°C x 1 wk	10.6 (2.2) ^{b}	20.0 (3.0) ^{c}	9.1 (2.1) ^{a}
-20°C x 1 mth	9.8 (0.6) ^{ab}	19.2 (1.1) ^{ac}	8.3 (0.5) ^{a}
Bovine milk heated at 70°C for 10 min after storage			
37°C x 1 d	8.4 (0.5) ^{ab}	15.8 (4.0) ^{ac}	7.0 (0.1) ^{a}
37°C x 7 d	8.9 (0.7) ^{ab}	16.2 (0.8) ^{ac}	7.7 (0.8) ^{a}
-20°C x 1 wk	8.5 (0.9) ^{ab}	15.7 (1.7) ^{ac}	7.4 (0.9) ^{a}
-20°C x 1 mth	8.0 (0.6) ^{a}	15.1 (0.3) ^{ab}	6.9 (0.7) ^{a}

Table 4.6 Mean value of backscattering and peak thickness of bovine milk during gravitational creaming over 24 hours. Results are presented as mean (Standard deviation). TSC: Trisodium citrate, PK: Peak thickness of cream layer.

Samples	Mean value of ΔBS (%)		PK (mm)
	Cream layer	Clarification layer	
Fresh bovine milk			
Control	15.7 (0.7) ^{ac}	-8.0 (0.4) ^{b}	3.1 (0.1) ^{ac}
65°C x 10 min	15.3 (0.2) ^{ac}	-7.3 (0.4) ^{b}	3.6 (0.3) ^{bc}
70°C x 10 min	13.7 (0.8) ^{a}	-6.6 (0.5) ^{b}	3.2 (0.2) ^{ab}
75°C x 10 min	14.8 (0.9) ^{ab}	-7.3 (1.4) ^{b}	3.0 (0.1) ^{a}
80°C x 10 min	13.6 (1.3) ^{a}	-6.5 (0.4) ^{b}	3.2 (0.2) ^{ab}
85°C x 10 min	14.4 (0.7) ^{ab}	-6.7 (0.8) ^{b}	3.2 (0.2) ^{ab}
TSC 20 mM	18.0 (0.8) ^{c}	-10.7 (0.6) ^{a}	3.6 (0.2) ^{ab}
Sonicated	14.4 (0.1) ^{ab}	-7.0 (0.1) ^{b}	3.1 (0.4) ^{ab}
Raw bovine milk (Control) after storage			
37°C x 1 d	16.3 (1.6) ^{ac}	-7.0 (0) ^{b}	3.5 (0.1) ^{ab}
37°C x 7 d	17.0 (0.8) ^{bc}	-7.3 (1.1) ^{b}	3.7 (0.1) ^{b}
-20°C x 1 wk	15.8 (1.0) ^{ac}	-7.8 (0.6) ^{b}	3.2 (0.1) ^{ab}
-20°C x 1 mth	15.8 (0.5) ^{ac}	-7.9 (0.4) ^{b}	3.3 (0.1) ^{ab}
Bovine milk heated at 70°C for 10 min after storage			
37°C x 1 d	15.1 (1.9) ^{ac}	-6.7 (0.2) ^{b}	3.3 (0.3) ^{ab}
37°C x 7 d	15.9 (0.9) ^{ac}	-7.7 (0.7) ^{b}	3.4 (0.2) ^{ab}
-20°C x 1 wk	14.3 (1.0) ^{ab}	-7.1 (0.8) ^{b}	3.1 (0.1) ^{ac}
-20°C x 1 mth	14.3 (0.5) ^{ab}	-6.8 (0.6) ^{b}	3.0 (0.2) ^{a}

As shown in **Fig. 4.5**, compared with gravitational creaming at 20°C, creaming of fresh bovine milk at 5°C showed lower TSI during the first 4 h, whereas the value was higher during creaming from 4 to 24 h. Similarly, there was a lower rate of change of ΔBS in the first 2 h of creaming at 5°C; the slope increased sharply from 2 to 4 h, and the final change of ΔBS at 24 h of creaming was even higher compared with milk creaming at 20°C. Thus, in terms of peak thickness, there was an evident lag phase (~ 90 min) before separation began, i.e., the peak thickness remained lower than 5% ΔBS of creaming at 5°C, whereas the lag phase was not apparent at 20°C. Furthermore, the peak thickness after creaming for 24 hours at 5°C (3.4 mm) was higher than that after creaming at 20°C (3.1 mm).

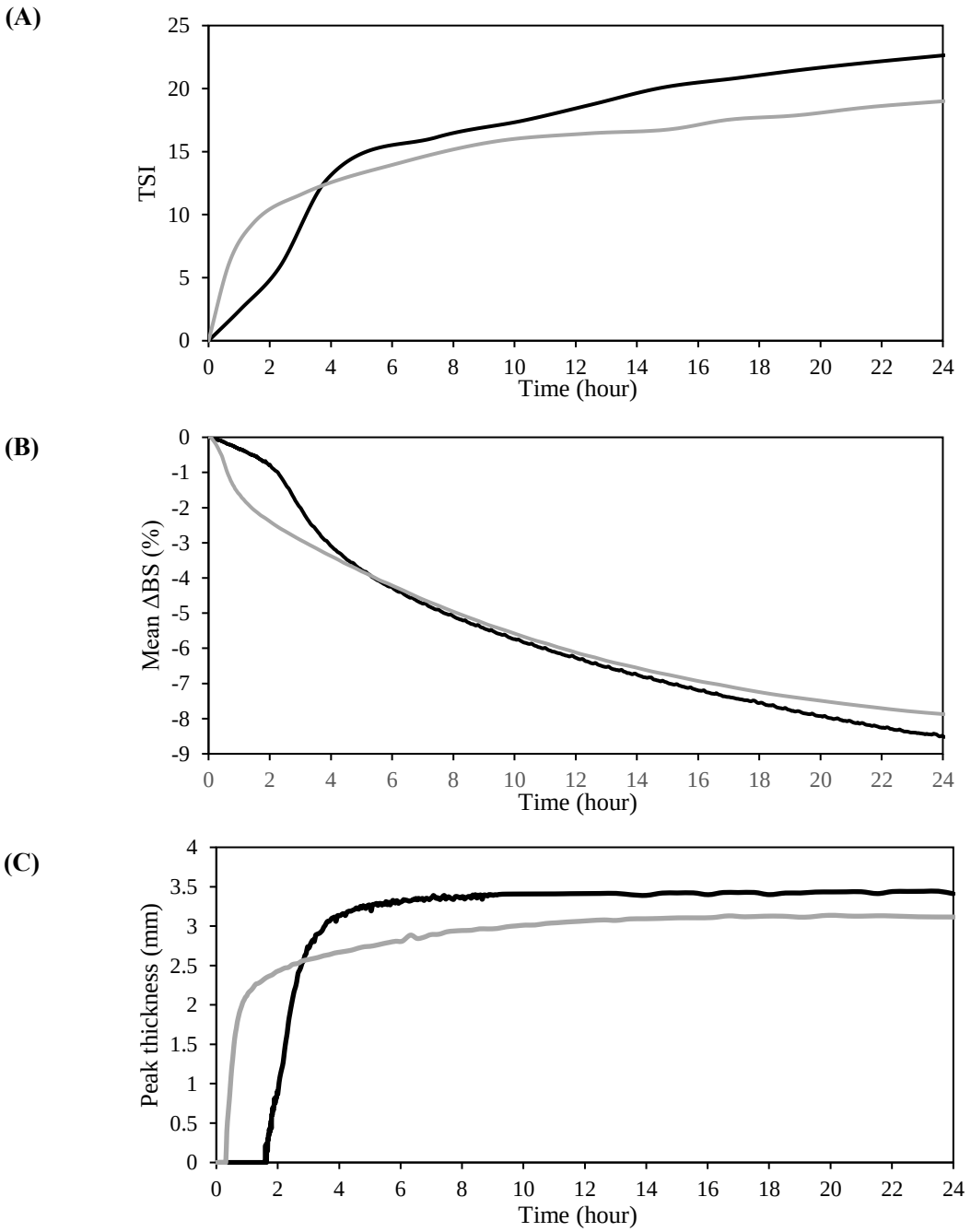


Fig. 4.5 Profiles of (A) TSI, (B) backscattering in clarification layer and (C) peak thickness during gravitational creaming of fresh bovine milk at 5°C (black line) and 20°C (grey line). The profiles are representative for multiple measurements.

4.3.5 Proteolysis during incubation at 37 °C

Proteolysis of raw and heated bovine milk during incubation was examined by

Urea-PAGE electrophoresis (**Fig. 4.6**). It can be seen that β-casein in both raw and heated milk remained intact after incubation for 1 day, while this protein was hydrolysed to a greater extent after incubation for 1 week. The products of degradation of β-casein, especially γ_2-, and γ_3- caseins increased after incubation for 1 week. There was also a breakdown of α_s-caseins after incubation for 1 week, but not as dramatic as the hydrolysis of β-caseins. Thus, changes seen in creaming during incubation were paralleled by a progressive decrease in the level of casein present, and rates of proteolysis were similar in raw and heated milk.

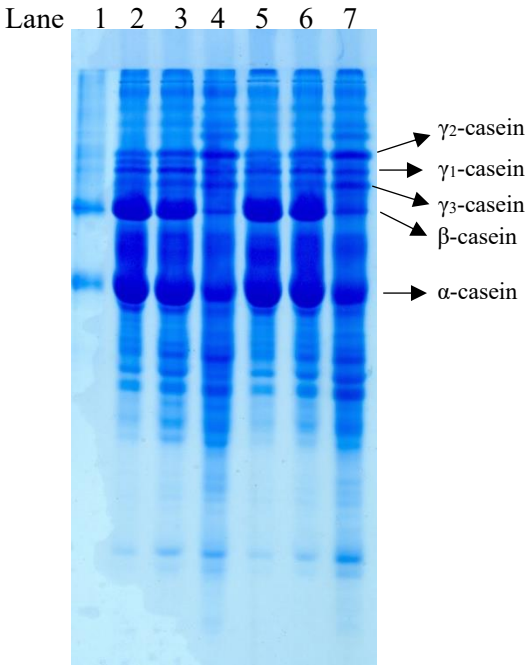


Fig. 4.6 Urea-polyacrylamide gel electrophoretogram of bovine milk samples incubated at 37 °C for 7 days. Lane 1: Sodium caseinate; Lane 2-4: Control/untreated bovine milk; Lane 5-7: samples preheated at 70 °C x 10 min; Lane 2 & 5: No incubation/ day 0. Lane 3 & 6: After incubation for 1 day; Lane 4 & 7: After incubation for 7 days.

4.4 Discussion

4.4.1 Effect of heating and sonication on milk creaming

Heat treatment (at temperatures from 65°C to 85°C for 10 min) did not change fat globule size distribution significantly, i.e., no flocculation or coalescence occurred, which is consistent with previous reports (Bezie, 2019; Dolby, 1953). The unchanged particle size distribution has been explained through the electrostatic and steric repulsion between the fat globules being strong enough to inhibit changes (Liang et al., 2017).

In this study, sonication led to partial homogenization of milk due to cavitation, i.e., the formation and collapse of air bubbles caused by pressure fluctuations. The collapse of air bubbles releases shock waves, causing disruption of fat globules (Taylor & Richardson, 1980). As expected, sonication increased the proportion of small fat globules, as reported by previous studies (Ertugay et al., 2004). During creaming, as shown in **Table 4.3**, the creaming rate of sonicated milk was the lowest due to the decreased size of fat globules, as predicted by Stokes' law. Sonication also increased the surface area of milk fat globule membrane (MFGM) required for much higher numbers of smaller globules, and thus the original level of available MFGM was not sufficient to cover them; whey proteins and caseins then adsorb onto the fat droplets, which increases the density of the MFG and further improves their stability during creaming (Michalski et al., 2002). In addition, homogenization of milk, resulted from sonication can denature immunoglobulins, the factor that clustering fat globules, therefore possibly decreasing the effective diameters of fat globule clusters and decreasing creaming rate (Chen & Chang, 1998; Fox, 2011).

No significant change in creaming rate during accelerated creaming was found after heating. However, control milk and milk heated at 65°C for 10 min showed higher instability index after 4 h of creaming (**Table 4.4**) and higher ΔT_{clar}, i.e., more MFG migrated to the top of samples during accelerated creaming (**Fig. 4.3**) compared with other heated milk samples. Similarly, after gravitational creaming at 20°C for 24 h, TSI values and mean ΔBS in selected regions of control and milk heated at 65°C were higher than that of milk heated from 70 to 85°C. These results suggested that heating at temperatures higher than 70°C decreased the creaming rate, possibly due to: (1) whey proteins, especially β-lactoglobulin, unfolding and exposing sulfhydryl groups when heated at temperature above 65°C leading to complexation with MFGM proteins or caseins attached to MFGM, and thus increasing the density of MFG (Kim & Jiménez-Flores, 1995; Raikos, 2010); and (2) denaturation of immunoglobulins that could link

MFG to initiate flocculation, therefore leading to MFG migrating individually and giving a decreased creaming rate (Walstra & Jenness, 1984).

After gravitational creaming at 20°C for 24 h, milk heated at 65°C showed higher peak thickness, i.e., depth of cream layer, compared with control milk or milk heated at higher temperatures, which agrees with previous findings that milk heated at 60 - 63°C had higher thickness of cream layer than fresh raw milk during creaming at 15°C (Mulder & Walstra, 1974). However, a much thinner cream layer for milk heated >70°C than raw milk at 15°C was found by Mulder and Walstra (1974), whereas no significant difference ($p>0.05$) was found in our study. Different time length of the observation (24 h vs 6 h) and observation conditions (20°C vs 15°C) may cause the variance in results.

Changes of MFG size distribution in the cream layer after mixing with SDS suggested that flocculation of MFG occurred to different extents (**Fig. S4.1**): MFG clustered to a higher extent in the cream layer of milk heated at 80°C or 85°C compared with other samples after creaming, leading to much closer packing MFG in the cream layer (Rowland, 1937). However, this seems not to have occurred at the beginning of creaming, but after milk fat globules moved into cream layer and came in close contact with each other. In addition, there may be an influence of the storage temperature on the flocculation or coalescence of MFG in cream layer. It has been considered that MFGM may be weakened due to pressure and local volume changes when the temperature increases from 5°C to 20°C, and there is also more liquid MFG to allow coalescence at 20°C. Sonicated MFG in the cream layer were also highly clustered (**Fig. S4.1**). This agrees with the hypothesis that clustering strongly increases with fat content in homogenized dairy products, whereas clusters are rarely formed when fat content is lower than 8% (Mulder & Walstra, 1974).

4.4.2 Effect of freezing on milk creaming

There were slightly higher levels of ΔT_{clar}, i.e., changes of transmission in the clarification region. of 1-week or 1-month frozen milk samples compared with fresh raw milk at each time point during accelerated creaming (**Fig. 4.4** B), which may result from increased D [4,3], i.e., increased particle size, after freezing at -20°C (Table 4.1); however, these changes were not significant ($p > 0.05$). The increased D [4,3] value was expected as slow freezing could induce the formation of ice crystals that damage the MFG (Webb & Hall, 1935), therefore leading to coalescence. Some studies have suggested that rapid thawing at room temperature could be better than thawing in a fridge for long time in terms of less changes in creaming profiles (Tribst et al., 2019; Walstra et al., 2005). However, our results showed that differences between frozen and

fresh milk were not significant even after thawing in the fridge overnight, according to parameters calculated from transmission and backscattering profiles. The only exception was that the initial creaming rate calculated from the cream layer of milk frozen for 1 month (2.0 ± 0.2 mm h^{-1}) was significantly lower compared with the control (3.1 ± 0.4 mm h^{-1}) during gravitational creaming.

Notably, the mean particle size D [4,3] of frozen heated samples were higher compared with non-heated samples frozen for the same period (**Table 4.1**), accompanied by a higher level of flocculation in the cream layer after creaming (**Fig. S4.1**). Freezing and thawing has been reported to disrupt the MFGM and allows access to the fat by the major lipase of bovine milk, lipoprotein lipase, whereas most of the lipase could be inactivated after heating at 70°C for 10 min (Deeth, 2006). Therefore, the MFG after heating and freeze-thaw cycle could have partially disrupted MFGM but have no lipolysis, leading to a higher level of flocculation compared with frozen control.

4.4.3 Difference in gravitational creaming at 20°C or 5°C

Interestingly, a lag phase presented before formation of a cream layer during gravitational milk creaming at 5°C, whereas not showed up when creaming at 20°C (**Fig. 4.5**). As also seen from ΔBS profiles, creaming at 5°C seems to be separated into three stages. In the first stage (the first 1 to 2 h), peak thickness was below the threshold (hard to detect with the naked eye) and clarification was slow; MFG may be clustering in this stage by agglutination. At the second stage, from 2 to 4 h, peak thickness and creaming rate highly increased, which may be due to highly clustered MFGs. In the third stage, after 4 h, peak thickness was nearly unchanged, whereas clarification was still ongoing. When creaming at 20°C, the first stage was not apparent, while the second (0 – 2 h) and the third stage remained. In addition, creaming at 5°C gave a higher final peak thickness compared with creaming at 20°C. This may be because temperature-dependent agglutinins were highly involved in MFG clustering when creaming at 5°C and a looser but thicker cream formed compared with the cream layer formed when milk creamed at 20°C (D'Incecco et al., 2018; Mulder & Walstra, 1974).

4.4.4 Effect of proteolysis and casein dissociation on milk creaming

Studies on the influence of proteolysis and dissociation of casein micelles on milk creaming are limited. In our study, milk was incubated at 37°C, the temperature at which plasmin has optimal activity to digest caseins. Heating at 70°C for 10 min could

inactive plasminogen activator inhibitors and plasmin inhibitors, whereas the plasmin and plasminogen activators are heat-stable and retain their activities (Prado et al., 2006). However, proteolysis of caseins occurred in both control and milk heated at 70°C with similar levels of proteolysis in our study (**Fig. 4.6**).

The particle size did not change significantly after incubation. However, the clarification was faster during accelerated creaming (**Fig. 4.4** A) as the mean ΔBS of cream layer increased after gravitational creaming with the length of incubation, i.e., with the degree of proteolysis. The peak thickness of the control increased significantly after incubation for 7 days, which suggests that incubation and proteolysis increased the creaming capacity.

Similarly, adding TSC did not alter the size distribution of milk MFG, but did accelerate creaming (**Fig. 4.3**). After creaming, no significant clustering of MFG was found in the cream layer of milk with added TSC as well as milk after incubation compared with heated or sonicated milk. This suggests that the dissociation of casein micelles accelerates creaming, whereas the denaturation of whey proteins (after heating) slows down the creaming. Proteolysis of caseins may lead to casein fragments with low density that attach to MFGs and form weak linkages between MFGs; therefore, the creaming rate increased and a thicker cream layer formed. In contrast, heating at high temperature leads to denaturation of heavier whey proteins that attach to MFGM proteins, which slows down the rising speed of MFGs by increasing their densities. These MFG with denatured whey proteins arrived in the cream layer more slowly and formed clusters of milk MFG with stronger linkages.

4.4.5 Comparison of accelerated and gravitational creaming

Centrifugal tests allow multiple samples to be tested simultaneously and save time when testing large numbers of samples. However, it has been considered that centrifugal forces may make cream layers more compact, form a fat plug, and increase the amount of MFG rising to the top (Anema, 2019). Also, coalescence and/or aggregation may occur during long-time gravitational creaming, which may not occur in the short-time centrifugation test (Anema, 2019). Therefore, the lowest centrifugal speed (200 g) and a longer time (4 h) were applied in our study to avoid the imprecise prediction caused by centrifugation. One drawback, however, was that the information on changes in the cream layer is limited from transmission profiles, for example, peak thickness of the cream layer was unknown from centrifugal test.

In contrast, backscattering profiles of gravitational creaming from the Turbiscan

gave more complete information but took longer times (24 h) with a single sample. The ranking was similar, especially in terms of ΔT_{clar} during accelerated creaming and ΔBS of cream layer during gravitational creaming (TSC added milk > Incubated milk control > Frozen control > CT, 65°C heated milk > heated milk (higher than 70°C), sonicated milk). However, the mechanistic details were different; for example, the initial creaming rate of control and frozen milk calculated from accelerated creaming profiles (**Table 4.4**) showed no significant difference, whereas differences were found if calculated from the cream layer of gravitational creaming profiles, which may suggest that natural gravity-driven technique is better for the creaming analysis. In addition, the particle size distribution of the cream layer (**Fig. 4.2**) and clarified layer (data not shown) also exhibited differences when comparing between the gravitational and accelerated creaming methods.

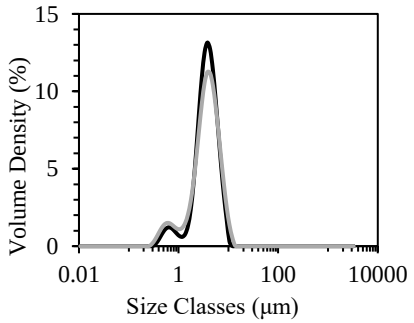
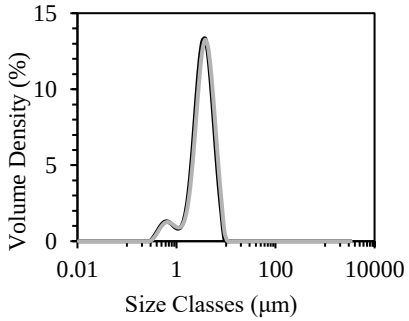
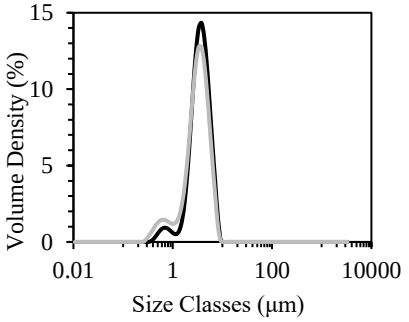
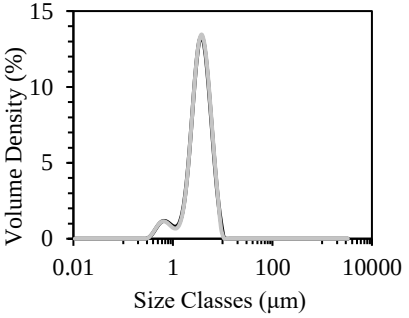
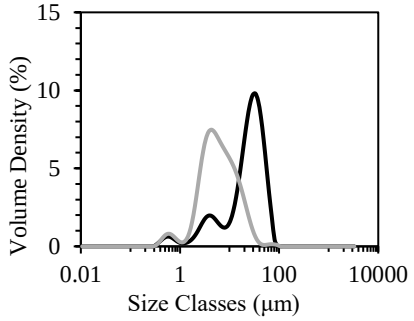
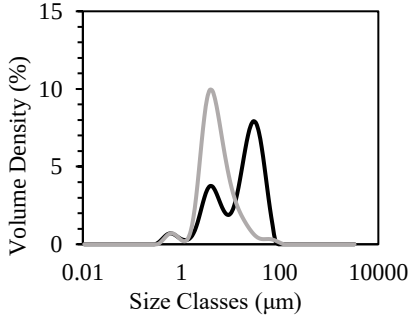
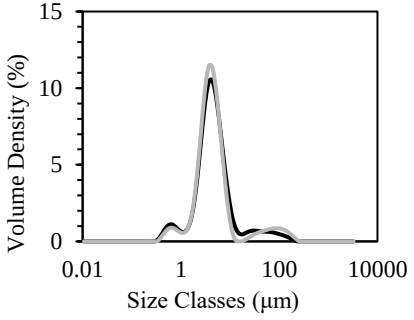
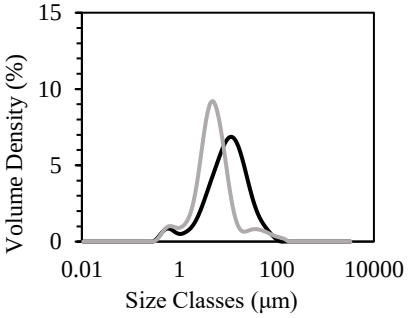
4.5 Conclusion

The creaming capacity of raw bovine milk did not change significantly after heating at temperatures from 65 to 85°C for 10 min. Sonication of the milk decreased creaming rates significantly, while adding TSC and incubation at 37°C increased creaming rates. A significant increase in instability of raw milk after freezing at -20°C for 1 week and of milk heated to 70°C after freezing for 1 month were found during gravitational creaming for 24 hours, but not found from accelerated creaming tests. This study overall updates knowledge on milk creaming as relates to common milk processing operations and illustrates relative advantages of choosing specific techniques to study milk creaming.

Supplementary Tables and Figures

Table S4.1 Levels of macronutrients during freezing and incubation. Results are presented as mean and standard deviation (SD).

Samples	Levels of macronutrients (%)		
	Fat	**Protein**	**Lactose**
Control (Untreated milk)			
Fresh	4.30 (0.08)	3.44 (0.06)	4.32 (0.02)
Frozen 1wk	4.20 (0.08)	3.42 (0.04)	4.29 (0.03)
Frozen 1mth	4.27 (0.08)	3.42 (0.03)	4.26 (0.01)
37°C x 1wk	4.29 (0.11)	3.44 (0.03)	4.26 (0.03)
70°C x 10min Preheated milk			
Fresh	4.20 (0.11)	3.42 (0.05)	4.26 (0.01)
Frozen 1wk	3.98 (0.02)	3.43 (0.03)	4.27 (0.01)
Frozen 1mth	4.10 (0.12)	3.41 (0.02)	4.24 (0.00)
37°C x 1wk	4.20 (0.10)	3.43 (0.03)	4.25 (0.00)

CT	65°C x 10min	70°C x 10min	75°C x 10min
			
80°C x 10min	**85°C x 10min**	**TSC 20mM**	**Sonicated**
			

(Continued)

Fig. S4.1 *(Continued)*

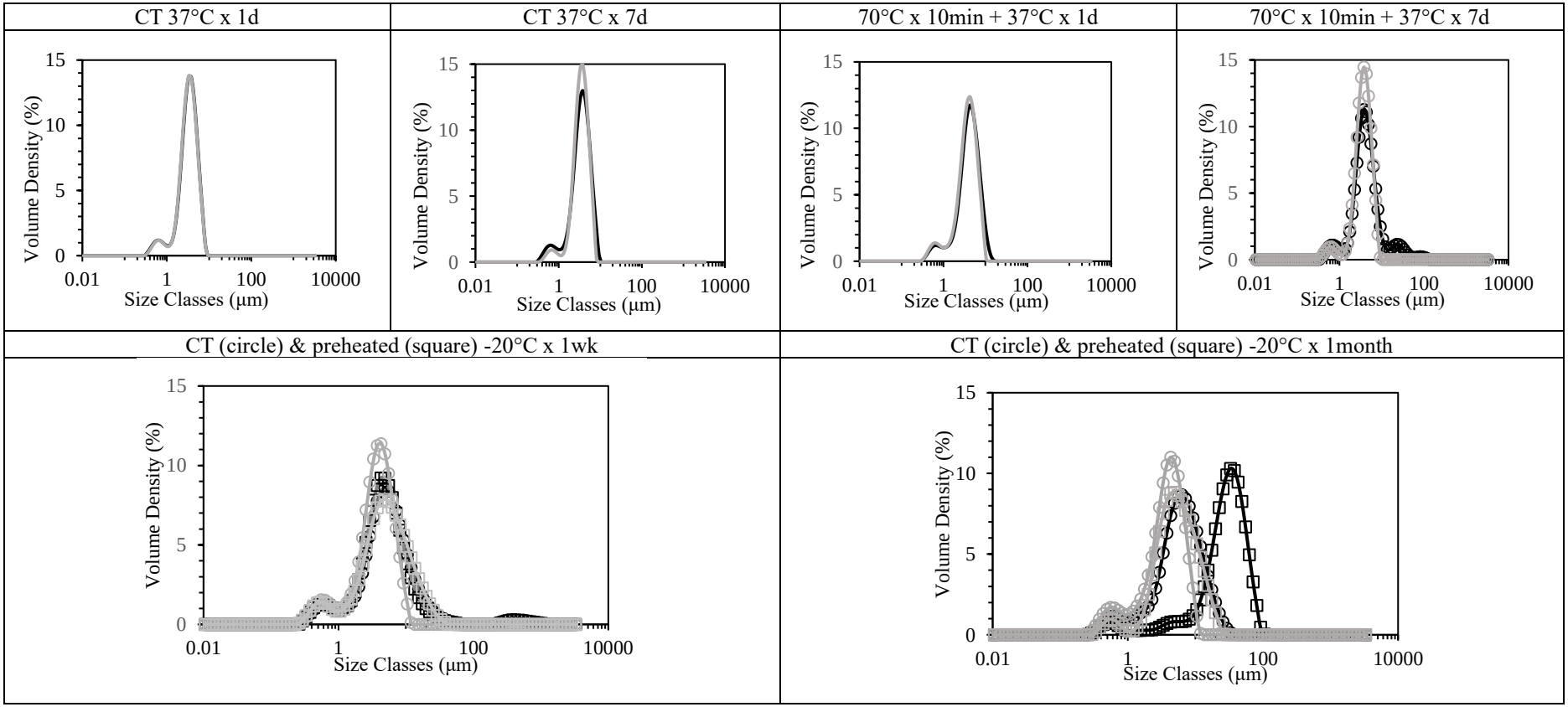


Fig. S4.1 Distribution of fat globule sizes in the cream layer (after creaming at 20°C for 24 h) of milk samples before (black) and after (grey) addition of 1% SDS.

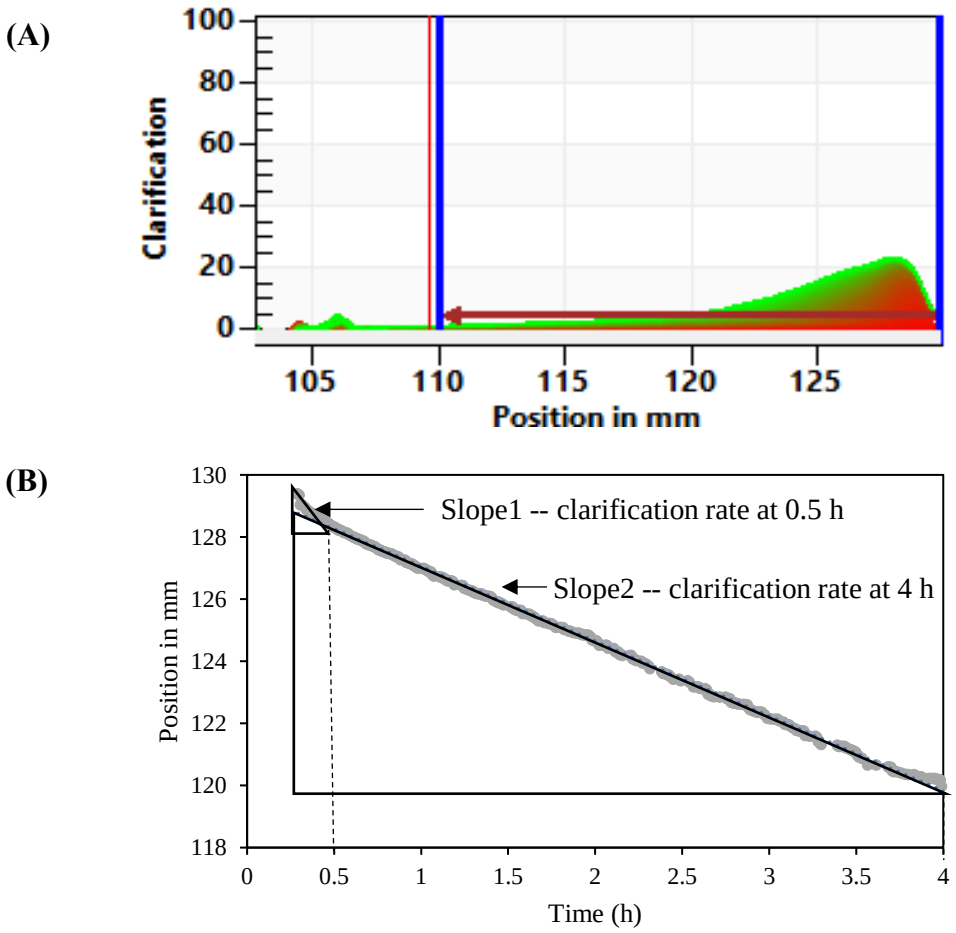


Fig. S4.2 Illustration of (a) clarification profile of bovine milk creaming and (b) calculation of clarification rate from front tracking profiles. In the clarification profile, the first scan was used as reference scan. The colour of scanned transmission profiles from red to green represents the time of creaming from 0 to 4 h. The red vertical line is set as the meniscus of samples. Clarification region was selected from 110 to 130 mm (between blue lines). Red horizontal line represents the threshold for front tracking is set as 5% ΔT and the arrow indicate the creaming direction. Clarification rates are calculated from the slopes of the front tracking profile within selected creaming time.

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CHAPTER 5

The Stability of Casein Micelles in Human Milk

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Authors Contributions:

Fanyu Meng undertook all experiments and drafted the manuscript, with the exception that Karen Burns conducted trials on heat-induced changes in human milk.
Valérie Rofidal, Sonia Hem, and Véronique Santoni performed identification of peptides by mass spectrometry.
Therese Uniacke-Lowe and Alan Kelly critically reviewed the manuscript.

Abstract

Caseins in human milk are an important nutritional source for infants. In this study, the stability of human casein micelles was examined, including their ethanol stability, heat stability, and chymosin-/pepsin-induced coagulation, which have not been reported previously. Human casein micelles had a lower ethanol stability (~75% at natural pH) compared to the ethanol stability of bovine milk. Human milk also showed higher resistance to heat-induced coagulation compared with bovine milk and Type B heat stability profiles of human milk were found. Heat-induced changes in human milk in terms of colour, protein profiles, and protein stability are reported. In addition, proteolysis of casein micelles in human milk was studied by addition of two protease inhibitors, aprotinin and pepstatin. Human milk exhibited high individual variance in plasmin activity; less intact caseins and a higher level of peptides were found in human milk with high plasmin activities. During incubation at 37°C, pepstatin prevented protein degradation in human milk, whereas aprotinin did not, suggesting that a major contribution to protease activity in human milk is aspartyl peptidases such as cathepsin D.

5.1 Introduction

Milk proteins can be divided in two groups: whey proteins and caseins (Kunz & Lönnerdal, 1992). Caseins are a group of acid-insoluble phosphoproteins that are an important nutritional source for mammalian infants to meet their growth and energy requirements. The level of caseins in mature human milk is approximately 2.4 - 4.2 g L^{-1}, which is lower compared with milk from other species, including cow (24 - 28 g L^{-1}), buffalo, camel, sheep, goat, deer, horse, and donkey (Claeys et al., 2014). In human milk, the level of caseins decreases over lactation, and exhibits similar longitudinal patterns to the levels of total proteins (Liao et al., 2017; Molinari et al., 2013); however, the proportion of casein within total protein increases from 20 % in colostrum to 40 % in mature human milk (Kunz & Lönnerdal, 1992).

In bovine milk, 4 isotypes of caseins, β-, α_{s1}-, α_{s2}-, and κ-casein, are present in approximate ratios 4: 1: 3.5: 1.5 (Rehan, Ahemad, & Gupta, 2019). In human milk, no α_{s2}- casein has been detected and the predominant casein is β-casein (~85%), followed by κ-casein (~15%) and trace amounts of α_{s1}-casein (Guo, 2021). A further difference between caseins in bovine and human milk is the degree of phosphorylation of β-casein. The major isoform of β-casein in bovine milk is 5P and occasionally, 4P, whereas β-casein in human milk has six isoforms depending on the phosphorylation degree, from 0P to 5P (Sood & Slattery, 2002), which has been considered to have benefits in increasing gastrointestinal digestibility in neonates (Rehan et al., 2019).

Caseins are present in milk as supramolecular aggregates, called casein micelles (CMs), which can stabilize high levels of calcium and phosphate in milk (Farrell et al., 2006). In terms of the structure of CM, it has been generally agreed that κ-casein is on surface of CM, while the internal structure can be perturbed in a variety of different ways (Holt, 2016). From bovine milk studies, casein micelles have an open structure and micellar integrity is maintained by hydrophobic interactions and bridging by colloidal calcium phosphate (CCP) (De Kruif & Holt, 2003). In general, less is known about the structure of human than bovine casein micelles. Interactions within human CMs have been studied through cooling-induced dissociation, chaotropic agent induced-dissociation (EDTA and lithium chloride), and self-association (Sood & Slattery, 2003; Sood, Herbert, & Slatter, 1997). Sood and Slattery (2003) proposed that human CMs are formed and held together through a combination of hydrophobic interactions and salt bridges involving protein phosphate esters and CCP.

Little is known on the stability of CMs in human milk, which is one of fundamental aspects of human milk protein properties, as well as human milk chemistry. To our

knowledge, there are no reports on ethanol stability or heat stability of human CM. Ethanol influences milk systems by destabilizing micelles through changes in the confirmation of κ-caseins and precipitation of calcium phosphate (Pierre, 1985), and was used as another probe to evaluate the stability of human CMs. It has been reported (Horne, 2003) that the precipitation of calcium phosphate increases when ethanol concentration increases, and thus the dissociation of CM increases and the size of CM decreases; meanwhile, the partial collapse of κ-casein leads to aggregation of CM (Horne, 2003).

Heat stability of milk refers to the ability of milk to withstand certain high temperatures without coagulation (Singh & Fox, 1987). In bovine milk studies, two types of response to heat are observed for different milk samples, referred to as type A and type B, that differ according to their heat coagulation time (HCT) - pH profiles. Type A milk shows a maximum and a minimum heat stability at around pH 6.6-6.8 and pH 6.9 -7.0, respectively; heat stability of type B milk on the other hand increases with increasing pH (Dumpler, Huppertz, & Kulozik, 2020). Removal of whey proteins or salts (by dialysis) from type A milk converts the HCT – pH profile to type B, which indicates that the difference of heat stability of milk can be related to salt and whey proteins, especially β-lactoglobulin (β-lg), which is not present in human milk (Singh & Fox, 1987). Lower heat stability at pH < 6.9 in bovine milk is caused by salt-induced coagulation that κ-caseins (hairy layer) collapse due to charge neutralization and the decrease of electrostatic repulsion (Dumpler et al., 2020). Heat treatment is applied to breast milk in human milk banks, typically 62.5 °C for 30 min (Holder pasteurization; HoP) to eliminate the bacteria and pathogens in the milk so that it can be given to infants who required the milk in hospitals (Moro et al., 2019). However, knowledge on the heat-induced changes, such as casein stability, flavour and colour is limited.

In terms of the digestion of CM, it is well-known that the Phe_{105}-Met_{106} site of κ-casein can be specifically cleaved by chymosin in the calf stomach, which is also a key step in cheese-making (Farrell et al., 2006). However, a chymosin-like enzyme has not consistently been detected in the stomach of human infants and one study reported it was present (at early stages of lactation, within 11 days after birth) only in some infants (Henschel, Newport, & Parmar, 1987). Instead, pepsin is the major enzyme in human infants' stomach that digests proteins at acid pH (Henschel et al., 1987). Pepsin also acts preferentially on κ-casein and has been reported to have a higher proteolytic activity and milk-clotting activity due to its higher non-specific proteolytic activity compared with chymosin. The cleavage of κ-casein by such enzymes could lead to coagulation of CM and formation of curds that influence the digestion of infants (Huppertz & Chia, 2020). In addition, chymosin displays an indirect correlation with

colostrum-dependent immunoglobulin G (IgG) transfer due to the mild enzymatic environment of the calf's digestive tract, which protects IgG from digestion, and the presence of trypsin inhibitors in bovine colostrum, which contribute to the protection of IgG in the small intestine (Foltmann, 1992; Lopes-Marques et al., 2017). Chymosin might be inactive in humans, which is considered to result from the acquisition of maternal IgG transfer and may indicate the evolution of the passive transfer of IgG (Lopes-Marques et al., 2017).

Proteolysis of caseins in human milk by proteases not only occurs in infants' gastrointestinal tract but could start from the synthesis of milk in mother's mammary gland (Nielsen, Beverly, & Dallas, 2017). A complex protease system is present in human milk, including proteases, zymogens, activators, and inactivators. Proteases that have been shown to be active in human milk include plasmin, elastase, thrombin, kallikrein, and amino- and carboxypeptidase (Armaforte et al., 2010; Demers-Mathieu et al., 2017; Monti, Mermoud, & Jolles, 1986). Some proteases, such as cathepsin D and trypsin, have been detected in human milk by ELISA assay (both active enzyme and zymogen can be detected); however, no activities were shown from spectrophotometric substrate assays (Demers-Mathieu et al., 2017; Monti et al., 1986). The complexity of the protease system has been considered to function in controlling proteolytic action at different feeding stages, as well as possibly providing pre-digested peptides and amino acids to infants (Armaforte et al., 2010). Specifically, proteolysis can potentially influence changes in the stability of CMs during storage, but there is limited information on this.

This study aims to expand knowledge on the stability of human CM, especially in terms of ethanol stability, heat stability, chymosin/pepsin induced coagulation, and proteolysis by major proteases in human milk. In addition, heat-induced changes in colour, protein profiles and proteolysis of human milk, which could occur during thermal preservation in human milk banks, were also studied.

5.2 Materials & Methods

5.2.1 Human milk samples

The use of human milk samples in this study was approved by Cork Research Ethics Committee. Fresh mature term human milk samples used to measure the physical stability of caseins were donated by one mother at different lactational stages. The milk

was transported at 4°C after the expression from the donor within 24 h, stored at 4°C, and used within 1 week for analysis. Fresh term human milk samples from 6 individuals were used to study the proteolysis of caseins. These samples were transported at 4°C after expression from mothers within 24 h and stored at -80°C until analysed.

To study heat-induced changes, term human milk from Irvinestown Human Milk Bank stored at -20˚C was used. Three pools of human milk were prepared from 6, 4, and 5 individuals (~ 300 mL each), respectively. According to bovine milk studies, freezing at -20°C has little influence on the stability of most milk proteins while, if destabilization occurs, it can be primarily due to the casein fraction (Wendorff & Rauschenberger, 2001).

Skimmed human milk was prepared by centrifuged at 6000 g at 4°C for 20 min. Raw bovine milk was purchased from local market. All chemicals were obtained from Merck KGaA (Darmstadt, Germany) unless otherwise stated.

5.2.2 Measurement of total protein, pH, and colour

Total protein level in human milk was determined using a human milk analyser (HMA; MIRIS, Uppsala, Sweden) based on mid-infrared (mid-IR) transmission spectroscopy principles. Samples were warmed to 40 °C and homogenized using a MIRIS sonicator (MIRIS, Uppsala, Sweden) before analysis. A Mettler Toledo FiveEasy™ pH meter, equipped with a LE pH electrode, was used for pH measurement at 20°C. A Minolta CR-300 colorimeter (Minolta Corp., Osaka, Japan) was used to measuring changes in the light scattering of milk samples; L* (lightness), a* (greenness to redness), and b* (blueness to yellowness) values were recorded.

5.2.3 Observation and determination of size of casein micelles

Fresh skimmed milk was diluted 1:50 with 0.01M $CaCl_2$ (Harwalkar, Allan-Wojtas, & Kalab, 1989) and stained with 20 g L^{-1} phosphotungstic acid (negative staining) on carbon film (400 mesh; Agar Scientific) and observed using a Jeol 2000FXII Transmission Electron Microscope (TEM; Jeol Ltd., Tokyo, Japan), at an accelerating voltage of 80 kV. Digital acquisition of areas of interest from TEM images was carried out using a Megaview-III digital camera and AnalySIS software (Olympus Soft Imaging Solutions GmBH., Munster, Germany). The average size of casein micelles in skimmed human milk at their natural pH was also measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, UK) according to

the method of McCarthy, Kelly, O'Mahony, and Fenelon (2014). Milk samples were filtered through Whatman No.40 paper immediately prior to analysis.

5.2.4 Stability of casein micelles in human milk

5.2.4.1 Sedimentation of casein micelles at different temperatures

Skimmed human and bovine milk samples were ultracentrifuged at 20 or 4°C at 100,000 g for 1 h. The milk was centrifuged at natural pH in an Optima LE80 K preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA). The supernatant was removed carefully and the pellets were redispersed in simulated milk ultrafiltrate (SMUF). The supernatants and pellets, suspended in SMUF, were analysed by urea-polyacrylamide gel electrophoresis (urea-PAGE). Casein pellets used in the following sections were all ultracentrifuged at 100,000 g for 1 h at 4°C.

5.2.4.2 Dissociation with urea, citrate and lithium chloride

Prior to analysis, milk dialysate was prepared as diluent by exhaustive dialysis of a 70 g L^{-1} lactose solution for human milk or 48 g L^{-1} lactose for bovine milk against 2 x 20 volumes of human or bovine milk, respectively for 48 h at 4°C. Sodium azide (0.5 g L^{-1}) was added as preservative. Human casein pellets, prepared as described in **Section 5.2.4.1**, were resuspended in human milk dialysate at levels of 10 g L^{-1} or 20 g L^{-1} protein. Bovine casein pellets were resuspended in bovine milk dialysate at 35 g L^{-1} protein.

To study the stability of human casein micelles, human and bovine casein micelles, resuspended in dialysate, were made to concentrations of 0.0 to 8.0 mol L^{-1} urea, 0.0 to 0.1 mol L^{-1} trisodium citrate, or 0 to 4 mol L^{-1} LiCl. Samples were vortexed and left to stand for 10 min (with urea or citrate) or 1 h (with LiCl) before centrifugation at 1,000 x g for 10 min at 20°C. The supernatant was filtered (0.45 μm) and diluted 1:5 with distilled water. The absorbance of the suspensions in a 1 cm path-length glass micro-cuvette at 600 nm and 20°C was measured using a Cary IE double-beam UV-Vis spectrophotometer (Varian Scientific Instruments Inc., CA, USA).

5.2.4.3 Determination of ethanol stability

Ethanol stability of fresh human milk was determined using the method of Davies and White (1958). Aqueous ethanol (4 mL) from 0 to 95% (v/v) at 5% intervals was added to human milk samples (4 mL) in Petri dishes. The minimum concentration of

ethanol necessary to destabilise the milk proteins (visible flocculation) was recorded as the ethanol stability (Horne & Parker, 1981). Control samples were prepared as described above but with addition of distilled water instead of ethanol. Ethanol-induced changes of casein micelles in skimmed human milk adjusted to pH 6.2 were also monitored by colorimetry, as described in **Section 5.2.2**.

