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Understanding the effect of processing and species on milk proteins during digestion

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

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Abstract

Milk is an important source of protein in a balanced human diet. Milk proteins not only have high nutritive value but also have biological properties. Milk composition and structure vary based on factors such as species, processing methods, and lactation stage. These differences are believed to affect digestion by influencing the breakdown of milk proteins, fats, and carbohydrates, as well as the rate and efficiency at which nutrients are absorbed in the gastrointestinal tract. The overall objective of this PhD thesis was to investigate how milk proteins from different species (cow, sheep, goat, and deer) are affected by digestibility under varying processing treatments (heating and homogenisation). Digestibility was assessed by the amount and types of bioaccessible peptides generated during gastrointestinal digestion. A dynamic *in vitro* digestion model (human gastric simulator (HGS)) was used for this study. Size exclusion chromatography was employed to measure the amount of peptides generated throughout digestion, with significant differences determined by a p-value threshold of 0.05. Mass spectrometry was used to analyse the types of peptides, requiring peptides to be present in at least two-thirds of the samples for inclusion. To assess the validity of the results obtained using the HGS model, comparisons were made with the peptide profiles generated using an *in vivo* (pig) digestion model. In addition, further work was undertaken looking into the protein composition of deer milk throughout the different lactation stages.

This study investigating digestibility found differences in the amount and types of bioaccessible peptides generated throughout gastric digestion in milk from different species. Overall, deer milk produced the most peptides, while goat and sheep milk produced the least. Ruminant species also affected which regions of the parent protein were resistant to digestion as well as their bioactive properties. In contrast, processing treatment did not have as significant an effect on the amount and types of bioaccessible peptides but did affect the digestion kinetics. Differences were only observed during early digestion and appeared to be species dependent. Similarities were found in the peptides released throughout gastric digestion between the HGS model and the *in vivo* pig model, which suggests that the HGS model is suitable for the study of gastric digestion of protein-rich food. However, the peptide profiles differed during the intestinal stage indicating that the intestinal step attached to the *in vitro* model needs improving to fully mimic the dynamic nature of *in vivo* digestion.

The study investigating deer milk proteins found that proteins related to transport *e.g.* apolipoprotein E and vitamin D-binding protein and immunity *e.g.* osteopontin, immunoglobulin J and lactotransferrin were found to change throughout lactation. This is thought to reflect the changing needs of the newborn as well as the development and protection of the mammary gland over lactation. Proteins were investigated using mass spectrometry, and significant differences throughout lactation were determined using simple linear regression calculations and log fold change calculations, comparing protein levels between week 3 and week 16 of lactation.

The results from this thesis will contribute to the knowledge of how milk composition and structure impact protein digestibility throughout gastrointestinal digestion. The information gained from this study may have important consequences for developing dairy products that deliver superior digestive and nutritional outcomes to targeted consumer groups.

Contents

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List of common abbreviations

α-LA	Alpha-Lactalbumin
α_{s1}-CN	Alpha-S1-Casein
α_{s2}-CN	Alpha-S2-Casein
AA	Amino acid
β-LG	Beta-Lactoglobulin
β-CN	Beta-Casein
CN	Casein
HGS	Human gastric simulator
HPLC	High pressure liquid chromatography
κ-CN	Kappa-Casein
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MFGM	Milk fat globule membrane
MS	Mass spectrometry
MW	Molecular weight
OPA	o-phthaldialdehyde
PAGE	Polyacrylamide gel electrophoresis
PTM	Post translational modifications
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
UHT	Ultra-high temperature

Chapter 1 - Introduction

Milk proteins are a valuable source of amino acids which are necessary for maintenance and growth of body protein. During food manufacture and digestion these proteins are broken down into a range of peptides with distinct biological functions. These bioactive compounds are not only beneficial to the neonate, but also form the basis for functional foods, which can offer additional health benefits beyond those of basic nutrients. The peptides produced from milk during digestion are influenced by factors such as species, breed, lactation stage and processing treatments, which are likely due to differences in milk composition and structure. The main proteins in milk include caseins (alpha-S1, alpha-S2, beta, and kappa) and whey proteins like alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG). Differences in milk composition such as the ratio of casein to whey proteins and casein micelle size can affect how these proteins are digested and the types of peptides formed. A better understanding of how milk composition and structure affects gastrointestinal digestion would contribute to the development of food products that deliver superior digestive and nutritional outcomes tailored to specific consumer groups.

Traditionally, Western dairy industries have been centred around cow milk. However, there is an increasing interest in non-cow milks such as goat, sheep and even deer milk driven by consumer perceptions that these milks have better digestive properties. Whilst it is established that milk composition varies between species, less is known about how this affects protein digestion. Whilst numerous studies have investigated protein digestion in cow milk, there are limited studies comparing this to digestion of proteins in non-cow milks. The available research comparing species mainly focuses on infant digestion, often with the goal of identifying milks that resemble human milk, rather than exploring the unique properties of these milks for use in functional foods. Most studies comparing the milk digestion across species have used static *in vitro* digestion models. However, to understand how milk composition and structure affects the digestion of milk proteins the use of dynamic *in vitro* digestion models is beneficial.

Prior to consumption, milk is processed either to kill microbes, preserve shelf life, or to prepare it for further production. Studies have indicated that processing treatment affects the physical structures of cow milk. However, there are conflicting results on the influence of processing treatments on digestion of proteins in cow milk. There are only limited studies investigating the effects of processing treatments in non-cow milks or comparing the effects processing

treatments have between the different species. Understanding how processing treatments affects the protein breakdown in the different species will aid in determining whether different treatments are more suited for milks from species.

In the current study milk from different species (cow, sheep, goat, and deer) was collected from farms in New Zealand and either left unprocessed or processed (heating and/or homogenisation). Digestion of milk was simulated for humans using a dynamic *in vitro* gastric digestion model followed by static intestinal digestion. The digesta were analysed using different proteomic techniques to determine the rate of digestion and peptide profile of the different milks. The objective of this study was to investigate how processing treatments affect protein structure and digestion of milks from ruminant species, which will enable a better understanding when developing milk products.

In vitro models are predominantly used to simulate digestion of milk. This is due to the comparative ease and reduced ethical issues compared to *in vivo* models. To improve the validity of results from *in vitro* digestion models it is important to determine how they align with *in vivo* models. To date there are very few studies that have directly compared how milk proteins are digested in *in vitro* models with *in vivo* models. These studies differed in the type of *in vitro* and *in vivo* digestion models used. To ensure the accuracy of the *in vitro* experiments in this study, a comparison between the *in vitro* (human gastric simulator) and *in vivo* digestion (pig model) using commercially produced cow milk was performed. Pigs were used to model *in vivo* digestion due to the similarity between the pig and human digestive system. The rate of digestion and peptide profiles for the *in vitro* and *in vivo* models were compared using different proteomic techniques.

The development of red deer dairy farming in New Zealand is relatively recent. There are comparatively fewer studies done on deer milk compared to the other species. This research has mainly focused on the overall milk composition and shows that deer milk has a higher concentration of protein compared to other ruminant's milk. In the current study the composition of proteins in deer milk is further studied as well as the effect lactation stage has on the proteins. Increasing the knowledge of deer milk proteins and the effects of lactation will aid in utilising deer milk for novel products and applications.