5.2.4.4 Dynamic measurement of chymosin- and pepsin- induced coagulation

Fresh human milk samples (25 mL) were pre-warmed in a water bath at 32° C for 20 min. Pepsin (1750 U mg^{-1} protein; final concentration: 1 g L^{-1}) or 1:10 (v/v) dilution of Maxiren 180 (250 μL) were added immediately prior to analysis. Dynamic oscillatory analysis of human milk was carried out using a controlled shear stress AR-G2 rheometer equipped with a 40 mm parallel plate geometry (TA Instruments, Leatherhead, Surrey, U.K.). The storage modulus, G', of the sample was recorded continuously at a low amplitude shear strain (0.1 %) and at 0.1 Hz frequency over 4 h.

5.2.4.5 Influence of chymosin-induced coagulation on particle size

Changes of the particle size distribution of skimmed human milk at controlled pH (6.2 or 6.6) were monitored using a Malvern Mastersizer 3000 laser particle size analyser (Malvern Instruments, Malvern, UK) equipped with a He–Ne laser (λ=633 nm). The refractive indices of the particles in milk and of the dispersant (water) were taken as 1.45 and 1.33, respectively, and the absorbance of fat globules was taken as 0.001.

5.2.4.6 Determination of heat stability

The heat stability of unconcentrated or concentrated skimmed human milk was determined as a function of milk pH (pH 6.3- 8.2; adjusted using 1.0 M NaOH or 1.0 M HCl) by the subjective method of Davies and White (1966). The time taken to cause the first sign of coagulation of a milk sample, as indicated by flocculation of the milk proteins, was recorded as the HCT.

5.2.5 Heat-induced changes in human milk

To study the effect of temperature on human milk, pooled samples were heated at a range of temperatures from 63°C-130°C. Human milk (~ 3 mL) was placed in a narrow glass test tube. Each tube was clipped horizontally onto a rocking platform and placed in an oil bath at the desired temperature for the desired period of time. Samples

at 63°C were heated for 30 min to simulate the holder pasteurisation treatment applied to human milk samples at human milk banks. Samples heated at 70°C to 130°C were heated for 12 minutes. All milk samples were allowed to cool to room temperature before further analysis.

The colour of human milk (at natural pH) heated at temperatures from 63°C to 130°C was determined by colorimetry as described above and photographs were taken. Protein profiles of human milk after heat treatments was examined by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE). Human milk was also incubated at 37° (Stuart Scientific, Orbital Incubator SI50) for 7 days to study the proteolysis, which was evaluated by electrophoresis. Ten µL of 10% sodium azide was added to each milk sample (1 mL) prior to incubation to prevent microbial growth.

5.2.6 Measurement of plasmin activities in human milk

Plasmin activities in milk were measured using the modified method from Richardson and Pearce (1981). Reaction mixtures of sample (100 µL), coumarin substrate (40 µL) and 50 mM tris-HCl buffer (pH 7.5) (60 µL) were added into wells of black 96-well microplates (VWR International Ltd, Luttervorth, UK). The fluorescence intensity was determined at a wavelength of 380 nm (excitation) and 460 nm (emission) using a plate reader (Spectrafluorplus, Tecan). One unit of plasmin activity is defined as the activity necessary to release 1 nmol AMC per mL of milk per minute under the conditions of the assay.

5.2.7 Inhibition study on proteolysis of human milk

Ten µL of 1000 mg L^{-1} aprotinin (from bovine lung) was added to 990 µL human milk (with 0.05% w/v sodium azide), to a final concentration of aprotinin of 10 mg L^{-1}. Similarly, pepstatin A (1000 mg L^{-1} in acetic acid and methanol buffer; ratio 1:9) was added to milk to achieve a final concentration of 10 mg L^{-1}. MiliQ water (10 µL) was added to 990 µL human milk as control. Samples were incubated at 37°C for 7 or 10 days and the profiles of proteolysis during incubation were monitored by urea-PAGE and ultra-performance liquid chromatography (UPLC).

5.2.8 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

Protein interactions were studied by SDS- PAGE. SDS-PAGE was carried out according to the method of Laemmli (1970) with modifications. Precast 4-20% gels (4-20% PreciseM, Tris-Glycine, Fisher Scientific Ireland Ltd., Ballycoolin, Dublin) and a

BioRad Mini Protean Tetra Cell were used for SDS-PAGE. A molecular weight marker (7.5 μL10 to 250 kDa; Fisher Scientific Ireland Ltd.) was added to an individual lane on each gel and 7 μL of sample was added to each lane. The gels were Coomassie-stained overnight (BioRad Biosafe-Coomassie G-250 stain) before being destained in deionised water until the background was clear and protein bands were visible. Each gel was scanned with a standard desktop scanner (HP Scanjet G4010).

5.2.9 Urea - polyacrylamide gel electrophoresis

Urea-PAGE (12.5 % total monomer, 4 % crossing-linking agent as percentage of total monomer, pH 8.9) of the human milk was performed using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, UK) according to the method of Shalabi and Fox (1987). The gels were stained directly with Coomassie Brilliant Blue G-250 and destained using distilled water. Human milk or resuspended casein pellets in SMUF was mixed with sample buffer using a ratio of 1:1. The change in protein levels was quantified densitometrically using gel analysis software Total Lab Quant 1D version 14.1 (Total Lab Ltd., Newcastle-upon-Tyne, UK).

5.2.10 Profiles of proteolysis using ultra - performance liquid chromatography

To obtain peptide profiles, milk samples were filtered at 14,000 g through 10-kDa Microcon centrifugal filters (Merck Millipore Ltd., Tullagreen, Ireland) for 30 min at room temperature for three times with 100 μL MiliQ water added each time. Filtrate with peptides less than 10-kDa was collected, freeze-dried, and resuspended into 80 μL buffer consist of 2% acetonitrile and 0.1% trifluoroacetic acid (TFA).

Peptide profiles were examined using a Waters Acquity UPLC H-Class Core System (Waters, Milford, MA, USA), comprising a Waters Acquity UPLC TUV Detector (dual wavelength; Waters) interfaced with Empower 3 software was employed. Analysis was performed on an Acquity UPLC Peptide CSH C18 column (1.7 μm, 2.1 × 100 mm) using gradients prepared from Solvent A (0.1 % trifluoroacetic acid in deionised water) and solvent B (0.1 % TFA in acetonitrile). The separation was performed at 40°C and the elution was initialized with 97% solvent A and 3 % solvent B, followed by a linear gradient to 40 % solvent B over 45 min, followed by a linear gradient to 85 % B over 2 min, elution at 85 % B for 3 min, followed by a linear gradient to 3 % B over 2 mins, and finally 3 % B over 8 min. Eluate was monitored at 214 nm and the flow rate was maintained at 0.20 mL min^{-1}.

5.2.11 Identification of protein bands in urea-PAGE electrophoretograms using mass spectrometry

After urea-PAGE separation, ten bands were excised manually using a scalpel, transferred to 1.5 mL Eppendorf tubes with 500 µL of HPLC grade acetonitrile, and stirred for 5-10 min. Acetonitrile was then removed by pipetting. Samples were sent to the MSPP platform of the BPMP laboratory in France for analysis on dry ice and stored at -80°C until analysis.

Each sample was solubilized by 10 µL of 2% formic acid, then injected for on-line analysis using a nano-HPLC (U3000 system, Thermo Fisher, Waltham, USA) coupled to a Q-TOF mass spectrometer equipped with a nanoelectrospray source (Maxis Impact, Bruker Daltonik, GmbH, Bremen, Germany). Six µL of milk was injected for the samples with low staining intensity, while 3 µL of milk was injected for the samples with high staining intensity. Samples were loaded to a pre-column (Thermo Scientific PepMap 100 C18, 5 µm particle size, 100 Å pore size, 300 µm i.d. x 5 mm length) linked to an Ultimate 3000 autosampler with 0.1% FA for 3 min at a flow rate of 20 µL/min. Separation of peptides was performed on a Thermo Scientific reverse-phase nano column (Thermo Scientific PepMap C18, 3 µm particle size, 100 Å pore size, 75 µm i.d. x 25 cm length) at a flow rate of 300 nL min^{-1}. After a 3 min period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1% FA and solvent B was 80% ACN, 20% water + 0.1% FA. The gradient employed was 4-25% of solvent B in 103 min, then 25-40% of solvent B from 103 to 123 min, and finally 40-90% of solvent B from 123 to 125 min. The total run time was 150 min, including a high organic wash step and re-equilibration step. Peptides were transferred to the gaseous phase with positive ion electrospray ionization at 1.8 kV.

The Mass spectrometry data analysis was performed on the Swissprot databank in "human" taxonomy using the Mascot search engine via the ProteomeDiscoverer 1.4 software. The results were validated on classical criteria; a protein is identified if it contains at least 2 peptides identified with a Mascot score> 26 and a False Discovery Rate< 1%.

5.3 Results and Discussion

5.3.1 Total protein, pH, colour and casein micelle size

As shown in **Table 5.1**, total protein levels of human and bovine milk were 1.09 ± 0.04 % and 3.44 ± 0.06 %, respectively, which were in the expected range (Feng et al., 2016; Grote et al., 2016; Nommsen et al., 1991; Yamawaki et al., 2005). Total protein level of human milk has been reported to decrease during lactation, whereas the percentage of caseins in total proteins increases to around 40% in mature human milk (Kunz & Lönnerdal, 1992). Compared with human milk, mature bovine milk contains higher levels of both total protein and caseins (~ 80% of total proteins) (Ng-Kwai-Hang et al., 1982). Calculated from typical percentages of caseins in total proteins above, human and bovine milk in our study contained approximately 0.436 % and 2.75 % caseins, respectively. Lower levels of total proteins and caseins in human milk compared with bovine milk reflects the differing nutritional requirements of human infants and calves.

Table 5.1 Total protein and selected physical-chemical properties of human milk. Results are presented as average (SD).

	Human milk (n=3)	**Bovine milk (n=3)**
Total protein (%)	1.09 (0.04)	3.44 (0.06)
pH	6.92 (0.03)	6.82 (0.02)
Average micelle diameter (nm)	52.8 (6.50)	162.1 (0.81)
Colour of skimmed milk		
L*	41.22 (5.99)	75.49 (0.03)
a*	-0.86 (0.14)	-6.29 (0.03)
b*	-5.27 (0.32)	1.02 (0.03)

The average pH of human milk, measured at 6.92 ± 0.03 at 20°C, was slightly lower than the typical pH (7 - 7.5) in the literature (Morriss et al., 1986), but in agreement with a higher value pH than that typically reported for bovine milk (6.5 - 6.7; Fox et al., 2015). pH value is crucial to milk as it controls the protein confirmation and enzyme activities in milk and could be an indicator of microbial growth (Rafiq et al., 2016).

The size of human CM (average diameter: 52.8 ± 6.5 nm) was smaller than that of bovine CM (average diameter: 162.1 ± 0.8 nm); from TEM images (**Fig. 5.1**), the average diameters for human and bovine CM were around 50 and 188 nm, respectively. The fact that lightness (L*) of skimmed human milk (41.2 ± 5.99) was lower than that of skimmed bovine milk (75.5 ± 0.03) also perhaps suggests indirectly a smaller size of human CM compared with bovine CM. The mean size of human and bovine CM has been reported previously as 64 nm and 150 – 182 nm (Holt, 1992; Malacarne et al., 2002), which were similar to our results. Human CM have been reported to be not only smaller than bovine CM, but also smaller than CM from goat, sheep, deer, camel, and horse milk, which may result from a lack of α_{s2}-casein, as well as the fact that α_{s1}-casein levels are low in human milk (Farrell et al., 2006; Roy et al., 2020).

The only study measuring human CM size using electron microscopy was reported by Carroll et al. (1985), who found an average diameter of human CM as 42.4 nm with a broad size distribution. Our observation was simliar to the TEM image from Carroll et al. (1985); human CM showed comparable appearance compared with bovine CM (**Fig. 5.1**) but with a looser structure. Some finer CMs were also found in our study, but not as many as found by Carroll et al. (1985), which may due to the different sample preparation; longer staining times and skimmed human milk rather than casein pellets were used in our study compared with the previous study. Carroll et al. (1985) considered that their image from TEM supports the 'submicellar hypothesis' that caseins interact with caseins first as submicelles and traps together with cacium phosphate to form CM (Farrell et al., 2006). However, in bovine milk studies, the results from small-angle scattering analysis suggested that CM are homogeneous and the submicellar hypothesis is improbable (De Kruif, 2014). After more models are proposed, images from TEM of human milk may need reevaluation with a view to exploring the relationship of theories such as the dual-binding model to the structure of the human casein micelle.

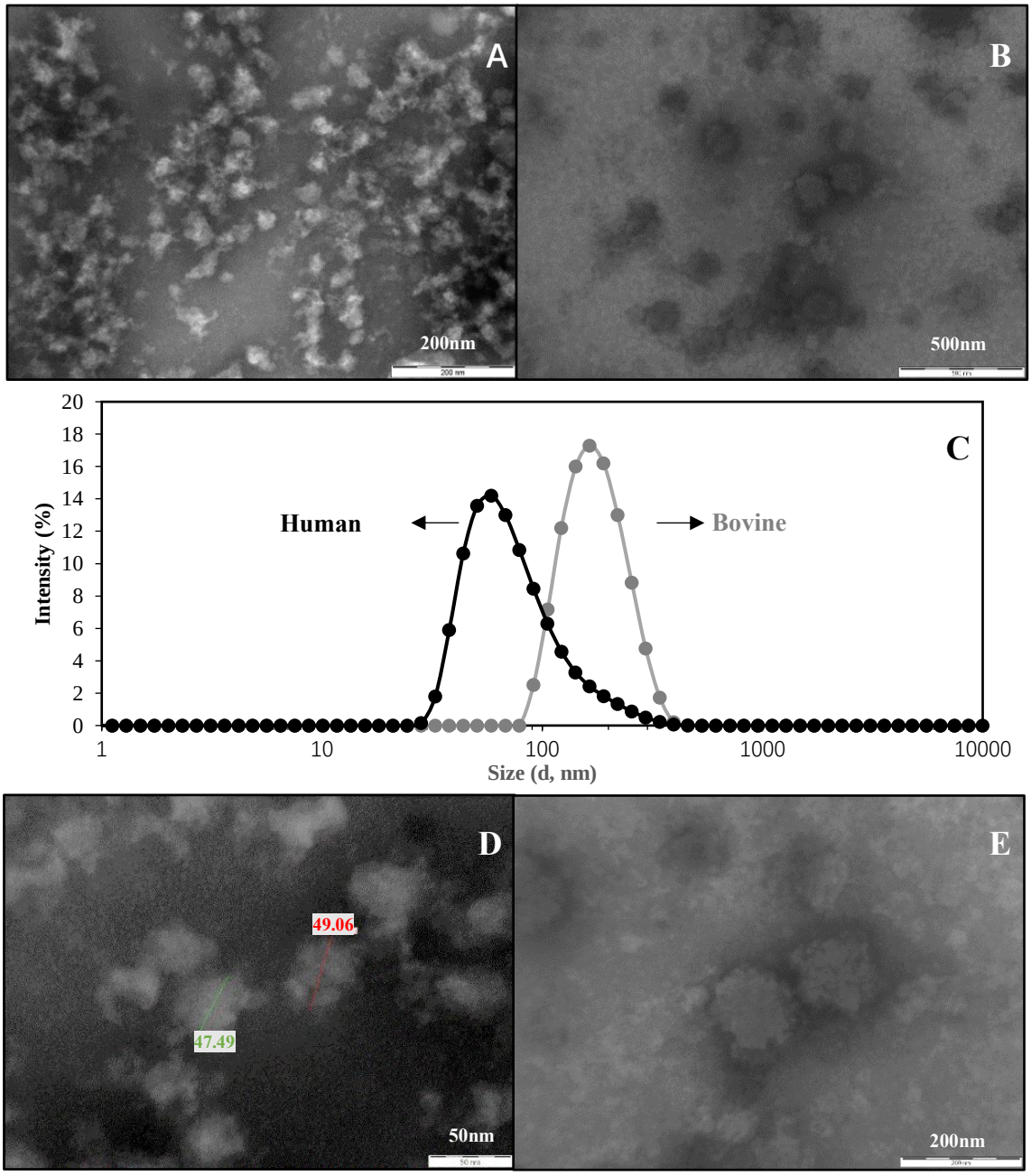

Fig. 5.1 Observation of human (A, D) and bovine (B, E) casein micelles from TEM and representative size distribution profiles of human (black line) and bovine (grey line) casein micelles (C) from Zetasizer analysis. Magnification: A, E: 120 K; B: 60 K; D: 400 K.

5.3.2 Sedimentable and non-sedimentable caseins of human milk

Cold storage of milk leads to the solubilization of caseins, especially β-casein, from casein micelles, which is evident from a higher levels of caseins remaining in an ultracentrifugal supernatant (Dalgleish & Law, 1988). Solubilization of milk caseins has been considered to result from decreased hydrophobic interactions (Davies & Law, 1983) and the solubilisation of calcium phosphate at low temperatures (de la Fuente, 1998). Differences in solubilization/dissociation behaviour between caseins in human milk and caseins in bovine milk at low temperature (4°C or 20°C) are of interest in terms of implications for micelle structure.

As shown in **Fig. 5.2**, several human β-casein phosphorylated isoforms, as identified based on the identification of Molinari et al. (2013), as well as bovine β- and α_{s1}- casein, were apparent on electrophoresis of human milk. Quantitative image analysis of samples after centrifugation showed that 92.2 % and 69.6 % β-casein remained in the supernatant of human milk centrifuged at 4°C and 20°C, respectively, i.e., a higher level of β-caseins dissociated at 4°C compared with at 20°C. The non-sedimentable caseins in bovine milk were measured as 64.4 % and 27.1 % at 4°C and 20°C, respectively, which concurs with previous reports (Uniacke-Lowe, 2011). The result suggested that human milk caseins dissociate to a greater extent compared with bovine milk at low temperatures. Sood et al. (1997) also reported a high level of dissociation of human β-casein when cooled at 4°C, suggesting that human β-casein is only loosely bound and easily dissociated from human casein micelles.

In terms of phosphorylated isoforms of β-casein, our results shows that the recovery of 0P β-casein from casein pellets was higher, whereas the recovery of 2P and 3P β-casein was lower at 20°C compared with at 4°C (**Fig. 5.2**). This finding (i.e., low retention of 0P β-casein in urea-gel at 4 °C) conflicts with the report from Sood et al. (1997) of a higher level of retention of 0P β-casein during cooling at 4°C compared to other β-casein isoforms. In previous bovine milk studies, weakened hydrophobic bonds were proposed as the reason for the dissociation of β-caseins during cooling (Ono et al., 1990). Phosphorylated bovine casein has been reported to show lower solubility than native casein (Matheis et al., 1983). However, the solubility is pH-dependent; slightly higher solubility was reported for non-phosphorylated proteins at pH 7, whereas solubility was lower at pH 6 compared to phosphorylated proteins, according to a buffalo milk study (Nayak et al., 2006). The influence of pH on the recovery of phosphorylated isoforms of human β-casein may require further study.

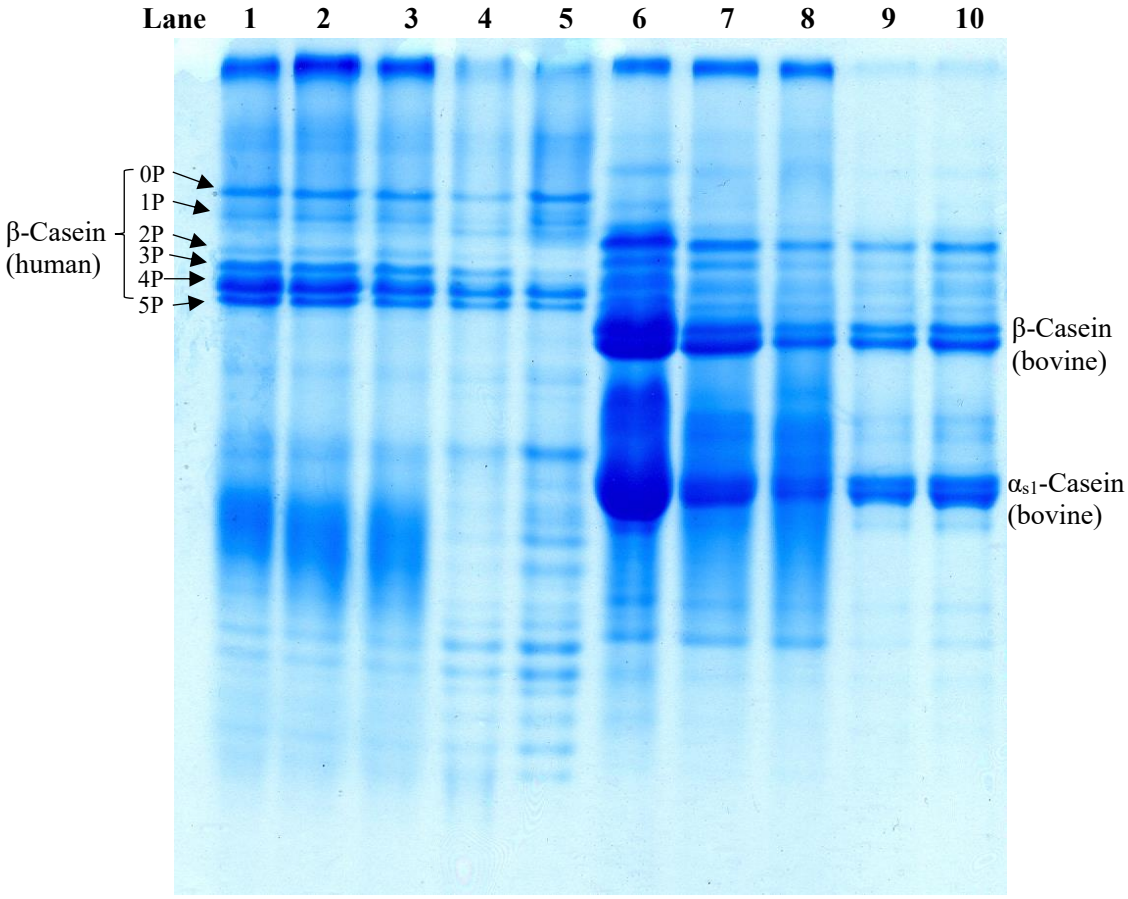

Fig. 5.2 Urea-polyacrylamide gel electrophoretogram of the sedimentable and non-sedimentable caseins of human and bovine milk at 4 and 20°C at natural pH. Samples: Skim milk (**Lane 1**: human & **Lane 6**: bovine); Supernatant at 4°C (**Lane 2**: human & **Lane 7**: bovine); Supernatant at 20°C (**Lane 3**: human & **Lane 8**: bovine); Pellets of milk at 4°C (**Lane 4**: human; resuspended in human milk dialysate & **Lane 9**: bovine; resuspended in bovine milk dialysate); Pellets of milk at 20°C (**Lane 5**: human; resuspended in human milk dialysate & **Lane 10**: bovine; resuspended in bovine milk dialysate).

5.3.3 Dissociation by urea, citrate, and lithium chloride

As well as during cold storage, caseins can also be dissociated by addition of chaotropic agents, such as urea, citrate, and lithium chloride, the impact of which have not been well studied in terms of caseins in human milk. As shown in **Fig. 5.3**A, the turbidity of the human CM suspension decreased, i.e., the dissociation of CM in human milk increased, when the concentration of urea increased, which was similar to the dissociation of bovine CM. However, the initial absorbance at 600 nm of the human CM suspension was much lower compared with bovine CM suspension, due to the smaller size of human CM. On adding 4 mol L^{-1} urea, the optical density of the bovine CM suspension was lower than that of human CM suspension with 20 g L^{-1} proteins,

indicating that the dissociation of bovine CM was much more extensive compared with that of human CM. It has been shown that bovine CM can be disrupted after addition of urea to a concentration of 6 mol L^{-1} (McGann & Fox, 1974).

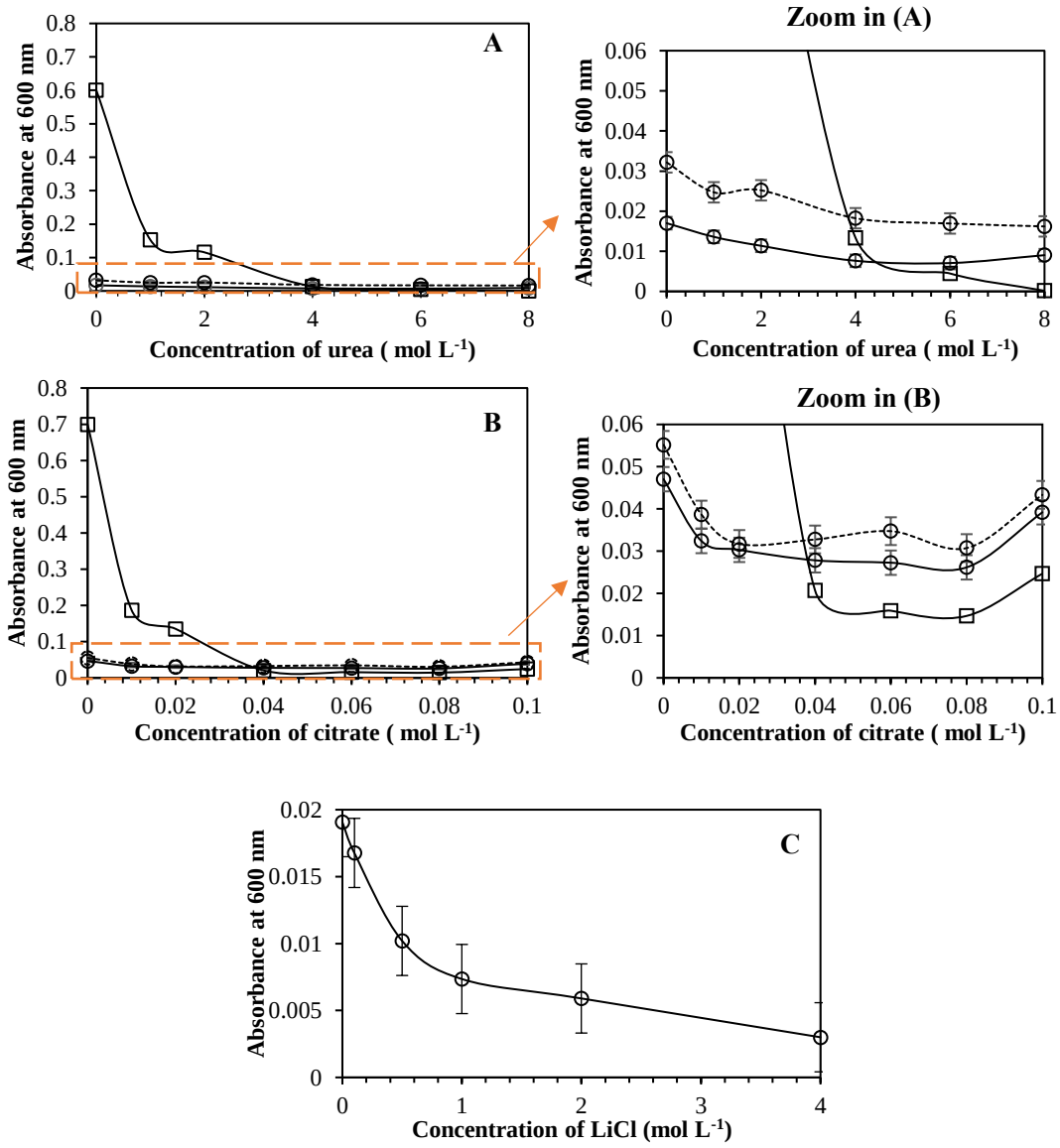

Fig. 5.3 Dissociation of casein micelles in milk samples by (A) urea, (B) citrate, and (C) lithium chloride; human caseins were suspended in dialysate at 10 g L^{-1} proteins (○, solid line) or 20 g L^{-1} proteins (○, dashed line), and bovine caseins were suspended in dialysate at 35 g L^{-1} proteins (□). Error bars represent one standard error of means.

Urea dissociates CMs by breaking hydrogen and hydrophobic bonds without rupturing the linkages between casein and calcium phosphate (Holt, 1992). Therefore, the difference between the effects of urea addition on human and bovine CM may reflect a lower level but higher strength of hydrogen and hydrophobic bonds in human CM compared to bovine CM. Furthermore, Day et al. (2017) reported, from small angle

x-ray scattering study, that urea addition has more effect on the surface of CM rather than its internal structure. This result may thus also indicate a different surface structure of human CM compared to bovine CM.

Addition of trisodium citrate caused a reduction in the turbidity for human CM suspension, which reached a plateau at 0.2 mol L^{-1} citrate, with a slight increase at 1 mol L^{-1} added citrate (**Fig. 5.3** B). Citrate is a calcium chelator that can decrease free calcium and colloidal phosphate with a loss of micelle integrity, leading to the solubilization of caseins (Pouliot et al., 1994). The decrease in turbidity of human CM was not as dramatic as that of bovine CM, i.e., human CM was less affected by the citrate-induced dissociation compared to bovine CM. This could be explained by the trace level of α_s-caseins in human milk. It has been reported that chelation of calcium has little effect on β- and κ-caseins, whereas α_s-caseins are greatly affected due to the high level of basic and acidic amino acids that can link to calcium and CCP (Holt, Davies, & Law, 1986; Smialowska et al., 2017).

LiCl is a mild chaotropic agent; Li^+ can displace the Ca^{2+} in the CM and it is also a potential disrupter of hydrogen bonds (Sood & Slattery, 2003). As shown in **Fig. 5.3** C, the addition of LiCl caused a reduction in the turbidity of the human CM suspension, i.e., dissociation of human CM occurred, which is in agreement with the results of Sood and Slattery (2003). However, the reduction of optical density at 600 nm was very small: from 0.019 to 0.003 following 4 mol LiCl addition and incubation for 1 hour at room temperature, which may agree with the report from Day et al. (2017), based on bovine CM, that the action of LiCl leads to a minor shrinkage at the surface of the CM by an increase in the shielding of the negative charges on the glycosylated sections of κ-casein.

5.3.4 Ethanol stability

The ethanol stability of fresh human milk at natural pH (6.92) was determined as 75%, v/v (**Fig. S5.1**). The ethanol stability of human milk was slightly lower compared with bovine milk, but higher compared with that of equine, ovine, deer or caprine milk reported previously (de la Vara et al., 2018; Uniacke-Lowe, 2011). de la Vara et al. (2018) reported the ethanol stability of bovine milk as 83%, v/v at pH 6.76, while a stability of 70-75%, v/v, at pH 6.7 has also been reported (Huppertz & de Kruif, 2007). Ethanol stability of bovine milk was inversely correlated with concentration of ionic calcium, whereas positively correlated with pH in a sigmoidal shaped curve (Horne, 2003). The concentration of ionic calcium has been reported as 3 mmol L^{-1} in human milk and 2 mmol L^{-1} in bovine milk (Neville, 2005), which is consistent with our results

that the ethanol stability of human milk (higher levels of Ca^{2+}) was lower than bovine milk (lower levels of Ca^{2+}).

As expected, the ethanol stability of skimmed human milk decreased to around 60 % when pH adjusted to 6.2. As shown in **Fig. 5.4**, L*, a*, and b* was monitored on addition of ethanol at different concentrations to skimmed human milk at pH 6.2. L* values decreased as ethanol concentration increased, due to the dissociation of CMs, and reached a minimum at 60 % ethanol. Above the critical ethanol concentration, the L* value increased, indicating aggregation of CM (Horne, 2003). Meanwhile, L* values was relatively stable between 80-100 % ethanol added (1:1 v/v), with a maximum (43.7) at 80% ethanol and minimum (43.0) at 85% ethanol. Interestingly, b* values decreased from 4.8 to 0.92 as ethanol concentrations increased from 5 to 50 %, followed by an increase to 1.44 at 60 % ethanol concentrations (the ethanol stability point). A similar trend was also reported previously in equine milk (Uniacke-Lowe, 2011).

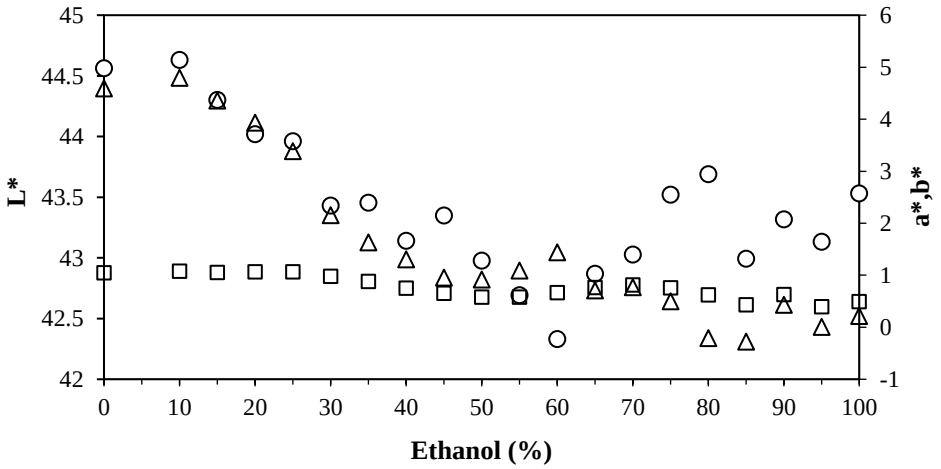

Fig. 5.4 Effect of ethanol concentration on the L* (○), a* (□), and b* (△) values of a 1:1 mixture of skimmed human milk and ethanol from concentrations at 0 to 100% at 20°C.

5.3.5 Chymosin- and pepsin- induced coagulation

When chymosin was added to skimmed human milk (at normal protein content) adjusted to pH 6.2, the storage modulus (G' value), which describes elastic (solid) behaviour, increased (**Fig. 5.5** A). However, skimmed human milk seems to form only a very weak gel after chymosin addition at pH 5.8, 6.0 or 6.6. Compared with chymosin, the coagulation induced by pepsin was even weaker (**Fig. 5.5** B). The weak gel formed by pepsin may be due to the pH value being higher than its optimal pH range (pH 2 -

5). Chymosin-induced coagulation of human milk was also showed by monitoring particle size changes after chymosin was added at pH 6.2. As shown in **Fig. 5.5**C, particle size increased dramatically during 4 hours holding, indicating aggregation of CM.

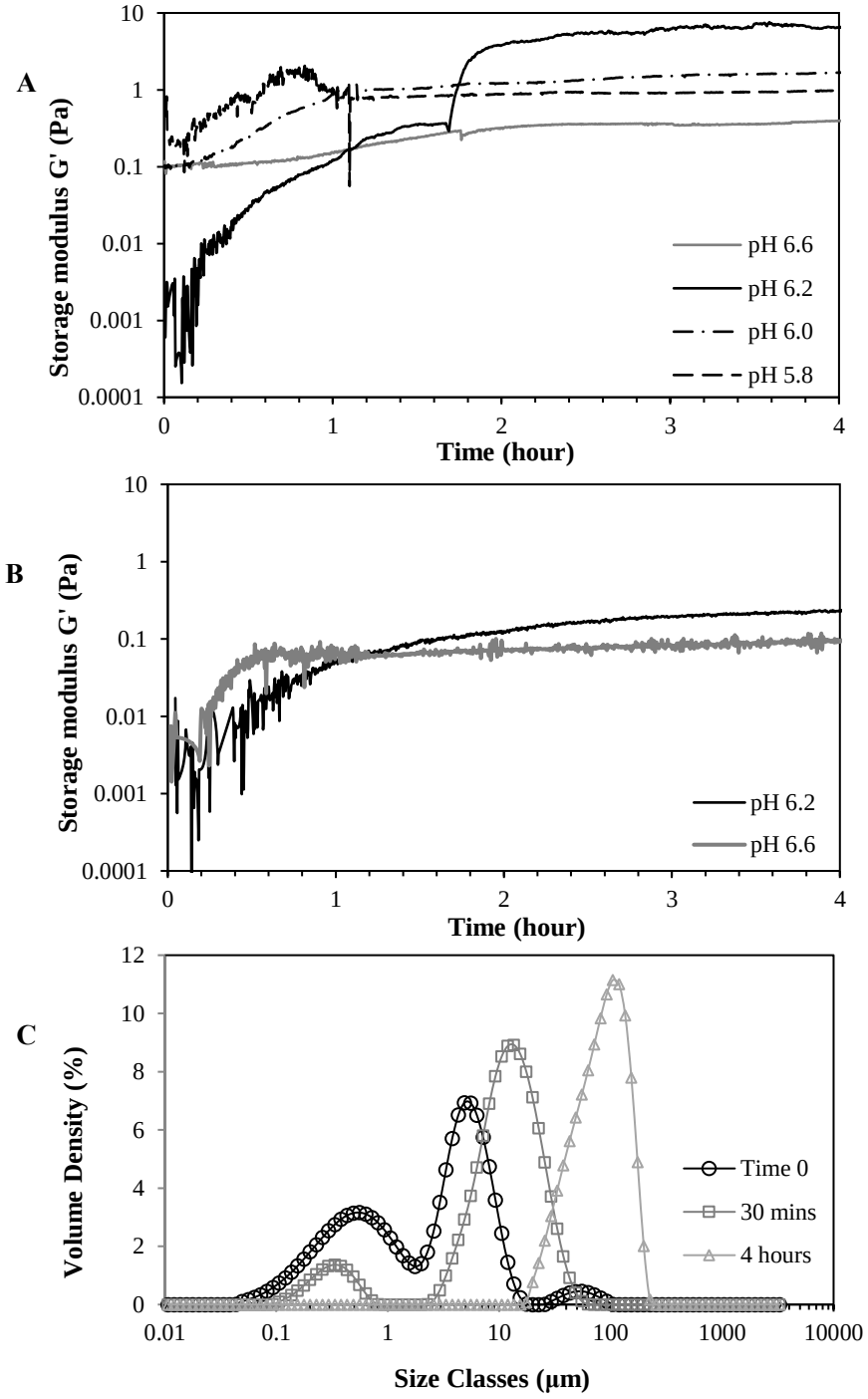

Fig. 5.5 Coagulation of skimmed human milk by **(A)** chymosin at pH 5.8, pH 6, pH 6.2, and pH 6.6 and by **(B)** pepsin at pH 6.2 (black line) and pH 6.6 (grey line); and **(C)** changes of particle size distribution after chymosin addition at pH 6.2 at 0 hour (control), 0.5 hour, and 4 hours.

The weak coagulation of human milk by chymosin and pepsin might due to the species specific reasons as neither chymosin nor pepsin were human source. Or it may reflect the demand of infants' digestion, as curd formation of milk in the stomach delays the degradation of proteins. In contrast, bovine milk can form a much firmer gel after chymosin addition due to the higher level of casein and calcium (total calcium: 29.4 mmol L^{-1}), compared with human milk (total calcium: 7.5 mmol L^{-1}) (Neville, 2005).

5.3.6 Heat stability

As shown in **Fig. 5.6**, the HCT of human milk increased with pH. Increased pH leads to increased charge of caseins, as they are increasingly above their isoelectric points, which is likely the major reason the HCT increased with pH (Mohammed & Fox, 1986). Shorter HCT at pH < 6.6 is likely because the neutralization of surface charge of CMs led to κ-caseins collapsing, leading to a tendency to aggregate. Similar to bovine caseins, human caseins showed long HCT, i.e., high resistance to heat, at natural milk pH (human milk: pH 6.8-7; bovine milk: pH 6.6 -6.8) resulting from the lack of secondary and tertiary structure of caseins (Rehan et al., 2019).

Longer HCT required for human milk coagulation compared to that for bovine milk at the same pH may be due to low levels of casein in human milk (Fox & Brodkorb, 2008). When proteins were concentrated, the shape of HCT-pH curves was similar to that of unconcentrated milk; however, HCT was shorter at each pH value (Fox & Brodkorb, 2008).

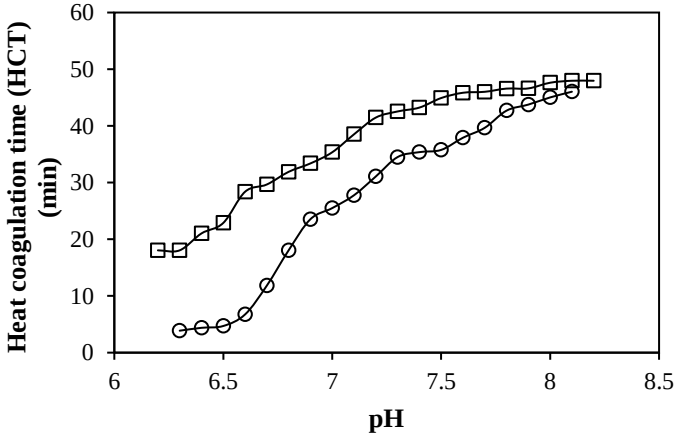

Fig. 5.6 Heat coagulation time-pH profiles of skimmed term human milk (□), and concentrated skimmed term human milk (3 x proteins, ○).

The heat stability profile of human milk was similar to the type B profile found in bovine milk studies because of different profiles of whey proteins (which are more heat-sensitive than caseins), especially the fact that no β-leg exist in human milk, which increases the extent of κ-casein dissociation and thus coagulation of CM (Dumpler et al., 2020). When β-lg is present, i.e., in type A bovine milk, denatured β-lg attaches to CM at high pH (pH > 6.7 in bovine milk) and aggregate together with CMs. In contrast, at low pH (pH < 6.7), denatured β-lg can form complexes with κ-casein as a steric barrier on the surface of CM against aggregation, and thus the HCT can be longer (O'Connell and Fox, 2003). Notably, although β-lg is absent from human milk, other whey proteins, including serum albumin and α- lactalbumin, have been reported to play similar roles (Fox & Hearn, 1978). According to the theory above, human milk exhibited similar short HCT at pH < 6.5, which may partly be caused by less steric barrier as no β-lg exist, while at high pH a competition may occur between whey proteins to accelerate the coagulation and repulsion between CMs.

5.3.7 Heat-induced changes in colour of human milk

The colour of human milk subjected to different heating conditions is shown in **Fig. 5.7**A. It is clear that the colour of milk changed from a milky white to a yellow/ brown colour with increasing heating temperature. From L*a*b* measurement, the increase of the yellowness is consistent with increased b* values (**Fig. 5.7** B), especially in milk heated at 100 to 130°C. However, the b* value decreased slightly in human milk heated between 63°C and 100°C. Compared with human milk, the increase of b* value of bovine milk started at a lower temperature, 80°C. The values of L* (lightness) and a* (green to red) of both human and bovine milk were relative constant when milk was heated at temperature from 63°C to 110°C. The dramatic decrease of L* and increase of a* occurred at 120°C and 130°C for both heated human and bovine milk.

At high temperature, changes in milk colour results from non-enzymatic (Maillard) browning, which is consistent with the L* value decreasing while the a* and b* values increased (Lewis & Heppell, 2000). Lactose, levels of which are high in human milk, and lysine residues in milk proteins (mainly casein) are the reactants of the Maillard reaction and the yellow-brown colour is caused by coloured compounds, including melanoidins produced from the final stage of the reaction (Arena et al., 2017; Van Boekel, 1998). Meanwhile, heat-induced denaturation of whey proteins and their aggregation with CM, dephosphorylation/breakdown of caseins, and precipitation of soluble calcium phosphate on CMs may all lead to a reduction in the whiteness of milk (Parmar et al., 2018).

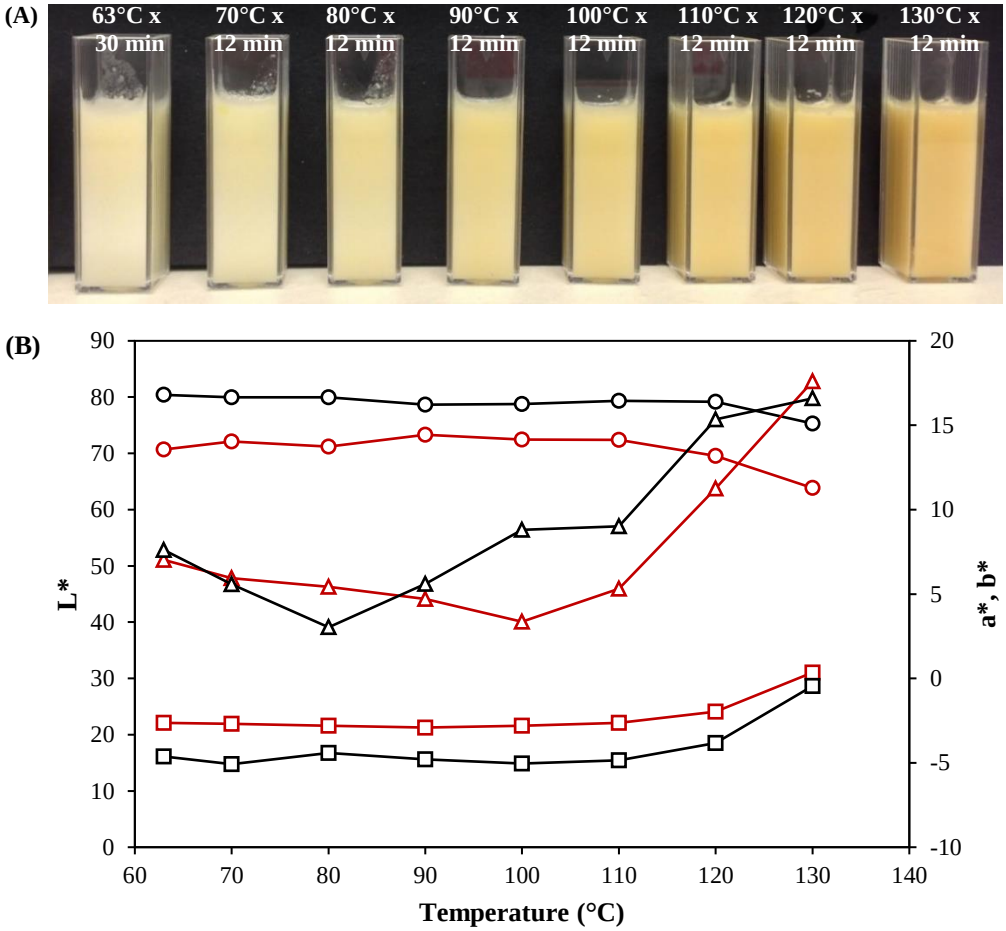

Fig. 5.7 Heat-induced colour changes of human and bovine milk. (A) Images of human milk samples heated from 63 to 130°C with the heating condition labelled; (B) L* (○), a* (□), and b*(△) changes of human (red) and bovine (black) milk. Results are from one pool of human milk or bovine milk but representative for others.

5.3.8 Effect of temperature on protein profiles and proteolysis in human milk

The impact of heating on proteolysis in human milk is was addressed in this study. As shown in **Fig. 5.8**, under non-reducing conditions, the bands of lactoferrin and serum albumin disappeared gradually during heating from 63°C to 130°C, and the disappearance of the α-lactalbumin band occurred after heating at 110°C. The bands of lactoferrin and serum albumin in milk heated at 110°C and the bands of α-lactalbumin in milk heated at 130°C reappeared under reducing condition, suggesting that these whey proteins are involved in disulphide-mediated interactions (Lowe et al., 2004). Furthermore, little evidence of protein bands of lactoferrin and serum albumin being resolved at 130°C was found under either reducing and non-reducing conditions, suggesting that the irreversible denaturation and heat-induced coagulation of proteins occurred.

Compared to lactoferrin and serum albumin, β-caseins are more heat-stable, as the bands of caseins only disappeared gradually after heating at 110°C, which could result from the absence of disulphide linkages and therefore little secondary or tertiary structure of caseins (Rehan et al., 2019).

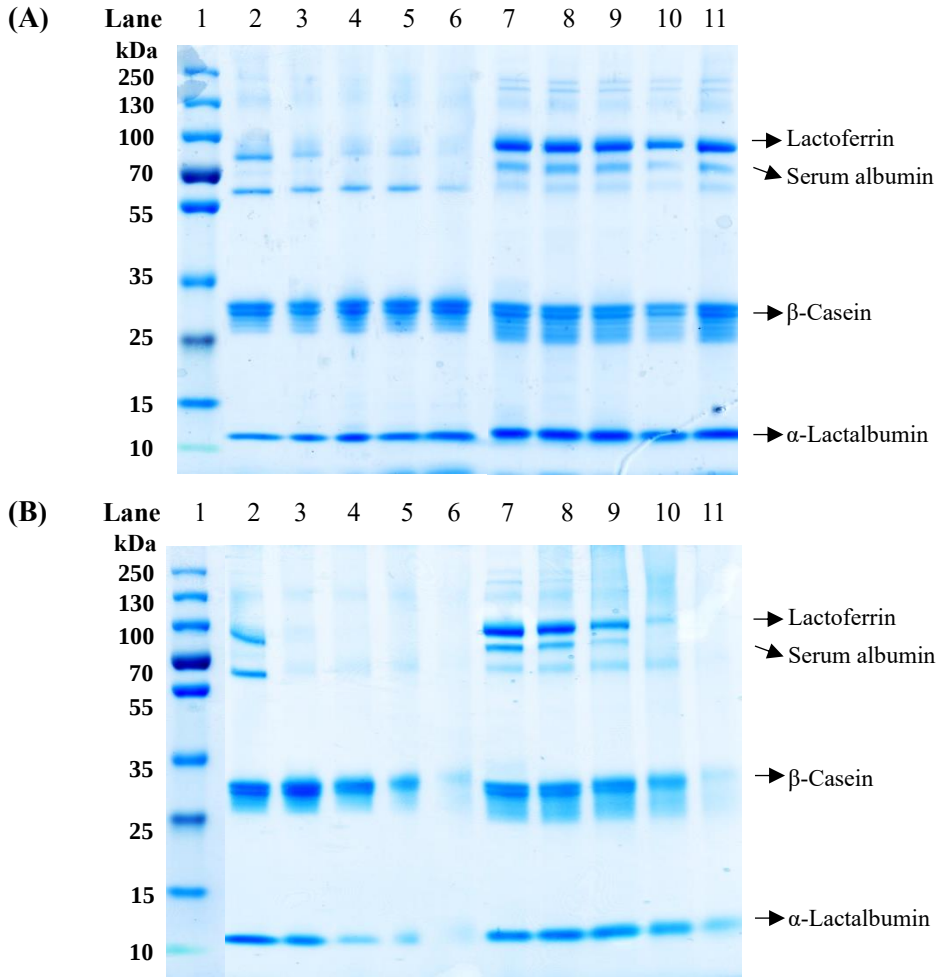

Fig. 5.8 Representative SDS-polyacrylamide gel electrophoretograms of human milk. Human milk samples were heated at (A) 63 – 90 °C or (B) 100-130°C before being separated by SDS-PAGE under non-reducing (Lane 2 – 6) or reducing conditions (Lane 7 – 11). Human milk control was unheated. The most abundant proteins in human milk are labelled on the gel. (A) **Lanes**: **1**: Marker; **2 & 7**: Control; **3 & 8**: 63°C x 30 min; **4 & 9**: 70°C x 12 min; **5 & 10**: 80 °C x 12 min; **6 & 11**: 90 °C x 12 min. (B) **Lanes**: **1**: Marker; **2 & 7**: Control; **3 & 8**: 100°C x 12 min; **4 & 9**: 110°C x 12 min; **5 & 10**: 120 °C x 12 min; **6 & 11**: 130 °C x 12 min.

Milk samples from different heat treatments were also incubated at 37°C to examine the heated-induced changes in proteolysis. Over a 7-day period, there was a progressive breakdown of β-caseins in the non-heated sample and sample heated at 63°C for 30 mins, whereas higher levels of β-caseins retained in milk heated from 80°C to 110°C for 12 mins (**Fig. 5.9**).

The intact β-casein suggests that proteolysis did not occur in the samples heated at 90°C or higher, which may be due to the inactivation of plasmin, the major milk protease active against β-casein. Plasmin is a heat-stable protease the activity of which has been shown to increase following high-temperature short-time (HTST; 72°C x 15 s) pasteurisation due to the inactivation of the thermally unstable plasmin activators inhibitors (PAI) (Prado et al., 2006; Richardson, 1983). From bovine milk studies, further heating leads to a decrease of plasmin activity due to disulphide interactions of plasmin and plasminogen with denatured β-lg (Burbrink and Hayes, 2006). Notably, human milk does not contain β-lg; however, the plasmin activity appeared to be lost at high temperatures (90 -120°C). In addition, a constant band (labelled as band 1 in **Fig. 5.9**) that remained in milk heated at 63 to 80°C disappeared in milk heated from 90 to 130 °C, which suggested the band could be whey proteins. Combined with findings from **Fig. 5.8** and **Fig. 5.9**, it seems that human milk heated at 80°C for 12 mins is better for stability of proteins when stored for longer periods with concomitant low modification of the colour (**Fig. 5.7**).

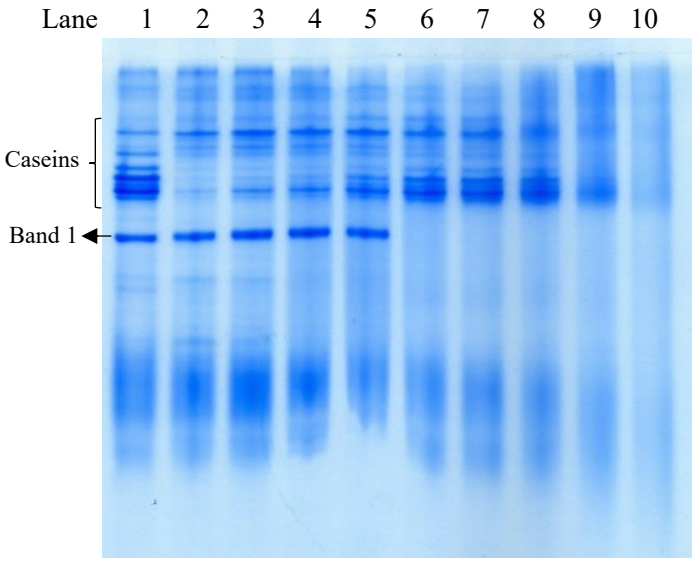

Fig. 5.9 Representative Urea polyacrylamide gel electrophoretogram of human milk heated from 63°C to 130°C and incubated at 37°C for 7 days. Samples: Control (untreated; no incubation, **Lane 1**); Unheated milk (**Lane 2**); Milk was heated at 63°C for 30 mins (**Lane 3**); Milk heated at 70°C (**Lane 4**), 80 °C (**Lane 5**), 90 °C (**Lane 6**), 100°C (**Lane 7**), 110°C (**Lane 8**), 120 °C (**Lane 9**), and 130 °C (**Lane 10**), respectively, in all cases for 12 mins.

5.3.9 Inhibition of proteolysis of caseins in human and bovine milk

A preliminary experiment involving the identification of bands from mass spectrometry (**Fig. 5.10**) showed that there are individual differences in human milk both in terms of initial protein profiles and the speed of protein breakdown during incubation at 37 °C. Proteins bands that changed during incubation were identified according to peptide-spectrum match (PSM), i.e., number of times that the mass spectrometer saw the peptides (**Fig. 5.10** B). In general, lactotransferrin was shown at the top of the urea-gel; caseins presented at the top of the gel under the lactotransferrin; osteopontin resolved at the middle of the gel (most likely band F); while α-lactalbulmin was found at the bottom of the gel. Accroding to the high levels of α-lactalbulmin, the wide band under band J could be α-lactalbumin.

The difference of protein breakdown in human milk could result from individual differences in plasmin activity. Human milk samples were subsequently grouped into high and low plasmin activity groups with average plasmin activities at 0.29 ± 0.016 and 0.012 ± 0.0042 nmol AMC mL^{-1} min^{-1}, respectively (**Table 5.2**). Armaforte et al. (2010) reported a lower plasmin activity in term human milk (0.045 - 0.104 AMC units mL^{-1}) compared with preterm human milk (0.147 - 0.397 AMC units mL^{-1}). Our results showed that high individual difference exists in terms of plasmin activity in human milk, even at a similar lactational stage. In parallel, bovine milk was incubated at the same condition as a comparison to human milk and its plasmin activity was measured as 0.11 ± 0.011 nmol AMC mL^{-1} min^{-1}, which was slightly lower than that reported previously (0.193 AMC units mL^{-1}) (Enright et al., 1999), but in agreement with the higher level compared with human milk (Korycha-Dahl et al., 1983).

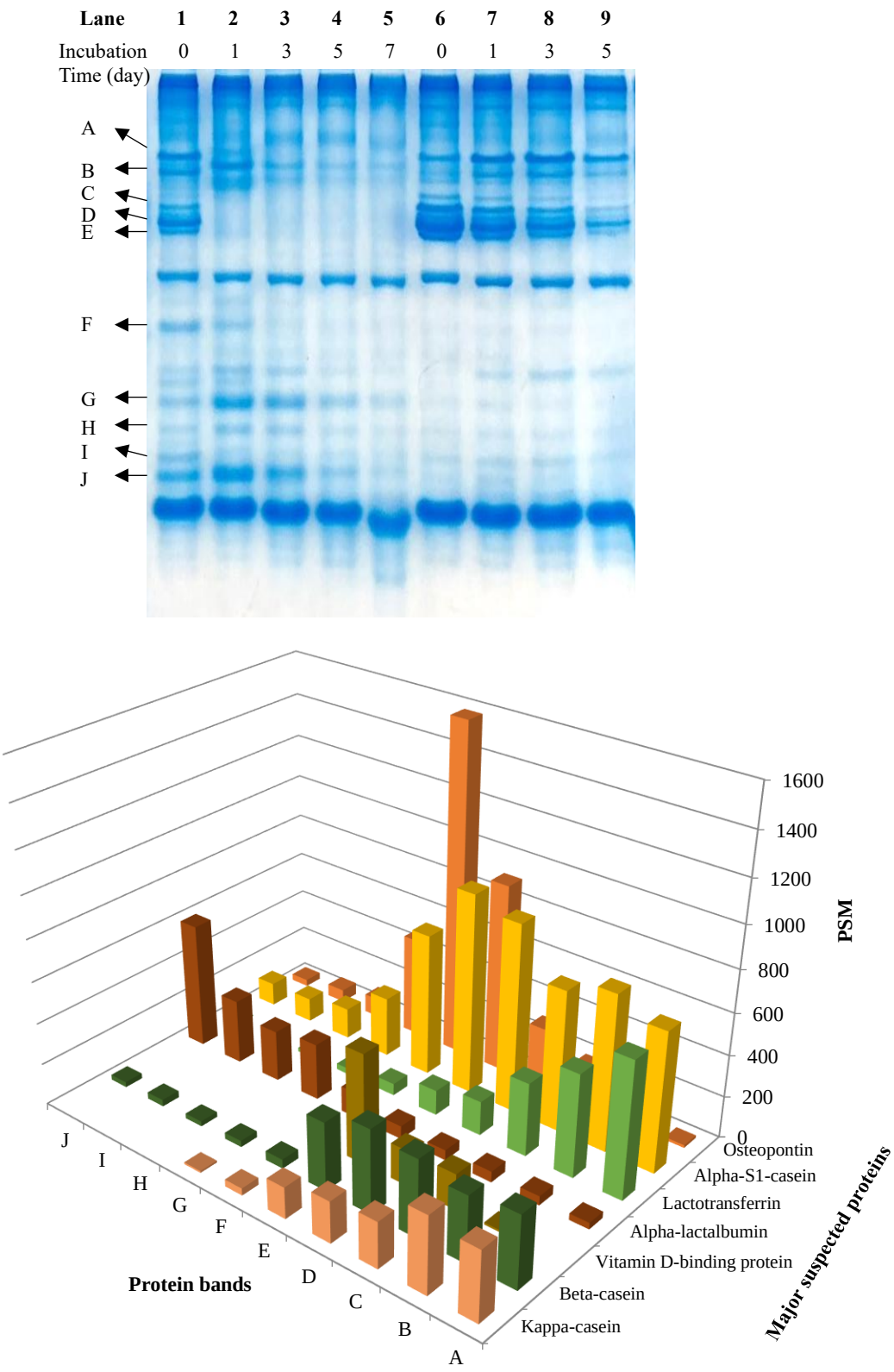

Fig. 5.10 (A) Protein breakdown of human milk during incubation at 37°C using urea-PAGE and (B) protein band identification using mass spectrometry. (A) Lane 1-5: human milk with relatively higher plasmin activity; Lane 6-9: human milk with relatively lower plasmin activity. PSM: peptide-spectrum match.

Table 5.2 Quantitative analysis of degradation of β-caseins in human and bovine milk. Data is presented as means ± standard deviation.

Samples	**PA (nmol AMC mL^{-1} min^{-1})**	**Inhibition/ Treatments**	**Percentage of total β-caseins before incubation (%)**				
			Incubation Time (day)				
			0	1	4	7	10
Human milk (Low PA)	0.012 ± 0.0042	CT	100	81 ± 2	66 ± 7	44 ± 29	44 ± 21
		+ aprotinin	100	82 ± 12	45 ± 19	33 ± 28	21 ± 22
		+ pepstatin	100	94 ± 11	66 ± 37	63 ± 37	74 ± 24
Human milk (High PA)	0.29 ± 0.016	The bands of β-caseins were barely present.					
Bovine milk	0.11 ± 0.011	CT	100	81 ± 27	51 ± 34	53 ± 1	26 ± 7
		+ aprotinin	100	89 ± 9	77 ± 19	80 ± 18	83 ± 14
		+ pepstatin	100	100 ± 16	52 ± 27	47 ± 19	37 ± 5

PA: Plasmin activities.

As expected, human milk with low plasmin activity showed more complete casein profiles before incubation and slower proteolysis during incubation at 37°C for 10 days (**Fig. 5.11** Lane 1) compared with human milk with high plasmin activity (**Fig. S5.2**). The variation of casein profiles before incubation has been reported to be due to the pre-digestion of milk proteins, mostly by plasmin in the mammary gland (Nielsen et al., 2017). Interestingly, aprotinin, which inhibits proteolysis of caseins by plasmin in bovine milk (**Fig. S5.3**) did not inhibit proteolysis of β-caseins in human milk (**Fig. 5.11**). In contrast, pepstatin clearly inhibited proteolysis in human milk, but has less impact on inhibition of proteolysis in bovine milk compared with aprotinin. Quantitative analysis (**Table 5.2**) showed that around 74% of β-casein remained in human milk with pepstatin addition after incubation at 37°C for 10 days, whereas only 21% of β-casein remained in the milk with aprotinin addition. In contrast, approximately 83% of β-casein was retained after the aprotinin addition, while only 37% of β-caseins remained after incubation of bovine milk with pepstatin at 37°C for 10 days. The results suggest that the major proteases, active during human milk incubation at 37°C, could be aspartyl peptidases, for example, cathepsin D, rather than serine protease, such as plasmin, kallikrein, elastase or thrombin.

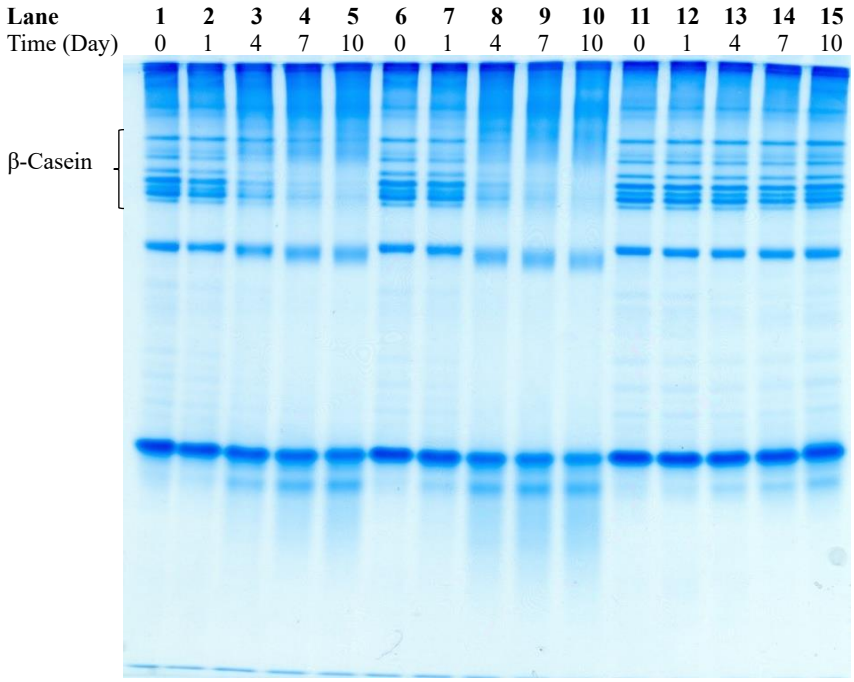

Fig. 5.11 Urea polyacrylamide gel electrophoretograms of human milk with low plasmin activity with no inhibitors (control; Lane 1-5), or with aprotinin (Lane 6-10), or pepstatin A (Lane 11-15) during incubation at 37°C for 10 days. The electrophoretograms are from individual human milk but representative for others.

As shown in UPLC chromatograms (**Fig. 5.12**), the initial peptide profiles in individual human milk samples were different, which may relate to the difference of plasmin activities in milk. Higher levels of peptides presented in human milk with high plasmin activity compared with human milk with low plasmin activity at D0 (before the incubation). Peptides in human milk with low plasmin activity increased after incubation at 37°C for 10 days. After aprotinin or pepstatin addition, fewer peptides were found; however, the profiles are different, especially the peptides eluted at retention time at around 30 mins. Peptides eluted between retention time from 15 to 35 mins disappeared after incubation at 37°C for 10 days. The results, once again, showed a clear difference of human milk protein profiles between individuals that could affect infant digestion.

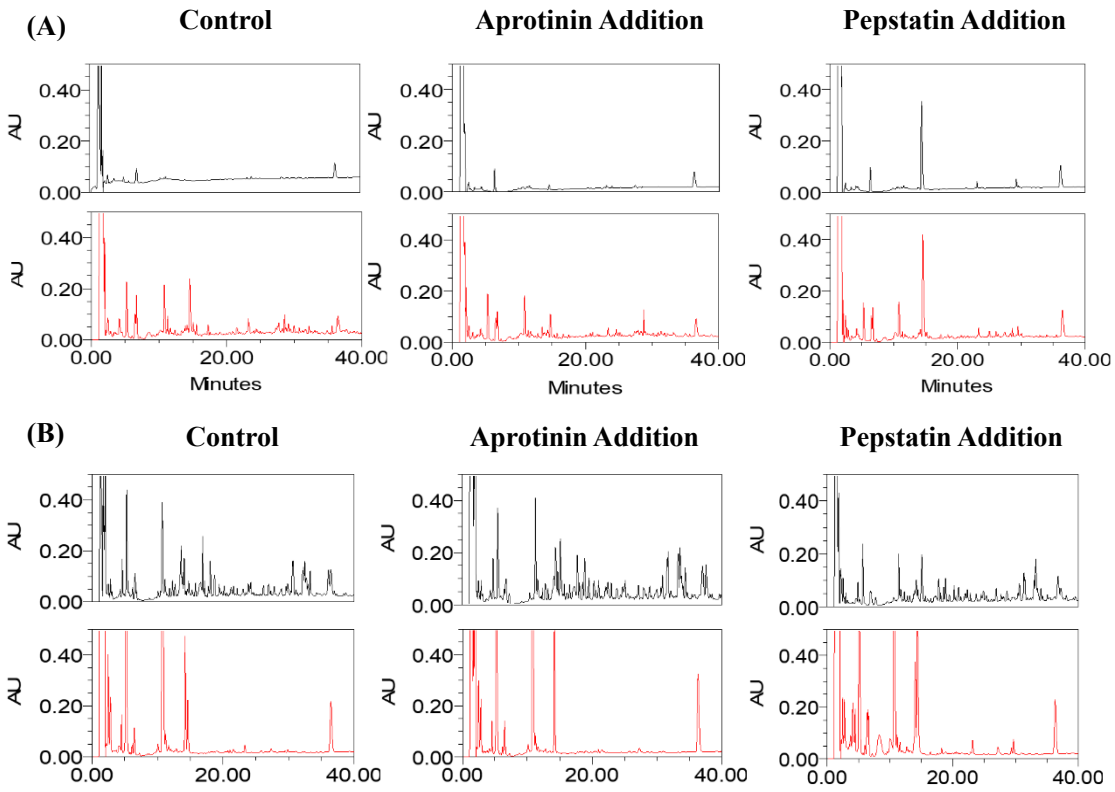

Fig. 5.12 Reversed-phase ultra-performance liquid chromatography chromatograms of peptides (< 10 kDa) in human milk with low plasmin activity (A) and high plasmin activity (B) after 10-day incubation (red) and control (before incubation, black).

5.4 Conclusion

This work studied the structure and physio-chemical properties of CMs in human milk, including ethanol stability, heat stability and stability to chaotropic agents, and expands our knowledge of the properties and stability of the casein system in human milk. Compared to CMs in bovine milk, when studying typical destabilising factors, human milk CMs were seen to be smaller with a lower ethanol stability and a type B HCT-pH profile. Meanwhile, human CM was shown to be coagulable by bovine chymosin to form a soft curd. Heating induced changes in colour and proteolysis of human CMs and an inhibition study on human milk proteolysis suggested that aspartyl peptidases may be major proteases in milk with low plasmin activity when incubated at body temperature. One limitation of this work is the small sample size; milk from different lactation times was supplied by only one mother to study the physical stability of caseins. In future studies, greater numbers of samples need to be studied for more robust results of the stability of human casein micelles.

Supplementary Figures

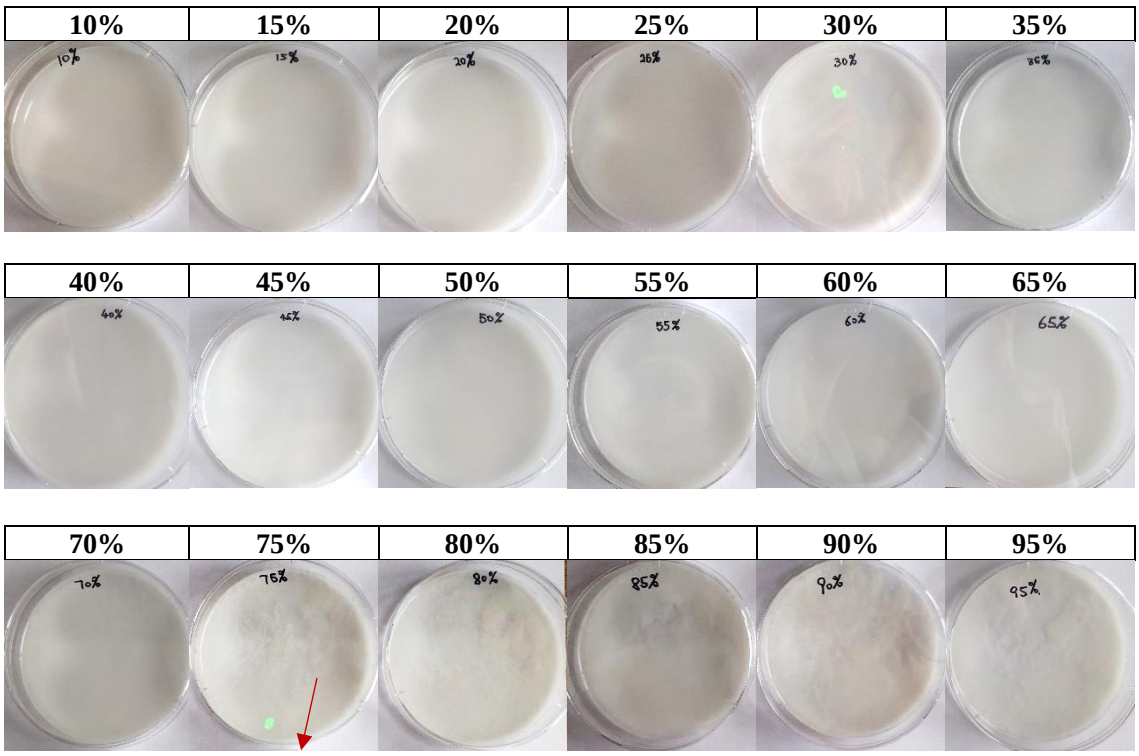

Fig. S5.1 Assessment of the ethanol stability of human milk. Percentage indicated ethanol concentrations (v/v).

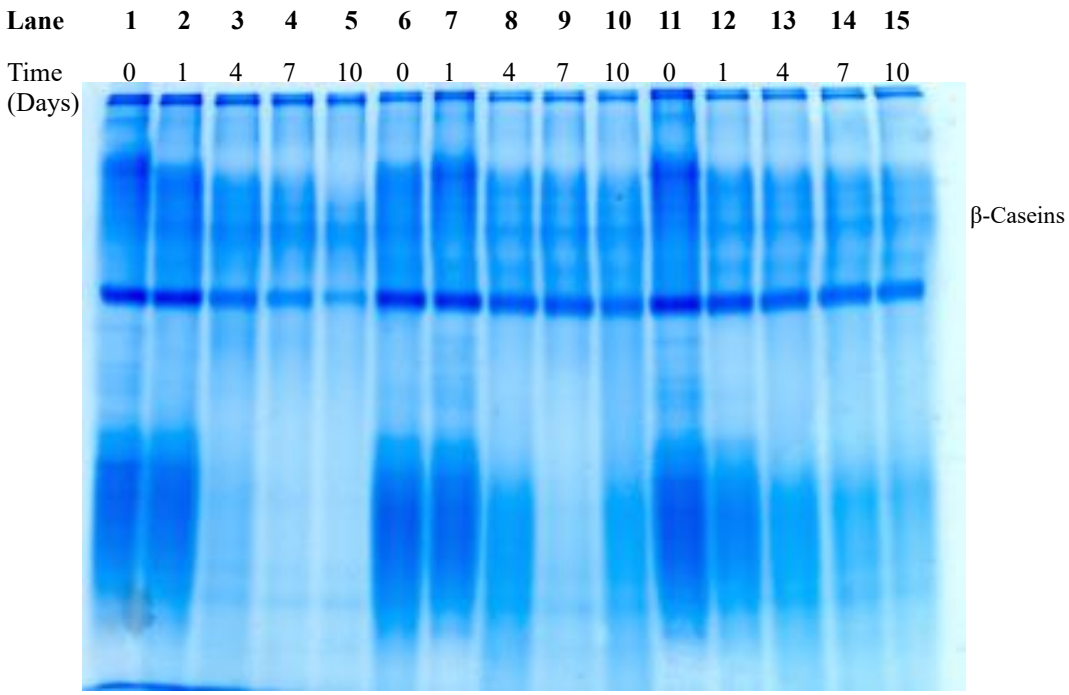

Fig. S5.2 Urea polyacrylamide gel electrophoretograms of human milk with high plasmin activities with no inhibitors (control; Lane 1-5), with aprotinin (Lane 6-10), and with pepstatin A (Lane 11- 15) during a 10-day 37°C incubation. The electrophoretograms are from individual human milk but representative for others.

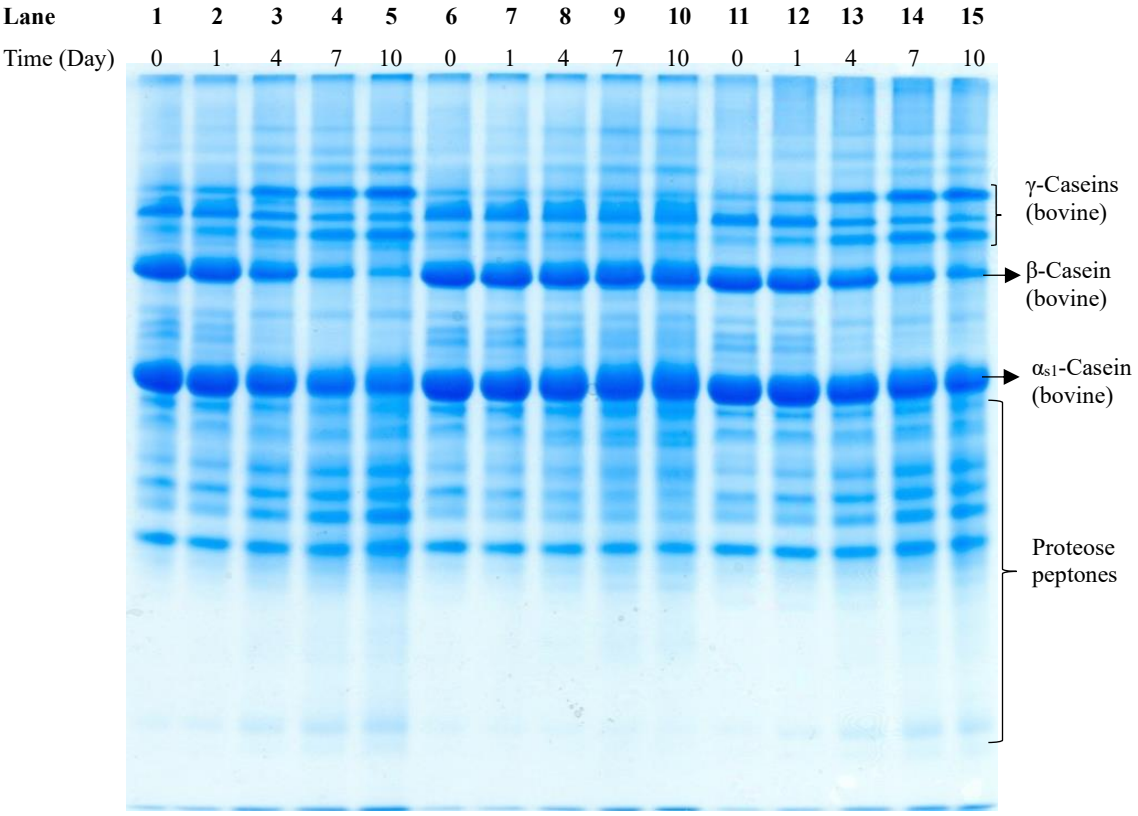

Fig. S5.3 Urea polyacrylamide gel electrophoretograms of bovine milk with no inhibitors (control; Lane 1-5), with aprotinin (Lane 6-10), and with pepstatin A (Lane 11- 15) during a 10-day 37°C incubation. The electrophoretograms are from individual bovine milk but representative for others.

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CHAPTER 6

Effects of Holder Pasteurization, High-pressure Processing, and Storage on the Physio-chemical Stability of Human Milk

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Abstract

Currently, holder pasteurization (HoP; 62.5°C for 30 min) is applied as a standard approach to preserve donated human milk, which affects some immunological and bioactive compounds in the milk. This study aims to study the impact of an alternative to HoP, high-pressure (HP) treatment, on human milk composition and stability. HP treatments was carried out at 200, 400 and 600 MPa for 10 min. Levels of macronutrients, enzyme activities, and some physico-chemical properties were measured before and after treatments, followed by monitored storage at 4°C for one week. Our results showed that lactoperoxidase activity was low in all samples before the storage (4.9 U L^{-1} in control); HP treatments preserved LPO activity. Higher levels of lysozyme and plasmin activities remained after HP treatments compared to HoP. Cathepsin D activity remained after HP treatments at 200 MPa or 400 MPa, whereas it was lost after HoP or HP treatment at 600 MPa. The stability of human milk after treatments changed during storage and the creaming rates decreased in all milk samples. The results suggest that HP treatments, which showed comparable perseveration of macronutrients and a better preservation of enzymes in human milk, can be a potential alternative to HoP in human milk banks. HP treatments at 400 MPa for 10 min may be the best preserving methods among treatments in our study that ensure both microbial and bioactive quality of milk.

6.1 Introduction

Human milk is a functional and dynamic fluid that provides infants with essential nutrients, as well as bioactive and immune molecules, such as cytokines, growth factors, and oligosaccharides, which aid their growth and development (Ballard & Morrow, 2013). Although fresh mothers' own milk is regarded as the optimal food for newborn infants (Eidelman & Schanler, 2012), donor human milk (DHM) is recommended by the World Health Organization (WHO) when mothers' own milk is not available (Arslanoglu et al., 2013; WHO, 2018). The levels of macronutrients in DHM have been reported by Wojcik et al. (2009), Michaelsen et al. (1990), and Mills et al. (2019). DHM is pooled from human milk, normally donated from mothers who have given birth to a term infant, which may not have sufficient macronutrient content or energy density to sustain a preterm infant with low birth weight (Wojcik et al., 2009; Underwood, 2013). In addition, freezing and thawing may influence human milk by slightly decreasing energy and fat content (Kim et al., 2019), decreasing pH, and increasing free fatty acid levels (Handa et al., 2014).

Holder pasteurization (HoP, 62.5°C x 30 min) is the current preservation method applied to DHM as a critical step in human milk banks to inactivate viral and bacteriological agents (Arslanoglu et al., 2010; Moro et al., 2019). Although the HoP process kills bacteria and inactivates viruses (Lima et al., 2017; Unger et al., 2020), it may negatively influence some of the nutritional and biological components in human milk (Chang et al., 2013; Moro et al., 2019). It has been shown that functional enzymes, including lipoprotein lipase, bile salt-stimulated lipase, and glutathione peroxidase (Evans et al., 1978; Guerra et al., 2018; Henderson, Fay, & Hamosh, 1998) and bioactive components, such as immunoglobulins (Escuder-Vieco et al., 2018), cytokines (Giorgi et al., 2018), hormonal compounds (Escuder-Vieco et al., 2018; Vass et al., 2020) may be damaged or destroyed as a result of this heat treatment. In addition, there have been varying reports on the impact of HoP of breast milk on the macronutrient and micronutrient content (Delgado et al., 2014; García-Lara et al., 2013; Lima et al., 2018; Moltó-Puigmartí et al., 2011; Peixoto et al., 2020).

Alternatives to HoP, including both thermal and non-thermal methods have been studied for persevering microbial-safe human milk with better nutritional and bioactive

compound. Among thermal methods, high temperature short time pasteurization (HTST; 72°C x 15s), which is the commercial pasteurization method used in dairy industry has been applied in human milk. A higher retention of total antioxidant capacity in human milk after HTST compared to HoP was reported by Silvestre et al. (2008). However, the retention of immunoglobulins in milk following HTST has been reported to be higher (Daniels et al., 2017; Escuder-Vieco et al., 2018) or lower (Castro et al., 2017) than in milk after HoP. Pasteurization at lower temperature (57°C) for 30 min has been demonstrated to retain high levels (> 90%) of secretory immunoglobulin A, lysozyme and lactoferrin (Czank et al., 2009), however, cytomegalovirus is known to persist at this temperature (Moro et al., 2019).

In recent years, high-pressure (HP) treatment has drawn attention as a non-thermal preserving method to be applied in human milk (Wesolowska et al., 2019). HP treatment using pressures in the range of 100 - 800 MPa has been demonstrated to inactivate pathogenic and spoilage bacteria (Balasubramaniam, Martinez-Monteagudo, & Gupta, 2015). The treatment can be conducted at ambient or chilled temperatures and has minimal impact on the composition of milk. It has been reported that HP treatments retains higher levels of heat-labile compounds, including immunoglobulins (Delgado et al., 2014; Permanyer et al., 2010; Viazis, Farkas, & Allen, 2007), lysozyme activity (Mayayo et al., 2016; Sousa, Delgadillo, & Saraiva, 2014; Viazis et al., 2007) and vitamin C (Moltó-Puigmartí et al., 2011) in human milk than HoP. Also, HP treatments does not affect the levels of certain cytokines (Delgado et al., 2013) and free nucleotide monophosphates (Mateos-Vivas et al., 2015). These compounds, such as immunoglobulins, lysozyme, and cytokines are essential to immune systems of infants by providing anti-bacterial and anti-inflammatory effects (**Chapter 1**).

This objective of this study was to investigate the efficiency of HP treatments at different conditions (200 MPa, 400 MPa, and 600 MPa) as an alternative to HoP in preserving macronutrients and some important enzymes, including lactoperoxidase (LPO), lysozyme, plasmin, and cathepsin D. In addition, the storage of HP-treated human milk at 4°C for one week on the nutritional quality as well as physical stability of human milk was investigated.

6.2 Materials & Methods

6.2.1 Human milk samples

The use of samples in the study was approved by Cork Research Ethics Committee. Unpasteurised human milk samples were collected from Cork University Maternity Hospital and one sample was donated with consent.

All human milk samples were stored at -20°C until analysis. Prior to analysis, frozen human milk was thawed at 4°C overnight and pooled under sterile conditions. Duplicated trials were carried out in our study: milk from 4 participants was pooled to achieve a total volume of 750 mL in the first trial, and milk from 6 participants was pooled to achieve a total volume of 1 L in the second trial.

In each trial, pooled milk was inverted to achieve a good mix without influencing the physical stability properties of milk, and then divided into five groups, depending on the treatment to be performed: control (untreated), HoP-treated, HP-treated at 200 MPa, HP-treated at 400 MPa, and HP-treated at 600 MPa. Following treatments, human milk was stored at 4°C for 1 week and analysis was performed at 0, 1, 4, and 7 days (D0, D1, D4, D7, respectively) of the storage. At each time point, milk in each treatment group was sub-aliquoted for determination of macronutrients levels, plasmin and cathepsin D activities, LPO and lysozyme activities, and physical stability testing. All chemicals in the study were obtained from Merck KGaA, Darmstadt, Germany unless otherwise stated.

6.2.2 Treatments

6.2.2.1 High-pressure processing

Human milk samples (100-150 mL) were vacuum-packed into sterile stomacher bags. The sterile stomacher bag was then placed into a polyethylene bag and vacuum sealed, which was repeated 3 times. HP treatment was performed with a Stansted Fluid Power Iso-lab 900 High Pressure Food Processor (Stansted Fluid Power, Stansted, Essex, UK). A 90:10 mixture of ethanol and castor oil was used as pressurising fluid. Pressure was increased at an average rate of 120 MPa min^{-1} to a value in the range 200–

600 MPa and maintained at the desired pressure for 10 min. Detailed profiles of temperature and pressure during treatment are shown in **Fig. S6.1.**

6.2.2.2 Holder pasteurisation

For HoP treatment, human milk (150 to 200 mL) was transferred into sterilized Duran bottles and placed into a preheated water bath (Grant Instruments Ltd, Cambridgeshire, UK). A control bottle, containing a thermometer and filled with an equal volume of water as the test bottle, was used to monitor the temperature of the water bath. Once the temperature reached 62.5°C, it was held for 30 min and then cooled.

6.2.3 Determination of levels of macronutrients in human milk

Total fat, protein, carbohydrate and energy content of human milk was determined with a human milk analyser (MIRIS, Uppsala, Sweden) based on mid-infrared (mid-IR) transmission spectroscopy principles. Samples collected for compositional analysis were frozen at -80 °C prior to analysis, thawed at 4°C overnight, warmed to 40°C and homogenized using a MIRIS sonicator (MIRIS, Uppsala, Sweden) before analysis.

6.2.4 Assay of enzyme activities

Prior to the assay of lysozyme and LPO activities, skimmed human milk was obtained by centrifugation at 6,000g for 20 min at 4°C. To measure the cathepsin D and plasmin activity, human milk was defatted according the method followed prior to the analysis.

Lysozyme activity was determined by the method of Shugar (1952). Skimmed human milk (0.1 mL) was mixed with a 0.015% (w/v) *Micrococcus lysodeikticus* ATCC 4698 cells suspension (2.5 mL) and the turbidity of the mixture was measured at A450 nm using a spectrophotometer (Varian Cary 50 UV-Vis, Agilent, Santa Clara, CA). One unit of lysozyme activity was expressed as the amount of lysozyme required to produce a decrease of A450 of 0.001 per minute at pH 6.24 at 25 °C using a suspension of M *lysodeikticus* as substrate.

LPO activity was determined by the method from Zarei et al. (2016). Skimmed milk (10 μL) was mixed with 210 μL of 100 mM potassium phosphate buffer (pH 5.5) and 70 μL of 10 mM 2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) in a UV-transparent 96-wall microplate (VWR International Ltd, Luttervorth, UK). The reaction was then initiated with 10 μL freshly prepared 0.025% H_2O_2 solution and the absorbance was determined at A436 nm using a microplate reader (SpectrafluorPlus, Tecan, Männedorf, Switzerland) for 5 min. The blank was a human milk sample heated at 95°C for 1 min. One unit of LPO enzymatic activity was expressed as the amount of enzyme needed to oxidize 1 μmol ABTS min^{-1}. The millimolar extinction coefficient of ABTS at 436 nm was 29.3 min^{-1} cm^{-1}.

Plasmin activity in milk was measured using the modified method of Richardson and Pearce (1981). A reaction mix of the sample (100 μL), coumarin substrate (40 μL) and 50 mM tris-HCl buffer (pH 7.5, 60 μL) were added into a well of black 96-well microplates (VWR International Ltd, Luttervorth, UK). The fluorescence intensity was determined at 380 nm (excitation) and 460 nm (emission) using a plate reader (Spectrafluorplus, Tecan). One unit of plasmin activity is defined as the activity necessary to release 1 nmol AMC sample per mL per min under the conditions of the assay.

The cathepsin D activity of samples was measured as described by Hurley et al. (1999). Skimmed human milk (70 μL) was mixed with 30 μL 1 mg mL^{-1} aqueous solution of a synthetic heptapeptide substrate (Pro-Thr-Glu-Phe-[NO2-Phe]-Arg-Leu; Bachem Feichemikalien AG, Switzerland) and 200 μL of 100 mM sodium formate buffer (pH 3.2, with 0.05% sodium azide) and incubated at 37 °C for 5 hours. A Waters Acquity UPLC H-Class Core System (Waters, Milford, MA, USA), comprising a Waters Acquity UPLC TUV Detector (dual wavelength; Waters) interfaced with Empower 3 software was employed for cathepsin D analysis. A Waters UPLC Peptide CSH C18 column (1.7 μm, 2.1 × 100 mm) was used with gradients prepared as described by Hurley et al. (1999) and generated as follows: 15-45% B over 8 min, 45%-95% B over 1.2 min, maintaining at 95% B for 0.8 min, and finally returning to equilibration conditions over 1.2 min. Eluate was monitored at 300 nm and the flow rate was maintained at 0.21 mL min^{-1}. The heptapeptide substrate eluted as a single peak before the incubation (peak 1), while the product produced by cathepsin D eluted

as another peak (peak 2). The cathepsin D activity was calculated by the percentage of peak area of peak 2 relative to peak 1.

6.2.5 Measurement of physio-chemical properties: pH, colour, and physical stability

pH measurements were conducted on human milk samples using a FiveEasy Plus Benchtop FP20 pH/mV Standard Kit (Mettler Toledo, Leicester, UK) at 4°C. The colour of skimmed human milk (defatted by centrifugation at 3,000 g for 20 min at 4°C) was measured using a Minolta chromameter CR-400 microcolour tristimulus colorimeter (Konica Minolta Sensing Inc., Milton Keynes, UK) and presented as L*, a* and b* values. L*, a*, b* represents the lightness from black (0) to white (100), colour from green (−) to red (+), and colour from blue (−) to yellow (+), respectively.

The physical stability of human milk was measured using the method described by Meng et al. (2020). The Turbiscan Lab^{TM} (Formulaction, Ramonville St. Agne, France) with an 800-nm near infrared (NIR) light source based on multiple light scattering theory was employed to study particle migration in milk samples. Each sample was gently inverted 20 times before a 10-hour scanning at 5°C. As creaming is the major phenomenon occurring during the storage of milk, the Turbiscan stability index of the cream layer (TSI_{cream}), a parameter to describe the changes of particles in the cream region during creaming, was recorded. The cream region was defined as approximately 20% of the sample height at the top of samples with manual adjustments according to the sample's real cream layer.

6.3 Results

6.3.1 Macronutrients

As shown in **Table 6.1**, DHM in the study had 3.4 (± 0.25) % fat, 7.1 (± 0.07) % carbohydrate, and 1.4 (± 0.07) % protein on average. No changes were found in macronutrient levels after HoP or HP-treated. The levels of total protein and carbohydrate, and energy, were also stable after all processing methods during storage at 4°C. There was a minor decrease in fat content during the 7-day storage at 4°C for control and all treatments, except after HP treatment at 600 MPa for 10 min, which remained unchanged (**Fig. 6.1**).

Table 6.1 Macronutrients, energy, pH and colour changes of human milk immediately after processing. Data is presented as average (SD).