The key objectives of this research and their subsequent research questions were:

Objective 1: To determine whether the protein digestion in milk varies depending on the species origin (cow, sheep, goat, and deer) and according to processing treatment (unprocessed, pasteurised, pasteurised-homogenised, high-heat (yoghurt treatment) and UHT).
Hypothesis 1: Milk from different species will exhibit distinct protein digestion patterns, and process treatments will differentially affect the digestion of proteins from each species, potentially altering the peptide profiles produced during digestion.

Objective 2: To investigate the peptide fraction of milks produced during *in vitro* digestion of commercial cow milk and align the results with those observed *in vivo* in the digesta of pigs.
Hypothesis 2: *In vitro* digestion of cow milk will reflect the *in vivo* digestion process, but there will be some discrepancies in the rate of digestion and peptide profiles, highlighting the need for more refined *in vitro* models.

Objective 3: To provide further insight into the protein composition of deer milk and determine whether lactation stage affects protein composition of deer milk at the individual protein level.
Hypothesis 3: Deer milk will exhibit unique protein composition, with the relative abundance of key proteins such as caseins and whey proteins changing as lactation progresses. These variations in protein abundance are likely to reflect changes in the milks nutritional and bioactive properties throughout the lactation period.

Figure 1 outlines how the chapters address these objectives. This study provides up-to-date information on the effects milk composition and structure have on the digestion of milk proteins from different species and processing treatments and contributes to understanding how these milks can be used to develop novel products and functional foods.

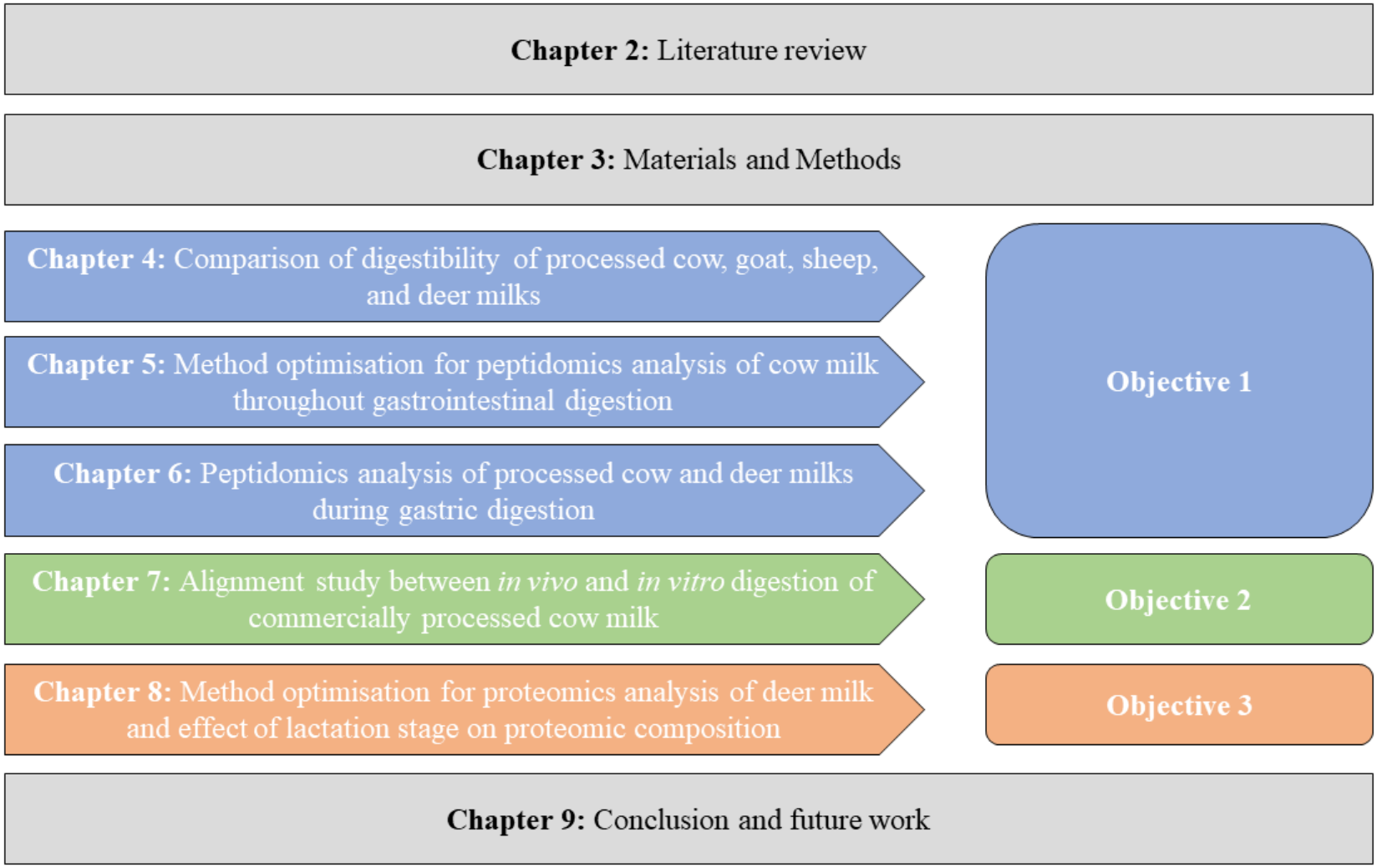

Figure 1. Layout of thesis chapters.

Chapter 2 - Literature Review

The aim of this chapter is to review the published work on the composition, effect of processing and digestive dynamics of milk proteins from four ruminant species – cow, sheep, goat, and deer.

2.1 Milk Composition

Milk is a nutrient-rich fluid secreted by the mammary glands of all female mammalian species. Milk's primary role is to meet the complete nutritional requirements of the neonate. This includes providing them with energy, essential amino acids and amino groups for the biosynthesis of non-essential amino acids in the form of proteins, essential fatty acids, carbohydrates (primarily lactose), vitamins, minerals and water (Boland & Singh, 2019). The exact protein composition of milk varies at a genetic level between different species and breeds. It also varies at a physiological level at different lactation stages, due to nutritional factors such as feed energy value and composition, as well as environmental conditions, such as location and season (Boland & Singh, 2019).

Investigations into compositional variations between species and other factors have been conducted primarily to either a) improve the quality of milk products such as infant formula, b) find alternatives to cow milk as a stratagem against allergens, or c) aid in rearing the young of other species in captivity. Most studies have predominantly focused on highlighting the differences between human (non-ruminant) and ruminant milks, as these differences are more pronounced (Claeys *et al.*, 2014; Gantner *et al.*, 2015). Consequently, the subtle compositional differences between ruminant species, such as cow, sheep, goat and deer, are often overlooked in the literature. While cow, sheep and goat milk are commonly included in compositional comparison studies, deer milk is typically excluded, further contributing to the gap in understanding the diversity among ruminant species. Published compositional information on deer milk draws on multiple species e.g., red deer, reindeer, fallow deer and thus contains a wide range of values (Claeys *et al.*, 2014; Gathercole, 2022). Table 1 illustrates the minimum and maximum values of milk components found in the literature for cow, sheep, goat, and deer milk.