	Fat	**Carbohydrate**	**Protein**	**Energy**	**pH**	**Colour**		
	%	**%**	**%**	**kcal dL^{-1}**		**L***	**a***	**b***
Pre-treatment								
	3.4 (0.25)	7.1 (0.07)	1.4 (0.04)	66.5 (2.8)	6.5 (0.06)	28.45 (0.63)	-0.21 (0.24)	-1.57 (0.62)
Post-treatment (D0)								
CT	3.4 (0.25)	7.1 (0.07)	1.4 (0.04)	66.5 (2.8)	6.4 (0.04)	28.45 (0.63)	-0.21 (0.24)	-1.57 (0.62)
HoP	4.8 (1.63)	6.9 (0.21)	1.4 (0.18)	77.5 (14.8)	6.6 (0.02)	35.22 (1.54)	-0.53 (0.59)	-6.08 (0.25)
200 MPa	3.5 (0.39)	7.1 (0.04)	1.5 (0.14)	67.0 (4.2)	6.4 (0.03)	28.93 (0.77)	-0.31 (0.25)	-1.86 (0.90)
400 MPa	3.9 (0.78)	7.0 (0.00)	1.5 (0.21)	69.8 (8.1)	6.5 (0.03)	29.02 (1.33)	-0.36 (0.18)	-1.87 (0.80)
600 MPa	3.8 (0.64)	7.0 (0.04)	1.4 (0.18)	69.3 (7.4)	6.5 (0.02)	29.04 (0.10)	-0.21 (0.18)	-2.32 (0.52)

CT: Control; HoP: Holder pasteurization; 200MPa: HP treatment at 200 MPa for 10 mins; 400MPa: HP treatment at 400 MPa for 10 mins; 600MPa: HP treatment at 600 MPa for 10 mins.

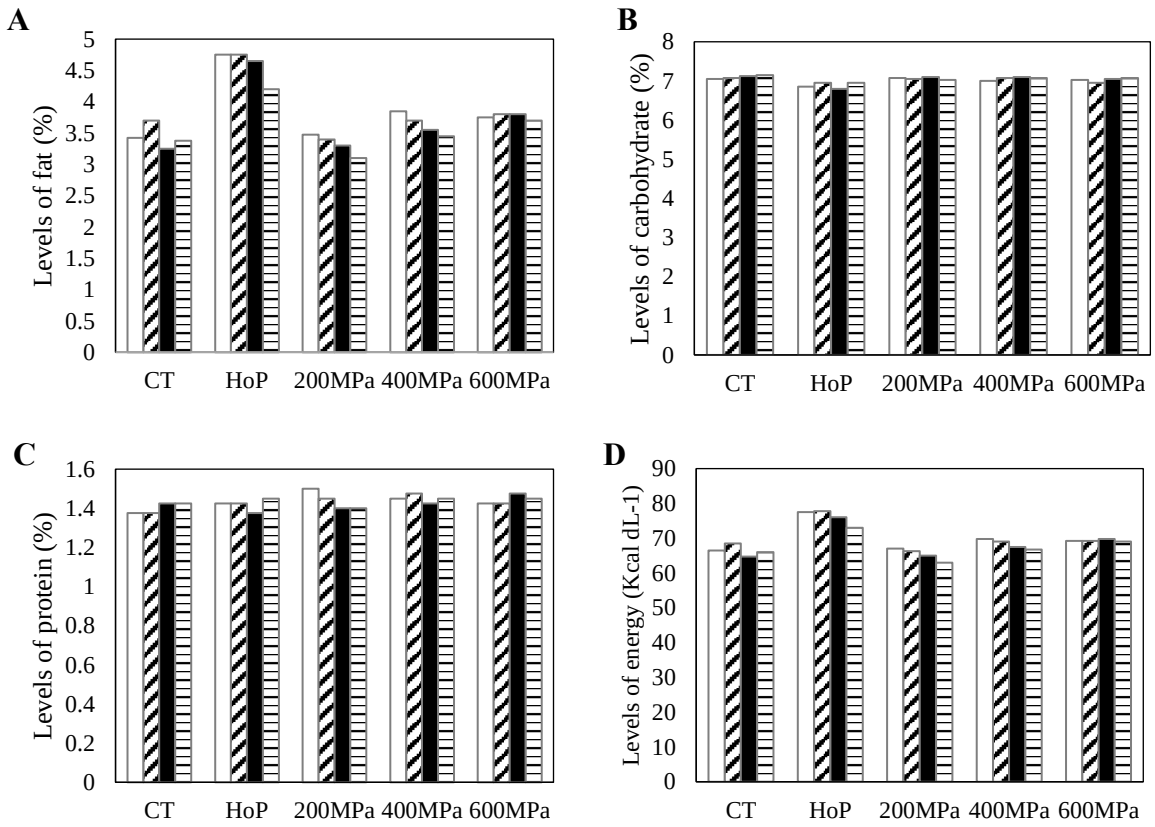

Fig. 6.1 Effect of high-pressure (HP) treatments and holder pasteurization on levels of fat (A), carbohydrate (B), protein (C) and energy (D) of human milk during storage at 4°C for 0 day (white), 1 day (slash); 4 days (black), and 7 days (horizontal line). CT: Control; HoP: Holder pasteurization; 200MPa: HP treatment at 200 MPa for 10 mins; 400MPa: HP treatment at 400 MPa for 10 mins; 600MPa: HP treatment at 600 MPa for 10 mins.

6.3.2 Effect of processing and storage on enzyme activities in human milk

As shown in **Fig. 6.2**A, LPO activity was very low in DHM and decreased during storage in all samples at 4ºC for 7 days. It was totally lost in human milk after HoP treatment, whereas it was retained in the samples after HP treatments, including after treatment at 600 MPa for 10 min. HP-treated samples were found to contain higher LPO activities than untreated samples from day 0 to day 7 of the storage.

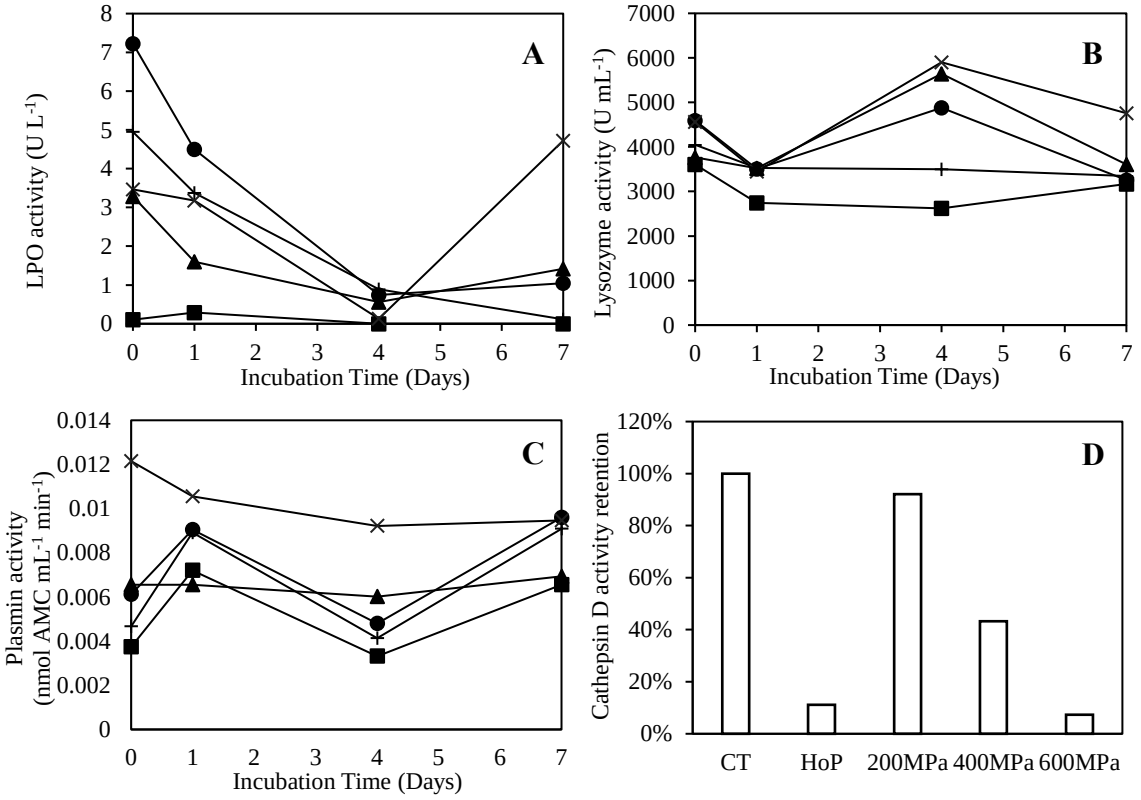

Fig. 6.2 Activities of LPO (A), lysozyme (B), plasmin (C), and cathepsin D (D) in samples after HoP (■), HP treatment at 200 MPa (●), HP treatment at 400 MPa (×), and HP treatment at 600 MPa (▲), and in control samples (+). Cathepsin D activities were measured only after treatment; other enzymes activities were also measured during a 7-day storage at 4°C.

The average lysozyme activity of pooled DHM was 4049 ± 1717 Units mL^{-1}. At D0, lysozyme activity decreased after treatments of HoP and HP-treated at 600 MPa (**Fig. 6.2** B). During storage, the activity remained stable for the control. However, for HP-treated samples, the lysozyme activity increased, especially after storage for 4 days.

After one-week refrigerated storage, the lysozyme activities are similar in all samples, except a higher level was found in samples after HP treatment at 400 MPa.

Plasmin activity in human milk was highest after HP treatment at 400 MPa for 10 min (**Fig. 6.2** C) compared to other processing methods. The plasmin activity decreased after HoP. In general, the plasmin activity was stable during storage at 4 °C for 1 week. HoP and HP treatment at 600 MPa decreased cathepsin D activity in DHM, while HP treatments at 400 MPa and 200 MPa retained 40% and 92% of the activity of that enzyme, respectively.

6.3.3 pH, colour and physical stability

Average pH of pooled DHM was 6.5 and processing did not lead to changes in pH (**Table 6.1**). **Fig. 6.3**A shows a reduction in pH during storage at 4°C after all processes. The pH of milk after HoP was the most stable among samples after different treatments, and remained at pH 6.5 at D7 of the storage, while the pH of the control samples decreased the most, from pH 6.4 to pH 6 at D7.

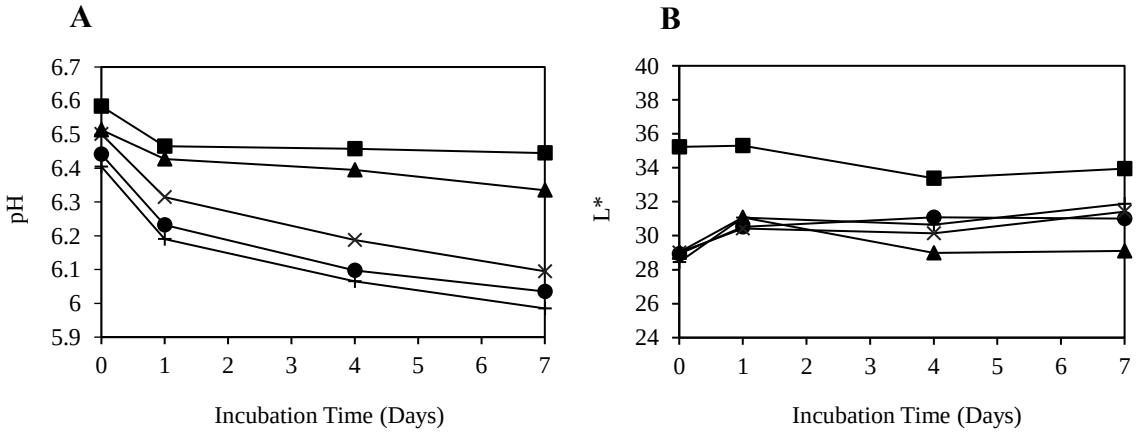

Fig. 6.3 Changes in pH (A) and lightness (B) of DHM control (+) and DHM after HoP (■), HP treatment at 200MPa (●), HP treatment at 400MPa (×), and HP treatment at 600MPa (▲) during storage at 4°C.

The colour of skimmed human milk after HoP treatment had higher absolute L* values, i.e., higher lightness compared with milk after other treatments (**Table 6.1**). Although **Fig. 6.3**B shows no clear trend for the colour of skimmed milk after

processing during storage, DHM after HP treatments at 600 MPa showed lowest L* values compared to DHM after other treatments at day 4 and 7 of the storage, while L* values of samples after HP treatments at 200 or 400 MPa were similar to the control at each time point.

The TSI_{cream}, i.e., the stability index to compare how fast the particles moved to cream layer between treatment groups is shown in **Fig. 6.4**. At D0, TSI_{cream} values of milk after 600 MPa treatment at 1, 4, and 10 hours of the creaming were lower than control and milk after 200 MPa or 400 MPa treated, indicating that HP treatment at 600 MPa decreased the creaming rate of milk. After storage for four days (D4), milk samples treated with HoP, HP at 400 MPa and HP at 600 MPa had similar TSI_{cream} values during creaming for 10 hours at 5°C, which were higher compared to the control and milk treated with HP at 200 MPa. After storage for one week (D7), the TSI_{cream} values were similar between groups of control, HP-treated at 200 MPa, and HP-treated at 400 MPa, but lower than HP-treated 600MPa and HoP groups. It is clear that in milk treated at 600 MPa, the influence of storage on the creaming rate according to the TSI_{cream} was minor. In contrast, the creaming rate of milk decreased during refrigerated storage in other treatment groups, especially in control and milk after HP treatment at 200 MPa after storage for 4 days.

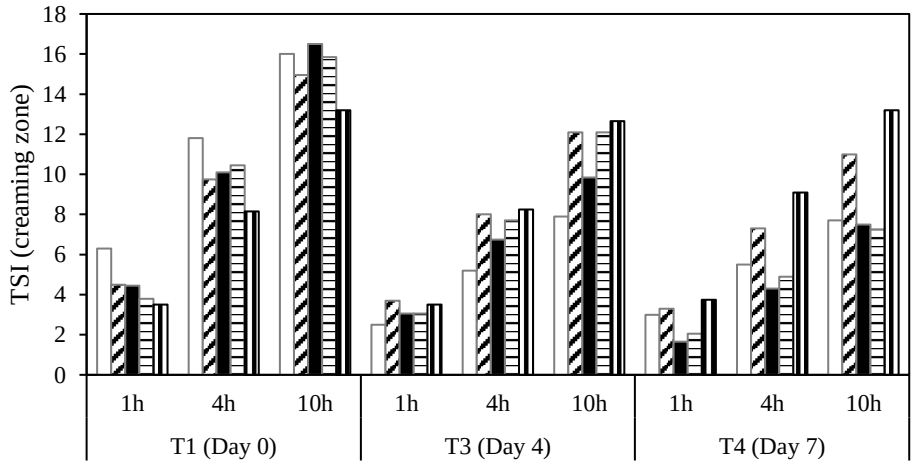

Fig. 6.4 Changes in TSI_{cream} of human milk during creaming at 1, 4, and 10 hours at 5°C. Samples: human milk after no treatment (control, white), HoP (slash), HP treatment at 200 MPa (black), HP treatment at 400 MPa (horizontal line), and HP treatment at 600 MPa (Vertical line) before storage (D0) and after storage at 4°C at D4 and D7. **Note:** TSI_{cream}: Turbiscan Stability Index of the cream region of milk; HoP: Holder pasteurization; HP: High-pressure.

6.4 Discussion

Optimising and maintaining nutritional quality of DHM is very important, as the milk will be provided to infants who cannot receive human milk from their own mothers. These infants are frequently preterm, low-birth-weight neonates in the hospitals that require immunological support from human milk. However, decreased immunological and bioactive activity of DHM have been reported, which could result from a series of handling steps, including freezing, thawing, pooling, and pasteurization (Haiden & Ziegler, 2016). Among these steps, even though pasteurization (typically HoP) is critical to bacterial elimination, it can be the major reason for some immunological compounds to lose their activities (Moro et al., 2019), due to heat-induced structural modifications of some compounds, especially immunological proteins (Escuder-Vieco et al., 2018). The effect of HoP and its alternative non-thermal processing method, HP treatment, on these nutrients were therefore investigated in this study.

The macronutrients levels of DHM in this study are in the range of other reports (Wojcik et al., 2009; Michaelsen et al., 1990; Mills., et al 2019). It has also been reported that the levels of total protein and carbohydrate are stable after HoP (Hamprecht et al., 2004; Ley et al., 2011), in agreement with our results. The results of the present study also showed that protein and carbohydrate levels are stable after HP treatments. Levels of total protein, carbohydrate and lipid following HP treatments have not been reported to date, probably because it is general assumed that macronutrients won't change after such purely physical treatments (Moro et al., 2019).

There are some conflicting reports regarding total lipids in human milk after processing. Our results showed a slight increase of the fat concentrations after HoP, while no changes were observed after all HP treatments (**Table 6.1**). Lepri et al. (1997) also reported an increase of total lipid after HoP; however, no significant difference was found when compared with control. They considered the method they used to determine the fat content, i.e., using the total chromatographic peak area for the fatty acids by gas chromatography as more accurate than the traditional creamatocrit method. The creamatocrit method is a rapid method for clinical settings, which has been reported as highly correlated with fat measured by gravimetric reference methods, but tended to underreport fat content by 0.3 to 0.6 g dL^{-1}(Du et al., 2017). In contrast, García-Lara et

al. (2013) reported a significant reduction in lipid levels following HoP using a human milk analyser, as seen in the present study. Other studies have not found significant difference in total lipids after HoP (Góes et al., 2002). Heating, freezing and thawing may damage fat globule membranes, resulting in a higher exposure of triglycerides to lipase (Wardell, Hill, & D'Souza, 1981). However, decreased activity of lipase on HoP of the milk has been reported (Fredrikzon et al., 1978). Overall, it is possible that the lipid remained intact in milk after HoP due to limited lipolysis.

To our knowledge, there are no reports on changes in macronutrients during storage after processing. This is the first paper reporting a decrease in fat and energy during one-week storage at 4°C after HP treatments at 200 or 400 MPa for 10 min, whereas no change in either parameter was found after HP treatment at 600 MPa for 10 min. Decreased energy was likely largely driven by the decreased fat levels, based on the calculation used to calculate the energy value; however, the reasons for fat loss remain unclear and further research is required in this area. Total carbohydrate and total protein contents were stable after HP treatments during storage at 4°C for one week.

LPO plays a vital role in the natural host defence system against microorganisms. The level of LPO was very low in DHM, in agreement with reports of Gothefors & Marklund (1975) and Shin, Tomita, & Lönnerdal (2000). It has also been reported that there is no LPO activity in human colostrum (Sousa et al., 2014). To our knowledge, there are no reports on LPO activity after HP treatments, while loss in activity ranging from 50–88% after HoP has been reported (Akinbi et al., 2010). In this study, LPO activity was totally lost after HoP treatment, while it remained after all HP treatments, providing further evidence that HP treatments is better than HoP in terms of preserving bioactive compounds.

The average lysozyme activity of pooled DHM (4049 ± 1717 U mL^{-1}) was lower than that reported for pooled raw human milk samples (7969 ± 1394 U mL^{-1}; Lima et al., 2017). This may be due to the frozen storage of milk reducing the lysozyme activity (Chang et al., 2013). Consistent with previous reports, lysozyme activity in human milk decreased after HoP, while it remained after a wide range of high pressures (Sousa et al., 2014; Viazis et al., 2007). Moreover, both the activities of lysozyme and LPO decreased significantly after storage for one day at 4°C.

Plasmin and cathepsin D, which can actively hydrolyse proteins at gastric pH, are important proteases in human milk helping infants' digestion (Demers-Mathieu et al., 2018). The relatively unchanged plasmin activity during storage at 4 °C for 1 week suggests that proteolysis may occur during storage, as also suggested by results from electrophoresis and liquid chromatography in **Chapter 7**. The major form of cathepsin D in human milk is its inactive form procathepsin D, which is inactive at milk pH (activation of procathepsin D is stopped at pH 7.4) (Wittlin et al., 1999). However, the presence of cathepsin D activity has been predicted through the identification of peptides with the specific cleavage sites from enzymes in pooled mature human milk (Khaldi et al., 2014). This is the first study to show that cathepsin D activity decreased after HoP, whereas it was better preserved after HP treatment at 200 or 400 MPa for 10 min.

As well as macronutrients and bioactive compounds, the present study also reported physiochemical properties that are parameters reflecting the quality of DHM during processing and storage routinely, including pH, colour, and physical stability. The average pH of pooled DHM in our study was lower than fresh raw human milk, reported to be pH 7 or higher (Morriss et al 1986), which may be due to prior frozen storage (Hamprecht et al., 2004). The pH of milk after HoP was the most stable among samples after different treatments, as well as HP treatment at 600 MPa, whereas the pH of the control sample and the sample HP-treated at 200 MPa showed a similar decrease. This may indicate that HP treatment at 200 MPa for 10 min is not sufficient for inhibiting microbial growth, which is consistent with the microbial result (data not shown) that growth of total bacterial, *Staphylococcus*, *Streptococcus*, and *Pseudomonas* were observed in milk after HP treatment at 200 MPa for 10 min.

In terms of colour, lower L* values reflect changes in the size or state of the casein micelles in milk, such as the extent of micelle dissociation in bovine milk studies (O'Sullivan, Kelly, & Fox, 2002). A decreased L* at 600 MPa HP-treated skimmed milk after storage for 4 days may be related to dissociation of casein micelles in DHM. It has reported, for bovine milk, that casein micelles are disrupted by pressure and that disruption increased with the pressure and time of HP treatments (Huppertz, Fox, & Kelly, 2004). Needs et al. (2000) reported a nearly completed disruption of casein micelle in bovine milk following HP treatments of 400 MPa and above, while Huppertz

et al. (2004) reported that increased pH level (to pH 7.0) increased the stability of bovine casein micelles to HP treatments. In addition, whey proteins in bovine milk, including β-lactoglobulin and α-lactalbumin, become denatured when milk is subjected to pressures higher than 100 MPa or 400 MPa, respectively, and interact with casein micelles. Fresh human milk has higher pH (average pH of term human milk: 6.8-7.3 as reported in **Chapter 2**) compared to bovine milk, while no β-lactoglobulin presents in human milk (Guo, 2021); HP treatments may thus have a different impact on human milk in terms of protein structures and interactions to those seen for bovine milk, and further studies are warranted.

Although the fat fraction has been reported as unchanged when exposed to HP treatments below 600 MPa, it should be noted that pressurization as a physical factor can alter the stabilization of milk fat globules and leads to a decreased milk fat globule size (Huppertz, Fox, & Kelly, 2003; Moro et al., 2019). The TSI_{cream} values, as an indicator of the stability of milk therefore, were tested. The TSI_{cream} was lower in milk after HP treatments at 600 MPa compared with other treatments before storage, indicating that the creaming rate decreased after treatment of milk at 600 MPa for 10 min (**Fig.6.4** D0). The result in agreement with a bovine milk study from Huppertz et al. (2003), who considered this decrease of creaming rate may be due to a decrease in the fat globule size after treatment at 600 MPa. In addition, it is possible that the decreased creaming rate may also be related to the denaturation of agglutinins, including immunoglobulin A (IgA) and immunoglobulin M, which can attach to fat globule membrane, cause fat globules to aggregate so that accelerate the natural creaming (**Chapter 3**). It has been reported that no IgA in human milk is destroyed after HP at 400 MPa for 5 min, whereas 72% and 69.3% IgA was retained after HoP and HP at 600 MPa for 5 min, respectively (Permanyer et al., 2010).

In a bovine milk study, creaming rate was reported to be increased after 200 MPa for 30 min after 4 hours creaming (Huppertz et al., 2003), which was not shown for human milk in our study. However, the study showed that the TSI_{cream} of human milk after HP treatments at 200 MPa for 10 min was slightly higher compared with control after creaming for 10 hours.

During storage at 4°C, TSI_{cream} values of human milk control and samples after all treatments decreased, which was consistent with previously reported in **Chapter 3**. After storage at 4°C for 4 days, TSI_{cream} values of the control and samples after HP treatments at 200 MPa decreased dramatically compared with other treatments, indicating that changes occurred in the control and milk after HP treatments at 200 MPa during storage, which could influence the phase separation of milk. In contrast, higher TSI_{cream} was seen after HoP or HP treatment at 600 MPa compared to milk after other treatments at D4 and D7, partly consistent with the fat content changes during storage (**Fig. 6.1** A). Milk HP-treated at 400 MPa showed similar TSI_{cream} profiles to milk after HoP or HP treatments at 600 MPa at D4 (**Fig.6.4** D4). However, at D7, the TSI_{cream} profiles of milk HP-treated at 400 MPa were similar to those of the control and milk HP-treated at 200 MPa, i.e., TSI values were much lower at each time point during creaming compared to that in D0. Considering this gradual change of 400 MPa HP-treated milk during storage, it is suspected that the changes in creaming rate and physical stability may have been related to the bacterial growth or changes of protein structure during the storage.

In a parallel microbial study (same treatments conducted with the same pooled human milk samples, unpublished data), HP treatments at 400 MPa or 600 MPa for 10 min provided comparable improvement in microbial quality of human milk to HoP, whereas HP treatments at 200 MPa for 10 min showed low ability to prevent the growth of bacteria, in terms of total bacterial, *Staphylococcus*, *Streptococcus*, and *Pseudomonas* counts. Combining the microbial results to this study, although treatment at 200 MPa preserved enzyme activities better than HoP, it may not be a promising preservation method in terms of microbial safety. Thus, overall, HP treatments at 400 MPa for 10 min is recommended as it has the ability to eliminate bacteria without compromising important immunological and bioactive compounds in human milk.

In addition, although both HP treatment and HoP are batch processes, HP treatment is faster and probably more convenient than HoP (Haiden & Ziegler, 2016). In the food industry, more than 352 commercial HPP units which can process 1.065 million metric tons/annum of HPP pasteurized foods are installed worldwide, including toll-processing systems in many countries (Priyadarshini et al., 2019). Although the application of HP treatments is increasing, the scaling down of the equipment and the

cost of an HP unit may still be key considerations for using it as a routine in human milk banks, which are non-profit organizations.

6.5 Conclusion

The study demonstrated that HP treatments could be a better option to HoP to preserve human milk concerning levels of nutrients, enzyme activities, as well as physical stability. The macronutrient contents were comparable after HoP and HP treatments, which remained stable during storage at 4°C for 1 week, although fat content may be affected. The activities of LPO, lysozyme, plasmin, and cathepsin D were preserved better in milk after HP treatments, especially after HP treatments at 200 MPa or 400 MPa for 10 min, compared to the milk after HoP during refrigerated storage for one week. HP treatment at 600 MPa for 10 min decreased the creaming rate; however, such milk was more stable in terms of less change of stability index during storage at 4°C for 7 days compared to control milk or that HP-treated at 200 MPa or 400 MPa. Combining the microbial and nutritional results, HP treatment at 400 MPa for 10 min could be the most promising potential alternative to HoP of human milk. Our findings provide evidence for the potential of using HP treatments as an alternative to HoP in human milk banks, which could bring health benefits to infants who required donor milk from milk banks.

Supplementary Figure

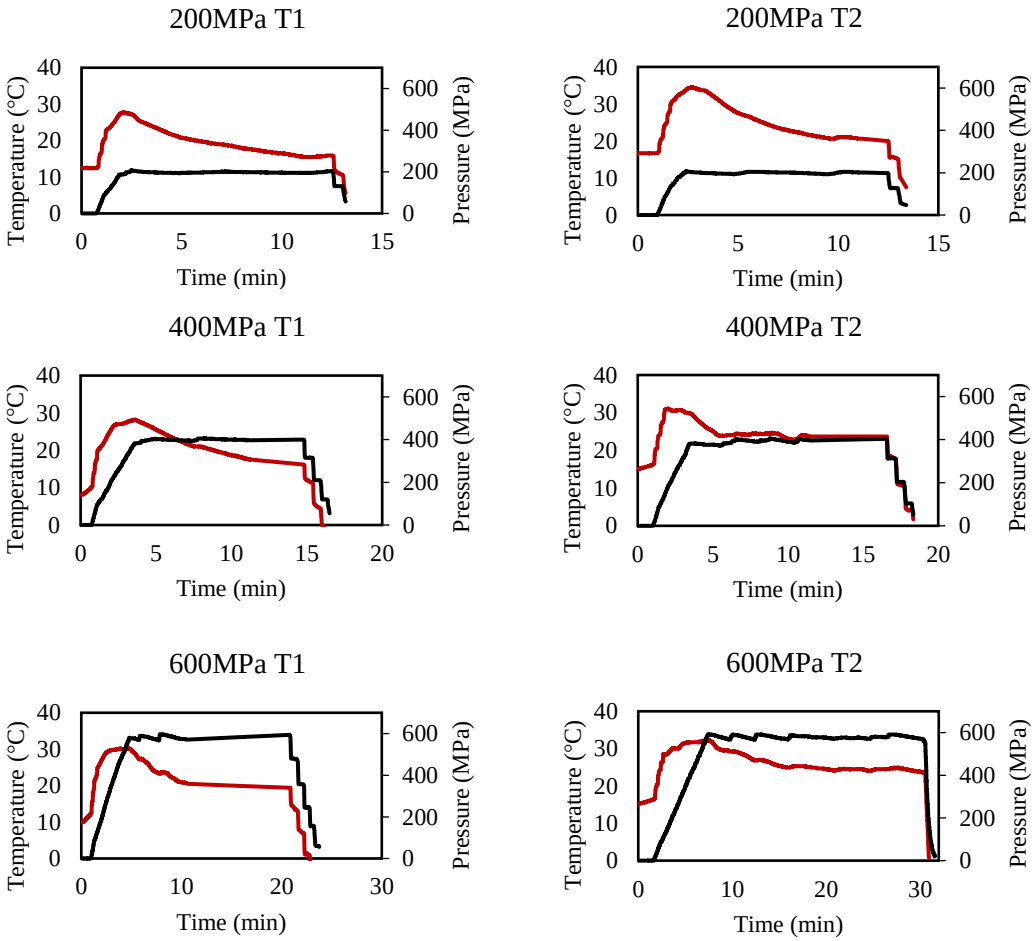

Fig. S6.1 Pressure (black line) and temperature (red line) profiles of high-pressure treatments in trial 1 (T1) and in trial 2 (T2).

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CHAPTER 7

Effect of Novel Processing and Cold Storage on Human milk: Macronutrients, Enzyme Activities, and Proteolysis

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Fanyu Meng undertook the experimental work and have drafted the manuscript.
Ruth Stephenson measured the total bacterial count.
Tony Ryan and Catherine Stanton helped with the study design.
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Abstract

Alternative methods to holder pasteurization on human milk are of interest because a higher bioactivity is optimal for neonates who cannot receive human milk from their own mothers. This study tested the influence of several preserving methods, including microfiltration, enzymatic methods, and bacteriocin treatments, combined with refrigerated storage, on the macronutrients content, pH, lysozyme and lactoperoxidase activities, and proteolysis in human milk. Macronutrients, enzyme activities, and protein profiles were unchanged after novel treatments, except that fat levels decreased as expected after microfiltration (as this was applied to skimmed milk). During storage at 4°C for 7 days, carbohydrate levels and the pH decreased to a lower content in the lactose oxidase treated group compared with other groups, and lysozyme activities decreased in milk after all treatments. Although enzymatic activities decreased in holder pasteurized milk, proteolysis did not occur during the storage, whereas it occurred in milk with novel treatments. Storage of milk at 4°C for prolonged time is not recommended due to the proteolysis that occurred and decreased antimicrobial activities. Although novel processing methods preserved the bioactive compounds in human milk, optimal strategies to ensure milk safety still require further study.

7.1 Introduction

Human milk requires handling and preserving in human milk banks, which can be provided to infants, especially preterm new-borns who are not always able to receive the milk of their own mothers. Collection, screening, storage, pasteurization, and distribution of human milk are critical steps currently applied in human milk banks (Haiden & Ziegler, 2016). Among these steps, pasteurization is a key step to keep balance between the microbiological safety and nutritional quality. Holder pasteurization (HoP), carried out in a water bath at 62.5°C for 30 minutes followed by rapid cooling, is currently recommended in international human milk banks guidelines and widely preformed in human milk banks (Peila et al., 2017). HoP preserves some important nutritional and biological components, such as oligosaccharides, lactose, glucose, polyunsaturated fatty acids, gangliosides, and vitamins A, D, E, and B2 (Wesolowska et al., 2019). However, levels or activities of some components decrease, including immunoglobulins (Igs), lactoferrin, lysozyme, alkaline phosphatase, lactoperoxidase, amylase, insulin, leptin, vitamin B6, B12 and C, some cytokines, and some growth factors (Wesolowska et al., 2019). In addition, the volatile profile may be modified in human milk after HoP due to lipid oxidation and the Maillard reaction (Contador et al., 2015).

Alternatives to HoP, for example, high-temperature short-time pasteurization (HTST, 72.5 °C x 15 s), high pressure processing, microwave irradiation, UV-C irradiation, ultrasound, and electric pulses has been studied as options to preserve human milk (Moro et al., 2019). These preservation methods had all been tested in the dairy industry and then applied in human milk. However, some non-thermal methods, such as microfiltration, enzymatic preservation methods, and bacteriocin treatments have not been applied in human milk so far.

Microfiltration (MF) is a membrane separation process that can separate fat, bacteria, spores, and somatic cells from the milk serum. Zhang et al. (2021) studied MF of skimmed bovine milk using 1.4 μm pore size; total bacterial counts (TBC) in skimmed bovine milk after was MF (~2 x 10^4 CFU mL^{-1}), which was lower than the milk after HTST (~5 x 10^4 CFU mL^{-1}) and did not increase after refrigerated storage for 12 days. The levels of coliform and spores were not detected after MF and almost

no influence on immunoglobulins and the LPO activities by MF was reported. A decrease of pore size to 0.45 μm leads to a slight decrease of casein micelles permeation rate, but a higher level of bacteria removal (Lindquist, 1998). In the dairy industry, MF skim milk can be remixed with the amount of heated cream (e.g., 95°C x 20s) for fat standardization (Saboyainsta & Maubois, 2000).

Addition of lactose oxidase (LO) to milk is an enzymatic preservation method where LO can act as an activator of the lactoperoxidase (LPO) system in milk (Kelly et al., 2021; Lara-Aguilar & Alcaine, 2019b). In the LPO system, LPO catalyses the oxidation of thiocyanate (TCN) with hydrogen peroxide (H_2O_2) to hypothiocyanite ion, which has antimicrobial activity. In bovine milk, the level of LPO (~ 30 mg L^{-1}) and the level of TCN (1-15 mg L^{-1}) are sufficient, whereas the concentrations of H_2O_2 can be too low to activate the LPO system (Lara-Aguilar & Alcaine, 2019a). LO can oxidize lactose to lactobionic acid with the concurrent reduction of O_2 to H_2O_2 and it has been demonstrated to be safely used in food products (Ahmad et al., 2004). It has been applied in bovine milk and the production of H_2O_2 from 1.2 g L^{-1} LO was sufficient to kill *Pseudomonas fragi* at 6°C (Lara-Aguilar & Alcaine, 2019a).

In human milk, lower LPO activities (~ 1-1.5 units mL^{-1}; ~ 5% of bovine milk) and lower thiocyanate concentrations (ranged from 0.4 to 228.3 μg L^{-1} with a mean of 35.6 μg L^{-1}) have been reported (Gothefors & Marklund, 1975; Kirk et al., 2007; Shin, Hayasawa, & Lönnerdal, 2001). The concentration of H_2O_2 is higher in human colostrum, at ~ 25 μM, compared to mature milk (~ 8.7 μM at 4 weeks *post-partum*) (Al-Kerwi, Al-Hashimi, & Salman, 2005). H_2O_2 is unstable at room temperature and can rapidly decrease from ~10^{-5} M to 0 M after 10 min at pH 6 (or 4 min at pH 7) (Cieslak et al., 2018). Application of LO to preserve human milk by increasing the H_2O_2 concentration has not been studied so far.

Another method has not been studied in human milk is bacteriocin treatment. Nisin, a hydrophobic peptide that has been licensed as a food preservative (E234), is the most widely used bacteriocin in food industry (Ibarra-Sánchez et al., 2020; Silva, Silva, & Ribeiro, 2018). Nisin has antimicrobial activity against Gram-positive bacteria, pathogens, and spore-forming bacteria (Silva et al., 2018). Arqués et al. (2011)

demonstrated that the growth of *Listeria monocytogenes* and *S. aureus* in bovine milk was prevented by nisin at refrigeration temperatures.

This study aims to apply these non-thermal technologies in human milk preservation using the HoP method as reference. The influence of treaments and subsequent storage at 4°C for 1 week on macronutrient contents, lysozyme activities, LPO activities, and proteolysis in human milk were also studied to examine if the important nutritional and biological components are preserved after these novel treatments and the storage.

7.2 Materials & Methods

7.2.1 Human milk samples

The use of human milk samples in the study was approved by Cork Research Ethics Committee. Unpasteurized term human milk was donated from two mothers with consent. Prior to analysis, all samples were stored at -20°C, thawed at 4°C overnight and pooled under sterile conditions.

7.2.2 Experimental design

Four trials were conducted to determine the effect of novel processing and storage on enzyme activities and proteolysis in human milk. As shown in **Fig. 7.1**, to achieve different microbial loads, 0% (control, no incubation), 5%, 100%, and 100% (v/v) of the volume of thawed milk samples were incubated at 10°C for 7 days prior to the treatment in the first, the second, the third, and the fourth trial (T1-T4), respectively. Each trial had 4 time-points; before storage (D0), after 24 hours (D1), 4 days (D4) and 7 days (D7), and samples were stored at 4°C between time-points. There were 6 treatment groups: holder pasteurization (HoP), microfiltration (MF), lactose oxidase (LO)-treated, nisin-treated, and combined treatment of HoP and nisin (HoP + Nisin), in addition to an untreated control group.

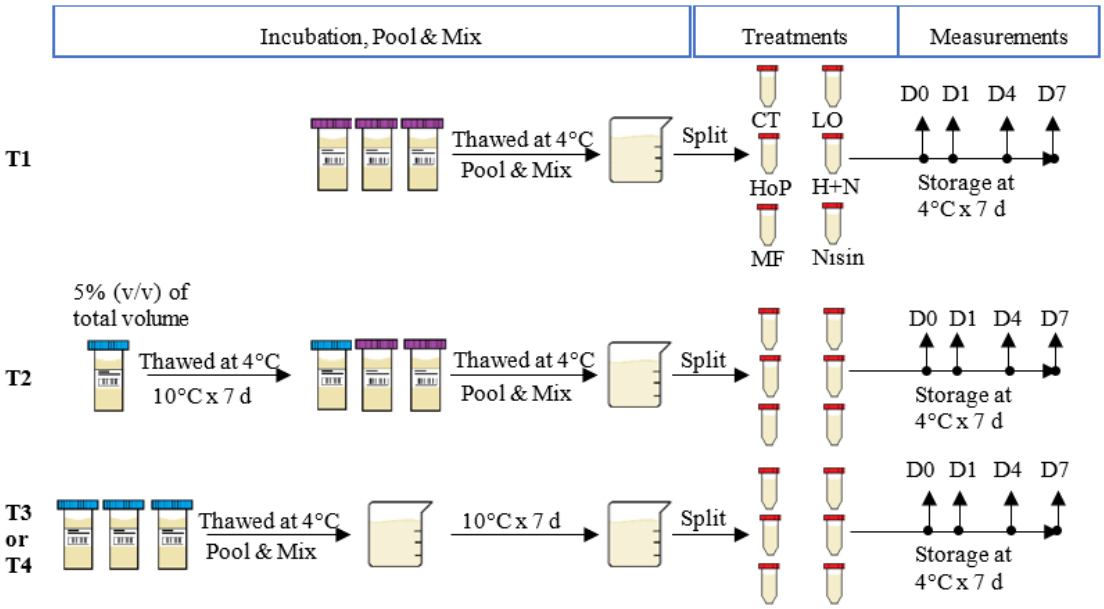

Fig. 7.1 Experimental design for studying the effect of novel processing and storage on enzyme activities and proteolysis in human milk. Abbreviations: CT: control; LO: Lactose oxidase- treatment; HoP: Holder pasteurization; H+N: Combination of holder pasteurization and addition of nisin; MF: Microfiltration.

7.2.3 Treatments

Pooled human milk (~ 450 mL) was split into treatment groups under sterile conditions. For HoP treatment, human milk (~ 70 mL) was transferred into a sterilized Duran bottles and placed into a preheated water bath (Grant Instruments Ltd., Cambridgeshire, UK). An equal volume of water in a control bottle was used to monitor the heating temperature. Once the temperature of the control reached 62.5°C, milk was held for 30 minutes and then cooled in iced water.

For MF treatment, milk was centrifuged at 6,000 g to remove fat and the serum phase was filtered through a membrane filtration unit with 0.45 µm pore size (VWR International Ltd, Dublin, Ireland). A Buchi V-700 Vacuum pump (Büchi Labortechnik AG, Flawil, Switzerland) was used to generate vacuum for the treatment, and the pressure was decreased from 500 to 200 mbar gradually to increase the vacuum and facilitate the passage of skim milk through the membrane.

In LO-treated group, LO was added to milk and vortexed to mix to a final concentration of 1.2 g L^{-1}. For nisin treated group, Nisin A was added to achieved a

final concentration of 1 g L^{-1}, while HoP was applied before nisin addition in the HoP+Nisin group.

7.2.4 Determination of total microbial count and Pseudomonas count

Measurement of total microbial count (TBC) and *Pseudomonas* count was described in **Chapter 6**. Briefly, human milk was diluted in maximum recovery diluent (1:9 v/v; Oxoid Ltd, Basingstoke, Hampshire, UK). TBC was determined by spread plating 100 μL of each serial dilution on a non-selective media made by Columbia Agar (Sigma Aldrich, Dublin, Ireland) supplemented with 5% defibrinated sheep blood (Sigma Aldrich). *Pseudomonas* was selected by Pseudomonas Isolation Agar (Sigma Aldrich). All plates were incubated aerobically at 37°C for 48 to 72 hours. Following incubation, bacteria in the plates with 30-300 colonies were counted and results recorded as colony-forming units (CFU) per mL of milk.

7.2.5 Determination of composition and pH of milk

To determine the concentrations of total fat, protein, carbohydrate and energy content of human milk, samples at each time points was frozen at -80°C, thawed at 4°C overnight, warmed to 40°C, and homogenized using a MIRIS sonicator (MIRIS, Uppsala, Sweden) before analysis. A human milk analyser (MIRIS, Uppsala, Sweden) was applied to the analysis based on mid-infrared (mid-IR) transmission spectroscopy principles. The levels of energy were calculated by MIRIS as: Energy (kcal L^{-1}) = protein (g L^{-1}) × 4.4 + fat (g L^{-1}) × 9.25 + carbohydrate (g L^{-1}) × 4. pH measurements of human milk were conducted on milk samples at each time point using a FiveEasy Plus Benchtop FP20 pH/mV Standard Kit (Mettler Toledo, Leicester, UK) at 4°C.

7.2.6 Measurement of enzyme activities

Enzyme activities were analysed in duplicate and all chemicals were obtained from Merck KGaA, Darmstadt, Germany unless otherwise stated. Prior to the measurement of enzyme activities, human milk was centrifuged at 14,000 rpm for 20 min to obtain a clear serum phase.

Lactoperoxidase activity was determined by the method of Al-Baarri et al. (2011)

with modification. Ten μL of milk serum, 90 μL of 1.0 mM 2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) in 10 mM acetate buffer (pH 4.4) and 90 μL 0.55 mM H_2O_2 in pure water were added into a well of a UV-transparent 96-well microplates (VWR International Ltd.). The optical intensity was determined at 412 nm using a plate reader (SpectrafluorPlus, Tecan, Männedorf, Switzerland), which read every 20 s for 20 min. One unit of LPO enzymatic activity was expressed as the amount of enzyme needed to oxidize 1 μmol ABTS min^{-1}. The millimolar extinction coefficient of ABTS at 412 nm was 32.4 min^{-1} cm^{-1}.

Lysozyme activity was determined by the method of Shugar (1952) with modification. Ten μL of milk serum was mixed with 250 μL 0.015% (w/v) *Micrococcus lysodeikticus* ATCC 4698 cells suspension in a well of UV-transparent microplates and the optical density was measured every 5 s for 5 min at absorbance of 450 nm. The unit is determined as the amount of lysozyme required to produce a decrease of ΔA450 of 0.001 per min at pH 6.24 at 25 °C using a suspension of M *lysodeikticus* as substrate.

7.2.7 Urea-polyacrylamide gel electrophoresis (Urea-PAGE)

Urea-PAGE (12.5 % total monomer, 4 % crossing-linking agent as percentage of total monomer, pH 8.9) of incubated human milk was performed using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, UK) according to the method of Shalabi and Fox (1987). The gels were stained directly with Coomassie Brilliant Blue G-250 and destained using distilled water.

7.2.8 Evaluation of proteolysis by ultra-performance liquid chromatography

Prior to analysis, human milk was frozen with 1% Halt protease cocktail inhibitor (ThermoFisher Scientific, Dublin, Ireland), thawed at 4°C overnight, vortexed, sonicated, and heated to 30°C. To obtain peptides profiles, 100 μL milk was ultrafiltered through 10-kDa Microcon centrifugal filters (Merck Millipore Ltd., Tullagreen, Ireland) at 14,000 g for 30 min at room temperature for three times with 100 μL MiliQ water added each time. Filtrate with peptides less than 10 kDa was collected, freeze-dried, and resuspended into 60 μL buffer consisting of 2% acetonitrile and 0.1% trifluoroacetic acid (TFA).

A UPLC using a Waters Acquity UPLC H-Class Core System (Waters, Milford, MA, USA), comprising a Waters Acquity UPLC TUV Detector (dual wavelength; Waters) interfaced with Empower 3 software was employed. Analysis was performed on an Acquity UPLC Peptide CSH C18 column (1.7 μm, 2.1 × 100 mm) using gradients prepared from Solvent A (0.1 % TFA in MiliQ water) and solvent B (0.1 % TFA in acetonitrile).

The separation was performed at 40°C and the elution was initialized with 97% solvent A and 3 % solvent B, then with a linear gradient to 40 % solvent B over 45 min, followed by a linear gradient to 85 % B over 2 min, elution at 85 % B for 3 min, followed by a linear gradient to 3 % B over 2 min, and finally 3 % B over 8 min. Eluate was monitored at 214 nm and the flow rate was maintained at 0.20 mL min^{-1}.