Table 1. Milk composition (g/100 g) from four different ruminants (Claeys *et al.*, 2014; Gantner *et al.*, 2015; Gathercole, 2022; Medhammar *et al.*, 2012; Potočnik *et al.*, 2011; Roy *et al.*, 2020a).

Properties	Cow	Sheep	Goat	Deer
Energy (kJ/100g)	263.0-284.3	403.8-443.9	280.0-290.0	554.1-843.6
Total solids	11.8-13.0	18.1-20.0	11.9-16.3	19.6-27.1
Protein	3.0-4.0	4.5-7.0	3.0-5.2	6.5-13.0
Fat	3.3-6.4	5.0-9.0	3.0-7.2	3.9-21.5
Lactose	4.4-5.6	4.1-5.9	3.2-5.0	1.2-6.2
Ash	0.3-0.8	0.8-1.0	0.7-0.9	1.0-2.7
Oligosaccharides	0.003-0.006	0.002-0.004	0.025-0.030	

In general, deer milk has the highest amount of energy, total solids, protein, fat, and ash followed by sheep milk with goat and cow milk having the lowest amount. Some studies suggest that cow and goat milk have similar protein levels (Claeys *et al.*, 2014; Roy *et al.*, 2020b) whilst others indicate that goat milk has a higher protein content than cow milk (Ceballos *et al.*, 2009). Lactose is the predominant carbohydrate in all ruminant milks. Deer milk has the lowest amount of lactose followed by goat with cow and sheep milk having the highest. Oligosaccharides are present in all four species. Cow and sheep milk have the lowest amounts of oligosaccharides whilst goat milk has comparable amounts to human milk (Roy *et al.*, 2020a). Although it has been shown that deer milk contains oligosaccharides no data is available on the amounts (Mineguchi *et al.*, 2018; Taufik *et al.*, 2014).

The range in the values is likely due to inconsistencies between different studies regarding genetic, physiological, and nutritional factors. The effect of breed on milk composition has been shown to be significant. In deer milk the amount of protein varies from 6.5-10 g/100g for fallow deer to 7.6-13.0 g/100g for reindeer (Gathercole, 2022). Indigenous Greek breeds of goat and international breeds show varying protein amounts of 3.88 g/100g and 3.19 g/100g, respectively (Moatsou *et al.*, 2008). Protein content in sheep milk has also been shown to differ between 5.29-8.40 g/100g depending on breed (Simos *et al.*, 1996). Lactation stage also influences milk composition. The protein and fat content increases throughout lactation in both cow and deer milk (Auldist *et al.*, 1998; Berruga *et al.*, 2021; Landete-Castillejos *et al.*, 2000; Li *et al.*, 2023). Goat milk contains a higher amount of fat and protein content during early and late lactation compared to mid-lactation (Fekadu *et al.*, 2005). Seasonal farming also affects

the composition of milk. In New Zealand cow milk, increases in fat and protein content were observed, following patterns consistent with those associated with different lactation stages (Li *et al.*, 2019). Differences in the amount of protein and fat was also observed in different years. In Italian sheep milk differences in protein and fat contents were observed between ewes which had lambed in autumn and those that had lambed in winter (Sevi *et al.*, 2004). They also showed different patterns throughout lactation stage for both protein and fat content.

2.2 Milk Proteins

Milk proteins provide the amino acids and nitrogen necessary for new-born growth and development and have specific functions such as enzyme and enzyme inhibitor activities, specific or non-specific nutrient binding properties, protective properties, mediator, hormone and growth factor activities and immunomodulatory effects (Boland & Singh, 2019; Tomé & Debabbi, 1998). Proteins in milk are classed into two main types, caseins (CNs) and whey/serum proteins (Boland & Singh, 2019). Table 2 shows a breakdown of the reported major protein components of milks from four ruminant species. These findings illustrate differences in the protein composition of different ruminant species.

Table 2. Protein composition (g/100g) of milk from four different ruminants (Claeys *et al.*, 2014; Roy *et al.*, 2020a).

Protein	Cow	Sheep	Goat	Deer
Casein	**2.46-2.80**	**4.18-4.60**	**2.33-4.63**	**5.70-8.40**
α_{s1}-CN	0.8-1.07	1.54-2.21	0.0-1.30	0.692
α_{s2}-CN	0.28-0.34	0.6	0.23-1.16	0.076
β-CN	0.86-0.93	1.56-1.76	0.0-2.96	
κ-CN	0.23-0.33	0.32-0.43	0.28-1.34	0.499
Whey	**0.55-0.70**	**1.02-1.61**	**0.37-0.70**	**1.1-1.5**
β-Lactoglobulin	0.32-0.33	0.65-1.35	0.15-0.50	
α-Lactalbumin	0.12-0.13	0.10-0.19	0.07-0.23	0.213
Lactoferrin	0.002-0.05	0.08	0.002-0.02	0.260
Serum albumin	0.03-0.04	0.04-0.06	0.51-2.15	0.768
Lysozyme	(7.0-60.0) x 10^{-5}	1.0 x 10^{5}	2.5 x 10^{-5}	
Immunoglobulins	0.5-1.0	0.7	0.46-2.14	0.768
CN/Whey ratio	**4.7**	**3.1**	**3.5**	**4-5**

2.2.1 Caseins

Caseins (CNs) are a family of phosphoproteins that are synthesised in the mammary gland in response to lactogenic hormones and other stimuli (Boland & Singh, 2019; Ginger & Grigor, 1999). The main biological functions of CN are the control of calcium phosphate precipitation via amorphous calcium phosphate sequestration, suppression of harmful amyloid fibril formation, and nutrition of the neonate (Holt *et al.*, 2013). The amount of total CN differs between the four species. It is greatest in deer milk, followed by sheep, with goat and cow having similarly low amounts (Table 2).

Four proteins make up the CN fraction of milk, they are: α_{s1}, α_{s2}-, β- and κ-CN. Although the type of CN remains consistent in all four species the proportions differ. The predominant CN in cow milk is α_{s1}-CN, making up 38% of total CN (Park, 2004). In goat milk the predominant CN is β-CN making up approximately 54.8% of total CN (Park, 2004). Goat milk contains much less or nondetectable amounts of α_{s1}-CN compared to cow milk (Korhonen, 2009). The predominant CN in sheep milk is uncertain. Some studies have found that sheep milk has a lower proportion of α_{s1}-CN than cow milk (Roy *et al.*, 2020b) whilst others indicate that the predominant CN in sheep's milk is α_{s1}-CN as seen in cow milk. Limited studies have been published on the proportion of CNs in deer milk. Ha *et al.* (2014) used reverse phase – high pressure liquid chromatography (RP-HPLC) to analyse milk from the four species. Their findings indicated that deer milk had a lower concentration of α_{s1}-CN and κ-CN than the other three species, whilst it had a similar level of α_{s2}-CN as goat milk. However, the concentration of β-CN could not be determined due to a shift in the elution profile. Roy *et al.* (2020b) also was unable to determine the proportions of all the CNs in deer milk due to different and higher electrophoretic mobilities than those of the other species. The results suggested that deer milk had lower proportions of α_{s2}-CN than the other species, however α_{s1}-CN, β-CN, and κ-CN were unable to be separated on the SDS-PAGE gel. The difference in the amino acid sequence of proteins between the four ruminant species is minimal (Claeys *et al.*, 2014). Comparisons of the amino acid sequence using UniProt (Consortium, 2022) show a similarity of 70-99 % between milk proteins from different species with the sequence of goat and sheep milk being the most similar to each other and the sequence of deer milk being the most dissimilar to the other ruminant species

CNs are flexible, rheomorphic molecules with very little stable secondary structure (Boland & Singh, 2019; Głąb & Boratyński, 2017). CNs typically aggregate together to form a stable CN micelle. This has predominantly been studied in cow milk, with limited studies on the other species (Holt *et al.*, 2013). A recent study used small-angle X-ray scattering to study CN micelles in cow, goat, and sheep milk. The authors reported similarities in the structure of the proteins and calcium-containing nanoclusters (Ingham *et al.*, 2018). CN micelles are primarily composed of protein with approximately 6% colloidal calcium phosphate and large quantities of water (approximately 2 grams for every gram of protein) (Livney, 2010). The exact structure of the micelle is still unknown. One popular model states that the α_{s1}-, α_{s2}- and β-CNs are mainly present in the interior whilst the κ-CNs form a 'hairy' layer that stabilises the micelle sterically due to the hydrophilic glycosylation of κ-CN (Głąb & Boratyński, 2017; Holland, 2014). Other proposed models include coat-core, subunit (sub-micelles) and internal structure (Phadungath, 2005). The size, hydration and mineralisation of CN micelles differs among the four species (Table 3). Cow milk has the lowest CN micelle diameter whilst sheep and goat milk have the lowest hydration and highest mineralisation (Park *et al.*, 2007; Roy *et al.*, 2020a). It is believed that the hydration level of CN micelles is negatively correlated with mineralisation of micelles.

Table 3. Comparison of CN micelle structure between four different ruminants (Park *et al.*, 2007; Roy *et al.*, 2020a).

Characteristics	Cow	Sheep	Goat	Deer
CN micelle size (nm)	**150-182**	**180-210**	**180-301**	**190**
Hydration (g H_2O/g protein)	**1.92-3.7**	**1.71-1.93**	**1.43-2.05**	
Mineralisation (g/ca/100 CN)	**2.9**	**3.7**	**3.6**	

A potential reason for the variation in the amount of CNs and the micelle size between the different species is their heterogeneity. CNs are heterogenous due to genetic polymorphism, post translational modifications (PTMs) including phosphorylation and glycosylation, disulphide bonding of α_{s2}- and κ-CNs and hydrolysis via plasmin (Boland & Singh, 2019; Holland, 2014). Genetic variation has been linked to differences in the levels of CN synthesis. The level of genetic variation ranges from single nucleotide substitutions, deletions, and large insertions. Currently eight identified alleles in goat milk are associated with a high level of α_{s1}-CN, two with medium levels, two with low levels and two with no α_{s1}-CN (Park *et al.*, 2007).

2.2.2 Whey proteins and other minor proteins

Whey proteins, unlike CNs, are highly structured. They are globular molecules with a high content of α-helix motifs with a fairly balanced distribution of acidic/basic and hydrophobic/hydrophilic amino acids along their polypeptide chain (Madureira *et al.*, 2007). The amount of total whey proteins differs between the milk of cows, sheep, goats and deer (Table 2). Deer and sheep milk have comparable high amounts of total whey proteins whilst goat and cow milk have similarly low amounts of whey protein. The whey fraction is predominantly made up of β-Lactoglobulin (β-LG) and α-Lactalbumin (α-LA).

β-LG is the principal whey protein in ruminant species milk. Sheep milk have the highest amount of β-LG followed by goat and cow milk (Table 2). Although it has been shown that deer milk contains β-LG, the amount is unknown (Ha *et al.*, 2015; Roy *et al.*, 2020b). The exact function of β-LG is unclear; however, it is part of the lipocalins family. Lipocalins are classified as small (160-180 amino acids) extracellular proteins sharing several common molecular recognition properties e.g. binding of small, principally hydrophobic molecules (such as retinol); binding to specific cell-surface receptors; and the formation of covalent and non-covalent complexes with other soluble macromolecules (Flower *et al.*, 2000). β-LG is a small, highly structured protein of 162 amino acid residues that fold up into an 8-stranded, antiparallel β-barrel with a 3-turn α-helix on the outer surface and a ninth β-strand flanking the first strand (Kontopidis *et al.*, 2004). Genetic polymorphism is found in β-LG, thus far 10 genetic variants of β-LG have been identified in bovine species (A-J with A and B being the most common), three have been identified in sheep and two in goat (Boland & Singh, 2019; Pena *et al.*, 2000). β-LG is very resistant to proteolysis due to its stable conformation, and it is also one of the major allergens in milk due to its lack of presence in human milk.

α-LA is a component of the enzyme that catalyses the final step in the biosynthesis of lactose; lactose synthetase (Boland & Singh, 2019). There is a direct correlation between the concentration of α-LA and lactose in milk. α-LA is synthesized in the mammary gland. It is a small, compact metalloprotein containing 123 amino acid residues with a mass of approximately 14 kDa. The primary structure of α-LA is very similar to lysozyme, with 54 identical amino acid residues between the two and 23 structurally similar amino acids. α-LA is extremely heat stable due to its interaction with calcium. The amount of α-LA varies between the different ruminant species. Ha *et al.* (2014) reported that sheep milk had the greatest amount

of α-LA followed by goat, cow, and deer milk. Less genetic variation is found in α-LA compared to β-LG with only one genetic variant being found in *Bos taurus* breeds, however minor inter-species differences are found in the composition and the properties of the protein (Boland & Singh, 2019).

Although the principal whey proteins found in milk are α-LA and β-LG, a wide range of minor whey proteins are also present, many of which are biologically active. The number of proteins identified in milk has grown recently due to increased capabilities to detect proteins of low concentration using mass spectrometry (MS). Cow milk has been the most extensively studied with MS. The experimental design and type of MS have great impact on the number of proteins identified in milk. For instance, Nissen *et al.* (2013) employed a wide range of protein fractionation methods combined with two dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS), identifying 376 unique proteins in cow milk. In contrast, Vincent, Ezernieks, *et al.* (2016) used different extraction methods and nano-liquid chromatography electrospray ionisation tandem mass spectrometry (nLC-ESI-MS/MS), which resulted in the identification of only 186 unique proteins in cow milk. Goat and sheep milk have also been studied using MS, however, to a lesser extent. Chen *et al.* (2019) and Sun *et al.* (2023) identified 310 and 344 proteins in goat milk, respectively. An in depth search of the proteins in milk whey of sheep involving both in-gel digestion using 1D-PAGE and in-solution digestion followed by OFFGEL isoelectric focusing fractionation prior to LC-MS/MS identified 669 proteins (Ha *et al.*, 2015). In comparison, 783 proteins from unique genes have been identified in the whey of cow milk (Ha *et al.*, 2015).The minor whey proteins of deer milk have not been well studied. Techniques such as chromatography and SDS-PAGE gels have typically been used to study deer milk (Ha *et al.*, 2014; Li *et al.*, 2023) and studies that have used MS typically focus on one protein such as lactoferrin (Wang *et al.*, 2021). Table 4 illustrates the key properties of a number of these minor whey proteins.