7.3 Results

7.3.1 Total bacterial and Pseudomonas count, composition, and pH before storage

As shown in **Table 7.1**, the initial TBC in human milk measured at D0 in T1, T2, T3, and T4 was 2.7 x 10^3, 7.2 x 10^2, 1.4 x10^6, and 4.5 x 10^2 CFU mL^{-1}, respectively. *Pseudomonas* was high in T3 (3.54 x 10^8 CFU mL^{-1}), compared to other trials. The TBC and *Pseudomonas* count were low in human milk without incubation, i.e., in T1. After incubation at 10°C for 1 week, the TBC and *Pseudomonas* count increased in pooled human milk (T3) or in one trial remained low (T4).

The levels of total protein and carbohydrate in untreated human milk were uniform among each trial; the levels of total protein ranged from 0.9 % (T2) to 1.1 % (T1), and the carbohydrate levels ranged from 7.0% (T1) to 7.1% (T2, T3, T4). In contrast, the levels of total fat of untreated samples varied between trials, from 2.6 % (T3) to 3.5 % (T2), while energy levels ranged from 56.5 kcal dL^{-1} (T3) to 65 kcal dL^{-1} (T2).

As expected, at D0, the levels of lipids, proteins, and energy decreased after MF to 0.2 - 0.4 %, 0.7- 0.9 %, and 33 - 36 kcal dL^{-1}, respectively, while the concentrations of carbohydrate increased slightly to 7.2 - 7.4 %. These changes occurred due to

molecules larger than 4.5 μm in human milk being excluded after MF. Although milk after MF is remixed with the appropriate amount of heated cream in conventional dairy processing (Saboyainsta & Maubois, 2000), this was not done in this study due to the low volume of human milk samples. Therefore, only MF of skimmed milk was studied. Concerning milk after HoP, LO, HoP+Nisin, and Nisin treatments, the levels of energy and macronutrients remained relatively constant at D0.

Table 7.1 Composition and pH of human milk after treatments but before storage at 4°C.

	Treatments					
	CT	HoP	MF	LO	HoP +Nisin	Nisin
T1 *(no incubation before treatments)*						
TBC/*Pseudomonas* (CFU mL^{-1})			2.77×10^3 / 4.25×10^2			
Fat (%)	2.9	2.9	0.4	3.0	2.9	2.6
Total protein (%)	1.1	1.1	0.9	1.0	1.0	1.1
Carbohydrate (%)	7.0	7.0	7.2	7.0	6.9	6.9
Energy (kcal dL^{-1})	60.0	59.5	36.0	60.0	58.5	57.0
pH	6.4	6.6	6.7	6.5	6.6	6.4
T2 *(5% of milk incubated at 10°C for 7 days before treatments)*						
TBC/*Pseudomonas* (CFU mL^{-1})			7.20×10^2 / 50			
Fat (%)	3.5	3.4	0.2	3.4	3.5	3.5
Total protein (%)	0.9	0.8	0.9	0.8	0.8	0.9
Carbohydrate (%)	7.1	7.0	7.4	6.9	6.8	6.9
Energy (kcal dL^{-1})	65.0	62.0	35.0	62.5	62.0	63.0
pH	6.4	6.8	7.1	6.5	6.9	6.8
T3 *(100% of milk incubated at 10°C for 7 days before treatments)*						
TBC/*Pseudomonas* (CFU mL^{-1})			1.43×10^6 / 3.54×10^8			
Fat (%)	2.6	2.6	0.2	2.5	2.6	2.6
Total protein (%)	1.0	1.0	0.7	0.9	1.0	1.0
Carbohydrate (%)	7.1	7.1	7.2	7.2	7.0	7.0
Energy (kcal dL^{-1})	56.5	56.0	33.5	56.0	56.5	56.0
pH	6.2	6.2	6.4	6.1	6.1	6.1
T4 *(100% of milk incubated at 10°C for 7 days before treatments)*						
TBC/*Pseudomonas* (CFU mL^{-1})			4.50×10^2 / 2.45×10^2			
Fat (%)	2.9	2.8	0.2	2.8	2.9	3.0
Total protein (%)	1.0	0.9	0.7	0.9	1.0	1.0
Carbohydrate (%)	7.1	6.8	7.2	7.4	7.1	7.1
Energy (kcal dL^{-1})	59.5	57.0	33.0	59.5	60.0	60.5
pH	6.0	6.1	6.3	6.1	6.0	6.0

Note: CT: Control; HoP: Holder pasteurization; MF: Microfiltration; LO: Lactose oxidase treatment; HoP + Nisin: Combination of holder pasteurization and Nisin treatment. TBC: Total bacterial count.

The pH of samples without incubation or with 5% (v/v) of the volume of milk incubated (Control: pH 6.4 - 6.5) were slightly higher compared to samples fully incubated at 10°C for 7 days (Control: pH 6.0 - 6.2). The pH increased after MF, while it was relatively consistent after other treatments at D0 in all trials, except in T2 where the pH of milk after HoP, HoP + Nisin, and Nisin treatments was higher compared to control before storage at 4 °C for 7 days.

7.3.2 Effect of processing and storage on composition, pH and Pseudomonas of human milk

After storage at 4°C for 7 days, the levels of total fat in control and nisin-treated samples decreased, while it remained unchanged in MF and HoP + Nisin treated samples (**Fig. 7.2**). In LO-treated samples, the levels of fat increased after storage in T2, T3, and T4, but not in T1 (stable). In milk after HoP, the fat concentrations were constant at D0 and D7, except which decreased in T2.

As also shown in **Fig. 7.2**, levels of proteins remained stable during storage at 4°C for 7 days, apart from levels of protein in the LO-treated samples, which decreased to 0.7 % in T2, T3, and T4. The levels of carbohydrates also decreased in LO-treated samples in all trials, except in T1. In MF-treated samples, carbohydrate concentrations varied between 6.8 % (T2) and 7.5 % (T3) among treatments after storage at 4°C for 1 week. Interestingly, although the concentrations of macronutrients changed after storage, the energy levels, which was calculated from the levels of macronutrients, remained unchanged compared to the levels before cold storage (**Fig. 7.2**).

pH of milk samples after all treatments decreased slightly in T1 after storage for 7 days (**Fig. 7.3**). In T1, pasteurized and MF treated milk presented higher pH compared to unpasteurized milk (CT, LO, Nisin) after the cold storage. In other trials, pH of LO treated milk decreased dramatically after the storage at 4°C, and was measured as low as pH 3.73 (T3) at day 7. In contrast, MF-treated milk showed higher pH levels compared to milk with other treatments during the storage.

Furthermore, the count of *Pseudomonas* was measured after treatments and storage in all four trials (Data not shown). In T1 (low *Pseudomonas* counts), *Pseudomonas* counts were negligible after all treatments and during storage. In T3 (high

Pseudomonas counts), *Pseudomonas* was inactive after heat treatments (counts were zero after HoP and after combination of HoP and nisin addition) or after MF treatment (zero count), whereas it was still present in control and milk after other treatments. After storage at 4°C for 7 days, the counts of *Pseudomonas* in control, LO-treated milk, nisin-treated milk were 1.09×10^8, 7×10^5, and 1.06×10^7 CFU mL^{-1}, respectively.

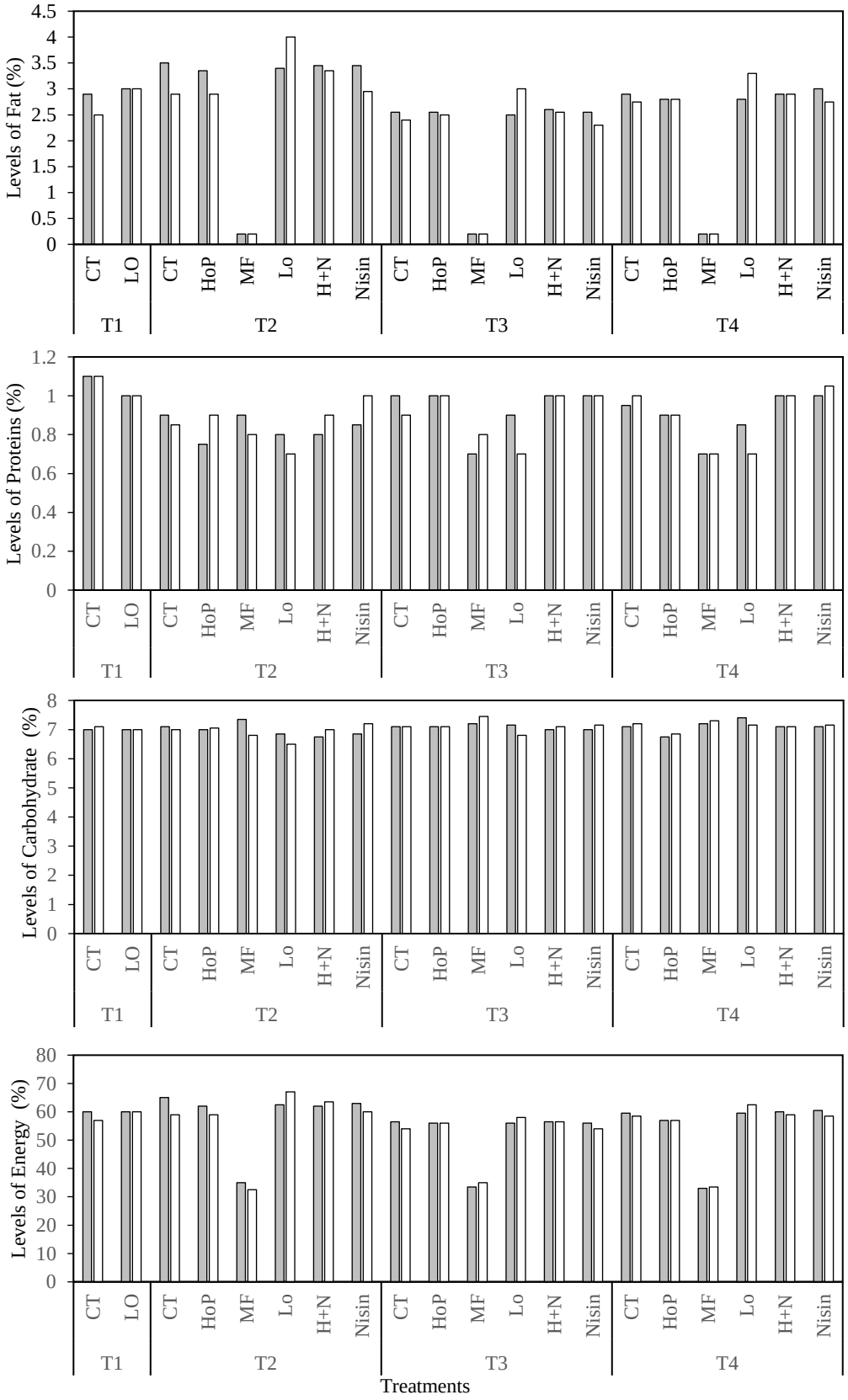

Fig. 7.2 Composition of human milk change before (D0; grey bar) and after storage at 4°C for 7 days (D7; white bar). CT: Control; HoP: Holder pasteurization; MF: Microfiltration; LO: Lactose oxidase treatment; H + N: Holder pasteurization followed nisin treatment. Nisin: Nisin treatment.

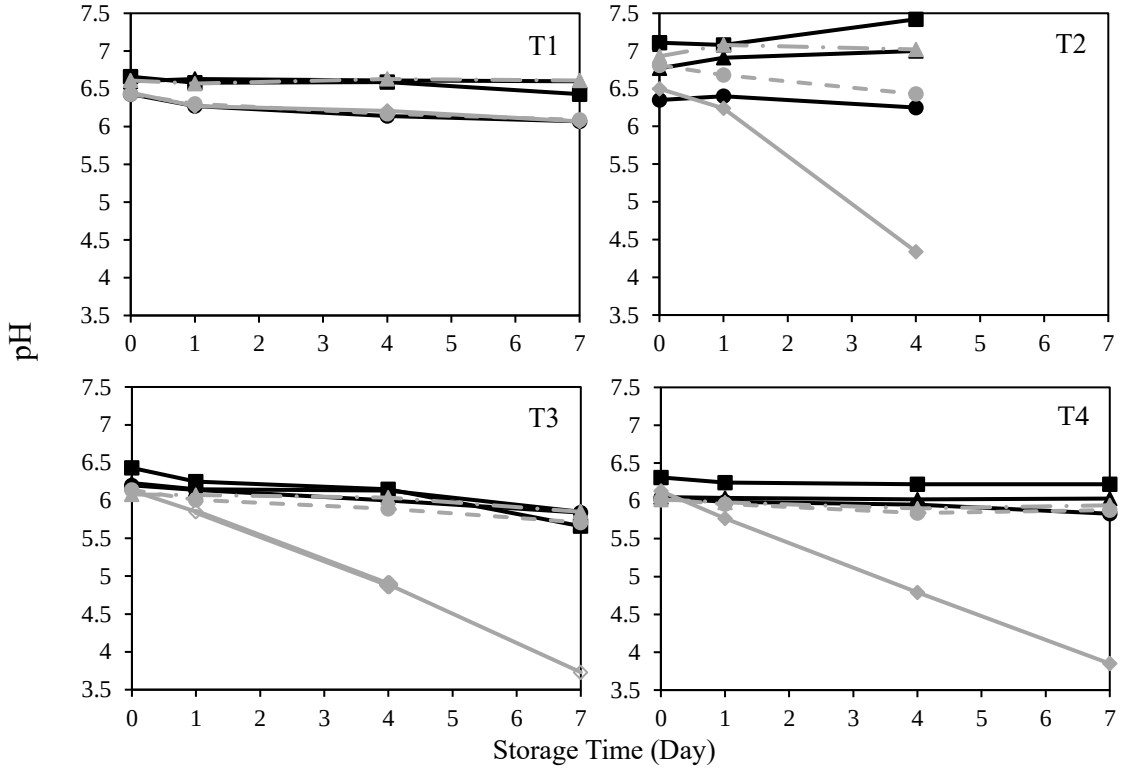

Fig. 7.3 Changes in pH of human milk after treatments during storage at 4°C for 7 days in trials (T) 1 to 4. Treatments: Control (—●—), Holder Pasteurization (—▲—), Microfiltration (—■—), Lactose oxidase treatment (—◆—), Holder pasteurization followed nisin treatment (—▲- -), and nisin treatment (- -●- -).

7.3.3 Effect of processing and storage on activities of lysozyme and lactoperoxidase in human milk

As shown in **Fig. 7.4**, milk after HoP exhibited lower initial LPO activities, while LO- and Nisin- treated milk exhibited a slightly higher initial LPO activities in all trials. In T1, LPO activities of human milk increased during storage at 4°C, especially in the case of LO-treated samples (from 0.003 Units mL^{-1} at D0 to 0.02 Units mL^{-1} at D7). However, the trend was not shown in other trials. The LPO activities in LO-treated milk increased from D0 to D4, followed by a decrease from D4 to D7 in T2 and T4. Meanwhile, there were slight increases in LPO activities in LO- and MF-treated milk during storage. In addition, a higher LPO activity in Nisin-treated milk was shown from D0 to D4 in T3. Overall, the LPO activities remained constant, with low levels, in human milk after all treatments in T2, T3, and T4.

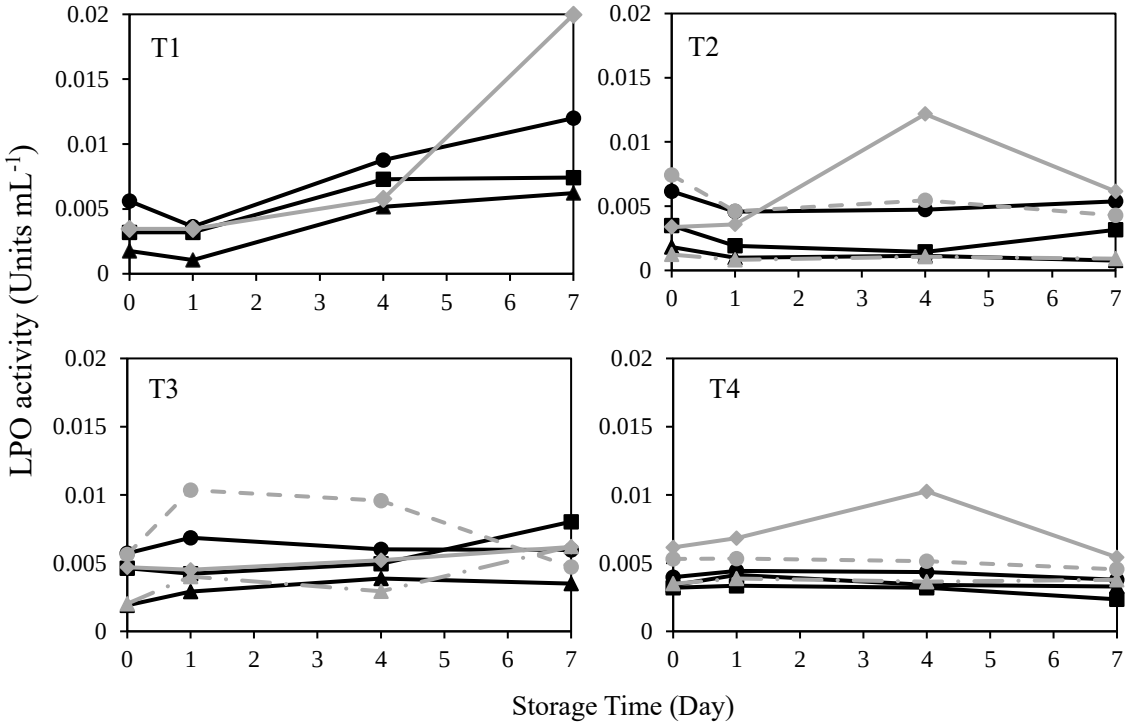

Fig. 7.4 Changes of lactoperoxidase activities in human milk after treatments during storage at 4°C for 7 days in trials (T) 1 to 4. Treatments: Control (—●—), Holder Pasteurization (—▲—), Microfiltration (—■—), Lactose oxidase treatment (—◆—), Holder pasteurization followed nisin treatment (—▲ - -), and nisin treatment (- - ● - -).

Lysozyme activities of human milk after all treatments decreased over storage at 4°C (**Fig. 7.5**). The initial lysozyme activities before storage of untreated milk samples were measured as 2080.0, 748.8, 448.5, and 579.8 units mL^{-1} in T1, T2, T3, and T4, respectively. Lysozyme activities were highest in T1, which may be due to the fact that samples were not incubated before treatments compared to other trials. Lysozyme activities decreased after HoP, especially in T1. However, the activities were similar among samples with all treatments after storage at 4°C for 7 days.

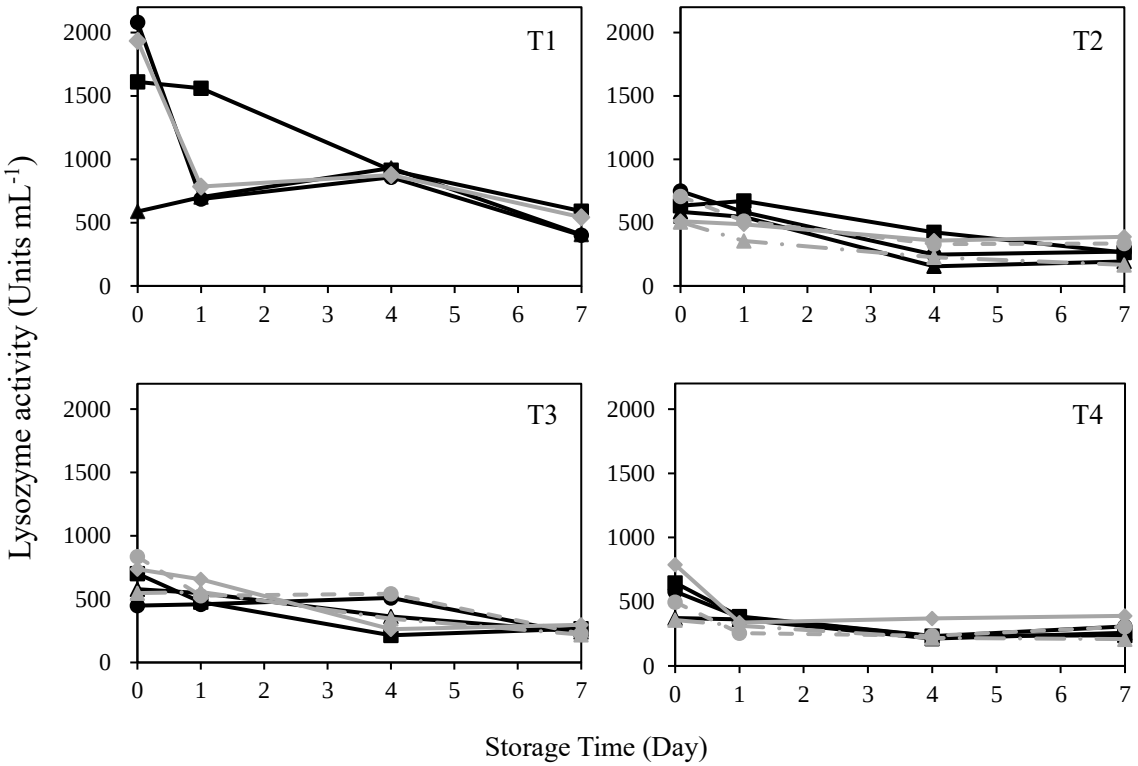

Fig. 7.5 Changes of lysozyme activities in human milk after treatments during storage at 4°C for 7 days in trials (T) 1 to 4. Treatments: Control (—●—), Holder Pasteurization (—▲—), Microfiltration (—■—), Lactose oxidase treatment (—◆—), Holder pasteurization followed nisin treatment (—▲ - -), and nisin treatment (- - ● - -).

7.3.4 Effect of processing and storage on proteolysis in human milk

As can be seen from the electrophoretograms (**Fig. 7.6** Lane 1 & 2), there were slightly decreased levels of caseins during storage for one week in CT in T1, T2 and T4, indicating that proteolysis occurred with low extent in human milk during cold storage. In contrast, the major caseins bands disappeared in control after storage at 4°C for 1 week in T3 (milk with the highest TBC), showing that the proteolysis occurred dramatically in that sample compared with milk in other trials.

In T3, samples were incubated at 10°C for 7 days and the initial TBC was higher compared with samples in the other trials. All casein bands in Nisin treated milk in T3 were disappeared in D7 (**Fig. 7.6** T3 Lane 12), indicating that proteolysis occurred at a higher extent compared to the control. There were also decreased levels of casein bands in MF-treated samples, but not as dramatic as control and Nisin-treated milk. In contrast, protein bands remained in samples after HoP, LO, and HoP+Nisin treatments in T3.

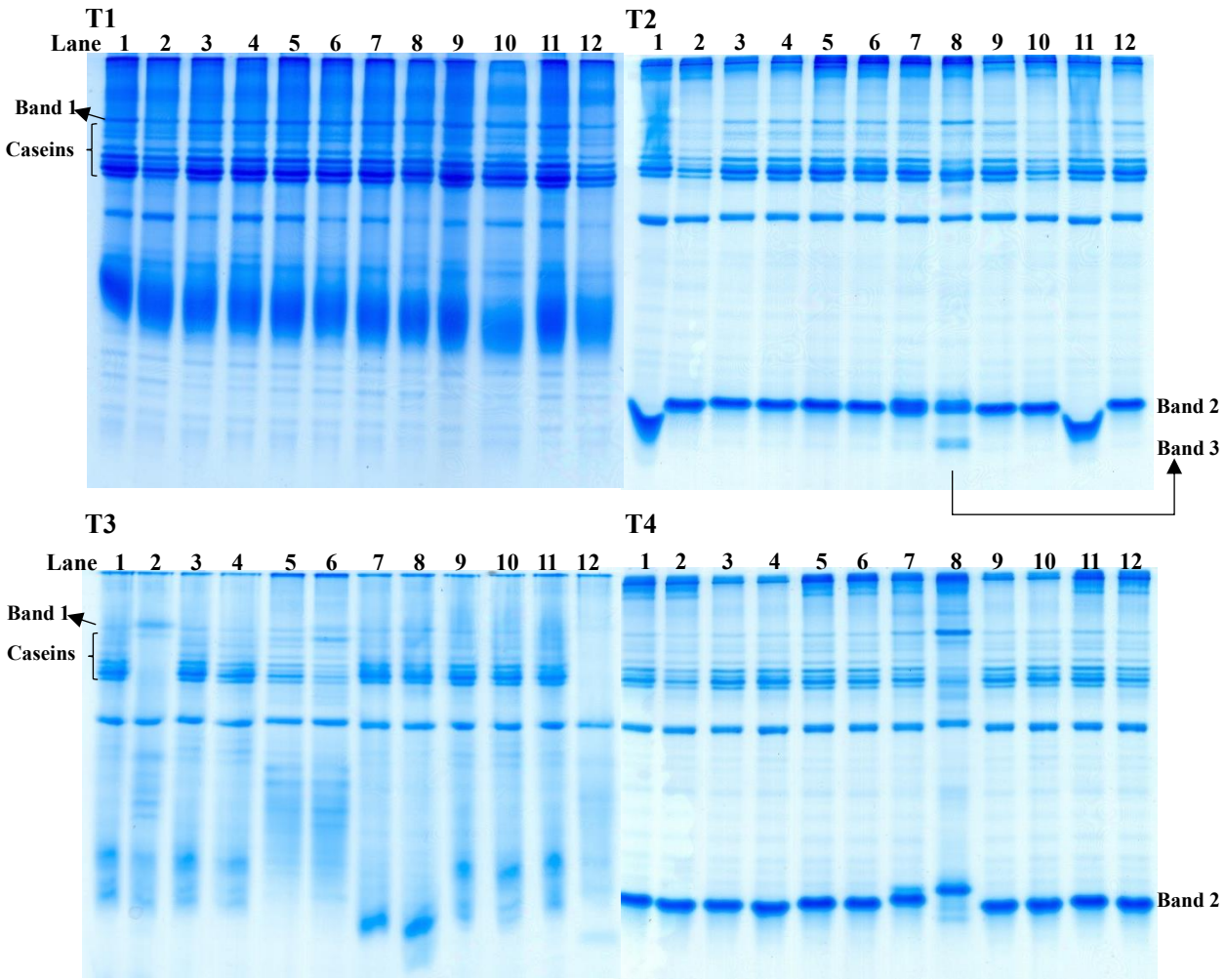

Fig. 7.6 Urea-polyacrylamide gel electrophoretograms of proteolysis during storage at 4°C in T1 - T4 at D0 (Lane 1, 3, 5, 7, 9, & 11) and D7 (Lane 2, 4, 6, 8, 10, & 12). Lane 1-2: Control; Lane 3-4: Holder-pasteurized; Lane 5-6: Microfiltered; Lane 7-8: Lactose oxidase treated; Lane 9-10: Holder pasteurized and nisin treated; Lane 11-12: Nisin treated.

In trials using human milk with low TBC (T1, T2, T4), no apparent proteolysis occurred in samples after all treatments in T1, whereas proteolysis occurred in LO treated milk in T2 and T4 during the storage. Major changes of LO-treated milk were: (1) levels of caseins decreased, whereas band 1, which could be lactotransferrin (see Chapter 5, **Fig. 5.10**) increased as indicated in **Fig. 7.6**; (2) the position of band 2, which could be lactalbumin (see Chapter 5), as labelled in the electrophoretograms with high concentrations moved and band 3 near to band 2 appeared.

Peptide profiles of milk samples in T1 (low TBC) and samples in T3 (high TBC) was further analysed using reversed-phase liquid chromatography (**Fig. 7.7**). It was

clear that the number of peaks increased, i.e., peptides were released from proteins due to proteolysis in samples with high TBC (CT, MF, and Nisin treated groups in T3). In contrast, no apparent proteolysis occurred in samples with low TBC (T1). There were four peaks (peak 8, 9, 13, 14) with high levels at approximately 15, 23, 30, and 38 min, respectively (details of the peak area and the peak height can be seen from **Table 7.2**).

In T1, peptide profiles were similar between treatment groups, except that MF treated milk had a higher level of peak 3, Nisin and MF treated milk had a higher initial peak 8, and HoP and HoP+Nisin groups had higher peaks 10 and 12. After storage at 4°C for 1 week, peak 14 decreased in milk after MF, LO, HoP + Nisin, or Nisin treatment, whereas it increased in CT and HoP groups. Peak 10 was slightly decreased in CT samples, while peak 13 increased during storage in untreated milk and milk after HoP or LO treatment.

In T3, peak 12 that was present in T1 samples disappeared, and may have co-eluted with peak 11 or 13 (**Fig. 7.7**). Compared to milk samples in T1, the levels of peak 3, 8 and 11 were higher in samples in T3. In the control with high TBC, new peptides were produced during cold storage; peaks appeared at retention times around 9 and 11 min, as well as a higher level of peaks after 15 min, i.e., more hydrophobic peptides produced in the milk after the storage. Similar to the control, a higher number of peptides were produced in the MF and Nisin-treated milk after the storage, which was even more aggressive compared with the control, especially in Nisin-treated samples.

In LO-treated milk, there were some new peaks at retention time around 31, 32, 34 and 38 min in the milk at D0, which disappeared after the cold storage. After storage, most peptides decreased, except the peak 14. The peptide profiles in human milk after HoP or HoP + Nisin treatments did not change significantly after storage at 4°C for 7 days.

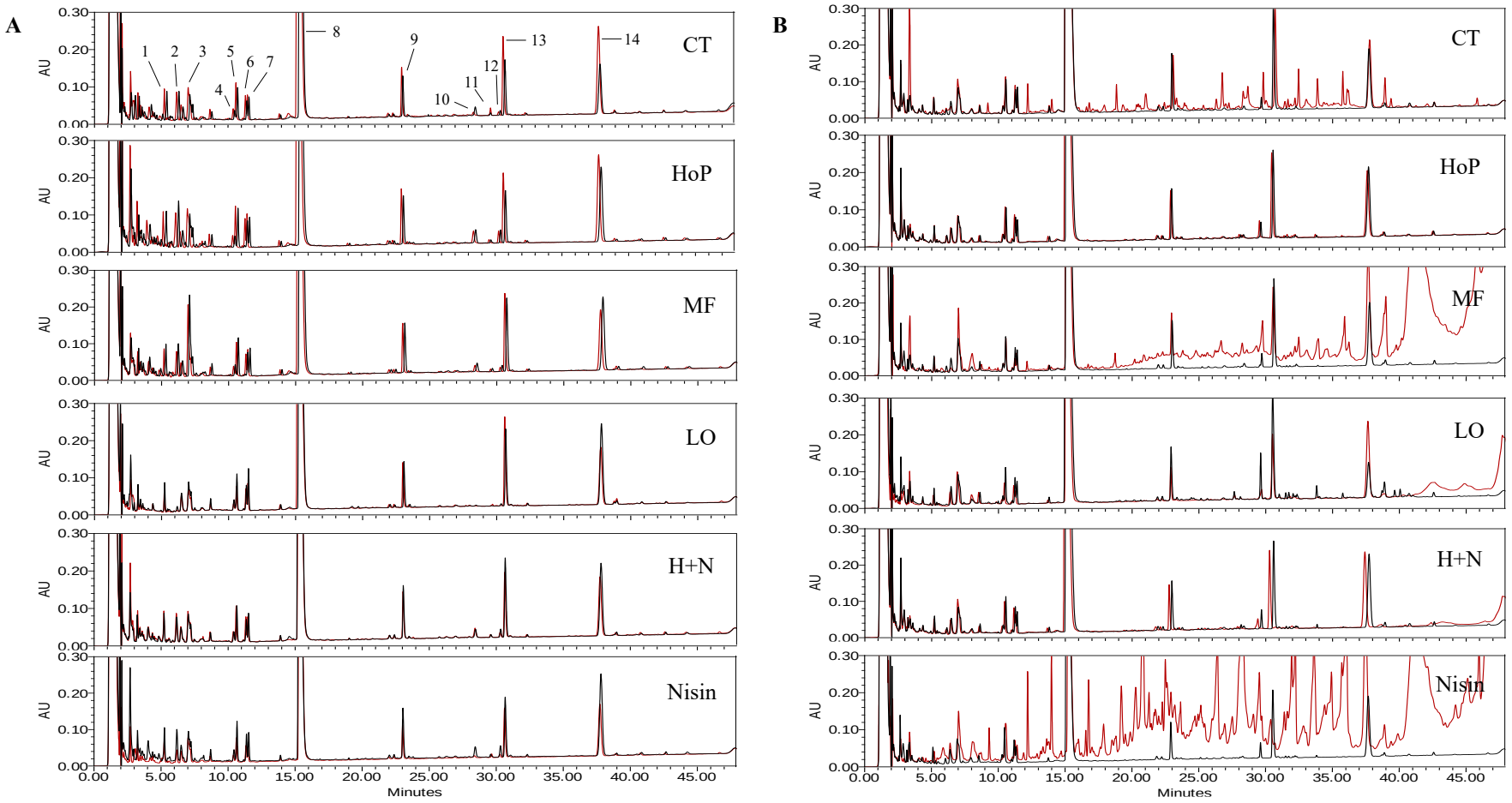

Fig. 7.7 Reversed-phase ultra-high-performance liquid chromatography chromatograms of peptides fractions (<10 kDa) in human milk with low initial total bacterial count (A; T1) and high total bacterial count (B; T3) before (D0; Black line) and after (D7; Red line) storage at 4°C for 7 days. Major peaks were labelled in chromatograms from 1 to 14. CT: Control; HoP: Holder pasteurization; MF: Microfiltration; LO: Lactose oxidase treatment; H + N: Holder pasteurization followed nisin treatment; Nisin: Nisin treatment.

Table 7.2 Area and heights of major peaks from liquid chromatography chromatograms. The background colour indicates the magnitude of area or heights.

Pk No.	Retention Time (min)	Area (x 10^3 AU·min)						Height (x 10^3 AU)					
		Treatments (T1)											
		CT		HoP		MF		CT		HoP		MF	
		D0	D7	D0	D7	D0	D7	D0	D7	D0	D7	D0	D7
1	5.2-5.4	565	390	579	714	424	434	84	79	95	105	80	76
2	6.4-6.6	356	317	354	633	269	335	41	36	44	49	35	37
3	7.0-7.2	667	993	492	1495	1815	1673	74	84	88	111	215	195
4	10.4-10.5	203	184	190	257	182	169	28	28	29	35	28	26
5	10.6-10.7	533	560	595	706	576	529	89	98	104	113	102	91
6	11.3-11.5	300	359	402	472	352	322	52	64	70	79	62	58
7	11.4-11.7	327	339	414	502	381	367	63	65	80	92	75	71
8	15.2-15.3	15509	20836	17466	21625	20421	21267	811	987	1121	1039	957	992
9	23.0-23.1	769	901	953	1083	931	949	111	132	132	150	136	136
10	28.4-28.5	233	96	392	350	231	205	24	9	37	34	24	19
11	29.5-29.7	49	123	76	74	63	54	8	20	10	10	9	8
12	30.3-30.4	94	67	265	234	123	92	13	9	36	32	17	13
13	30.6-30.8	1172	1740	1127	1539	1636	1768	149	211	141	188	200	213
14	37.7-37.9	1821	3323	2780	3296	2769	2314	133	235	200	234	199	165
		Treatments (T1)											
		LO		HoP + Nisin		Nisin		LO		HoP + Nisin		Nisin	
		D0	D7	D0	D7	D0	D7	D0	D7	D0	D7	D0	D7
1	5.2-5.4	434	262	513	747	501	413	83	54	83	98	91	64
2	6.4-6.6	514	394	461	725	381	391	53	44	43	53	42	36
3	7.0-7.2	1059	783	1024	1278	982	974	83	61	79	93	80	73
4	10.4-10.5	233	126	207	244	188	165	31	19	29	31	29	21
5	10.6-10.7	623	373	599	645	629	421	101	65	98	99	107	68
6	11.3-11.5	410	339	361	416	400	275	70	59	61	68	71	46
7	11.4-11.7	611	390	415	380	391	232	114	74	77	68	76	42
8	15.2-15.3	16962	21226	22445	18783	19417	14373	879	1015	1008	909	1062	781
9	23.0-23.1	836	826	1007	874	967	634	124	121	143	126	138	94
10	28.4-28.5	27	20	213	267	282	53	3	2	22	25	29	5
11	29.5-29.7	16	43	59	57	64	60	2	5	8	8	8	10
12	30.3-30.4	51	18	170	91	230	66	7	2	23	13	31	9
13	30.6-30.8	1700	2034	1762	1391	1331	1088	208	240	212	173	164	136
14	37.7-37.9	3029	2164	2703	2208	3159	1997	218	154	194	157	225	142

(Continued)

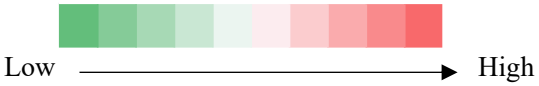

Table 7.2 *(Continued)*

Pk No.	Retention Time (min)	Area (x 10^3 AU·min)						Height (x 10^3 AU)					
		Treatments (T3)											
		CT		HoP		MF		CT		HoP		MF	
		D0	D7	D0	D7	D0	D7	D0	D7	D0	D7	D0	D7
1	5.1-5.2	217	414	346	350	308	167	46	58	53	55	52	38
2	6.4-6.5	309	572	516	479	403	116	35	32	45	47	42	10
3	6.9-7.0	885	1623	1052	1079	1188	1712	73	106	77	79	95	174
4	10.3-10.4	146	279	194	196	152	257	22	30	25	25	22	23
5	10.4-10.6	554	924	613	657	576	616	94	109	97	97	96	93
6	11.1-11.3	389	559	417	440	387	225	67	85	70	76	66	38
7	11.3-11.4	390	478	352	325	320	349	73	74	63	61	60	52
8	15.0-15.2	29427	28587	24175	23457	23980	21590	1219	1193	1100	1083	1089	1037
9	22.8-23.2	1169	1228	957	918	916	1776	158	158	137	131	132	165
10	28.2-28.3	111	520	111	89	21	1567	11	55	11	9	4	84
11	29.5-29.8	212	761	297	336	210	2563	34	105	44	47	38	146
13	30.3-30.4	2468	2920	1991	1933	2055	3597	284	293	237	229	243	238
14	37.7-37.9	2296	2770	2643	2509	2459	7804	162	187	187	177	173	340
		Treatments (T3)											
		LO		HoP + Nisin		Nisin		LO		HoP + Nisin		Nisin	
		D0	D7	D0	D7	D0	D7	D0	D7	D0	D7	D0	D7
1	5.1-5.2	339	340	405	246	292	197	56	43	59	44	50	35
2	6.4-6.5	586	751	548	350	389	659	58	49	51	40	41	55
3	6.9-7.0	1332	1396	1194	1139	946	1947	90	102	80	96	69	140
4	10.3-10.4	197	181	203	159	154	111	25	18	26	20	22	16
5	10.4-10.6	675	483	669	529	567	960	102	62	104	87	95	102
6	11.1-11.3	430	342	441	422	367	343	73	53	75	70	62	55
7	11.3-11.4	339	252	331	296	233	286	63	39	61	52	43	42
8	15.0-15.2	29279	17259	24697	22563	17907	11818	1223	896	1114	1046	922	734
9	22.8-23.2	1093	608	954	841	675	2310	148	91	136	125	101	132
10	28.2-28.3	68	55	81	66	59	9082	9	5	14	7	6	364
11	29.5-29.8	736	267	375	235	245	3185	127	27	54	27	42	210
13	30.3-30.4	2811	1679	2077	1884	1522	1231	311	177	243	216	184	83
14	37.7-37.9	1416	3218	2870	3072	2308	6696	97	210	202	208	163	405

Note: Peak 12 was excluded in T3 as it did not show up in most of samples in T3. CT: Control; HoP: Holder pasteurization; MF: Microfiltration; LO: Lactose oxidase treatment; HoP + Nisin: Holder pasteurization followed nisin treatment; Nisin: Nisin treatment.

7.4 Discussion

Human milk samples examined in these studies had a lower initial TBC (10^2 -10^3 CFU mL^{-1}) and pH compared with fresh human milk, which was reported as 10^3 - 10^5 CFU mL^{-1} and pH 7- 7.5 (Ahrabi et al., 2016; Khodayar-Pardo et al., 2014; Marín et al., 2009). The low TBC and pH could be caused by the storage of human milk at -20°C for more than 1 month before analysis (Ahrabi et al., 2016). The differences in levels of total fat, and similar levels of total protein and carbohydrate in milk between trials were consistent with those in previous studies (Fischer Fumeaux et al., 2019; John et al., 2019).

The slight decrease of carbohydrate level and the dramatic drop of pH of human milk during cold storage after the LO treatment may indicate that extensive oxidation of lactose occurred and the lactobionic acid was produced, which could potentially affect the flavour of the milk (Ahmad et al., 2004). A separate trial on bovine milk showed a decrease of pH in bovine milk caused by LO addition (1.2 g L^{-1}) from 6.70 to 6.48 after storage at 6°C for 7 days, in agreement with reports from Lara-Aguilar and Alcaine (2019a), and the most dramatic decrease of pH in bovine milk was after storage for 1 day, to pH 6.58. Human milk, in contrast, showed much more dramatic decreases in pH after LO addition in T2, T3 and T4 (pH 3.73 at D7 in T3). We hypothesise that such decreases could result from a higher level of substrate, lactose, in human milk. In contrast, the pH of LO-treated human milk without incubation (in T1) showed a slower drop compared with other trials (from 6.5 to 6.09 after storage at 4°C for 7 days). Notably, this difference among trials may not relate to initial TBC in milk, but other reasons, as milk in T2 (5% (v/v) incubation) and T4 (100% (v/v) incubation) had lower TBC before treatments compared with T1. The impact of LO on milk pH is clearly a major consideration in evaluating possible applications of this enzyme for preservation of human milk.

Low LPO activities in human milk were in agreement with previous studies (Gothefors & Marklund, 1975; Shin et al., 2001) as well as **Chapter 6**, which suggests that the LPO system is less effective as a preservative mechanism compared with bovine milk. In addition, no LPO activity has been detected in human milk in some studies (Ford et al., 1977; Sousa, Delgadillo, & Saraiva, 2014). Although LPO activities were low, H_2O_2 produced by LO may still improve the quality of human milk. It has been demonstrated that H_2O_2 alone can inhibit the growth of Gram-positive *Staphylococcus aureus* at concentrations of 25 - 100 μM (Al-Shehri et al., 2015). In addition, a higher concentration of H_2O_2 (10^{-3} M) may benefit infants by promoting gastric motility,

according to an *in vitro* rat study (Fajardo et al., 2018), however, H_2O_2 can also be potential biologically harmful at higher concentrations.

The initial lysozyme activities in human milk used in our study were lower than in the fresh human milk reported in literature using the same method (Lima et al., 2017). Chang et al. (2013) considered this low level could be due to milk being stored frozen at -20°C, as they reported a 39.8% loss in milk frozen for at least 1 month. Lysozyme activity was retained after non-thermal processing, whereas it decreased after HoP, in agreement with previous studies that reported lysozyme activity loss after HoP (Chang et al., 2013; Czank et al., 2009). In our study, storage at 4°C or 10°C negatively affected lysozyme activities in human milk, as also has been suggested previously (Chang et al., 2013; Silvestre et al., 2006). We found a 58.7 % (T3) to 65.1 % (T4) loss during pre-incubation at 10°C compared to milk without incubation (T1). After storage at 4 °C for 7 days, the loss of lysozyme in unpasteurized milk was 63.3 - 80.8%, 23.9 - 63.7%, 50.4 - 72.8%, 38.6 - 63.0%, respectively. Silvestre et al. (2006) reported that total bactericidal capacity, which was partly contributed by lysozyme, decreased by 13% after storage at 4°C for 72 h.

In addition, proteolysis occurred during storage at 4 °C, which could be caused be native proteases, as well as possibly bacterial proteases. Many native proteolytic enzymes are present in human milk, including plasmin, cathepsin D, kallikrein, thrombin, aminopeptidase, carboxypeptidase and elastase (Chan, Beverly, & Dallas, 2021). However, not all of them are active in the milk, due to reasons such as the presence of inhibitors and unfavourable pH. It has been reported that only cathepsin D and plasmin are active at pH 4.5 among all proteases in human milk; however, plasmin activity is much lower at pH 4.5 compared to at pH 6.5, as it is an alkaline protease with a pH optimum of 7.5 (Demers-Mathieu et al., 2018). Therefore, it is proposed that the difference of protein profile in urea-gel of LO-treated milk (**Fig 7.6** T2-T4 Lane 8) could be caused by lower pH (pH of LO-treated milk can be lower than pH 4 after a 7-day storage) favouring cathepsin D activities as well as favouring the activation of procathepsin D (Wittlin et al., 1999). In contrast, in T1, the pH of LO-treated milk after storage at 4 °C for 7 days was not as low as in other trials (**Fig. 7.3**), which lead to a different profile of proteolysis in LO-treated milk in T1 compared with other trials. Cathepsin D is able to degrade all types of caseins and α-lactalbumin in bovine milk (Larsen et al., 1996). From this point of view and the protein band identification results in **Chapter 5**, band 2 in urea-gel (**Fig. 7.6**) could possibly be α-lactalbumin, which is abundant in human milk as a major target of cathepsin D.

In terms of the possible relationship between proteolysis and TBC levels in milk (**Fig. 7.7**), almost no proteolysis occurred in milk with low TBC, whereas proteolysis occurred in a high extent in milk with high TBC. The high initial TBC in human milk could be related to either individual differences or the milk collection methods. Noticeable, the higher TBC (10^6 CFU mL^{-1}) in the study is similar to the TBC in fresh human milk (Zhang et al., 2020). Storage of human milk at 4 °C for 1 week influences both protein integrity and the lysozyme activities in human milk, which may lead to differences compared to feeding infants with fresh human milk. Although knowledge on how the protein integrity in milk influence the infant's digestion is limited (Wada & Lönnerdal, 2020), lysozyme is an important bactericidal protein that protects human infants from microbial infections (Ballard & Morrow, 2013). Storage of human milk in a refrigerator for 1 week is therefore, not recommended for infants' feeding.

A higher level of peptides was produced in the milk after non-thermal treatments compared to the milk after HoP and LO-treated milk in the high TBC groups (**Fig. 7.7**). Fewer peptides were produced in the LO-treated milk, may be due to pH, which does not favour the proteolytic activities in the milk. Compared to milk after HoP, high levels of psychrotrophic bacteria, which mostly can be Gram-negative bacteria, may be presented in unpasteurized milk (Júnior et al., 2018). These bacteria can produce proteases and/or thermoresistant extracellular lipases at a minimum bacterial cell counts at 10^6–10^7 CFU mL^{-1} according to bovine milk studies (Ribeiro et al., 2018). For example, *Pseudomonas* spp., and *Yersinia enterocolitica* can produce alkaline metalloprotease (aprX) (Júnior et al., 2018). As we measured in the study (T3), the growth of *Pseudomonas* can be partly prevented by LO (7 x 10^5 CFU mL^{-1} at D7), whereas not be prevented by nisin treatment (1.06 x 10^7 CFU mL^{-1} at D7), which was consistent to the high extent of proteolysis in nisin-treated milk compared to that in LO-treated milk. Although *Pseudomonas* was excluded and did not grow in milk after MF, proteolysis occurred in MF-treated milk in T3 (**Fig. 7.7**), which could due to the presence of proteases or other bacteria. *Pseudomonas* proteases may also contribute to overall plasmin activity by acting as plasminogen activators to convert plasminogen to plasmin (Fajardo-Lira et al. 2000). In addition, psychrotrophic bacterial proteases readily hydrolyse caseins, which can lead to bitter flavour and increased viscosity of milk (Ribeiro et al., 2018; Zhang et al., 2019). The bitter flavour resulted from the generation of hydrophobic peptides by hydrolysis of caseins (Marchand et al., 2017).

To our knowledge, this is the first study to evaluate the preservation of human milk using MF, an enzymatic method, and the bacteriocin method. In general, the three methods are all non-thermal, the benefits of which preserve heat-sensitive compounds as well as LPO and lysozyme activities in human milk. However, each method has their

own advantages and limitations. Compared to LO- or nisin-treated milk, MF is a non-preservative-added membrane-based method that provided higher bactericidal effects on human milk, for example, *Pseudomonas* was inhibited after MF, but not after LO- or nisin-treated samples. However, proteolysis was not inhibited in the milk after MF. Meanwhile, the MF process is slow compared to the other two methods, a membrane filtration facility is required, and trouble-shooting, such as solving fouling problems, is required (Gul, Hruza, & Yalcinkaya, 2021). In dairy industry, cross-flow membrane filtration is applied in the MF treatment and the pore size could be larger than was used here, which may speed up the process, but increase the chance of contamination (Fernández García, Álvarez Blanco, & Riera Rodriguez, 2013).

Addition of LO is a simple and quick enzymatic method that could inhibit the proteolysis in human milk during the storage. The drawback of LO treatment was that it was not as effective as HoP or MF on elimination of bacteria. Low pH of human milk resulted from LO treatment can be another issue, which could active the Cathepsin D and destabilise the milk system.

Nisin is a hydrophobic peptide that widely approved for use as a food preservative (over 50 countries) (Favaro, Penna, & Todorov, 2015). The acceptable daily intake of nisin for infants (12 weeks–11 months) can be 1 mg kg^{-1} birth weight per day, which is safe for using nisin as a food additive (Younes et al., 2017). However, it did not effectively prevent the growth of Gram-negative bacteria and led to proteolysis in human milk, which could result from bacterial proteases in human milk. The ineffective prevention by nisin could result from the low solubility of nisin at natural pH of milk (Ibarra-Sánchez et al., 2020). The combination of treatments to both create hurdles for bacteria growth and preserve heat-sensitive compounds in human milk may be required.

The study was limited as frozen milk, with small sample sizes, was used and the treatments were on a laboratory scale. In future studies, some treatments may be improved, for example, cross-flow membrane filtration pilot units may be applied in the MF treatment and cream can be heated and remixed. The specific bacterial count measurement during storage is required (which was actually conducted as a parallel study, data not shown) and peptides produced during storage need to be identified by mass spectrometry.

7.5 Conclusion

Novel processing methods, including MF, LO treatment, and nisin treatment for preservation of human milk were explored. Macronutrients were largely retained after different treatments and storage, except that lactose was oxidised after LO treatments. The activities of LPO and lysozyme were higher in milk after novel treatments compared to the milk after HoP. During storage at 4°C for 7 days, the LPO activities remained low, which may influence the ability of the LPO system to produce active antibacterial compounds, while lysozyme activities decreased. In addition, proteolysis occurred during refrigerated storage to an extent dependent on the initial TBC of human milk. Among novel processing methods, nisin treatment may be worthy of futher study, as it can prevent microbial growth better than other methods and it did not change levels of macronutrients. The study proves the potential of novel processing methods in maintaining nutrients and bioactive compounds and highlights potential problems with the storage of human milk for extended periods in the fridge.

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CHAPTER 8

General Discussion

8.1 Introduction

Human milk is ideal food for infant nutrition, as it provides short-term and long-term health benefits to infants. Most importantly, it supports infants' vulnerable immunological system in protecting them against several infective diseases, as well as helping the maturation of their gastrointestinal tract and brain development (Mosca & Giannì, 2017; Perrella et al., 2021). In previous chapters, the author reviewed human milk composition and some physiochemical properties (**Chapter 1**); measured changes of macronutrients, plasmin activities, fatty acid profiles, pH, and colour of milk during lactation (**Chapter 2**); studied the influence of temperature, cold storage, freeze-thawing, and pre-heating on physical stability (creaming) of human milk and compared this to bovine milk (**Chapter 3**); further investigated the impact of pre-heating temperatures, sonication, adding calcium-cheating agent on creaming in raw bovine milk using both accelerated and gravitational creaming methods (**Chapter 4**); studied the impact of heating, ethanol, chaotropic agents, enzymatic coagulation, and storage on casein stability in human milk (**Chapter 5**); monitored the changes in enzymes activities and physiochemical properties in human milk after high-pressure (HP) treatments followed by storage (**Chapter 6**); and attempted to find novel alternatives to holder pasteurization (HoP) of human milk, including microfiltration, addition of lactose oxidase, and addition of nisin (**Chapter 7**). In this chapter, key results of all previous chapters, some additional results and rethinking will be included. Finally, recommendations for further study from the viewpoint of both scientific research and recommendation for clinical practice were given.

8.2 Human Milk Composition

Human milk composition was measured in **Chapter 2** as part of a prospective observational cohort study aiming to analyse various components of human milk over time and to compare their contents in the preterm versus the term milk. The study was established following the previous study by Caldeo et al. (2020) with a longer observational time (up to 6 months of lactation) in a new cohort of infants in the hospital.

Human milk was individualised when considering fat levels, fatty acid profiles, as well as plasmin activities, as reported previously (Armaforte et al., 2010; Demmelmair & Koletzko, 2018). However, the levels of composition followed some patterns when considering lactational time, group by maternal characteristics (pre-pregnancy body mass index (BMI)), or group by offspring characteristics (gestational age and infant gender). Previous studies have reported a relationship between BMI, gender, or lactational stages and total fat and energy in human milk (Bzikowska-Jura et al., 2020; Ellsworth et al., 2020; Hascoët et al., 2019), as well as BMI or lactational stages and fatty acid profiles (de la Garza Puentes et al., 2019; Floris et al., 2020). Although the conclusions about this link between these factors and human milk composition are still controversial, several studies can be found. In contrast, limited studies reported the association between infant gender and fatty acid profiles (Miliku et al., 2019; Thakkar et al., 2013), which was found in our study, although bias may be involved due to the sample size limitation.

Maternal diet has been considered to impact on fatty acid profiles (Koletzko, 2016). Irish mothers have a diet habit with high dairy consumption and low fish consumption (**Chapter 2**). For this reason, it is hard to compare the group of mothers who consumed oily fish, white fish or butter to mothers without those intakes within 24 hr of milk expressed. Other food consumptions, including red meat, poultry, vegetable oil, olive oil, seeds, nuts, and supplements (generally vitamins and DHA supplementation) were correlated with fatty acid profiles during lactation among 56 term milk samples (with diet information). The cohort of mother with red meat intakes within 24 hr of milk expressed had milk with higher levels of heptadecenoic acid (C17:1), α-linolenic acid (C18:3n-3), and *cis*-11-eicosenic acid (C20:1), while milk contained higher lauric acid (C12:0) from mother had poultry intake within 24 hr of milk expressed. These results are consistent with previous findings that milk from mothers on high fat diets contained higher levels of α-linolenic acid (C18:3n-3) (Nasser et al., 2010). Nasser et al. (2010) also reported that lauric acid (C12:0) levels were significantly higher when consuming a low-fat diet. No association was found between palmitic (C16:0) or stearic acids (C18:0) and red meat, poultry or dairy products consumption by mothers, which was reported previously (Sinanoglou et al., 2017). In addition, term human milk from mothers with supplement intake had higher levels of C20:3 n-3 and C22:6, whereas levels of C12:0 and C22:2 were lower in such milk. The relationship between Irish diet and fatty acid profile in human milk requires further study. One recent study also reported that fatty acid profiles are

related to genetic and environmental (i.e., different between study sites) factors (Miliku et al., 2019), which may be also interesting for future study.

Microbiome data (Lyons, 2021) and microbiota data (unpublished, see preliminary results in Poulsen et al., 2019) from the same cohort of human milk during lactation were also collected. Limited studies have reported the relationship between microbiome, microbiota, and macronutrients in human milk. One study reported the association of the fatty acid profile with the microbiota, which revealed that monounsaturated fatty acids (MUFA) were negatively associated with *Proteobacteria*, while the genus of *Lactobacillus* was associated with MUFA (Kumar et al., 2016). Another study from Gómez-Gallego et al. (2018) reported the association of microbiome and microbiota; *Proteobacteria*, *Actinobacteria*, and *Bacilli* were most significantly associated with the milk metabolites (. Future output by pooling and interpretation the data from **Chapter 2**, microbiome data, and microbiota data will fill the gap on this area.

In addition, this study, as a part of the INFAMILK project, supported by Department of Agriculture, Food and the Marine, Ireland, also tried to elucidate the relationship between other components, for example, between plasmin activity and somatic cell counts (SCC) in human milk, whereas no relationships were observed (**Fig. 8.1**). This may be due to mothers with clinical mastitis, i.e., mothers with significant mastitis symptoms, being excluded in the study. However, the population of mothers with subclinical mastitis (no clinical manifestations with SCC ≥ 250,000 cells) was 32.6% in human milk samples measured in this study. In bovine milk, high plasmin activity is related to high SCC, as urokinase-type plasminogen activators are associated with somatic cells (Politis et al., 1989; White et al., 1995). The reason that plasmin activity may not be related to somatic cells in human milk need further confirmation and research is required to elucidate this relationship.

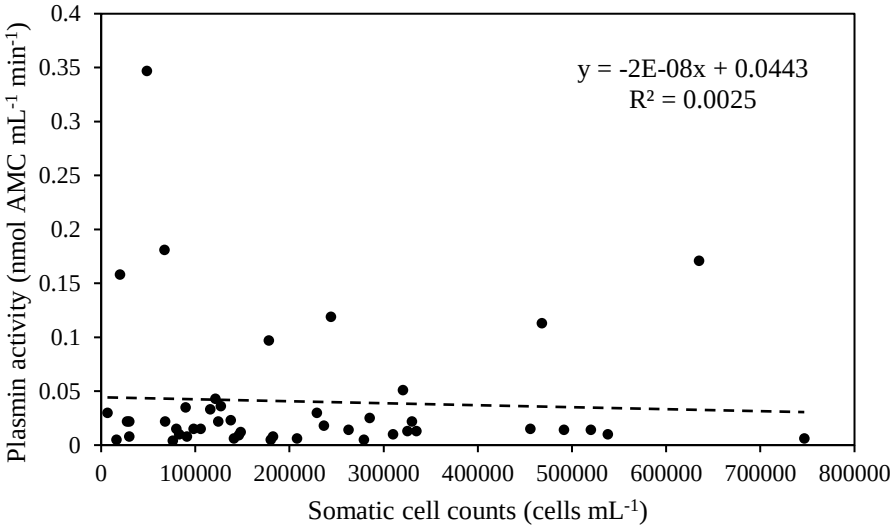

Fig. 8.1 Relationships between plasmin activities and somatic cell counts in human milk. **Note**: Somatic cell counts were measured by Angelopoulou (2019) by a direct microscopic method using the same cohort of samples for the INFAMILK project. Plasmin activity was measured as described in **Chapter 2**.

8.3 New Insights into Human and Bovine Milk Creaming

In **Chapter 3**, human milk creaming was studied and it was compared to bovine milk creaming. According to Stokes' law, it is understood the velocity of natural creaming can be influenced by the density difference between the droplets (major fat) and serum phases, size of fat globules, and viscosity of the serum phase. However, the prediction from Stokes' law was not accurate as it based in an ideal (Newtonian) liquid system. In the real milk system, milk fat globule (MFG) size is unequal and there are many other factors required to take into account, for examples, droplet (i.e., MFG) fluidity, concentration, polydispersity, charge and interactions, interfacial thickness, and no-Newtonian behaviour of continuous phase (McClements, 2015). Creaming at 5°C was of interest, as the actual creaming rate was much higher than the prediction by Stokes' law, especially in terms of bovine milk. Cold agglutination of MFG that caused by cold agglutinins, including immunoglobulin M (IgM) and immunoglobulin A (IgA) has been suspected as the reason for this higher creaming rate than the prediction. Interestingly, even the size of human MFG as measured was comparable to bovine MFG, and human

milk contains higher levels of IgA, the creaming rate was lower compared to bovine milk at 5°C. Further studies are required to determine the levels and distribution of IgA and IgM during creaming. Other proteins present at different levels in human and bovine milk, such as caseins may also be important as it has been reported that high levels of caseins enhance creaming rate in protein-stabilised oil-in-water emulsions (Liang et al., 2013). Meanwhile, crystallization of milk fat needs to be examined, which has been reported to occur in both human and bovine milk at 4°C (Lopez et al., 2002; Lopez et al., 2013).

Chapter 4 further investigated the creaming properties in bovine milk, which has not been fully understood. A lag phase appeared in bovine milk creaming at 5°C, whereas it not appears in creaming at 20°C. The temperature seems to influence the sequence of clustering and migration of MFG. The extent of clustering of milk fat globules may be interesting to monitor during creaming.

Meanwhile, in the dairy industry, it is a challenge to treat milk with harsh heat processing and maintaining good creaming stability during storage. The severity and the duration of heat processing may result in destabilisation of milk (McClements, 2015). Human milk goes through heat treatments in human milk banks. The influence of heating on both human and bovine milk were, therefore, also studied. Heating human milk at 70°C for 10 min reduced the peak thickness of the cream layer. Heating bovine milk at 80°C or 85°C for 10 min led to decreased creaming rate and more packed cream layer (higher levels of clustering of MFG), but the peak thickness of the cream layer was similar to the control when creaming at 20°C.

Heating can change the structure of proteins, including whey proteins and milk fat globule membrane (MFGM) proteins in milk, and thus influence the fat-fat and protein-fat interactions (Ye et al., 2004). The decreased creaming rate has been considered due to increased viscosity of serum phase and increased density of MFG (denatured whey protein attached) after heating. Concerning the final peak thickness of the cream layer, tightly packed MFG can form a thinner cream layer as the attraction between particles is relatively weak so that they can rearrange themselves over time. In contrast, a thicker cream layer forms due to loosely packed MFG when there is a strong attractive interactions between such MFG that they do not allow them to rearrange themselves once they have come into contact with their neighbours (Mcclements, 2007). Heating may therefore decrease this attraction between MFG by changing its surface properties.

Moreover, it was also found that the physical stability of human milk was stable after freezing at -20°C for 1 week as the fat globule size did not change, while the Turbiscan stability index (TSI) slightly increased after freezing at -20°C for 9 months. During storage at 4°C for 1 week, the TSI decreased, i.e., creaming was slower than before the storage (**Chapter 3**), which was consistent with the results in **Chapter 6** that the TSI decreased after storage of human milk after HoP or HP treatments at 4°C for 1 week. The results provide evidence that frozen milk for short term can be a better option to study the physical properties of human milk compared to storage of human milk in the fridge, when it is difficult to analyse all milk at fresh status. Meanwhile, the result is clinically significant for processing and storage of human milk in human milk banks and using human milk in hospitals.

8.4 Casein Micelle Stability

Human casein micelles exhibit difference compared to bovine milk, including size, heat stability, ethanol stability, and dissociation behaviour (**Chapter 5**). Concerning human casein micelle structure, studies are limited and can be sourced back to 20 years before, which may be due to the rare source of human milk and much smaller size of human casein micelles compared to bovine casein micelles. Similar graphs of casein micelles were obtained from transmission electron microscopy (TEM) compared to Carroll et al. (1985). However, author doubt their conclusion that the observations fit into submicellar hypothesis of the internal structure of casein micelles. The internal structural model may also fit into the observation, which proposes that casein constituents directly form the internal structure of the casein micelle, rather than forming submicelles first. Human casein micelles appeared to have a looser structure compared to bovine milk casein micelles.

Human milk has been shown to have lower ethanol stability than bovine milk and exhibit type B heat stability profile, where heat coagulation time increased as pH increased. Meanwhile, the dissociation of caseins in human milk can be different between 4°C and 20°C. These results expand the knowledge on human milk and help in understanding the changes of human milk during heating and cold storage. The analytical

methods for casein micelles were mostly based on those used in bovine milk studies, including ultracentrifugation, dissociation, and heat stability. For example, when measuring heating stability, browning reactions can occur before milk coagulation, which may due to the high levels of lactose in human milk, therefore, influencing the observation. Besides, when measuring dissociation of human casein micelles through spectrometry at an absorbance of 600 nm, the initial optical density (without dissociation agents addition) was perfect fit for bovine milk (0.6-0.7), but was low for human milk (0.03 - 0.06). Due to the volume of milk samples, these methods are not modified in the thesis, but they should be modified to be best applied in human milk studies in future research.

8.5 Proteolysis in Human Milk

In previous studies, we examined proteolysis in individual human milk or milk after heat treatments during incubation at 37°C (**Chapter 5**), and in human milk after novel processing methods followed by storage at 4°C for 1 week (**Chapter 6 & 7**). **In Chapter 5**, high individual differences in the proteolysis in milk were found, which was related to their plasmin activities. Milk was then incubated at 37°C for several days with inhibitors, aprotinin and pepstatin, to understand which enzymes will cause proteolysis. It was found that pepstatin can inhibit the proteolysis in human milk with low plasmin activity. It is proposed that aspartyl peptidases such as cathepsin D can be the major protease in proteolysis in certain human milk samples (low initial plasmin activity with higher number of protein/peptides bands in urea-gel). In contrast, the casein bands were greatly reduced in urea-gels in human milk examined with high plasmin activity, which indicated that proteins may be pre-digested in human milk. Human milk has been demonstrated to contain high levels of unique pre-digested peptides, which has been demonstrated to be produced by milk proteases within the mammary gland (Lopez et al., 2020; Nielsen, Beverly, & Dallas, 2017). It may be also interesting to know if the variation of the abundance of peptides causes differences in infants' digestion and when does this variation start, from the mammary gland or during the storage.

Processing, such as heating leads to denaturation of proteins in human milk (**Chapter 5**). Lactoferrin and serum albumin can be degraded after heating at 63°C for 30

min. The denaturation of lactoferrin and serum albumin can be irreversible after heating at 130°C for 12 min. β-caseins and α-lactalbumin are more heat stable compared to lactoferrin and serum albumin in human milk, the degradation of which occurred after heating at 110°C for 12 min. During storage, heating from 80°C for 12 min can preserve higher levels of caseins as well as some whey proteins compared to heating at other temperatures; low heat temperatures cannot inhibit proteolysis of caseins, while high heat temperatures lead to denaturation of proteins. In **Chapter 7**, proteolysis after different treatments and during storage at 4°C was also studied. The proteolysis during storage is related to the initial bacterial counts in human milk. The degree of proteolysis was comparable between milk with low bacterial counts after novel treatments (microfiltration, lactose oxidase treatment, and nisin treatment), HoP, and control (untreated). In contrast, the degree of proteolysis was different during storage of milk with high bacterial counts after those treatments. HoP inhibited proteolysis, whereas higher levels of proteolysis occurred in milk (high bacterial counts) after nisin treatment or microfiltration. The presence of *Pseudomonas* may lead to aggressive proteolysis in human milk, which suggests careful handling of human milk to avoid contamination can be important. Again, it may be interesting to know what peptides are produced from bacterial proteolysis and what impact these will have on infant health.

8.6 Alternatives to Holder Pasteurization

Several aspects, including nutrition, safety, supply and immune protection are taken into consideration when providing donor human milk to infants who cannot receive milk from their own mothers (Underwood, 2013). These infants are often premature, and require milk with higher levels of immunological compounds and nutrients similar to milk colostrum (e.g., high protein levels in milk after one week *post-partum*) compared to mature milk. However, milk in human milk banks is primarily donated from mothers who had term babies and have weaned their own infant but continue to express milk (Underwood, 2013). As reported in **Chapter 6** and **Chapter 7**, especially in **Chapter 7**, both levels of total protein (1-1.4 %) and energy (60 – 66 kcal dL^{-1}) in pooled donor milk were lower than that in colostrum of preterm milk (1.6 % protein and 69 kcal dL^{-1} energy) reported in **Chapter 1**. Concerning the safety of milk, human milk in milk banks is

bacterially screened and pasteurized to avoid transmission of infectious agent to infants (Haiden & Ziegler, 2016). The commonly used pasteurization method in human milk, HoP, has been demonstrated to inactive almost all bacteria and non-heat resistant viruses, but results in decreased levels of immunological compounds (Arslanoglu et al., 2010; Pitino et al., 2021; Underwood, 2013). Our results showed that LPO activity, lysozyme activity, and cathepsin D activity decreased, while plasmin activity remained unchanged, after HoP.

Alternatives to HoP were tested on human milk, including HP processing (**Chapter 6**), microfiltration (MF), lactose oxidase (LO) treatment and nisin treatment (**Chapter 7**) The effect of these non-thermal preserving methods on macronutrients, enzyme activities, proteolysis, and physical stability of human milk are evaluated and compared to the traditional HoP method. It was found that HP treatment at 400 MPa for 10 mins was the most promising alternative method to treat milk in terms of comparable macronutrient levels and higher enzyme activities compared to HoP or HP treatment at 600 MPa. In addition, the milk after HP treatment at 400 MPa for 10 min showed physical stability after cold storage for four days, less change of casein micelle size (indirect measurement through colour) compared to HP treatment at 600 MPa, and effective eliminate bacteria in human milk. Higher levels of lysozyme are also present following other novel treatments; however, these methods had their own limitations. The limitation for MF was that it is a slow process as milk was required to be centrifuged prior to the MF milk. After MF, fat is always required to be pasteurized and added back to the skim milk. The advantage of MF was that it was as effective as HoP in preventing the growth of microbiome. In terms of LO and nisin treatments, both of them are quick and easy to operate; however, the shortcomings were that carbohydrate levels and pH decreased in LO-treated milk, and bacterial proteolysis occurred in nisin-treated milk. A hurdle approach that combines these treatments, for example, combining HPP and Nisin, to preserve human milk with microbial safety and high immunological compounds may require further investigation.

8.7 Conclusion

Overall, the results reported in this thesis greatly expand our knowledge in several areas relating to human milk, including factors affecting its macronutrient composition and lipid profile, the physicochemical properties of key colloidal species in human milk (fat globules and casein micelles) and the impact of storage and processing on its stability. In addition, new knowledge on aspects of bovine milk was generated through its inclusion in studies as a reference material.

The work presented also has practical applications. For example, there are 270 active milk banks and 15 planned milk banks in Europe according to European Milk Bank Association website (European Milk Bank Association, 2021) while one personalized nutrition unit has been established in Spain to obtain proper nutrients for preterm infants (Luna, Martin, & Gómez-de-Orgaz, 2020). No milk bank in the Republic of Ireland has been established nor are there plans to establish one currently. Our results may provide useful data for Irish human milk banks in the future concerning the milk composition of Irish mothers, milk handling, and milk storage.

In conclusion, this work contributes to the understanding the factors affecting human milk components, especially milk fat and protein, and has potential to apply in instructions for mothers, human milk banks, and hospitals to improve infant feeding strategies.

8.8 Recommendations for Future Studies

1. Interpretation of the relationships among nutrients, such as fatty acids, somatic cells, the microbiome, and metabolites in human milk is required. As analysed in **Chapter 2**, macronutrients, plasmin activity, and fatty acid profiles change during lactation. Such data is currently being pooled with microbiome and metabolites data to explore their relationships and to explain the mechanism of changes of those components. In addition, attention can be also paid to other components, such as human milk oligosaccharides, phospholipids, and glycoproteins, which are present at low levels, but have high biological functions and may be related to the development of the infant gut microflora.

2. Further investigation is warranted on milk creaming with regards to microstructure of human milk fat globules, particularly in terms of proteins on human milk fat globule membrane (MFGM), and proteins which may attach to human MFGM, which could be studied through transmission electron microscopy or using specific stains and probes with Confocal Laser Scanning Microscopy. These observations can help to further develop creaming models in human milk.

3. The mechanism of proteolysis and the function of pre-digested peptides in human milk requires further studies. Changes of concentrations of plasminogen, cathepsin D and other proteases during lactation require further monitoring. Peptides were produced from these native proteases or from bacterial protease as detected from pervious chapters. However, it is unclear about if these peptides have impacts on the digestion of infants, which remains to be elucidated in the future.

4. Improvement of the methods of investigation for human milk is required, such as casein dissociation, heat stability testing, and enzyme activity tests. For example, quick, simple, and cheap tests may be required for detection of plasmin or other protease activities. For the detection of some enzymes at low levels in human milk, although enzyme-linked immunosorbent assays (ELISA) may be available, cheap methods with lower limit of detection, i.e., higher sensitivity, may be required to develop.

5. There is a need for further investigation of novel preservation methods on other milk components. Due to low volume of human milk, the investigation of novel preservation

strategies for human milk is still limited in regards of number of repeats, evaluation of important bioactive compounds e.g., immunoglobulins, and some structural changes of components, e.g., casein micelles. Meanwhile, some combinations of treatments were not studied, such as combining low heat temperatures of pasteurization with lactose oxidase or nisin treatments, which can be explored in future studies.

6. Further study could examine the importance of individualised human milk for infants' health outcomes. The study found high individual difference in regards to milk composition, such as plasmin activities and fatty acid composition. However, the relationships between these components and infant health outcomes may still need exploration in the future.

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APPENDIX

Publications

Trends in Food Science & Technology 112 (2021) 608–621

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: www.elsevier.com/locate/tifs

The composition and physico-chemical properties of human milk: A review

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ARTICLE INFO

Keywords:
Human milk
Human milk macronutrients
Physico-chemical properties
Human milk ingredients
Milk processing
Human milk banking

ABSTRACT

Background: Human milk is recommended as the optimal source of nutrition for babies up to six months of age, as it provides individualized, specific and abundant nutritional compounds, as well as immunological protection. Currently, there is wide interest in studying human milk because of this key role, as well as identification of biologically active compounds. In addition, human milk banks play an important role in supplying donor human milk to infants, especially premature infants, in hospitals, which involves processing and storage of such milk.

Scope and approach: This review summarises recent studies on macronutrients in human milk, including their structure, levels, influencing factors, and contributions to the health benefits of infants. Physico-chemical properties of human milk are also reviewed, including pH, colour, stability of casein micelles, size and zeta-potential of fat globules, heat stability, freeze-thaw effect, dehydration and phase separation, and the impact of preservation processes on these.

Key findings and conclusions: The levels of human milk macronutrients and factors that influence these, such as lactation and gestational age, are ongoing research topics. Studies on physicochemical properties of some human milk components, such as caseins, are comparatively few, as research has mainly focused on bioactive peptides, milk fat globule membrane and human milk oligosaccharides. Processing and preservation are applied to human milk and the effects of these methods on the retention of human milk nutrients for use in human milk banks and hospitals must be considered. Some physico-chemical properties, such as milk pH, colour, and physical stability, may be possible indicators of milk quality.

1. Introduction

Human milk from the infant's own mother is the optimal source of nutrition for infants because: (1) it provides core required nutrients, including proteins, lipids, carbohydrates and minerals; (2) it provides bioactive factors, especially immunological factors, to protect infants from invading microorganisms (Ballard & Morrow, 2013; Lee et al., 2018); (3) it helps the development of the infant microbiome and thus the newborns' immune system (Kulinich & Liu, 2016; Lyons, Ryan, Dempsey, Ross, & Stanton, 2020); and (4) some components in human milk are considered to influence the expression of genes in neonates, therefore influencing their health outcomes (Verduci et al., 2014). Breast-feeding also provides many other short-term and long-term benefits to infants, such as reducing the risk of necrotizing enterocolitis (in preterm infants) and obesity later in life (Boix-Amorós et al., 2019; Mosca & Giannì, 2017). Human milk from an infant's own mother is therefore recommended as the first-choice source of nutrition by the World Health Organization (WHO) (WHO, 2003).

When an infant's own mother's milk is unavailable or in limited supply, for example for vulnerable preterm infants, donor human milk (DHM) from milk banks is fed as a substitute because it can significantly reduce morbidity and mortality among vulnerable infants (WHO, 2003). DHM usually consists of pooled milk samples provided by lactating mothers who have been medically screened. It is then typically pasteurized, frozen and stored until required (DeMarchis, Israel-Ballard, Mansen, & Engmann, 2017; Weaver et al., 2019). As a result, some beneficial compounds, such as secretory immunoglobulin A (sIgA), lactoferrin, lysozyme, long-chain polyunsaturated fatty acids, cytokines and growth factors, may be present at lower levels than in fresh human milk (Malinowska-Pańczyk, 2020). Studies on how to minimize the nutrient gap between DHM and mothers' own milk, for example, finding alternatives to the major human milk banking preservation step, holder pasteurization (HoP, typically at 62.5 °C × 30 min), are ongoing.

There is also an increasing interest in innovative applications of human milk, for instance, the possibility that the human milk component sIgA can be used to treat the newly identified illness severe acute

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https://doi.org/10.1016/j.tifs.2021.03.040
Received 22 October 2020; Received in revised form 5 February 2021; Accepted 23 March 2021
Available online 28 March 2021
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respiratory syndrome coronavirus 2 (SARS-CoV-2) (Fox et al., 2020). Furthermore, human milk compounds should guide strategies for infant feeding, including identifying new critical ingredients for infant formula, such as oligosaccharides, components of the milk fat globule membrane (MFGM) and bioactive peptides (Koletzko, 2016; Sun et al., 2017; Wang et al., 2020). To achieve better preservation of bioactive compounds, in order to utilize the human milk more effectively, it is important to study how best to process and preserve human milk, as well as evaluating the underlying physico-chemical properties of human milk.

The physical and chemical properties of milk refers to attributes such as pH, colour, and density; these properties depend on both intrinsic compositional and structural factors in the milk and extrinsic factors such as temperature and treatments (McCarthy & Singh, 2009). To the best of our knowledge, there is no systemic review on the physico-chemical properties of human milk and their relationship to milk composition, which are important in understanding milk quality and the impact of processing and preservation. The purpose of this review is to compile existing knowledge and new findings on human milk composition and its physico-chemical properties, with the goal of identifying new insights into human milk processing and storage and, ultimately, enhancing feeding strategies for vulnerable infants. It should be noted that the extensive topic of the microbiome of human milk was not included in this review, but recent reviews in this area include those of Lyons et al. (2020), Togo et al. (2019), and Moossavi et al. (2019).

2. Macronutrients in human milk

Human milk provides infants with essential nutrients, including water, carbohydrates, lipids, proteins, vitamins and minerals, as well as bioactive molecules, such as cytokines, growth factors, and oligosaccharides (Boix-Amorós et al., 2019; Lee et al., 2018; Lyons et al., 2020). As illustrated in Fig. 1, most components in human milk, including casein, lactose, and human milk oligosaccharides (HMO), are synthesised and secreted from the specialized mammary epithelial cells (MEC) in the mammary gland of mothers (Monks & McManaman, 2013). However, not all of them are synthesised in the specialized MEC, but may originate from the maternal circulation, including immunoglobulins (Ig), immune cells, and some micronutrients (Monks & McManaman, 2013).

Typically, human milk contains 1–2% proteins, 3–5% fat, and 6–8% carbohydrate (Bauer & Gerss, 2011; Guo, 2021; John et al., 2019). The individual differences and *intra*-analysis variations in content are influenced by several factors, including gestational age, stage of lactation, maternal characterization (BMI, age, parity, diet), birth weight of infants, diurnal variation, and the stage of expression or feeding, e.g., whether the milk is foremilk or hindmilk (Bzikowska-Jura, Sobieraj, Szostak-Węgierek, & Wesołowska, 2020; Hascoët et al., 2019; Jensen, 1999; Paulaviciene, Liubsys, Molyte, Eidukaite, & Usonis, 2020).

In general, the level of human milk protein, fat, and carbohydrate decreases, increases, and remains consistent during lactation, respectively (Gidrewicz & Fenton, 2014). The impact of gestational age on macronutrients levels is not conclusively understood. The levels of macronutrients in milk from mothers of extremely preterm infants are of particular significance, due to the vulnerability of these babies, and there have been a number of studies comparing milk from extremely preterm, preterm, and term milk groups to understand best strategies for individualized nutrition for infants (Bauer & Gerss, 2011; Caldeo et al., 2020; Léké et al., 2019; Maly et al., 2019). Maly et al. (2019) reported no difference of levels of milk macronutrients between very preterm (24–30 GA; average protein levels: 1.7% during the first week of lactation) and preterm (31–35 GA; average protein levels: 1.6% during the first week of lactation) milk. Longitudinal data of changing protein levels among term, preterm and very preterm groups was also provided

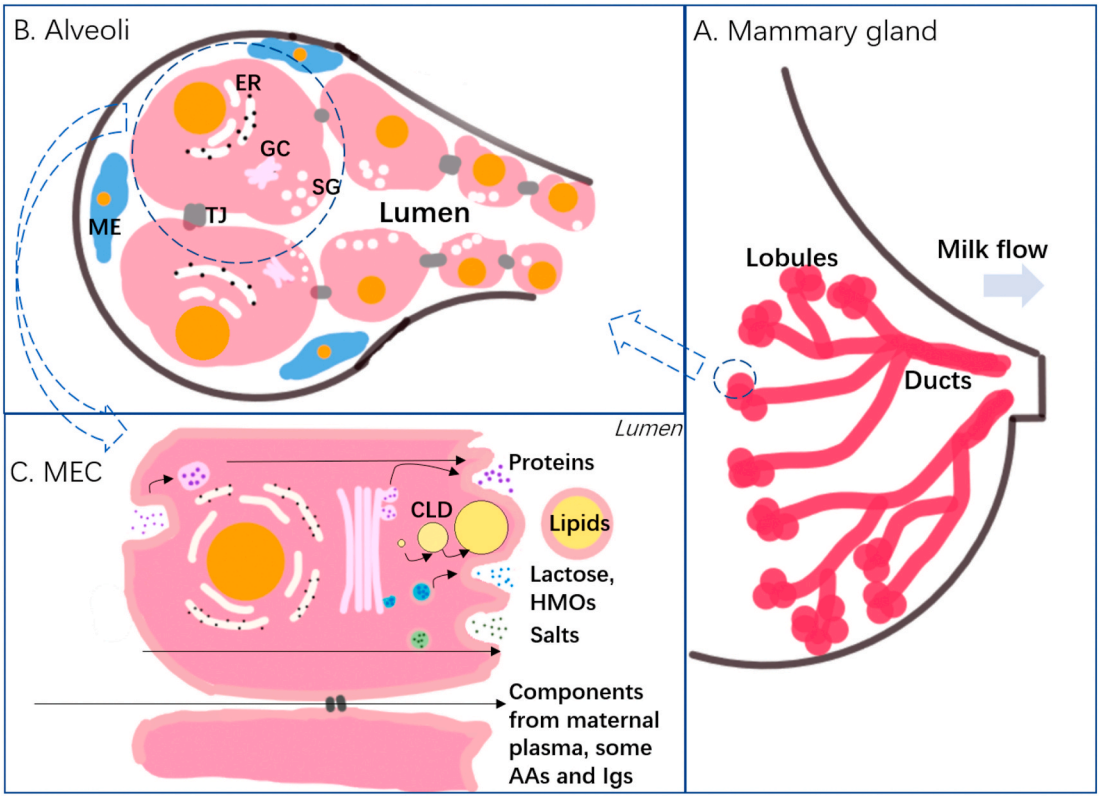

Fig. 1. Illustration of the structure of (A) mammary gland, (B) alveoli, and (C) specialized milk epithelial cell (MEC). ER: endoplasmic reticulum; GC: Golgi complex; SG: secretory granules; TJ: tight junctions ME: myoepithelial cells (help milk expression); CLD: cytoplasmic lipid droplets; HMOs: Human milk oligosaccharides; AAs: Amino acids; Igs: Immunoglobulins. Modified from Monks and McManaman (2013).

by Caldeo et al. (2020); significantly higher protein levels in preterm or very preterm milk compared with term milk was found during days 0–7 and months 1–2 of the lactation. Fischer Fumeaux et al. (2019) reported a decrease in average protein concentrations from birth to four months after birth, from 2.2 to 1.5% in preterm milk and from 2.5 to 1.7% in term milk. They also reported that preterm human milk contains a higher mean fat content in the first week of lactation, but a lower mean fat content during 3–8 weeks of lactation, compared with term milk. The carbohydrate level has been variously reported to be higher in term milk than in preterm milk (Léké et al., 2019), to not differ significantly (Caldeo et al., 2020), or to be higher (although not significantly) in preterm milk compared with term milk (Fischer Fumeaux et al., 2019).

2.1. Proteins and their structures

Based on proteomic studies, human milk contains more than 400 proteins, with functions in immune response, cellular metabolism and protein metabolism (Molinari et al., 2012; Zhu & Dingess, 2019). In general, human milk proteins can be classified into three groups: whey proteins, caseins, and MFGM proteins, accounting for 60–80%, 20–40%, and 1–4% of total proteins, respectively (Lönnerdal, Erdmann, Thakkar, Sauser, & Destaillats, 2017). Caseins and whey proteins can be separated due to their different solubilities; caseins are acid-insoluble, whereas whey proteins are acid-soluble. Unlike bovine milk, it is not easy to separate whey proteins and casein in human milk through the addition of acid alone and/or addition of calcium. A combination of adjustment of pH to 4.3, addition of calcium and ultracentrifugation has been used for whey protein and casein separation in human milk (Kunz & Lönnerdal, 1992). Futhermore, a method for the fractionation of caseins from human milk by addtion of calcium and secondary phosphate has been recently developed; the precipitation of caseins accompanied the precipitation of colloidal calcium phosphate (CCP), which results from the reaction of added calcium and soluble phosphate in the milk (Ten-Doménech, Simó-Alfonso, & Herrero-Martínez, 2018).

The whey protein to casein ratio changes progressively during lactation, from 90:10 in early lactation, to 60:40 in mature milk and 50:50 in late lactation, reflecting the changing requirements of the growing infant (Kunz & Lönnerdal, 1992). However, Liao et al. (2017) showed a high inter-individual difference of whey protein to casein ratio during lactation. In contrast to these changes in levels of whey proteins and caseins, the concentrations of MFGM proteins, i.e., proteins distributed on the membrane surrounding fat globules, are more stable during lactation (Lönnerdal et al., 2017).

2.1.1. Caseins and casein micelles

As shown in Table 1, caseins are a family of phosphoproteins found in the milks of all mammals that typically includes four isoforms: α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein. Among these, α_{s2}-casein, which exists in bovine milk, is not present in human milk (Martin, Ferranti, Leroux, & Addeo, 2003).

In human milk, 70% of total casein is β-casein (Carroll, Basch, Phillips, & Farrell Jr, 1985), a proline-rich amphipathic casein; the phosphorylation sites of human β-casein, at specific seryl and threonyl residues, were identified by Greenberg, Groves, & Dower (1984). Human β-casein has six isoforms depending on the phosphorylation degree, from 0 to 5 (Sood & Slattery, 2002), which differ in solubility, polymerization manner, and calcium-binding capacity (Azuma, Kaminogawa, & Yamauchi, 1985).

From liquid chromatography analysis, human κ-casein has been shown to exist as its dimeric form (37 kDa), in which disulphide bonds join the single cysteine residue of each monomer, and the molecule contains 6 to 7 phosphate groups (Ferranti et al., 2004). κ-casein is important as a heavily glycosylated protein in human milk. It can inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa and trigger the growth of *Bifidobacterium infantis* and *Lactobacillus bifidus,* thus protecting breast-fed infants against gastrointestinal disease (Wada & Lönnerdal, 2014).

α_{s1}-casein has been characterised in mature human milk, and exists naturally as a multimer in complex with κ-casein (Rasmussen, Due, & Petersen, 1995). Martin et al. (2003) reported that human α_{s1}-casein contains cysteine, which is usually found in α_{s2}-casein instead of α_{s1}-casein in milk of other species, which suggests that, even though α_{s2}-casein is absent from human milk, α_{s1}-casein plays its role, such as forming disulphide links with κ-casein (Rasmussen et al., 1999). In addition, α_{S1}-casein is present in human milk in an unphosphorylated or partially phosphorylated form with a lower level of phosphorylation than other species, which may benefit the infant immune system (Saenger et al., 2019). Unphosphorylated α_{S1}-casein can induce

Table 1
Differences in levels, properties, and stability between caseins in human and bovine milk.

Type of milk	Human[a]			Bovine[b]			
Major Caseins	β-Casein	κ-Casein	α_{s1}-Casein	β-Casein	κ-Casein	α_{s1}-Casein	α_{s2}-Casein
Level (g L^{-1})[c]	1.25 (0.04–4.42)	0.75 (0.1–1.72)	0.33 (0.04–1.68)	8.6–9.3	2.3–3.3	8.0–10.7	2.8–3.4
Molar Mass (kDa)[d]	23.9–24.2	19.0	21.0	23.9–24.1	19.0	22.1–23.7	25.2–25.4
Structure	Multi-phosphorylation (0–5P)	High glycosylation (carbohydrate weight:40%)	9 potential phosphorylation sites; -0P or partially phosphorylated form	Major -5P, occasionally -4P form	Carbohydrate weight:10%	Major -8P and -9P form	10–13P form
	Human CM (mean size: 64–80 nm)			Bovine CM (mean size: 150–182 nm)			
Influencing Factors							
pH	Fractionation at pH 4.3 with Ca^{2+} addition; β-casein (especially -0P and -1P form) dissociates from CM with increased pH (pH 7.6 to pH 9.9)			Fractionation at pH 4.6; CM is unstable at pH < 5.2; the size of CM decreases at pH < 5.5; CM dissociates at pH > 9			
Heating	NS			Partial disruption at 50 °C–90 °C			
Cooling/ Refrigeration	Cooling: Reversable solubilization; 0P β-casein increases; mean size of CM increases; Refrigeration: β-casein dissociates			Reversable solubilization during cooling; β-casein dissociates during refrigeration			
Freezing (−20 °C)	NS			Destabilization during freezing			
Pressurization	NS			Disruption at 400 MPa due to solubilization of CCP			

[a] Adapted from Liao et al. (2017), Azuma, Kaminogawa, and Yamauchi (1984), Ferranti et al. (2004), Carroll, Basch, Phillips, and Farrell Jr (1985), König et al. (2017), Crowley, Kelly, Lucey, and O'Mahony (2017), Kunz and Lönnerdal (1992), Sood, Herbert, and Slattery (1998), Sood, Herbert, and Slatter (1997), and Zhang et al. (2020).

[b] Adapted from Crowley et al. (2017), Swaisgood (2003), Schulte, Stöckermann, and Gebhardt (2020), Vaia, Smiddy, Kelly, and Huppertz (2006), Horne (2020), Fox and Brodkorb (2008), and Huppertz, Fox, and Kelly (2004b).

[c] Levels of caseins in human milk are presented as average (range).

[d] Molecular Mass is calculated from its amino acid sequence (carbohydrate free form). **Note**: CM: Casein micelle; CCP: Colloidal calcium phosphate; NS: Not studied/ Report not found.

secretion of proinflammatory cytokines *via* Toll-like receptor 4 and its co-factors, whereas phosphorylated α_{S1}-casein cannot do this *in vitro* (Saenger et al., 2019).

β-casein and κ-casein, with a small amount of α_{s1}-casein, can interact together with calcium ions creating a special dynamic and ordered structure, i.e., the casein micelle (CM) (Azuma et al., 1985; Martin et al., 2003). Human CMs can be observed from transmission electron microscopy as a cloud-like loose broadly spherical tangle (Fig. 2A). Based on electron microscopy studies, the size distribution of CMs ranges between 20 and 104 nm in mature human milk, with an average of 42 nm (Carroll, Basch, Phillips, & Farrell, 1985). Human CMs are thus smaller than those of bovine, caprine and equine CMs (Inglingstad et al., 2010). The size of human CMs is positively correlated with β-to κ-casein ratio; when the κ-casein fraction increases, the number and the surface area of the micelles increase, and thus the size of CMs decreases (Dev, Sood, Dewind, & Slattery, 1994). Although there has been no direct investigation of changes in the size of human CMs during lactation, the ratio of β-to κ-casein increases during lactation, suggesting that the size of CMs may increase during lactation (Cuilliere, Tregoat, Bene, Faure, & Montagne, 1999). The high level of κ-casein and small size of CMs in colostrum may compensate for the low digestive function of neonates (Cuilliere et al., 1999). To our knowledge, recent studies about human CMs, and a model for their structure, are lacking, and some key properties such the surface charge (zeta potential, an indicator of the stability of colloidal dispersions) of human CMs have not been reported.

Although the casein profile of human milk differs from that of bovine milk, the ultra-structure appears similar, with β-caseins constituting the core of the micelle through hydrophobic interactions and phosphoryl groups, while κ-casein is positioned on the surface, contributing to the stabilization of the micelle against precipitation by Ca^{2+} ions (Horne, 2020). Various models of internal bovine CM structure have been proposed from findings in bovine milk and they have been refined progressively. Among these, there are three detailed structural models of CMs (Fig. 2B–D): (1) the sub-micelle model (Slattery & Evard, 1973); (2) the nanocluster model of Holt (Holt, 1992); and (3) the dual-binding model (Horne, 1998, 2020).