A wide range of proteins are also associated with the milk fat globule membrane (MFGM) and have been implicated in a wide range of biological functions such as stabilisation of the globule as an emulsion, inhibition of pathogen adhesion and participation in antimicrobial defence (Lee *et al.*, 2018; Yang *et al.*, 2015). Studies investigating the proteins associated with the MFGM have typically used SDS-PAGE gel separation followed by MS and have been performed on cow (Bianchi *et al.*, 2009), goat (Cebo *et al.*, 2010) and sheep (Pisanu *et al.*, 2011) milk. Both Yang *et al.* (2015) and Cebo and Martin (2012) identified inter-species differences in the

proteins associated with the MFGM. To date no study on the MFGM proteins in deer have been carried out. Most of the proteins associated with the MFGM are present at trace levels, with the principal proteins including adipophilin, butyrophilin, lactadherin and xanthine oxidase (Cebo & Martin, 2012). Table 5 outlines the key properties of these proteins.

Table 4. Properties of a selection of minor whey proteins (Boland & Singh, 2019; Goldfarb, 1997; Lund *et al.*, 2009; Madureira *et al.*, 2007).

Protein	Molecular weight (kDa)	Amino acid residues	Functions
Immunoglobulins (A, M and G)	25,000 (light chain) 50,000-70,000 (heavy chain)	-	Antibodies that give immunity to the neonate whilst they are developing their own immune system.
Serum albumin	66,267	582	Fatty acid binding, anti-mutagenic function, prevention of cancer, disease protection via passive immunity.
Lactoferrin	80,000	700	Iron-binding protein that removes iron from milk making it unavailable to bacteria. Has antioxidant, antiviral, anti-inflammatory, immunomodulatory and anti-carcinogenic activity.
Lactoperoxidase	70,000	612	Enzyme, catalyses inactivation of a wide range of micro-organisms in the lactoperoxidase system, antimicrobial properties.
Osteopontin	25,000-75,000	300	Plays a critical role in neonatal development. It supports immune system modulation by influencing immune cell activity and cytokine production. It promotes gut health by enhancing intestinal barrier function and fostering maturation of the gut microbiome in neonates.
Alolipoprotein E	34,000	299	Lipid transport, aiding the efficient delivery of milk fat globules to neonatal tissues for energy and growth. It also plays a role in immune function by facilitating lipid metabolism in immune cells and contributing to the development of the neonatal immune system.

Table 5. Properties of the major proteins found in the milk fat globule membrane (Lee *et al.*, 2018).

Protein	Molecular Weight (kDa)	Function
Adipophilin	52	Milk fat globule secretion.
Butyrophilin	56	Milk fat globule secretion, immune system.
Lactadherin	43	Immune system.
Xanthine oxidase	145	Milk fat globule secretion, immune system.

Milk also contains approximately 70 endogenous enzymes. These proteins are responsible for producing the complex fraction of peptides found in milk called the proteose peptone fraction (Boland & Singh, 2019). The main enzyme found in milk is plasmin. Plasmin is a protease that is responsible for causing proteolytic fragments of α_{s1}-, α_{s2}- and β-CN by preferentially cleaving Lys-X sites (Baum *et al.*, 2013). Cathepsin B, D and G are endoproteases (they break peptide bonds of non-terminal amino acids), and like plasmin they target CNs, however, unlike plasmin they have a broad specificity. Other common enzymes include elastase and lipase. Endogenous enzymes have been studied in cow, sheep and goat milk and have been shown to vary throughout lactation and have implications on processing and quality of dairy products (Albenzio & Santillo, 2011; Gautam *et al.*, 2023; Moatsou, 2010). Differences have also been observed in the activity level of the enzymes in different species, for example, cathepsin D activity has been found to be higher in goat milk than cow milk and the plasminogen-derived activity was higher than the plasmin-derived activity in cow milk whilst the opposite was observed in goats milk (Gautam *et al.*, 2023).

Baum *et al.* (2013) has shown that some of the peptides found in raw milk have bioactive properties such as immunomodulating effect, antimicrobial activity and antioxidative effect. Bioactive peptides are found embedded in proteins and, once liberated through proteolytic digestion or processing induced fragmentation, exhibit a wide variety of health and physiological functions such as antihypertensive, antimicrobial, antioxidative, antithrombotic, opioid, anti-appetizing, immunomodulatory and mineral-binding activities (Park & Nam, 2015). These are typically released via gastrointestinal digestion and certain processing techniques such as fermentation. Differences in the amount and types of bioactive peptides have been observed between ruminant species (Albenzio *et al.*, 2017), and this is likely due to the differences in the amount and biochemical characteristics of the proteins in the species.

2.3 Processing treatments of milk for human consumption

Although the primary role of milk is as a food source for the young of the species, the human diet has incorporated milk from a wide variety of different species since approximately 8000 BC (Boland & Singh, 2019). As well as being a nutritious food source, the consumption of milk has also been linked to a wide variety of health benefits including bone health, weight maintenance, reduction of type 2 diabetes and cardiovascular disease even as an adult (Jung *et al.*, 2015). Milk production for human consumption today is around 600 x10^6 tonnes per annum comprising 85% cow, 11% buffalo, 2% sheep, 2% goat and small amounts produced from other species such as horses, donkeys, camels, yaks and deer (Boland & Singh, 2019). Food products generated from milk include liquid milk, cheese, milk powders, concentrated milks, fermented milk products, butter, ice cream, infant formula, cream, protein-rich products and lactose (Boland & Singh, 2019). Milk is typically processed prior to consumption or production into a food product. Common forms of processing include heat treatment, homogenisation, drying and storage (Michalski & Januel, 2006; van Lieshout *et al.*, 2020). Processing stages have been shown to induce changes in the intrinsic quality of milk as seen in Table 6. This review focuses on the effects that thermal processing and homogenisation have on protein structure and function of ruminant milks.

Table 6. Major changes in milk caused by processing (Michalski & Januel, 2006).

Process	Reaction	Consequence
Heat treatment	Destruction of microorganisms	Increased microbiological quality and shelf life.
	Whey protein denaturation	Formation of CN-whey protein complexes.
	Lactone formation	Enhanced flavour and taste.
	Enzyme inactivation	Increased quality and shelf-life.
	Destruction of water-soluble vitamins	Decreased vitamin B and C.
	Maillard reaction	Lactose-protein complexes, partial loss of lysine.
	Lactose isomerisation	Formation of lactulose.
Homogenisation	Fat globule disruption	Smaller fat globules with new interface.
	Dispersion of CN micelles	Formation of fat-protein complexes.
	Activation of some enzymes	Oxidised taste, rancid taste.

Drying	Maillard reaction	Lactose-protein complexes, partial loss of lysine.
Storage – cold	Dissolution of CN micelles	Aggregation of CN and calcium phosphate.
	Fat crystallisation	Alteration of the milk fat globule membrane.
	Lipolysis	Free fatty acids, rancid taste, oxidative off flavour.
	Proteolysis	Peptides, free amino acids.
– heat	Reactivation of enzymes	Organoleptic defects.
	Growth of psychrotrophic bacteria	Bitter taste due to proteolysis
	Destruction of water-soluble vitamins	Decreased vitamin B and C.
	Age gelation	Formation of protein-mineral complexes.

2.3.1 Thermal processing

Thermal processing of milk is performed to ensure biological safety and to prolong shelf life (Xiong *et al.*, 2020). Pasteurisation and sterilisation are the two main types of heat treatment. Pasteurisation is typically conducted at lower temperatures over a longer period of time, for example 72 °C for around 15 sec or 63 °C for 30 mins which makes milk stable at 4 °C for a few weeks, whilst sterilisation such as ultra-high-temperature processing (UHT) exposes milk to higher temperatures for a shorter period of time, for example above 140 °C for around 4 sec which makes it stable at ambient temperature for months (van Lieshout *et al.*, 2020; Wada & Lönnerdal, 2014). Thermal processing of milk is known to cause denaturation, aggregation, and post translational modifications (PTMs) such as glycation of milk proteins (Michalski & Januel, 2006; van Lieshout *et al.*, 2020; Wijayanti *et al.*, 2014).

Denaturation of proteins involves the unfolding of native protein structures, a reversible condition; but this is typically followed by aggregation which renders it irreversible (Wijayanti *et al.*, 2014). The extent of denaturation of milk proteins is temperature dependent. The degree of correlation between temperature and denaturation is still unclear, with some studies reporting little difference in denaturation between raw and pasteurised milk (Patel *et al.*, 2006; Qi *et al.*, 2015; Wada & Lönnerdal, 2014) whilst others show significant loss of native protein content in pasteurised milk (Xiong *et al.*, 2020). Differences between milk samples processed at ≤75 °C and at higher temperatures (>75 °C) such as UHT milk were more profound and consistent between studies. For example Qi *et al.* (2015) demonstrated that based on the

densitometry of the bands from SDS-PAGE gels the level of β-LG decreased from 50% in raw and pasteurised whey to 35% for UHT whey samples and Xiong *et al.* (2020) showed a significant decrease in the native antibacterial proteins in milk samples treated above 75 °C. A potential explanation for the differences in the literature regarding denaturation between raw and pasteurised milk is the holding times used in the studies. Different holding times ranging from 2 sec to 30 mins were utilised over a range of temperatures from 65 to 151 °C (Li *et al.*, 2019; Patel *et al.*, 2006; Qi *et al.*, 2015; Wada & Lönnerdal, 2014; Xiong *et al.*, 2020), comparable to industrial heating conditions. However, Oldfield *et al.* (1998) used a range of temperatures (70-130 °C) and holding times (3 sec-30 min) to show that the concentration of native whey proteins decreased with heating time at all temperatures studied.

Milk proteins have high heat sensitivity, and some are more sensitive than others. CNs are more thermally stable than whey proteins as they do not show the typical denaturation and aggregation characteristics when heated. This is attributed to their lack of tertiary structure. In contrast, whey proteins are susceptible to thermal processing due to their globular structure; it is believed that all whey proteins are fully denatured when exposed to 90 °C heat for 10 mins (Qian *et al.*, 2017). However, whey proteins vary in their susceptibility to heat. Xiong *et al.* (2020) used MS to determine the order of heat sensitivity of some common whey proteins and showed that lactoperoxidase was denatured at the lowest temperatures followed by lactotransferrin, immunoglobulin, bovine serum albumin, α-LA and β-LG. Many other studies focused only on the major whey proteins, α-LA, and β-LG, with the consensus being that α-LA is less heat sensitive than β-LG (Oldfield *et al.*, 1998; Qi *et al.*, 2015).

The aggregation of whey proteins with other whey proteins or with CNs typically coincides with denaturation via sulfhydryl or disulphide interchange reactions, hydrophobic and electrostatic interactions. The aggregation of β-LG mainly involves the formation of intermolecular disulphide linkages at 70-130 °C and α-LA aggregation, particularly below 80 °C, appears to involve hydrophobic interactions (Oldfield *et al.*, 1998). Aggregation is a consequence of heat-induced denaturation where internal disulphide bridges within protein molecules are broken followed by intermolecular disulphide bridge reformation. In skim milk, heat-induced binding of denatured proteins to CN micelles provokes pH-dependent increased micelle size and inter-micelle interactions. Heating induces complex formation between κ-CN and whey proteins such as β-LG or α-LA via hydrophobic interactions and disulphide bond formation (H. Liu *et al.*, 2019).

Heating has also been shown to cause non-enzymatic PTMs such as glycation, oxidation, cyclisation and racemisation of amino acids (van Lieshout *et al.*, 2020). Glycation (or Maillard reaction products) is the most researched PTM in milk. The Maillard reaction in milk typically involves a Schiff base reaction between the reducing sugar, lactose, and the NH_2-group of the essential amino acid lysine (Van Boekel, 1998; van Lieshout *et al.*, 2020). Methods used to analyse Maillard reaction products include a shift in molecular weight, decrease in available amino-groups and detecting unmodified lysine (van Lieshout *et al.*, 2020). New methods are developed regularly for the quantitation and characterisation of Maillard reaction products in milk, with continual improvement in MS playing a key role. However, novel Maillard reaction products and pathways are still being determined which hinders the ability to quantitatively analyse them (Renzone *et al.*, 2015; van Lieshout *et al.*, 2020).

The type of heating utilised affects the degree of glycation and the sites that are glycated. Fenaille *et al.* (2005) showed a positive correlation between protein carbonyl content (Maillard reaction product) and thermal treatment with the average protein carbonyl content being two-fold higher in UHT samples compared to pasteurised ones (milk powders) (Fenaille *et al.*, 2005). Milkovska-Stamenova and Hoffmann (2016) also showed that more severe heating treatments, such as those used in UHT processing and infant formula production, increased both the degree of lactosylation (Maillard reaction product) and number of detected lactosylation sites, compared to pasteurised milk. These modifications are believed to negatively affect digestion of these proteins by reducing susceptibility to proteolysis by digestive enzymes that cleave at lysine (e.g. trypsin) and in some cases increase the proteins allergenicity (Wada & Lönnerdal, 2014).

The effects of thermal processing in milk have been most heavily studied in cow milk, however, studies are present in other ruminant species. For example studies on goat milk have shown that although heat treatment causes similar denaturation and aggregation effects to cow milk, the distribution and formation of the whey-CN complexes are slightly different (Pesic *et al.*, 2012). Heat-induced complexes in goat milk were only micelle bound whereas in cow milk they were also found in the serum phase of milk. Whilst whey-CN complexes in cow milk mainly involved β-LG, α-LA and κ-CN, in goat milk they also involved α_{s2}-CN and β-CN. Xiangying Li *et al.* (2020) also reported that heat treatment caused significant changes to the structural and functional properties of goat milk proteins related to the denaturation and

aggregation of CN and whey proteins. In sheep milk studies have shown that the level of denaturation and association with CN micelles for β-LG and α-LA increased with increasing heating temperature (Pan *et al.*, 2022). Heating resulted in an increase in the size of the CN micelles. This is thought to be due to aggregates forming between whey proteins and κ-CN depleted CN micelles (Pan *et al.*, 2023). Meanwhile Li *et al.* (2023) observed that the heat treatment of deer milk also increased the size of the CN micelle as observed in other species milk. Deer, goat, and sheep milk have also been reported as being less stable to heat treatments than cow milk and thus less suited to UHT treatment (Li *et al.*, 2023; Pan *et al.*, 2023; Raynal-Ljutovac *et al.*, 2007).