In terms of bovine CMs, the 'dual-binding' model suggests that micelle structure is governed by a balance of hydrophobic interactions and CCP-mediated cross-linking of hydrophilic regions. The nanocluster model fits well with all changes of milk during processing, suggesting that not only the hydrophobic interactions, but also main-chain and side-chain H-bonds and other interactions play roles in the formation of the internal structure. All these models still have limitations in explaining the behaviour of caseins in reality (Li & Zhao, 2019).

From human milk studies, the 0P and 1P forms of β-casein require a much lower level of κ-casein to be suspended compared with the 4P and 5P forms, when Ca^{2+} is present, which reflects the fact that high phosphorylated forms are major β-caseins involving in forming intermolecular cross-links *via* calcium ions in micelles (Sood & Slattery, 2002). The extent to which κ-casein molecules in the human micelle shield the other proteins is less than that in bovine micelles, and so β-casein molecules may have greater potential mobility from the core to the surface of human CMs (Dev et al., 1994). Such an open structure may provide accessibility to digestive enzymes, and consequently, a large number of peptides could perhaps be released during proteolysis or digestion.

The formation of CMs can be influenced by many factors *in vitro*, such as ion concentration and the degree of pholysphorylation of caseins. A lower level of calcium is required for human CM formation than for bovine CMs, which partly corresponds to the lower content of calcium in human milk than in bovine milk (Azuma et al., 1985). Sood and Slattery (2003) studied the nature of protein-protein interactions in human CMs through use of a mild chaotropic agent, lithium chloride, which can extract nondenaturing proteins from complexes. Their results suggest that the 0P form of β-casein may participate in interactions that are not available to the phosphorylated forms and that human CMs are formed and maintained through a combination of hydrophobic interactions and

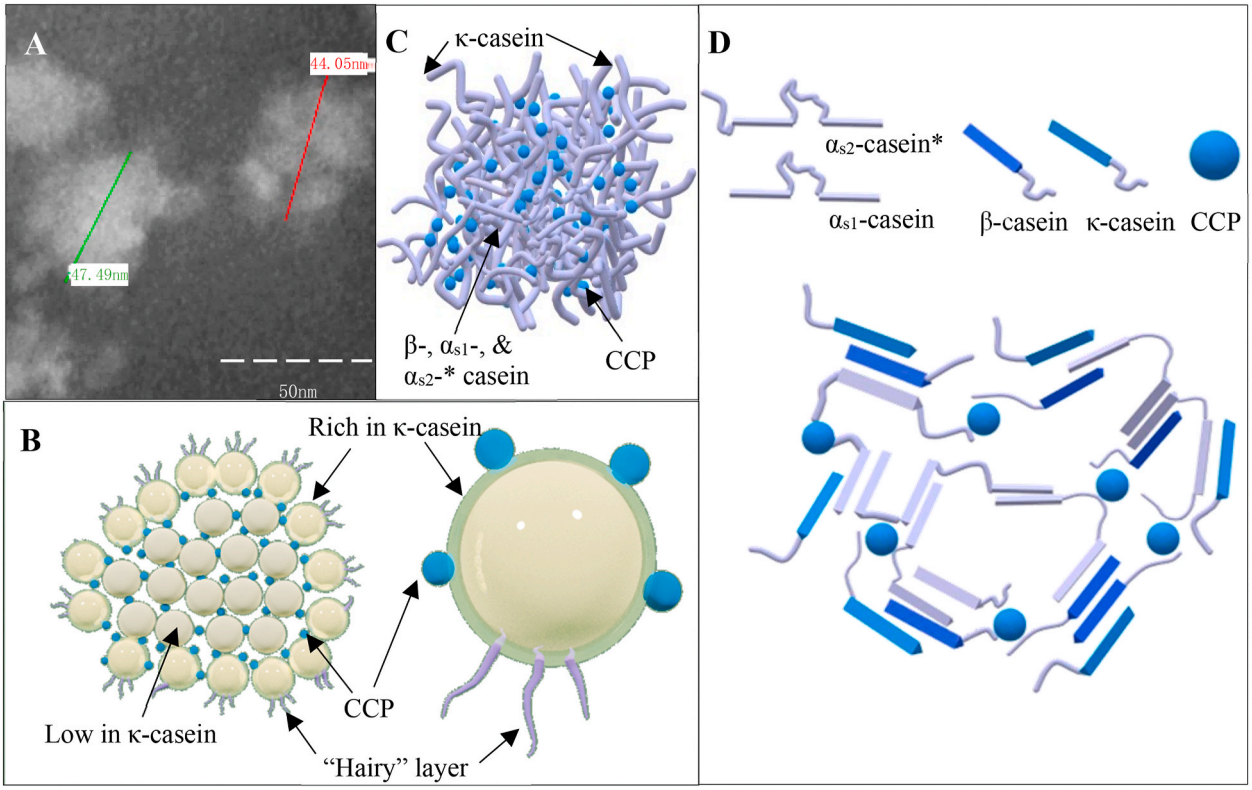

Fig. 2. Human casein micelles (A) observed by transmission electron microscopy of fresh skimmed human milk diluted 1:50 with 0.01M $CaCl_2$ stained with 20 g L^{-1} PTA (negative staining; unpublished data), and illustration of casein models from bovine milk studies: (B) the sub-micelle model (Slattery & Evard, 1973), (C) the nanocluster model of Holt (Holt, 1992), and (D) the dual-binding model (Horne, 1998, 2020). *α_{s2}-caseins present in bovine milk, but not in human milk.

salt bridges involving protein phosphate esters and CCP.

The effects of other chaotropic agents, cooling, and pH (from pH 5 to 10) on the dissociation of human CMs have also been studied (Sood, Herbert, & Slattery, 1997). Similar to bovine milk, human β-caseins can be released during cooling with even higher extents (Table 1; Sood et al., 1997). However, CMs are quite resistant to dissociation by chaotropic agents at 4 °C due to their stabilizing salt bridges (Sood & Slattery, 2003). Bovine CMs are stable at high temperatures, but can be destabilized by freezing due to a decrease in pH and increasing level of ionic calcium in the unfrozen phase of milk, according to studies (Chen & Yamauchi, 1969); the corresponding information for human CMs, including the effects of freeze-thawing and heating, is currently lacking in the literature.

2.1.2. Whey proteins

The most abundant whey proteins present in human serum are α-lactalbumin, lactoferrin, lysozyme, IgA, and serum albumin (Lönnerdal et al., 2017). β-lactoglobulin, which is the dominant whey protein in bovine milk, is not present in human milk, but has been detected therein most likely as an allergen arising from the maternal diet (Dekker et al., 2020). The differences between major human and bovine whey proteins are summarized in Table 2.

α-lactalbumin is a 14-kDa globular protein stabilized by four disulfide bounds and a strong single calcium-binding site (Lönnerdal & Lien, 2003). Ca^{2+} can strongly bind to α-lactalbumin, but is released by low pH or addtion of chaotropic agents (Permyakov, 2020). Bovine α-lactalbumin shares similar amino acid compositions (89%) with human α-lactalbumin, but with lower levels of tryptophan and cysteine (Layman, Lönnerdal, & Fernstrom, 2018). In a clinical trial, term infants fed with low-protein formula fortified with bovine α-lactalbumin showed similar growth outcomes to human milk-fed infants (Trabulsi et al., 2011).

Lactoferrin is an 80-kDa glycoprotein, structured as a single polypeptide chain folded into two globular lobes. It contains two iron-binding sites and has a significant iron-binding capability; however, iron-free lactoferrin is the most common form in human milk (Furmanski, Li, Fortuna, Swamy, & Das, 1989). Recombined human lactoferrin (rhLF) has been produced from the milk of transgenic goats and cows due to the high cost of lactoferrin isolation from human milk (Wang et al., 2017). Although some studies have shown that rhLF has similar physicochemical and biological properties to human lactoferrin, the glycosylation patterns of rhLF were suggested to be more similar to bovine lactoferrin than human lactoferrin, which may influence the function of lactoferrin (Parc et al., 2017). Despite some clinical studies demonstrating the protective effect of lactoferrin against infections of preterm infants, recent large trials showed no clear relationships between supplementation with bovine lactoferrin and reducing the risk of mortality/morbidity or the late-onset infection in preterm infants (Asztalos, Barrington, Lodha, Tarnow-Mordi, & Martin, 2020; Griffiths et al., 2019).

Lysozyme in human milk is a 15-kDa acid-resistant glycoprotein containing 130 amino acids. Lysozyme and α-lactalbumin are similar in primary and tertiary structures; however, the former exhibits much higher cell lytic activity than the latter (Ho, Nadeem, & Svanborg, 2017). Lysozyme attacks the outer cell wall of gram-positive bacteria, and it also works together synergistically with lactoferrin to kill

Table 2
Differences in levels, properties, and stability between major whey proteins in human and bovine milk.

	α-Lactalbumin[b]		Lactoferrin[c]		Lysozyme[d]		IgA[e]	
Milk Type	Human	Bovine	Human	Bovine	Human	Bovine	Human	Bovine
Level (g L^{-1})[a]	1.9–3.4	1.2–1.3	1.5–2.0	0.02–0.5	0.1–0.9	Trace	0.96	0.05–0.14
Molar Mass (kDa)	14.1	14.2	75–82.4	80–84	15	16.5–18	162 (415 ± 60) [f]	160 (385–410) [f]
Structure	Contains a secondary Ca^{2+} binding site	Less Ca^{2+} bound	Glycoprotein; low degree of iron saturation (~10%)	Different *N*-glycan profiles; higher degree of iron saturation	Both are c-type lysozymes; the enzymatic activity of human lysozyme is 10 times higher than bovine lysozyme		sIgA (effective IgA in human milk): SC attaches to dimeric structure to prevent adhesion of bacteria; free SC was isolated from bovine milk	
Influencing Factors								
pH	Ca^{2+} can release and bind to oleic acid at low pH	Stable at low pH	Iron-binding capacity and antimicrobial activity remain at pH 2.0	Maximum exposure of hydrophobic regions at pH 3.0	pH optimum: 6.35; more thermolabile at pH 7.2 than at pH 4.2	pH optimum: 7.9	Stable at low pH	NS
Heating	Heat stable when Ca^{2+} bound	The presence of β-lg or α-CN decreases the stability	HoP affects proteolysis during gastric digestion; more heat-resistant than bovine lactoferrin	No significant effect after HTST; iron-binding ability decreases after UHT	Heat stable; Decreased activity after HoP	Heat stable as c- type lysozyme	Decreased level after HoP;	No structural change after HTST; 80 °C × 25 min inactivates IgA
Refrigeration	NS	NS	Levels remain for 5 d	NS	Stable for 96 h (PHM)	NS	Stable for 48 h	NS
Freezing (−20 °C)	NS	Stable	Decreased level	NS	Decreased level	NS	No change	NS
Pressurization	NS	Stable up to 400 MPa	Decreased level	The structure is stable and the activity remains at 400 MPa	Maintained level up to 600 MPa	NS	Decreased level	NS

[a] Adapted from Crowley et al. (2017).
[b] Adapted from Lönnerdal and Lien (2003), Permyakov (2020), Wehbi et al. (2005), Huppertz, Fox, and Kelly (2004a) and Rodiles-López et al. (2010).
[c] Adapted from Furmanski et al. (1989), Karav, German, Rouquié, Le Parc, and Barile (2017), Sreedhara et al. (2010), Rollo, Radmacher, Turcu, Myers, and Adamkin (2014), Suzuki et al. (2003), and Franco, Pérez, Castillo, Calvo, and Sánchez (2013).
[d] Adapted from Castillo, Franco, Pérez, Calvo, and Sánchez (2011), Chandan, Parry Jr, and Shahani (1965), Parry Jr, Chandan, and Shahani (1969), Malinowska-Pańczyk (2020).
[e] Adapted from Cakebread, Humphrey, and Hodgkinson (2015), Mainer, Sanchez, Ena, and Calvo (1997), Ustunol and Sypien (1997), Ramírez-Santana et al. (2012), and Malinowska-Pańczyk (2020).
[f] Molar mass of sIgA, modified from Kerr (1990) and Korhonen and Marnila (2009). **Note**: sIgA: Secretory immunoglobulin A; SC: Secretory component; HoP: Holder pasteurization; HTST: High temperature short time pasteurization; HP: High pressure; β-lg: β-lactoglobulin, UHT: Ultra-high temperature pasteurization; PDM: Pasteurized donor milk; NS: Not studied/Report not found.

gram-negative bacteria (Lönnerdal et al., 2017). Zavaleta et al. (2007) showed that oral rehydration solution fortified with recombinant human lysozyme (rhLY) and rhLF decreased the duration of diarrhoea of children hospitalized with acute diarrhea. Supplementing rice powder with rhLY and bovine lactoferrin improved the gut health of 2-year-old children (Cheng et al., 2019). Lysozyme has also been shown to inhibit the growth of HIV *in vitro*, but in human milk it may only act upon the free virus and not on cell-associated virus (Lönnerdal et al., 2017).

The most abundant and effective immunoglobulin in terms of anti-inflammatory effect is sIgA. sIgA can recognize receptor-binding domains (e.g., reovirus type 1 Lang) as well as possibly affecting the virulence of bacteria directly. This immunological inhibition of the microbial colonization and restriction of antigen uptake is commonly known as "immune exclusion" (Brandtzaeg, 2013). Immune exclusion by sIgA is activated by a broad-spectrum of commensal bacteria, as well as potentially allergenic antigens, and it is thus important to the human immune system. It has been found that both sIgA and immunoglobulin G (IgG) can form complexes with antigens in human milk (Verhasselt, 2010). However, sIgA-antigen complexes cannot transfer to the child, whereas the IgG-antigen complex can easily cross the barrier of the infant gut (Verhasselt, 2010). A recent study reported that an immune response involving sIgA was found in human milk against the COVID-19 virus (Fox et al., 2020). This finding suggests that sIgA could be used as a novel therapeutic antibody; however, the efficacy and durability of the protein requires further studies.

sIgA is specific because of an epithelial glycoprotein called secretory component (SC) which attaches to its dimeric structure. SC helps sIgA to block toxins adhering to intestinal epithelium (Brandtzaeg, 2013). Also, this structure contributes significantly to the high resistance of sIgA to digestion, as it has been shown to be stable even at pH 2 *in vitro* (Chatterton, Rasmussen, Heegaard, Sørensen, & Petersen, 2004) and it remains intact in infant fecaes (Lönnerdal et al., 2017).

Other whey proteins are still being explored, and several of them have been relatively well described due to their specific functions and relatively high concentrations, including haptocorrin (a vitamin B12-binding protein) and osteopontin (Demmelmair, Prell, Timby, & Lönnerdal, 2017). These proteins are generally glycosylated, which contributes to their biological functions such as antimicrobial activities.

2.1.3. Milk fat globule membrane proteins

There is significant interest in MFGM proteins (Fig. 3), which represent 1% of the total fat globule mass (Liao, Alvarado, Phinney, & Lönnerdal, 2011). Compared with milk from other species, more than 100 types of MFGM proteins are uniquely present in human milk, including bile salt-stimulated lipase, lactotransferrin and clusterin. The dominant molecular function of MFGM proteins has been described as GTP-binding and lipoprotein binding (Lu et al., 2016). They are also crucial for lipid synthesis and secretion and many of them have been described as bioactive proteins (Demmelmair et al., 2017). Well-described MFGM proteins in human milk, including Mucin 1 (MUC1), clusterin, lactotransferrin, and the enzyme complex, xanthine oxidoreductase (XOR), are discussed here briefly.

Glycoproteins, such as MUC1 and lactadherin, exhibit antiviral ability *in vitro* (Demmelmair et al., 2017). MUC1 blocks the interaction between pathogens and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), which is only found in human milk, but absent from infant formula and bovine milk (Koning et al., 2015). Clusterin, another highly glycosylated protein, is likely to provide a chaperone-like activity to protect other proteins against damage; its exact function is still under investigation (Hettinga et al., 2011).

Human MFGM lactotransferrin content has been reported to be 100-fold higher than that in bovine and caprine MFGM (Lu et al., 2016). It shows bacteriostatic activity because it is only partly saturated with iron in its native state, which is different from serum transferrin, and it can thus compete with bacteria for iron (Farnaud & Evans, 2003). XOR in human milk, an enzyme as well as an endogenous metalloflavoproteins, has been suggested as generating hydrogen peroxide and reactive nitrogen species, therefore contributing to antimicrobial activity (Harrison, 2006).

2.1.4. Proteolysis

Endogenous peptides present in human milk before infant consumption are mainly produced by indigenous or microbial proteases in the mammary gland or during storage (Armaforte et al., 2010; Guerrero et al., 2014). After consumption, indigenous proteases in human milk, together with infant proteases, produce peptides throughout the whole gastrointestinal tract, which can be considered as compensating for the low gastric acid level and protease activity of neonates (Dallas & German, 2017).

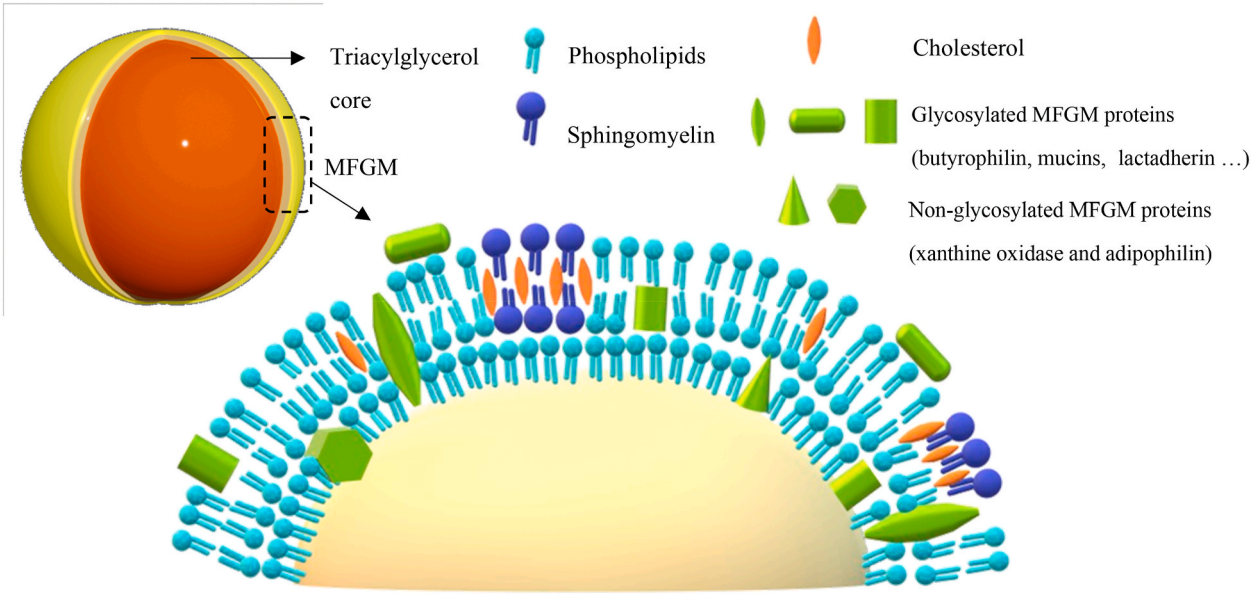

Fig. 3. Human milk fat globules and human milk fat globule membrane. Modified from Gallier et al. (2015).

The major proteases detected in human milk include plasmin, cathepsin D, elastase, thrombin, and kallikrein. Plasmin is the predominant native protease and it behaves in human milk as part of a complex system: its zymogen - plasminogen, plasminogen activators, plasmin inhibitors (e.g., α_1-antitrypsin) and plasminogen activator inhibitors are all present in the milk as well (Heegaard, Rasmussen, & Andreasen, 1994). Plasmin activities in human milk have been reported to be higher in preterm and very preterm milk compared with term milk (Armaforte et al., 2010; Caldeo et al., 2020; Demers-Mathieu, Nielsen, Underwood, Borghese, & Dallas, 2017). Other protease systems in milk may not be as active or as complete as the plasmin system. For example, activators of elastase, thrombin, and cathepsin D have not been detected in human milk to date, whereas inhibitors of these enzymes, such as antielastase, antithrombin III, and α_1-antitrypsin have been shown to be present (Dallas et al., 2015; Molinari et al., 2012).

In human milk, most peptides in milk originate from β-casein, α_{s1}-casein, osteopontin, and polymeric immunoglobulin receptor, due to their unfolded structure or the presence of regions for protease access (Guerrero et al., 2014). Some, but not all, pre-digested peptides are characterised as bioactive peptides or side products of activation processes (Guerrero et al., 2014). In addition, proteins and peptides can be digested by aminopeptidase and carboxypeptidase in human milk to free amino acids (5%–10% of total amino acids), which has been speculated to be a rapid source of amino acids to neonates without protein digestion by infants (Zhang, Adelman, Rai, Boettcher, & Lönnerdal, 2013).

The bioactive peptides produced from human milk proteins are well researched (Wada & Lönnerdal, 2014). In recent studies, antioxidant peptides, e.g., CAMP 211–215 (Wang et al., 2020) and PDC213 (Sun et al., 2017), from β-caseins have been identified. However, bioactivities, such as antimicrobial, immunomodulatory or opioid-like actions of these peptides have largely only been confirmed in synthesised peptides or *in vitro* digested peptides.

2.2. Lipids

2.2.1. Milk fat globules

By supplying 50–60% of total energy, human milk lipids (4.1 ± 0.7% in term mature milk; Bauer & Gerss, 2011) are essential to infant growth, as well as brain development, nerve function and vitamin absorption (Koletzko, 2016). As summarized in Table 3, human milk lipids are principally comprised of triacylglycerols (TG, 98%), phospholipids (PL, 0.8%), and cholesterols (0.5%) (Koletzko, 2016). The majority of these are found in human milk fat globules (87%) (MFG) (Jensen, 1999). As shown in Fig. 3, TG form the core of MFG, while they are surrounded by a triple-layer membrane, i.e., MFGM, comprising of PL, cholesterol and MFGM proteins.

TGs are the major type of apolar lipid in human milk, comprising a glycerol backbone with three linked fatty acids (Jensen, 1999). The structure of TG in human milk is unique partly due to positionally distribution of fatty acids. For example, short-chain fatty acids and medium-chain fatty acids are mainly esterified at *sn*-3 position (Chen et al., 2020), while most palmitic acid (16:0) is esterified to the *sn*-2 position of TG in human milk (Carnielli et al., 1995). This preferential distribution protects long chain palmitic acid from being hydrolysed to free fatty acids, the form indigestible for infants, as they tend to form insoluble soaps in the presence of calcium or magnesium (Carnielli et al., 1995). Therefore, the difference in positionally distribution of fatty acids in infant formula leads to impaired absorption of calcium, which may negatively influence early bone accretion of infants (Carnielli et al., 1995). Furthermore, faecal calcium-palmitate soap is associated with hard stools, which partly explains the greater stool softness in breast-fed infants than formula-fed infants (Béghin et al., 2019). Recent application of formulae enriched in sn-2 palmitate proved its benefits to stool consistency and bone mineral content in early infancy (Béghin et al., 2019).

The crystallization and melting properties of human milk TG, which occurs across a wide range of temperatures, were studied by Lopez, Briard-Bion, Bourgaux, and Pérez (2013). Lopez et al. (2013) reported that refrigerated storage of human milk induced the solidification of TG and that, after such storage of human milk, the final melting temperature of TG was reported to be 39.8 ± 1.5 °C, depending on the exact fatty acid composition.

PLs and other complex lipids are the key components of MFGM, including sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) (Koletzko, 2016). Interestingly, PLs are not evenly distributed on the human MFGM (Fig. 3); liquid-ordered domains that are rich in SM and cholesterol are present in the outer bilayer of the MFGM and surrounded by a liquid-disordered phase (PC, PI, PE, and PS) (Lopez & Ménard, 2011). The concentration of PLs is inversely correlated with MFG diameter (Zou et al., 2012). As well as a structural role, PLs have an impact on membrane functions and metabolism (Koletzko, 2016).

The principal sterol in human milk is cholesterol, the level of which (10–20 mg dL^{-1} at 3 weeks *postpartum*) is much higher than that in bovine or caprine milk (Yao, Zhao, Xiang, et al., 2016). Cholesterol is essential for all cell membrane structures, especially the myelin membrane in the nervous system, which is critical for brain growth (Saher et al., 2005). It also provides substrates for important modulators of infant growth, including bile acids, lipoproteins, vitamin D, hormones,

Table 3
Difference between major lipids in human and bovine milk.

Milk type	Human (Total lipids: 22–50 g L^{-1})			Bovine (Total lipids: 30–50 g L^{-1})		
Major lipids	TG	PL	Cholesterol	TG	PL	Cholesterol
Level (g L^{-1})	21.5–49	0.4–0.7	0.1–0.2	29.4–49	0.24–0.4	0.09–0.15
Structure	TG at the core of MFG; MFGM comprises of polar lipids, such as PLs and cholesterol and MFGM proteins					
	Human MFG (3–5 μm); zeta-potential: −5.6 to −8.2 mV; TG: positional distribution of FAs; PLs: SM dominate; high level of saturated FAs; higher levels and types of MFGM proteins			Bovine MFG (3–5 μm); zeta-potential: −13.5 to −20 mV; SM: sphingoid base and FAs are different from human SM		
Influencing factors						
pH	NS			The interaction of MFG with caseins is higher at acid pH		
Heat treatment	No impact/decrease after HoP			No impact on the level of total fat and fatty acids profiles		
Refrigeration	Crystallization of high-melting point TG in MFG			Lipid auto-oxidation; hydrolysis of MFG by indigenous and bacterial lipases		
Freezing	Activation of lipolysis			Freezing point: −0.564 to −0.516 °C; unaffected level		
Pressurization	Unaffected structure up to 600 MPa (10min)			100–600 MPa for 0–60 min has little effect on MFG size		
Dehydration	NS			Spray- and freeze-drying affect the physical, chemical and microstructure characteristics of MFGs		

Adapted from Koletzko (2016), Ma et al. (2019), Lopez and Ménard (2011), Zou et al. (2012), Michalski (2013), Lima et al. (2018), Lopez et al. (2013), Wesolowska et al. (2019), Obeid et al. (2019), O'connor and O'brien (2006), Prajapati et al. (2017), Huppertz, Fox, and Kelly (2003), and Yao, Zhao, Xiang, et al. (2016). **Note**: TG: Triglyceride; PL: Phospholipids; MFG: Milk fat globule; FAs: Fatty acids; SM: Sphingomyelin; MFGM: Milk fat globule membrane; HoP: Holder pasteurization; NS: Not studied/Report not found.

and oxysterols (Koletzko, 2016).

The size and available surface area of MFGs in milk influence the efficiency of lipolysis, and therefore digestion by infants (Berton et al., 2012; Garcia, Antona, Robert, Lopez, & Armand, 2014). Smaller micelle size and large available surface area of MFGs facilitate the digestion of human milk by infants (Armand et al., 1999). The average size of MFGs in human milk is approximately 3–5 μm. Compared with bovine MFG ($D_{4,3}$: 4.2 μm), human MFG ($D_{4,3}$: 5.0 μm) have been reported to be larger (Lopez, Cauty, & Guyomarc'h, 2015; Ma, Zhang, Wu, & Zhou, 2019) or to not be significantly different in size (Meng et al., 2020).

Studies agree that the size of human MFG increases from transitional milk to mature milk (Jiang et al., 2020; Michalski, Briard, Michel, Tasson, & Poulain, 2005; Rüegg & Blanc, 1981); however, the size of MFGs at the beginning of lactation is controversial. Michalsk et al. (2005) reported a decrease at 0–4 day *postpartum* and Zou et al. (2012) described a decrease from colostrum (1–5 day *postpartum*) to transitional milk (6–15 day *post-partum*). However, Rüegg and Blanc (1981) suggested an increase from colostrum (4 days *post-partum*) to transitional milk (9 days *post-partum*) and Jiang et al. (2020) noticed an increase from 3 to 12 days *post-partum*.

The zeta potential of MFG, i.e., the surface charge of the globules, which can influence its interactions with other milk components, such as proteins and lipases, was measured by Lopez and Ménard (2011), Zou et al. (2012), Michalski (2013), and Jiang et al. (2020); the absolute values (−5.6 to −8.2 mV) reported are lower than those reported for bovine milk (−13.5 to −20 mV; Michalski, 2013). This could be related to the presence of a glycoprotein 'coat', the sphingomyelin content of human MFGM, other compositional attributes of the MFGM, and the surrounding environmental mineral composition of the aqueous phase (Michalski et al., 2005).

2.2.2. Lipolysis and oxidation of human milk lipids

Human milk mainly contains two types of lipases responsible for lipolysis, bile salt-stimulated lipase (BSSL) and lipoprotein lipase (LPL). BSSL is the major lipase present in human milk (1–2% of total protein), but it is not present in bovine or caprine milk. Although LPL is also active in human milk, it shows much lower activity than BSSL (Olivecrona, Vilaro, & Olivecrona, 2003).

BSSL is a carboxyl ester lipase that not only catalyzes the hydrolysis of TG but also breaks the bond between palmitic acid and glycerol in the sn-2 position that many other lipases cannot, which partly resolves the question as to why breast-fed infants have higher efficiency of fat absorption than formula-fed infants (Lu et al., 2016). A higher level of BSSL in preterm milk than term milk may reflect a mechanism to enhance the digestion of lipids by preterm infants (Liao et al., 2011; Molinari et al., 2012). In addition, recombinant human BSSL (rhBSSL) expressed in hamster ovary cells has been supplied to preterm infants in clinical trials; however, whether there is an improvement in the growth of preterm infants after feeding with rhBSSL is still inconclusive, according to a phase III clinical trial (Casper et al., 2016).

The lipolysis of human milk lipids by BSSL is limited during storage at 4 °C for up to 96 h (Bertino et al., 2013). However, BSSL retains activity during storage at - 20 °C and the level of lipolysis increased after several freeze-thaw cycles. Free fatty acid level doubled in milk stored at −20 °C for 8 weeks (Penn et al., 2014).

Furthermore, oxidation of fatty acids in human milk has been reported to be much lower than in infant formula, because of protection from antioxidants, such as vitamin E, as well as the MFGM structure in human milk (Michalski, Calzada, Makino, Michaud, & Guichardant, 2008).

2.3. Carbohydrates

2.3.1. Lactose

Lactose is the principal sugar in human milk, which remains constant during lactation at levels measured as 6.2 ± 0.9% in mature term milk (Bauer & Gerss, 2011; Gidrewicz & Fenton, 2014). Besides supplying energy, lactose also plays a role in osmotic pressure regulation during milk production, as well as increasing the absorption of minerals, especially calcium (Ziegler & Fomon, 1983). This can be due to the ability of microbiota in the gastrointestinal tract to convert lactose into lactic acid, thus leading to a pH drop and a shift in the solubility of calcium (Ziegler & Fomon, 1983). Lactose is not hydrolysed in the upper gastrointestinal tract, and only to a very small extent in the proximal intestinal tract (Nommsen, Lovelady, Heinig, Lönnerdal, & Dewey, 1991).

2.3.2. Human milk oligosaccharides

Human milk contains a high level of human milk oligosaccharides (HMOs); free oligosaccharides are built of five monosaccharides: glucose, galactose, *N*-acetylglucosamine, fucose, and sialic acid, with high structural diversity (Morozov, Hansman, Hanisch, Schroten, & Kunz, 2018). The concentration of total HMOs in colostrum can be as high as 20–25 g L^{-1}, decreasing to 5–20 g L^{-1} in mature milk (Xu et al., 2017).

To date, over 200 types of HMOs have been identified, and more than 100 of them have been characterized and structurally resolved (Cheng, Akkerman, Kong, Walvoort, & de Vos, 2020; Kulinich & Liu, 2016). Different profiles of HMOs can be a result of specific transferase enzymes expressed in the lactocytes in the mammary gland (Ballard & Morrow, 2013). The production of HMOs is controlled by genes, especially Secretor and Lewis blood group genes (Totten et al., 2012).

HMOs are important bioactive factors, through directly altering gene expression in immune cells as immune modulators, or by modifying the microbiota in the gastrointestinal tract as prebiotics, thus indirectly affecting the immune system of infants (Ray et al., 2019; Totten et al., 2012). HMOs are resistant to low pH in the stomach and pancreatic brush enzymes (Engfer, Stahl, Finke, Sawatzki, & Daniel, 2000). Therefore, they can reach the large intestine to serve as substrates for bacterial metabolism. The protective mechanism by which HMOs prevent gastrointestinal infection by acting as receptor decoys is also crucial (Morozov et al., 2018). HMO and their derivatives are similar to the binding sites on cells, for example, DC-SIGN; they can, therefore, block the interaction between pathogens or viruses and their receptors by acting as receptor decoys. Many studies have also indicated that the branched chains of HMOs also contribute to blocking viral infection (Gao et al., 2020). The relationship between structures and antiviral effects of HMOs still needs further research.

HMO molecules can be synthesized chemically, *via* fermentation, and by enzymatic synthesis, which are still both expensive and challenging options (Cheng et al., 2020). Two HMOs, 2′fucosyllactose (2′FL) and lacto-*N*-neotetraose (LNnT) have been considered as novel foods by European Union in 2015 and been added to infant formula in clinical trials. However, the difference in the clinical outcomes between HMO-fortified formula and non-fortified formula is still not conclusive (Vandenplas et al., 2018).

3. Physico-chemical properties of human milk

3.1. Acidity

The major contributors to acidity in human milk are caseins, minerals and organic acids. However, acidity can further increase, i.e., pH can decrease, during storage and processing, primarily due to lactic acids and other organic acids being produced from microbial degradation of lactose. Therefore, human milk pH has been recommended as an indicator of the milk quality in human milk banks (Escuder-Vieco et al., 2016).

The typical pH of mature human milk ranges between 7 and 7.5 at room temperature, with a decrease during the course of lactation due to changes in composition, such as casein concentration (Morriss et al., 1986). In contrast, an increase in milk pH from 6.99 to 7.24 at room

temperature during the first 20 days of lactation has been reported (Sunaric et al., 2017). No significant difference of pH between term and preterm human milk has been reported by Caldeo et al. (2020). The pH of human milk decreases after freezing at −20 °C; Ahrabi et al. (2016) reported that it decreased from above pH 7 to pH 6.3 after storage at −20 °C for 9 months.

pH is an important factor in proteolysis and lipolysis as it influences the activity of enzymes such as lipase (Erickson, Gill, & Chan, 2013). Moreover, acidification of human milk to pH 4.5 is reported to cause a decrease in white cell count, possibly due to lysis of white cells during the acidification process (Erickson et al., 2013). However, there is still no conclusion as to whether milk pH is just the result of maternal mammary metabolism and of little importance to newborns, or if it is an important factor influencing the properties of milk and the health of infants (Demers-Mathieu, Nielsen, Underwood, Borghese, & Dallas, 2018).

3.2. Colour of human milk

The colour of milk mainly depends on CMs and MFGs that scatter light, and therefore result in milk appearing to be white (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). In bovine milk studies, it was reported that sonicated or homogenised milk had higher luminosity compared with raw milk, which was largely due to an increase of visible light scattering caused by smaller fat globules (Truong, Palmer, Bansal, & Bhandari, 2016). Meanwhile, the colour of skimmed bovine milk reflects the size change of CMs (Fox et al., 2015); no report on the colour of skimmed human milk has been found. Human milk can also take on different colours because of the food and medication intake of mothers; however, no conclusive research on food- and medication-induced colouration of milk exists (Anderson, 2018). Beta-carotene and other carotenoids in milk are considered to be related to dietary intake of beta-carotene-rich foods such as carrots and sweet potatoes, and lead to an orange colour of colostrum and the pale white to cream colouration of mature human milk (Anderson, 2018). A green colour of milk has occasionally been reported and could be due to: (1) high multivitamin and mineral (especially iron) intake; (2) low lactose and casein content; and (3) propofol administration (Anderson, 2018). Other colours have also been observed in human milk, including black due to minocycline administration and pink pigments caused by *Serratia* bacteria (Yazgan, Demirdöven, Yazgan, Toraman, & Gürel, 2012).

3.3. Phase separation: creaming and sedimentation

Breast milk is an emulsion of milk fat globules in milk plasma, which separates naturally into cream and serum layers under gravity. The creaming properties of human milk during storage were studied by Meng et al. (2020); human milk showed a faster creaming rate at 40 °C, but a slower creaming rate at 5 °C, compared with bovine milk. To our knowledge, the sedimentation of human milk, which usually refers to protein-rich material accumulating as a layer at the bottom of the container following expression and during the storage, has not been studied yet. Future studies are required as the sedimentation or creaming may result in protein and fat loss in the container on decanting and thus impair the nutritional value of human milk.

The milk emulsion can also be destabilized by the acidic pH of the stomach (Golding & Wooster, 2010). In an *in vitro* dynamic gastrointestinal system, aggregates formed during digestion, based on clusters of milk fat globules around which protein aggregates form, are prone to creaming in the gastric compartment. This early gastric creaming could be a protective mechanism favouring emptying and compensating for the immature gastric motility in preterm new-borns (de Oliveira, Deglaire, et al., 2016).

4. The effect of preservation methods on nutritional and biological properties of human milk

4.1. Refrigeration, freezing and thawing

Freezing may bring benefits to human milk as it has been regarded as an effective method for reducing bacterial colony counts and viral load (Handa et al., 2014). Most studies agree that the level of total protein is stable during freezing at −20 °C (Orbach, Mandel, Mangel, Marom, & Lubetzky, 2019). The level of carbohydrate has been reported to not significantly change, while the level of fat has been reported to decrease or not significantly change (up to 9 months) after freezing at −20 °C (Ahrabi et al., 2016; Orbach et al., 2019). The major hypothesis for the reduction in fat due to freezing is the oxidation of both free fatty acids and those bound to glycerol which occurs due to a reduction in antioxidants during freezing at −20 °C (García-Lara et al., 2013). Adhesion of human milk fat to container walls has also been considered as a possible reason for the loss of fat during freezing (Chang, Chen, & Lin, 2012; Lev et al., 2014). Others have proposed that slow freezing or long-term freezing can lead to the destruction of MFGM and allow access of lipases to the TG (Berkow et al., 1984). Deep freezing (−80 °C) for 1 week has a similar impact on the level of macronutrients as freezing at −20 °C (Handa et al., 2014); however, a higher level of fat and energy was maintained after 4, 12, and 24 weeks at −80 °C than at −20 °C (Orbach et al., 2019).

The level of IgA after freezing at −20 °C has been studied but is still controversial. Some studies reported no significant change of its level after freezing at −20 °C for 1, 3, and 9 months (Ahrabi et al., 2016). In contrast, a decrease in IgA level after 1 month at −20 °C was reported (Akinbi et al., 2010). Ramírez-Santana et al. (2012) also reported that the concentration of IgA decreased in colostrum after freezing at either −20 °C or −80 °C after 12 months. Moreover, there could be a decrease in total antioxidant status and an increase of rancid flavour compounds during storage of human milk at −20 °C (Hung, Hsu, Su, & Chang, 2018; Păduraru et al., 2018).

In terms of thawing methods, Handa et al. (2014) reported no significant difference between thawing in tepid water (37 °C, 10 min for thawing and 10 min for warming) and water-less thawing (in an enclosed chamber with warm air, no temperature indicated, time limit set automatically according to the volume of milk). In a recent study, room-temperature thawing, water-less thawing (in a bottle warmer) and microwave thawing showed different impacts on the levels of macronutrients (Kim et al., 2019). Compared with fresh milk, samples thawed at room temperature showed a significant decrease in fat content; in contrast, microwave thawing for 1 min led to no significant difference in fat, but levels of protein and carbohydrate increased significantly compared with fresh milk. Samples treated with the water-less thawing method showed significant differences in all macronutrients compared with fresh milk (Kim et al., 2019). Studies on the impact of thawing methods on human milk composition are limited partly because the impact of thawing is hard to isolate from that of the freezing method used. More detailed and complete description of freeze-thawing cycle may be useful for future study.

Compared with freezing at −20 °C (from 7 days to 2 months), short-term refrigeration (<72 h) retains more energy and macronutrients (Pramitasari, Sidiartha, & Pratiwi, 2019). However, a higher level of lipids was peroxidised in human milk during the storage at 4 °C than freezing (Miranda et al., 2004). Refrigeration also leads to the crystallization of high-melting point TG within MFG, therefore decreasing the rate of TG hydrolysis and solubilization, which could affect the absorption of milk fatty acids by infants (Lopez et al., 2013).

4.2. Heat treatment of human milk

For safety purposes, pooled donor human milk is frequently heated in human milk banks at 62.5 °C for 30 min, i.e., HoP, which allows a

good compromise between achieving microbiological safety and retaining quality of human milk. Macronutrient content is relatively unaffected after HoP (Lima et al., 2018).

It is well known that enzymes are sensitive to temperature and thus that heat treatment leads to decreased enzyme activity in human milk. Henderson, Fay, and Hamosh (1998) reported that LPL and BSSL were completely destroyed after HoP. The activities of glutathione peroxidase, a selenium-containing enzyme that plays roles in protecting cells against lipid oxidation-induced damage, also decreased after HoP (Guerra, Mellinger-Silva, Rosenthal, & Luchese, 2018). Immunological profiles were also altered after HoP, and IgG showed the highest retention levels, followed by IgA and IgM (Escuder-Vieco et al., 2018).

The impact of HoP on the gastrointestinal digestion of human milk has been considered, as different bioactive peptides were released from caseins in pasteurized milk compared to raw milk during intestinal digestion (Deglaire et al., 2019). In addition, a clinical trial demonstrated that HoP of preterm milk did not affect lipolysis, but affected the proteolysis of lactoferrin and α-lactalbumin during gastric digestion by preterm infants (de Oliveira, Bourlieu, et al., 2016).

High temperature short time pasteurization (HTST, 72 °C for 15 s), the principal dairy preservation technique, has been demonstrated to give a better retention of antiviral activity and antioxidant activity than HoP (Donalisio et al., 2018). The level of Ig retention in milk after HTST has been considered to be higher than in milk after HoP (Escuder-Vieco et al., 2018).

Other thermal methods, such as ultrasonic heating and microwave-assisted heating, have also been investigated. Homogenisation of milk (at 45 or 50 °C) occurred after 2 min of ultrasonic heating (20 kHz, 150 W) and temperature of milk increased 10.3 ± 0.4 °C, with bioactive components, including sIgA, lysozyme, lactoferrin and BSSL, being retained (Czank, Simmer, & Hartmann, 2010).

4.3. Non-thermal alternatives to holder pasteurization

Because thermal methods influence some of the nutritional and biological properties of human milk, and also eliminate some of the beneficial microbiota present in the milk, non-thermal treatments for preserving human milk have been studied (Peila et al., 2016).

High pressure processing (HPP) is a novel non-thermal method which can effectively reduce the bacterial population while retaining higher levels of Ig, lysozyme activities, and BSSL activities in human milk compared to the HoP method (Mayayo et al., 2016; Sousa, Delgadillo, & Saraiva, 2014; Wesolowska et al., 2019). The structure of TGs is unaffected by HPP at 600 MPa for 10 min (Wesolowska et al., 2019). However, control of pressure and time for HPP is crucial and the optimal conditions that should be applied to human milk still need to be defined.

Ultraviolet (UV)–C (200–280 nm) irradiation is also an emerging non-thermal technology applied in human milk preservation, which can reduce levels of bacteria in breast milk without losing activities of some heat-sensitive enzymes (Christen, Lai, Hartmann, Hartmann, & Geddes, 2013). Another non-thermal treatment is ultrasonic processing, which provides better retention of BSSL, lactoferrin, and lysozyme compared with HoP or a combination of ultrasonic processing and heat (40 or 50°C). However, application of ultrasonic processing alone is not as effective as when combined with heat in terms of inactivation of bacteria (Czank et al., 2010).

4.4. Stability to dehydration

Drying of human milk can be an option for human milk storage as it saves storage space, prolongs storage time, and also reduces the cost of refrigeration. Spray-drying and freeze-drying of human milk have been investigated in several studies; the profile of Ig was evaluated because they are essential to infant immune system, especially IgA as discussed in Section 2.1.2; however, all Ig are sensitive to heat. Spray-drying was associated with good retention of IgG (>88%) and IgM (>67%) but retained only 38% of IgA (Castro-Albarrán et al., 2016). Freeze-drying of milk showed even better retention of Ig compared with spray drying, as the highest product temperature reached is 40 °C (Castro-Albarrán et al., 2016). In addition, freeze-drying improved the retention of vitamins, fats, and antioxidant capacity compared with frozen unpasteurized milk after storage for 3 months (Lozano, Castellote, Montes, & López-Sabater, 2014). Hahn, Kim, Song, Park, and Kang (2019) also reported that HMO profiles were not affected by freeze-drying. Therefore, applying freeze-drying in human milk banks is suggested as an option to extend the life of milk while maintaining immunological properties that benefit the health of the newborn, presuming the material can be readily reconstituted in sterile water before use.

5. Conclusion

Levels, structures and functions of macronutrients in human milk, including fat, carbohydrate, and protein have been extensively evaluated. Macronutrients not only supply energy, but also have significant biological and immunological roles and consequently, bring short-term and long-term benefits to infants. Proteins, consisting of casein, whey and MFGM proteins, were a particular focus of this review; caseins are major digestible proteins, while many whey proteins, e.g., Ig, are responsible for the immunity of infants, and both have biological roles depending on their structures. There are still gaps in knowledge of physiochemical properties of milk proteins, such as zeta potential and the effects of freeze-thawing on casein micelles. In addition, MFGM proteins provide health benefits and remain to be further explored. The unique composition and structures of human milk lipids is also significant, as it leads to a higher digestion efficiency of infants for human milk compared to infant formula. Finally, physico-chemical properties of human milk may affect its stability during handling and storage, such as many thermal and non-thermal strategies for milk preservation, which may differ in retention of bioactive and immunological compounds. Knowledge on some such properties of human milk, for example, sedimentation during milk storage, is limited and future studies are required. Questions also remain about whether pre-digestion proteolysis and some physiochemical properties are important factors influencing the properties of human milk and the health of neonates, or if they are just the outcome of maternal mammary gland with no function as indicators of milk quality. Overall, the knowledge of some of these properties requires updating and deeper exploration in many areas.

Declaration of competing interest

The authors declare no competing financial interests.

Acknowledgment

Authors gratefully acknowledge the funding provided by Department of Agriculture, Food and the Marine, Ireland under its FIRM programme (grant number 15/F/721).