2.3.2 Homogenisation

Homogenisation prevents the separation of the fat phase in the milk by emulsifying the fat globules. Homogenisation can be carried out by: 1) forcing pressurised milk (8-20 MPa) between a valve needle and seat to dramatically reduce fat globule size due to shear stress, inertial forces and cavitation; 2) microfluidisation where milk is forced under high pressure in the reaction chamber and is divided into two jets colliding at 180 °; or 3) high pressure homogenisation which is operated at a higher pressure (>50-100 MPa) (Michalski & Januel, 2006).

Homogenisation causes disruption of the milk fat globules which leads to redistribution of the proteins on the fat globule surface (Michalski & Januel, 2006). Studies have shown that homogenisation of cow milk results in changes to the protein composition as well as significant secondary structure loss when combined with UHT treatment (Qi *et al.*, 2015). However, homogenisation alone, or combined with pasteurisation, only caused disruption in the tertiary structural environment of whey proteins. Lopez (2005) found that homogenisation of cow milk reduced the average size of the native fat globule which increased the fat surface area and caused partial disruption of the MFGM as well as adsorption of milk proteins. The homogenisation pressure is believed to affect the size of the CN micelle diameter, with a higher pressure causing smaller average CN diameters. Sandra and Dalgleish (2005) showed no reduction in micelle diameter at 41 MPa, however, increasing pressure to 114 MPa and 186 MPa significantly reduced the micelle diameter.

Chen *et al.* (2019) demonstrated that homogenised goat milk had smaller protein particle sizes that were more evenly distributed, with an increase in the regular arrangement of secondary structures. Significant changes were found in the homogenised-treated goat milk proteome that were related to glycolysis/gluconeogenesis metabolism. Patrignani *et al.* (2007) and Sert *et al.* (2023) found that high pressure homogenisation of sheep milk resulted in a decreased size of fat globules and increased surface area of fat globules. This leads to a redistribution of proteins on the surface, increases protein solubility and denaturation, changes the properties of CN micelles, activates milk enzymes as well as facilitates interactions between proteins and fat globules and makes the proteins more susceptible to proteolysis.

To date limited information is available about the effect of homogenisation on deer milk. Li *et al.* (2023) showed an increase in CN micelle size from 195 nm for raw deer milk to 244 nm for heated-homogenised deer milk (200/50 bar, 95 ºC for 5 mins). However, this increase is suggested to be due to the effects of denaturation and aggregation associated with heating rather than due to homogenisation. Due to the tendency to pair homogenisation with heat treatment it is often hard to distinguish what is caused by the heat treatment and what is a result of homogenisation.

2.4 Digestion models

Milk processing treatments, such as homogenisation and heat treatment, can influence the physical properties of milk, including its protein structures and micelle size. These changes may impact the behaviour of milk during digestion. For example, homogenisation and heat-induced denaturation could alter the accessibility and solubility of milk proteins, affecting their breakdown and absorption in the gastrointestinal tract. Understanding how milk processing influences protein structure is crucial, as it directly ties into how these proteins are digested and utilised by the body.

Digestion begins with oral digestion in the mouth and involves breaking food structures down into digestible chunks through mechanical action (chewing) and mixing with saliva. This is followed by gastric digestion in the stomach where the food is subjected to low pH for a varying length of time before moving to the small intestine (intestinal digestion) where it is neutralised and subjected to duodenal, jejunal and ileal environments on its passage to the large intestine (Singh *et al.*, 2015; Wickham *et al.*, 2009). Proteins are released from food during all stages of

ingestion and digestion depending on their solubility and accessibility. They are further broken down by different enzymes such as amylase during oral digestion, pepsin in the stomach (gastric digestion), pancreatic proteases such as trypsin and chymotrypsin in the duodenum, and additional proteases in the mucous layer covering the epithelium before being absorbed (intestinal digestion) (Antalis *et al.*, 2007; Singh *et al.*, 2015; Wickham *et al.*, 2009).To properly utilise proteins as a source of amino acids in the body, the proteins need to be digested to free amino acids, di- and tripeptides as well as bioactive and immunologically active peptides (Giromini *et al.*, 2019).

Understanding the fate of proteins during digestion is important for understanding the basis of food allergies and diseases, generation of bioactive peptides and to aid in the development of new nutritious food products (Singh *et al.*, 2015; Wickham *et al.*, 2009). A range of simulated digestion models including *in vivo* and *in vitro* methods have been developed which enable the study of how the structure of food is changed during digestion as well as the nutrient release and digestion, as shown in Table 7.

Table 7. Types of simulated digestion models.

Type	Features	Pros	Cons	Examples used in milk digestion
In vivo				
Human models	Utilises methods to either directly sample and/or visualise human stomach contents.	Most representative.	Expensive, Invasive, Ethical issues.	Pennings *et al.* (2011) Sullivan *et al.* (2014)
Animal models	Utilises either cannulation or slaughter methods to directly sample stomach contents.	Easier to access test subjects, Ability to do invasive tests.	Variability, Ethical issues, Expensive.	Dalziel *et al.* (2017) Miralles *et al.* (2020)
In vitro				
Static	Utilises laboratory-based methods to simulate enzymatic and pH changes of oral, gastric, and intestinal stages.	Simple, Relatively quick, Inexpensive, Ideal for assessments of digestibility of	No absorption, Does not mimic physical processes of the GIT.	Aalaei *et al.* (2021) Almaas *et al.* (2006) Hodgkinson *et al.* (2018) Kopf-Bolanz *et al.* (2014)

		isolated allergenic proteins.		Tagliazucchi *et al.* (2018)
Semi-dynamic	Utilises laboratory-based methods to simulate enzymatic, pH changes and physical movement of oral, gastric, and intestinal stages. Usually only the gastric stage simulates movement.	Enables evaluation of changes occurring in food structure and disintegration. Assessment of the rate and extent of nutrient bioaccessibility. Does not consume high amounts of expensive enzymes. Easy to operate.	Variability due to certain parameters such as amount of food, number of gastric emptying aliquots and mixing speed have to be fixed. Emptying could result in loss of sample. Non-physiologically relevant mechanical forces. Oral and intestinal phases are static.	Mulet-Cabero, Torcello-Gómez, *et al.* (2020)
Dynamic	Utilises laboratory-based methods to simulate enzymatic, pH changes and physical movement of oral, gastric and/or intestinal stages.	More accurately mimics human digestion as it mimics peristaltic movement.	Complex, Time consuming, Less accessible, Large quantities of expensive enzymes utilised, Often does not factor in absorption.	Deglaire *et al.* (2019) Xing Li *et al.* (2020) Sánchez-Rivera *et al.* (2015) Ye *et al.* (2016b)

2.4.1 *In vivo* digestion

Studying digestion *in vivo* involves examining the process of digestion in either human or animal subjects. Multiple techniques have been developed for examining the processes of digestion in humans, including methods to directly sample the gastric or intestinal contents using either a nasogastric or nasojejunal tube (Boutrou *et al.*, 2013; Sullivan *et al.*, 2014), visualisation of the stomach contents/processes via capsule endoscopy or magnetic resonance imaging (Mackie *et al.*, 2013; Sullivan *et al.*, 2014), and wireless telemetric systems such as the SmartPill® which can measure the gastrointestinal pH, temperature and pressure (Koziolek *et al.*, 2015). Although human studies are the most representative way for analysing the processes of human digestion, they are often not feasible. These experiments are often

expensive, invasive (such as the nasojejunal tube which can be painful), cause vomiting and non-migration through the pylorus in some people (Boutrou *et al.*, 2013) and are difficult to carry out. Thus they typically only have a small sample size and are used for when a very specific question needs answering or to compare with other experiments such as *in vitro* models (Sullivan *et al.*, 2014).