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International Dairy Journal 108 (2020) 104726

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Factors affecting the creaming of human milk

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ARTICLE INFO

Article history:
Received 6 December 2019
Received in revised form
1 April 2020
Accepted 6 April 2020
Available online 6 May 2020

ABSTRACT

The creaming properties of human milk have not been widely studied to date, and a mechanism for this phenomenon is not known. Here, the natural creaming of human milk, as affected by temperature and pre-treatments, was studied using dynamic light-scattering. The creaming rate of human milk increased with temperature in the range 5 °C–40 °C. Freezing human milk at −20 °C and thawing at room temperature had little influence on creaming. Compared with bovine milk, human milk showed a faster creaming rate at 40 °C, but a slower rate at 5 °C, suggesting a lack of cold agglutination; the mechanisms of creaming were also shown to differ in response to heat treatment. This study expands the current knowledge on milk creaming, and may have potential application to storage and handling of human milk in hospitals or homes, therefore supporting optimal nutrition of infants.

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1. Introduction

Human milk is essential as our first natural food, providing both nutrients and immunity to infants. Infants, especially preterm infants, who receive no or limited milk from their own mothers, can also be fed by human milk from donors as a substitute. Those milk donations are screened, received, pasteurised and stored in human milk banks (Hartmann, Pang, Keil, Hartmann, & Simmer, 2007). Moreover, working mothers may store their milk in the fridge (4 °C) from 24 h to 8 d or in the freezer (−18 °C) for even longer times (Hands, 2003; Weiss, 2005).

Natural creaming of milk occurs during storage because of the lower density of milk fat globules compared with milk serum, which leads to fat rising to the top under the influence of gravity. The most well studied subject of creaming has been bovine milk, since at least the work of Babcock (1889). Creaming properties of caprine (El-Ghannam, Attia, & Zeidanr, 1986), buffalo (Abo-Elnaga, 1966), carabao (Gonzales-Janolino, 1968) and camel (Farah & Rüegg, 1991) milk have also been studied, and all of these showed much slower creaming rates than bovine milk at refrigeration temperature. Human milk has been reported to cream more slowly than bovine milk at body temperature (Whittlestone & Perrin, 1954), but overall the creaming of human milk and the factors affecting this process have not been extensively studied to date.

The creaming rate, v, which is used to describe the creaming process in milk, is described by Stokes' Law (Walstra, 1995):

$$v = \frac{g\left(\rho_p - \rho_f\right)d^2}{18\eta_p} \quad (1)$$

where g is acceleration due to gravity, d is the diameter of the fat globule, ρ_p and ρ_f are the densities of the plasma and fat, respectively, and η_p is the viscosity of the plasma.

However, applying Stokes' Law solely based on the size of individual milk fat globules cannot exactly predict the real creaming rate. For example, fresh bovine milk creams much faster than predicted by Stokes' Law at cold temperatures, which is explained by a phenomenon called cold agglutination, caused by the flocculation of fat globules (Sharp & Krukovsky, 1939). Cold agglutination is facilitated by agglutinins, which attach to the milk fat globule membrane (MFGM) and cause fat globules to aggregate; these agents have been identified as immunoglobulin M (IgM) (Euber &

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https://doi.org/10.1016/j.idairyj.2020.104726
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Brunner, 1984; Payens, Koops, & Mogot, 1965), and immunoglobulin A (IgA) (D'Incecco et al., 2018; Honkanen-Buzalski & Sandholm, 1981) in bovine milk. A homogenisation-labile component, termed the skim milk membrane but now sometimes referred to as exosomes or extracellular vesicles (Benmoussa et al., 2017), was also reported to be involved in cold agglutination (Euber & Brunner, 1984). Other factors that have been reported to influence the rate of creaming of bovine milk include the presence of bacteria (Jenness, Shipe, & Sherbon, 1974) and somatic cells (Geer & Barbano, 2014), heating (>70 °C), and high pressure treatment (≥400 MPa) (Huppertz, Fox, & Kelly, 2003).

Moreover, techniques for measuring creaming have improved. Traditionally, the creaming of milk was measured as the volume of cream produced from a specified volume of milk in a glass tube, of specific dimensions, at a stated temperature after certain time intervals, usually up to 24 h (Dunkley & Sommer, 1944; Euber & Brunner, 1984; Hammer, 1916; Kenyon, Jenness, & Anderson, 1966). More recently, the Turbiscan instrument has been used to study the creaming behaviour of milk, based on multiple light-scattering principles; this gives information on creaming properties even when nothing is visible to the naked eye due to the opacity of milk samples, therefore removing the ambiguity of visual observation (Celia, Trapasso, Cosco, Paolino, & Fresta, 2009; Juliano et al., 2011).

In this study, the natural creaming properties of human milk under different temperature conditions were analysed using the Turbiscan stability analyser and compared with the creaming behaviour of bovine milk to understand the reasons behind any differences that might exist between them. In addition, the influence of different pre-treatments, such as freezing and pre-heating, which may be used in hospitals or milk banks to stabilise human milk, on the creaming were studied.

2. Materials and methods

2.1. Human milk samples

Ethical approval for this study was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland, and fresh human milk samples (collected after the infant was satisfied, and hence being largely mid-to hind-milk) were collected from Cork University Maternity Hospital, Cork, Ireland. Full-term individual fresh milk samples from healthy mothers who were one week after birth (i.e., first week of lactation) were collected and used within 24 h. Fresh raw bovine milk was obtained from a local market. Both human and bovine milk were stored at refrigeration temperatures until the creaming analysis.

2.2. Compositional analysis

The fat, protein, lactose and total solids content of human milk was determined with a human milk analyser (MIRIS, Uppsala, Sweden) based on mid-infrared (mid-IR) transmission spectroscopy principles. Subsamples of fresh human milk (n = 12) were collected for compositional analysis were frozen at −80 °C, thawed at 4 °C, warmed to 40 °C and homogenised using a sonicator (MIRIS) before analysis. The fat, protein, lactose and total solids content of fresh bovine milk samples was measured using a Milkoscan FT 120 (Foss Electric, Hillerød, Denmark).

2.3. Size distribution of milk fat globules

The size distribution of milk fat globules (MFG) in human and bovine milk samples was determined with a Mastersizer 3000 laser particle size analyser (Malvern Instruments, Malvern, UK) equipped with a He–Ne laser (λ = 633 nm). The refractive indices of the fat globule and of the dispersant (water) at 25 °C were taken as 1.458 and 1.33, respectively, and the absorbance of fat globules was taken as 0.01 (Michalski, Briard, & Michel, 2001). The surface area mean diameter D [3,2], the volume-weighted mean diameter D [4,3], and the standard percentiles values, D_v10, D_v50 and D_v90 were measured in triplicate, as described earlier (Ménard et al., 2010).

2.4. Measurement of density of milk fat and viscosity of skimmed milk

Bovine milk fat was obtained by melting commercial butter and allowing to stand, followed by recovery of the upper fat layer. Human milk fat was obtained from a sample of donor milk that was agitated at high speed to destabilise the emulsion and recover a fat-rich phase, which was separated as for separation of bovine milk fat from butter to obtain a fat sample. The density of these human and bovine milk fat samples was determined using a pycnometer (Sci-Labware Ltd., Stoke-on-Trent, UK) at 20 °C by following standard method ASTM D3505 (ASTM, 2018). To obtain skim milk, milk was centrifuged at 3000×*g* for 20 min at 5 °C. Flow time of human skim milk, bovine skim milk, and pure water were measured by a U-shape glass viscometer (VWR International Ltd., Dublin, Ireland) at 5 °C following standard method ASTM D445 (ASTM, 1997), and viscosities of human and bovine skim milk were calculated by:

$$\eta_{milk} = \frac{t_{milk}\,\rho_{milk}}{t_{H_2O}\,\rho_{H_2O}}\,\eta_{H_2O} \tag{2}$$

where t_{milk} and t_{H_2O} are the time (seconds) flow through the specific area of U-shape viscometer of milk and water, respectively; ρ_{milk} is the density of skimmed human (1.03×10^3 kg m^{-3}; Neville et al., 1988) or skimmed bovine milk (1.04×10^3 kg m^{-3}; Ma & Barbano, 2000) at 5 °C; ρ_{H_2O} is the density of water at 5 °C, which is 1.00×10^3 kg m^{-3} (Jones & Harris, 1992); and η_{H_2O} is the viscosity of water at 5 °C, which is 1.52 mPa s (Kestin, Sokolov, & Wakeham, 1978). Fresh milk samples were measured and each analysis was performed in triplicate.

2.5. Sample preparation for creaming analysis

For creaming analysis, five different temperatures, 5 [number of samples (n) = 11], 20 (n = 10), 37 (n = 13), 40 (n = 11) and 45 °C (n = 3) were selected in individual experiments for simulation of refrigeration, room temperature (RT), mammalian body temperature, and higher temperatures. Refrigerated milk samples were incubated in a water bath for 20 min to achieve the target temperature, and each sample (3 mL) was then inverted at least 10 times and placed in the Turbiscan immediately to make sure the back-scattering profile of the first scan was a flat line, and then scanned at each temperature for 10 h. That time was chosen as a compromise between the freshness and creaming duration of the milk, and because, after this time, the peak thickness in the cream layer of human milk reaches a plateau (as determined in preliminary work).

In the experiment with pre-heating, fresh milk samples were pre-heated in a water bath at 70 °C for 10 min. In a separate experiment, bovine whey protein isolate (WPI90, Carbery Group Ltd., Ballineen, Cork, Ireland) and immunoglobulins (Igs, isolated as described by McGrath, 2014), was added to human milk to achieve 1 g L^{-1} final Igs (average level of human and bovine Igs in milk). In a 'phase-reversal' experiment, human and bovine milk cream and serum were separated at 45 °C, which can concentrate agglutinins from the whole milk to the milk cream layer, and recombined with the opposite fraction as described by Jennes and Parkash (1971).

2.6. Creaming analysis

The Turbiscan LAB™ (Formulaction, Ramonville St. Agne, France) with an 800 nm near infrared (NIR) light source was used to study particle migration (creaming) in milk samples. The Turbiscan is based on multiple light scattering theory, where NIR photons are transmitted or back-scattered from the sample to transmission (T) or backscattering (BS) detectors (Carrentero, Villepin, Brunel, & Carries, 2005).

Parameters quantified to describe creaming were the Turbiscan stability index (TSI), the mean value (of ΔBS, %), the peak thickness (ΔH), and the migration rate. The TSI was calculated by summing up the changes in BS at all measured positions, based on a scan-to-scan difference, over total sample height or a selected zone (Carrentero et al., 2005). In this study, the selected zone is the area where the cream layer forms; when the TSI value increases, the stability of the system has decreased. The mean value represents changes in the concentration and size of particles, i.e., signal variation of ΔBS (%). It was calculated at the cream layer and for the bottom layer (lowest 20% of total height) of samples, and the ΔH measures the depth of the cream layer (represented by a peak in back-scatter data) that forms. A threshold value was set as 2% ΔBS. The slope of the curve of ΔH as a function of time reflects the migration of the fat globules moving upwards within samples; the migration rate, calculated as the slope of the initial linear part of the ΔH plot, was taken to represent the creaming rate.

2.7. Statistical analysis of data

Data were analysed using Minitab® v18 (Minitab Inc., State College, PA, USA). Prior to analysis, data were tested for normality using the Anderson-Darling test. All TSI data, which were not normally distributed, were transformed according to a Box–Cox analysis. GLM ANOVA or one-way ANOVA followed by a paired multiple comparison test (Tukey's test) were used as appropriate. Human and bovine fat globule size distribution parameters were analysed using a 2-sample T-test. For all statistical analysis, the level of significance, α, was set at 0.05.

3. Results

3.1. Composition, size distribution and density of milk fat globules of human and bovine milk

The averaged composition of fresh human milk samples and fresh bovine milk samples tested in this study is reported in Table 1 (details for the composition of milk samples from individual mothers are shown in Supplementary material Table S1). Compared with fresh bovine milk, human milk had lower levels of lipids and protein, but a higher level of carbohydrate and solids-not-fat content.

Differences in fat globule size between human and bovine milk were minor, with fat globules in human milk having a slightly lower average D [3,2] (2.3 ± 0.3 μm) and higher average D [4,3] (4.1 ± 0.9 μm) than those in bovine milk (2.4 ± 0.5 μm and 3.7 ± 0.3 μm, respectively) (Table 2). In case of the size distribution of human MFGs, 10% of particles were below 1.1 ± 0.3 μm, and 50% of particles below 3.5 ± 0.7 μm, both of which are lower than the values presented for bovine milk; however, the average D_v90 of human MFGs (7.1 ± 1.6 μm) was higher than that in bovine milk (6.6 ± 0.36 μm). Overall, statistical analysis of human and bovine MFG size distribution parameters showed no significant difference ($P > 0.05$) between the parameters measured for the two types of milk.

The densities of human and bovine milk fat at 20 °C were $0.88 \times 10^3 \pm 0.46$ kg m^{-3} and $0.88 \times 10^3 \pm 0.61$ kg m^{-3}, respectively. The viscosity of skimmed human milk at 5 °C was measured as 2.53 ± 0.08 mPa s, which was lower than that of skimmed bovine milk at 5 °C (3.45 ± 0.02 mPa s).

Table 1
Levels of macronutrients in human milk and bovine milk samples.[a]

Type of milk	Fat	Crude protein	Carbohydrate	Total solids
Human milk	31 (1.04)	16 (0.18)	69 (0.25)	119 (1.16)
Bovine milk	40 (0.31)	35 (0.27)	45 (0.05)	130 (0.55)

[a] Data (in g L^{-1}) are means (human milk, n = 12; bovine milk, n = 4) with standard deviation in parenthesis.

Table 2
Particle size distribution parameters of milk fat globules.[a]

Size distribution parameters	Human milk	Raw bovine milk
D [3,2]	2.27 (0.33)	2.35 (0.47)
D [4,3]	4.11 (0.92)	3.69 (0.32)
Dv10	1.09 (0.32)	0.97 (0.12)
Dv50	3.53 (0.74)	3.63 (0.32)
Dv90	7.10 (1.56)	6.64 (0.36)

[a] Data (in μm) are means (human milk, n = 7; bovine milk, n = 3) with standard deviation in parenthesis.

3.2. Creaming profiles of human and bovine milk at different temperatures

The formation of the layer of fat droplets (creaming) is illustrated in the back-scattering profiles of the samples (Supplementary material Fig. S1) as an increase in ΔBS at the top of samples over time, while a decrease of the back-scattering is indicative of clarification in the middle and lower phases of samples. As calculated from the backscattering profiles, the TSI values of cream layers (TSI_{cream}) in both types of milk increased with time (Fig. 1). In human milk, the TSI_{cream} increased with temperature from 5 to 40 °C, and there were significant differences ($P < 0.05$) between TSI_{cream} at 5 °C and TSI_{cream} at 40 °C at each time point.

Bovine milk held at 5 °C had a lower TSI_{cream}, i.e., a higher stability, than that at 40 °C during the first hour; however, instability increased dramatically between 1 and 4 h of measurement. The average TSI_{cream} values of human milk were lower at 5 °C but higher at 40 °C, compared with those of bovine milk at each time point during 10 h. However, no significant difference ($P > 0.05$) in TSI_{cream} was found between bovine and human milk at 10 h at either temperature.

The mean value of ΔBS in cream layer (MV_{cream}), which can be estimated from ΔBS profiles (Supplementary material Fig. S2), increased with temperature for human milk (Fig. 2A), i.e., the higher the temperature, the more rapidly the fat globules concentrated in the human milk cream layer. The averages of MV_{cream} for human milk ranged from 9.5% to 16.3% after 10 h from 5 °C to 40 °C. The average MV_{cream} for bovine milk after the same time at 5 °C and 40 °C were similar to each other (Fig. 2B). However, the time for bovine cream to reach the maximum ΔBS decreased from 6 h at 5 °C to 3 h at 40 °C. The rate of increase in ΔBS for milk from both species started to decrease after around 2 h creaming at all temperatures; however, this decrease in rate was more significant for bovine milk than for human milk.

The mean value in the bottom layers (MV_{bottom}) of the milk samples decreased from over time due to the fat globules moving upwards (Fig. 2C, D). In the bottom layer, human milk held at 20, 37, and 40 °C showed similar initial behaviour, while milk held at 5 °C had a lower initial rate of the MV_{bottom} curve. Interestingly, human

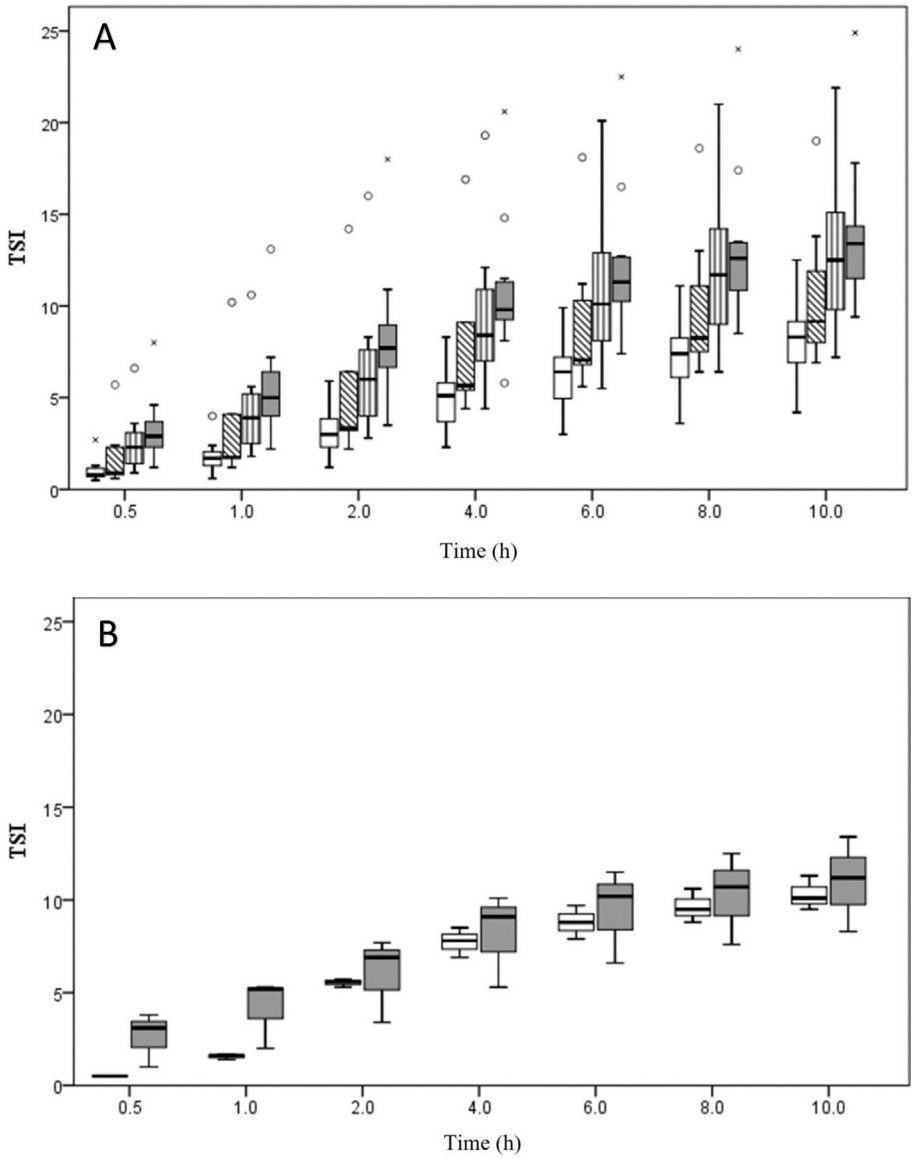

Fig. 1. Turbiscan stability index (TSI) distribution of the cream layer for fresh human milk (A) and fresh bovine milk (B) at 5 °C (□), 20 °C (▨), 37 °C (▥), and 40 °C (■) as a function of time. Horizontal lines inside boxes indicate the median value. Lines extending vertically from the boxes indicate variability outside the upper and lower quartiles. Mild outliers (○, values that lie between 1.5 and 3 times the interquartile range below the first quartile or above the third quartile) and extreme outliers (×, values that lie more than 3 times the interquartile range below the first quartile or above the third quartile) are indicated.

milk held at 20 °C had a higher decrease of MV_{bottom}, i.e., a more complete clarification, compared with that at 5 °C. Moreover, in the bottom layer of both human and bovine milk, the clarification was more extensive at 5 °C than at 40 °C.

The peak thickness (ΔH) of human milk cream layer varied due to creaming temperature at different time points. As seen from Table 3, there were significant differences ($P < 0.05$) in ΔH among temperatures at 0.5 and 4 h, whereas there was no significant difference ($P > 0.05$) in ΔH among temperatures at 10 h creaming. Compared with bovine milk (Fig. 3), ΔH values of human milk cream increased steadily to a maximum at the end of 10 h, whereas it reached a plateau for bovine milk after 2 h at 5 °C. There was a lag phase for bovine milk cream formation at 5 °C, which was not seen at 40 °C, while human milk started to cream earlier than bovine milk at both 5 °C and 40 °C. The final ΔH value of the human milk cream layer at 5 °C was lower than that at 40 °C, whereas it was notably higher for bovine milk at 5 °C than at 40 °C.

The average creaming rate, i.e., the migration rate of MFGs, or clusters thereof, of human milk increased with temperature (Table 4); there was a significant difference ($P < 0.05$) between rates at 5 °C and 40 °C. The rate was lower than that of bovine milk at 5 °C, whereas it was higher than that of bovine milk at 40 °C, suggesting different mechanisms of creaming in the milk of the two species. However, there was no significant difference ($P > 0.05$) in creaming rate between bovine and human milk at 5 °C or at 40 °C.

3.3. Influence of freezing-thawing and preheating on creaming

The TSI cream values of individual human milk samples after different pre-treatments as a function of time are shown in Fig. 4. Refrigeration for 1 week resulted in a lower TSI_{cream} at each time point, whereas a 9-month frozen and thawed milk had a higher TSI_{cream} at each time point. As seen in Fig. 4, short-term frozen storage (one week) has less impact on the physical stability of

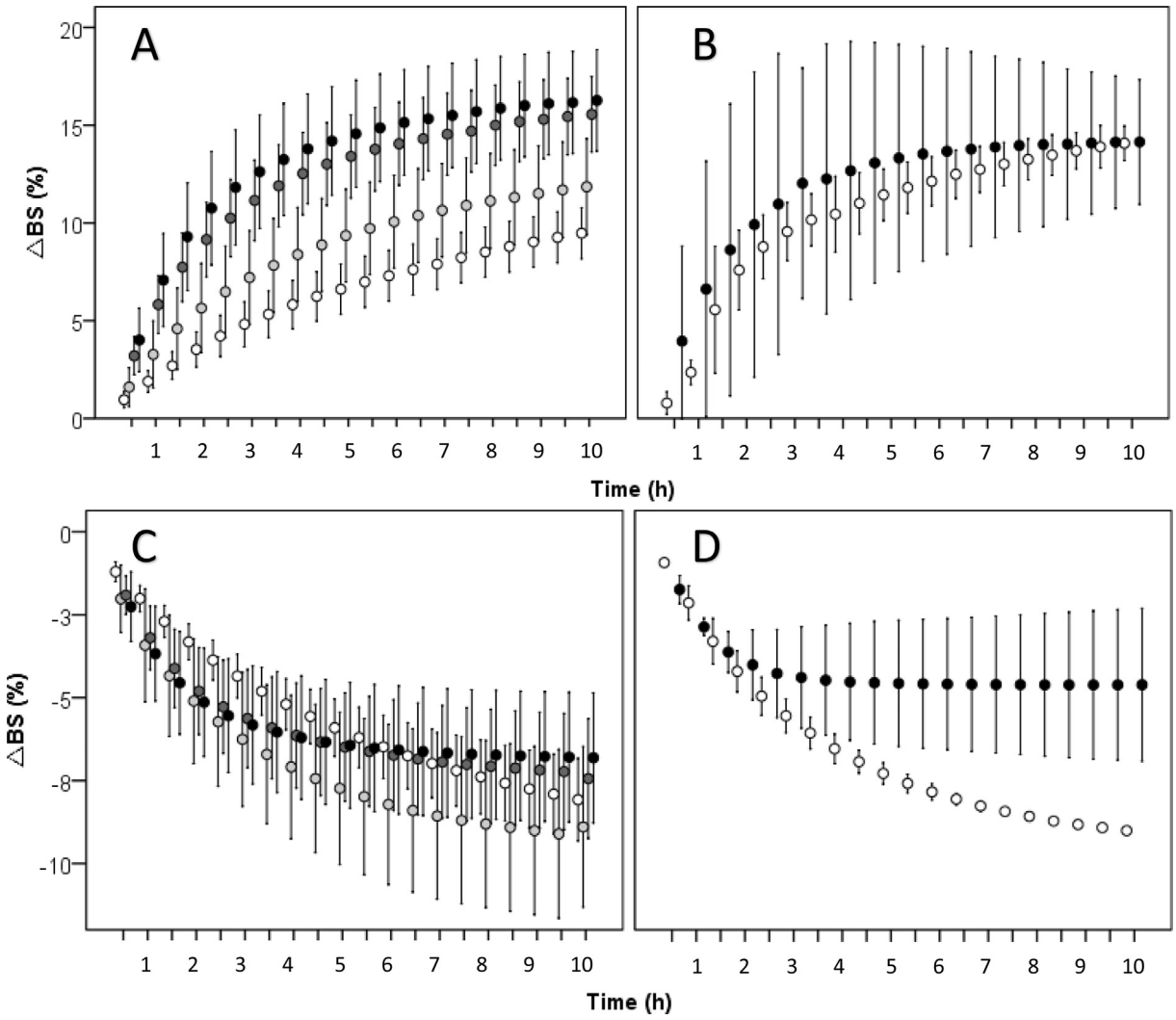

Fig. 2. Mean value of delta backscattering (%) of (A, B) cream layer for fresh human milk and the bottom layer (C, D) and cream layer for fresh human milk (A, C) and fresh bovine milk (B, D) at different temperatures as a function of time. Temperature are 5 °C (○), 20 °C (●), 37 °C (●), and 40 °C (●). Data points correspond to average mean value and error bars show the 95% confidence interval (CI).

Table 3
Peak thickness of human milk cream layer at different temperatures and time points.[a]

Temperature (°C)	n	Time		
		0.5 h	4 h	10 h
5	11	0.50 (0.37)^{a}	1.73 (0.50)^{a}	0.84 (0.82)^{a}
20	10	0.76 (0.63)^{b}	2.22 (0.90)^{ab}	1.65 (1.66)^{a}
37	13	0.94 (1.61)^{bc}	2.36 (0.76)^{ab}	1.78 (1.38)^{a}
40	11	1.17 (0.38)^{bc}	2.57 (0.53)^{ab}	1.97 (1.59)^{a}
45	3	2.38 (0.65)^{c}	3.28 (1.10)^{b}	3.29 (0.98)^{a}

[a] Data (ΔH; in mm) are means with standard deviation in parenthesis; n is the number of samples at each temperature.Different superscript letters indicate statistically significant differences at $P < 0.05$ between temperatures within each column.

human milk than refrigerated storage and longer term frozen storage. However, no significant difference ($P > 0.05$) was found among TSI_{cream} values for fresh milk, one-week refrigerated milk, one-week frozen milk, and 9-month frozen milk at all time points.

The effect of preheating on human milk creaming was also studied. Compared with the control, the TSI of human milk cream layer decreased significantly ($P < 0.05$) after preheating (Supplementary material Fig. S3). The final peak thickness of the cream layer was lower after heating at 70 °C for 10 min; combined with the fact that ΔBS also decreased significantly ($P < 0.05$) compared with the control, this suggests that heating human milk led to impaired creaming capacity.

3.4. Mechanistic studies of creaming of human milk

When human and bovine milk were mixed in a ratio of 1:1 (v/v), the mixture behaved in a manner closer to bovine milk than to human milk (Fig. 5A), with a lag in time before creaming commenced as observed above, but then the migration rate increased rapidly. When the cream from human milk was mixed with bovine skimmed milk, and vice versa, the recombined milk prepared by mixing human cream and bovine skim milk had higher TSI_{cream} (from 10 to 50 min, Fig. 5B), MV_{cream} (0–7 h, Fig. 5C), final ΔH of the cream layer, and initial creaming rate at each time point, compared with fresh bovine milk, fresh human milk, or milk recombined from skimmed human milk and bovine cream (Fig. 5D).

After the addition of bovine WPI (0.83%, w/v) to human milk, both the peak thickness and the mean ΔBS increased slightly. The

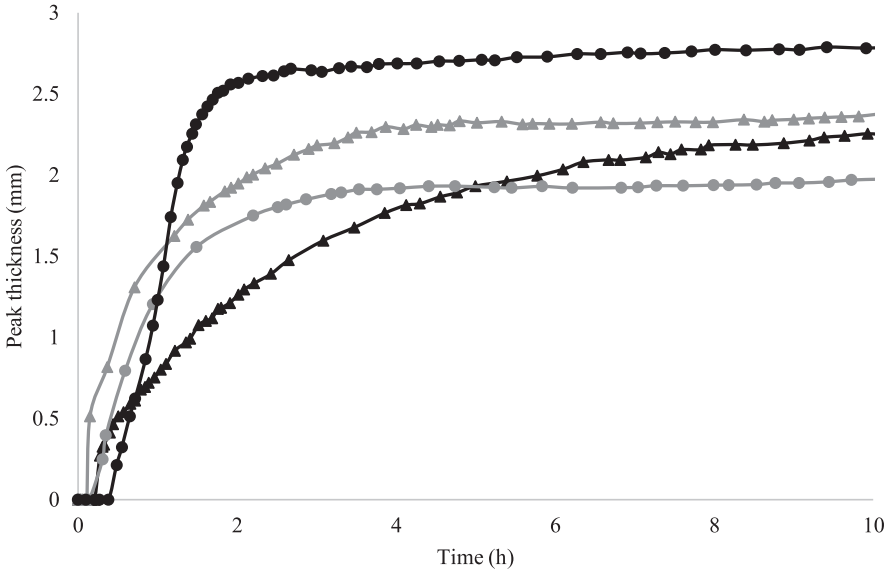

Fig. 3. Peak thickness of the cream layer for fresh human milk (▲, ▲) and fresh bovine milk (●, ●) at 5 °C (▲, ●) and 40 °C (▲, ●), respectively. Peak thickness profiles are from individual samples but are representative of data from others.

Table 4
Creaming rates measured in the cream layer and the clarification layer of human and bovine milk.

Temperature (°C)	n	Migration rate	
		Creaming zone	Clarification zone
Bovine milk			
5	3	2.37 (1.45)	1.34 (0.58)
40	3	2.38 (0.52)	2.69 (0.27)
Human milk			
5	11	1.25 (0.95)	0.61 (0.14)
20	10	1.81 (1.85)	1.46 (1.80)
37	13	2.27 (1.61)	2.18 (1.31)
40	11	3.34 (2.11)	3.33 (3.00)
45	3	6.06 (1.69)	6.88 (3.82)

Data (in mm h^{-1}) are means with standard deviation in parenthesis; n is the number of samples at each temperature. Zones are as defined in Supplementary material Fig. S1 (creaming, zone r_1; clarification, zone r_2).

initial creaming rates of human milk also increased after addition of both bovine WPI and bovine Igs, and the addition of bovine Igs had a higher impact on human milk creaming than the addition of WPI. However, the increases due to addition of bovine WPI and Igs were not significant ($P > 0.05$) in the case of TSI_{cream} (Supplementary material Fig. S4) at each time point.

4. Discussion

To our knowledge, this is the first detailed study on creaming behaviour of human milk, which contributes to knowledge of physico-chemical properties of human milk, specifically its creaming and cold agglutination behaviour. A similar or even higher creaming rate of human milk than that of bovine milk at warm temperature (40 °C) is inconsistent with the only report on human milk creaming, which suggested that the rate of creaming of human milk was lower than that in bovine milk at 37 °C (Whittlestone & Perrin, 1954). This inconsistency may be due to the high individual variation of human milk samples, more advanced analytical methodologies, and data coming from a larger sample size in our study. In this study, fresh human milk samples were obtained in the early stages of lactation (one week post-partum), when milk contains a lower fat content and a higher protein content than in mature human milk (Gidrewicz & Fenton, 2014). A lactational effect was noticed and samples at 8 and 24 weeks post-partum appear to have higher values of peak thickness than samples at 1 week post-partum (data not shown). Mid-to hind-milk, which was collected after the babies have been satisfied in this study, may contain a higher level of fat than foremilk (Mizuno et al., 2009); however, the milk fat globule size is not expected to be significantly different (Mizuno et al., 2009). The content of macronutrients of human and bovine milk measured was, however, consistent with the literature values (Anderson, Atkinson, & Bryan, 1981; Bauer & Gerss, 2011; Gidrewicz & Fenton, 2014; Guo & Hendricks, 2008). The lower average fat content and a higher average solids-not-fat content in human milk than in bovine milk samples may slow fat separation in the milk (Webb & Hall, 1935), and may partly explain the observed slower creaming rate of human milk than bovine milk at 5 °C.

The range of MFG size of human and bovine milk was similar to those reported by Rüegg and Blanc (1981), although some studies reported a higher MFG size of human milk than bovine milk (Lopez, Cauty & Guyomarc'h. 2015; Ma, Zhang, Wu, & Zhou, 2019). It has previously been noted that the human milk expressed from the fourth day to the first month of lactation, as for samples in this study, can have a lower average MFG size than that of colostrum or mature milk from the first month until the end of lactation (Michalski, Briard, Michel, Tasson, & Poulain, 2005). According to Stokes' Law, the creaming rate rises when the diameter of MFG increases. A small extent of fat globule clustering, which increases the effective diameter of MFGs, was found in human milk at 5 °C, as the average MFG size of human milk cream layer decreased after dilution with 1% SDS (Supplementary material Table S2), which has also been reported in bovine cream (Boode, 1992; D'Incecco et al., 2018).

Considering the average diameter D [3,2] of human MFGs during the first week of lactation (2.27 μm) and the average of bovine MFG diameter (2.35 μm), the creaming rate for individual MFGs can be predicted by Stokes' Law [Equation (1)]. In this estimation, it is assumed that densities of human and bovine milk serum at 5 °C are 1.03×10^3 kg m^{-3} and 1.04×10^3 kg m^{-3},

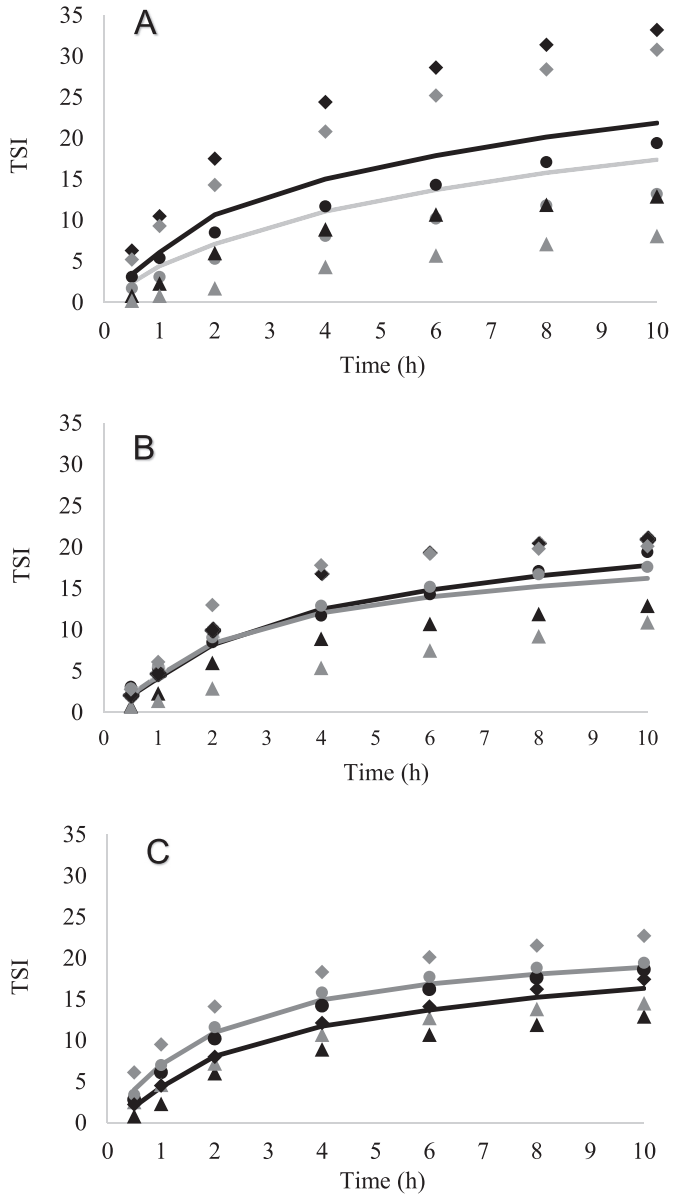

Fig. 4. TSI profiles of human milk cream layer for milk stored at (A) 4 °C for 1 week, (B) milk frozen at −20 °C for 1 week (thawed at RT), and (C) milk frozen at −20 °C for 9 months (thawed at RT) compared with fresh human milk before treatments. The TSI_{cream} before treatments (black symbols) and the TSI_{cream} after treatments (grey symbols) are shown. The solid lines are averages of the TSI_{cream} before and after treatments; ◆, ▲, ● represent three individual samples in each treatment.

respectively (Ma & Barbano, 2000; Neville et al., 1988), and, based on measurements in this study, densities of human and bovine milk fat are both 0.88 × 10^3 kg m^{-3}, and viscosities of human and bovine milk at 5 °C are 2.53 mPa s and 3.45 mPa s, respectively. On this basis, the calculated creaming rates of human and bovine milk are 0.60 mm h^{-1} and 0.51 mm h^{-1}, respectively. The predicted creaming rates for human and bovine milk are thus both lower than the measured averages (0.84 mm h^{-1} for human milk and 1.75 mm h^{-1} for bovine milk) at 5 °C, especially for bovine milk, which may reflect the occurrence of cold agglutination in bovine milk.

IgM and IgA are reported as major agglutinins to accelerate bovine milk creaming during cold storage (D'Incecco et al., 2018;

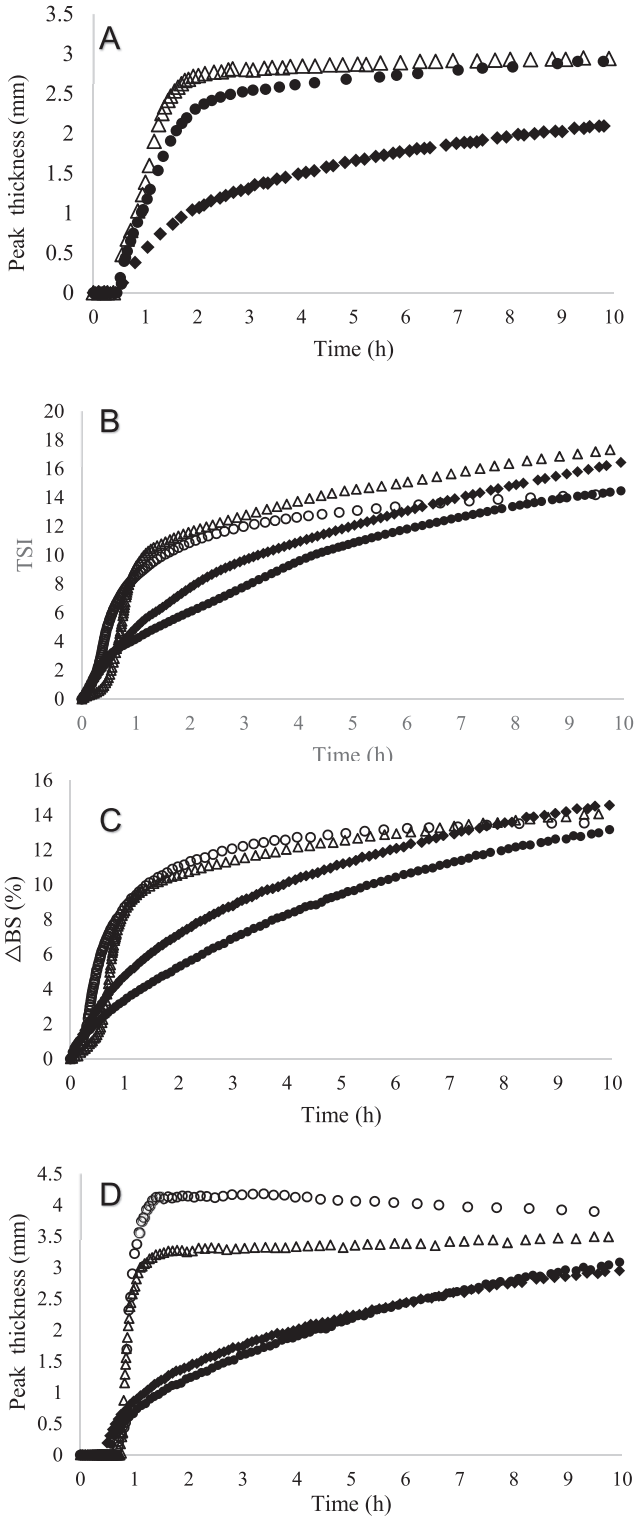

Fig. 5. The peak thickness profiles (A) of cream layer for fresh human milk (◆), fresh bovine milk (△), and bovine and mixed human milk (1:1, v/v; ●) and the TSI (B), mean value (C), and peak thickness (D) profiles of cream layer for fresh human milk, fresh bovine milk, recombined milk of human cream and bovine serum (○), and recombined milk of bovine cream and human serum (●) held at 5 °C, as a function of time. The profile is from an individual sample but is representative of data from others.

Hansen, Larsen, & Wiking, 2019), but remain in skim milk after separation at 45 °C (Jennes & Parkash, 1971). Although the protein content of human milk was lower than that of bovine milk, human milk is reported to contain a higher percentage of Igs (approximately 16% of total whey proteins) than bovine milk (approximately 10% of total whey proteins; Guo & Hendricks, 2008). Also, human milk has IgA-dominated Igs, whereas bovine milk Igs are IgG-dominated (Hurley & Theil, 2011). The level of IgA has been reported to be 0.13 kg m^{-3} and 1 kg m^{-3} for bovine and human milk, respectively, whereas the level of IgM is 0.04 kg m^{-3} and 0.1 kg m^{-3} for bovine and human milk, respectively (Hurley, 2003), i.e., human milk contains higher levels of agglutinins (IgA and IgM). However, the cream volume and the creaming rate of human milk at 5 °C were lower than that of bovine milk (Fig. 3, Table 4), which may result from a higher extent of agglutinins being associated with bovine milk fat globules than human milk. This is supported by the fact that the creaming ability of human milk was increased after mixing with bovine skim milk.

There are two possible reasons why human milk contains high levels of agglutinins, but has a lower creaming rate than bovine milk at 5 °C. Firstly, although hydrophobic interactions will be weakened at low temperatures, bovine milk has been reported to have the most hydrophobic secretory IgA among species, including humans, which can result in a higher level of bovine IgA associated with fat globules (Honkanen-Buzalski & Sandholm, 1981). Secondly, competition between soluble antigens and MFGM antigens to capture IgM may occur in human milk (Euber & Brunner, 1984). It is known that the interaction of IgM with MFG is specific and involves carbohydrate moieties (Euber & Brunner, 1984); therefore, milk oligosaccharides and κ-casein molecules containing carbohydrate moieties may be involved in the interaction of IgM. The oligosaccharides have not been considered as a factor in bovine milk due to their low concentration (Euber & Brunner, 1984), but should be considered in human milk due to their higher levels (Kunz & Rudloff, 1993). Moreover, the content of sialic acid, which has been demonstrated to inhibit milk creaming (Euber & Brunner, 1984), is much higher in human milk than in bovine milk (Wang & Brand-Miller, 2003; Wang, Brand-Miller, McVeagh, & Petocz, 2001). Further research on the reasons behind the difference of creaming between bovine and human milk appears warranted.

Human milk was stable following short-term freezing, which is consistent with an unchanged size distribution of human MFG before and after freezing (data not shown). It has been reported that no significant change in concentration occurs for IgA, the major agglutinin in mature human milk, at −20 °C for 1 month (Evans, Ryley, Neale, Dodge, & Lewarne, 1978) or 3 months (Reynolds et al., 1982). In contrast, a decrease in IgA level after 4 weeks at −20 °C has also been reported (Akinbi et al., 2010), and Ramírez-Santana et al. (2012) reported a decrease in IgA concentration in human colostrum when the frozen storage period was increased to 12 months, indicating that IgA can be temperature labile. Moreover, although the MFG can be resistant to lipolysis as long as its structure remains intact, slow freezing or long-term freezing can lead to the destruction of MFGM and allow access of lipases to the core of MFG, i.e., to triglycerides (Berkow et al., 1984; Munkwitz, Berry, & Boyer, 1933).

Heating human milk at 70 °C for 10 min slowed cream separation and decreased the volume fraction of milk fat in cream layer, which has also been found for bovine milk (Caplan, Melilli, & Barbano, 2013). One possible reason could be that heat treatment increased the viscosity of the serum phase and thereby reduced the rate of creaming. Also, it has been hypothesised that heat-induced denaturation of proteins can result in interactions with milk fat globules and therefore increase their density (Caplan et al., 2013). A thinner cream layer can be thus formed after preheating (Supplementary material Fig. S3), shown as a lower ΔH of cream layer in preheated human milk than the untreated sample.

5. Conclusion

The creaming behaviour of human milk was affected by temperature and pre-treatments, including freezing-thawing and preheating. The creaming capacity of human milk was correlated with the temperature in the range 5–40 °C. Short-term freezing-thawing had no effect on creaming while heating at 70 °C for 10 min slowed the creaming of human milk. Similarities and differences of human and bovine milk creaming behaviour at low temperature are discussed, and may be caused by the fat content, the properties of MFGM, the presence of agglutinins (IgA and IgM), and competition for attachment of agglutinins in milk serum. This study provides the information for both mothers and human milk banks in terms of handling and storing human milk and feeding infants with optimal methods in terms of ensuring proportionate levels of fat alongside other constituents.

Acknowledgement

The authors would like to acknowledge the funding provided by Department of Agriculture, Food and the Marine, Ireland under its FIRM programme (grant number 15/F/721).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2020.104726.

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