In vivo animal models are often used as an alternative due to the greater availability of test subjects and the ability to perform invasive tests and extensive tissue sampling. The ideal animal model for studying the digestion of food products should be simple, cost-effective, and not labour-intensive, while also being capable of detecting both stimulation and inhibition of digestive tract motility (Merwid-Ląd *et al.*, 2009). Additionally, selecting an animal model requires careful consideration of the research question to determine the most appropriate model, or whether an animal model is necessary at all. Common animal models include rodents such as rats and larger animals including pigs, chickens, dogs, and monkeys (Bornhorst & Singh, 2014; Ziegler *et al.*, 2016). In particular, pigs are often considered the best choice as they have many fundamental anatomical (glandular-type stomach), physiological, genomic, proteomic, immunologic and nutritional similarities to human beings (Ziegler *et al.*, 2016). Meals may be mixed with an indigestible marker, such as chromium oxide or titanium oxide, to determine the proportion of the original meal that is collected at each location. The collection usually involves either animal death or surgical approaches in which cannulas are placed into digestive organs to access the contents of the gastrointestinal tract (Bornhorst & Singh, 2014). Although cannulation enables sampling from the same animal over a period of time and the ability to reduce inter-animal variability by applying different experimental treatments to the same animal, the surgery required to insert the cannulas can cause changes in gastrointestinal secretions and motility, altering flow patterns and modifying the digestion process (Bornhorst & Singh, 2014; Knudsen *et al.*, 2006). The slaughter technique enables sampling from many different locations in the gastrointestinal tract but does not enable multiple sampling of the same animal which may increase the number of animals needed to produce statistical significances (Bornhorst & Singh, 2014; Knudsen *et al.*, 2006). The main experimental disadvantages of animal models include the great interspecies variability and that they may differ greatly in comparison with human subjects. Especially in recent years the ethical issues with these experiments have also been brought into question; thus, like with human studies, they are usually only used for comparison with *in vitro* models or when *in vitro* methods cannot fully answer a research question (Miralles *et al.*, 2020).

2.4.2 Static *in vitro* digestion

In vitro digestion is a technique that simulates the digestion process outside the human body by providing required conditions. They are often utilised as they do not require advanced techniques, such as magnetic resonance imaging or invasive techniques, are more cost effective and associated with fewer ethical concerns. Static *in vitro* digestion models in the literature are extremely diverse from single static models to multi-compartmental models. The various parameters such as the steps included in the digestion sequence *e.g.* oral, gastric, small intestinal and large intestinal and chemical composition of digestive solutions *e.g*. enzyme type and concentration, salts and buffers are also widely different in the literature, making it difficult to compare studies (Lucas-González *et al.*, 2018). Table 8 outlines some of the key features of a few of the static *in vitro* digestion methods found in the literature.

The INFOGEST static digestion protocol was established in 2011 by a collection of scientists from a range of disciplines including food science, nutrition, gastroenterology, and immunology to address this diversity in protocols. It was developed to provide a standardised *in vitro* digestion model that would enable studies to be compared between laboratories. It was published in 2014 by Minekus *et al.* (2014) and according to Scopus has received 3422 citations as of the 30th October 2023. Since then different iterations have been bought out including the 2016 model by Egger *et al.* (2016) which aimed to remove inter-lab variability and the 2019 version by Brodkorb *et al.* (2019) which further improved the protocol. In this improved method, food samples are subjected to sequential oral, gastric and intestinal digestion with parameters such as electrolytes, enzymes, bile, dilution, pH and period of digestion based on available physiological data (Brodkorb *et al.*, 2019). The method can be used to assess the endpoints resulting from digestion of foods by analysing the digestion products for example peptides/amino acids however it is not suitable for simulating digestion kinetics and does not show the dynamic nature of gastric secretion.

Table 8. Examples of static *in vitro* digestion methods.

Method	Description	Advantages	Disadvantages	References
One-step assay	Incubate directly with enzymes e.g., pepsin, trypsin, papain.	Direct comparison of treatments on single foodstuff.	Lower digestible protein values than those obtained *in vivo*,	Boucher *et al.* (2009)

			becomes more comparable if multiple enzymes used.	
pH Drop	Modified digestibility assay involving dropping the pH and plotting pH curves.	Good correlation with *in vivo* data. Reproducible.	Some food stuffs interfere with pH drop due to buffering capacity.	Almaas *et al.* (2006)
Immobilised digestive enzyme assay system	Stepwise assay involving acid solubilization, pepsin digestion, neutralization, trypsin, chymotrypsin, and intestinal peptidase digestion followed by analysis of newly exposed α-amino groups.	Estimates digestibility of protein and amino acids in feed. Good correlation with *in vivo* data.	Takes longer than other methods (2.5 days) and employs complicated system of proteases immobilised to glass beads (a kit is available which only takes 1 day).	Boucher *et al.* (2009)
INFOGEST™	Food samples are subjected to sequential oral, gastric and intestinal digestion with parameters such as electrolytes, enzymes, bile, dilution, pH and period of digestion based on available physiological data.	Standardised protocol developed to address variation in methodology.	Does not account for physical processes that occur in the stomach. Not suitable for simulating digestion kinetics.	Minekus *et al.* (2014) Egger *et al.* (2016) Brodkorb *et al.* (2019)

2.4.3 Dynamic *in vitro* digestion

Dynamic *in vitro* digestion models, in comparison, are highly complex, time-consuming, require higher amounts of expensive enzymes and are less accessible than static models. Although static models do not factor in the removal of digesta (absorption) or physical processes that occur during digestion such as shearing, mixing, hydration and changing conditions over time, dynamic model designs do attempt to simulate the changes in the physicochemical conditions that occur during digestion such as pH, ionic strength and digestive enzyme concentrations (Dupont & Mackie, 2015; Wickham *et al.*, 2009). At least 12 different dynamic *in vitro* digestion models have been developed over the last 30 years, with some being developed as commercially available simulators (e.g. TIM, SHIME) as outlined in Table 9. These models vary depending on how the stomach contents are mixed, how the gastric pH is controlled, and emptied, the digestive secretions utilised as well as whether there is a joint intestinal component (Dupont *et al.*, 2019; Sensoy, 2021).

Table 9. Dynamic *in vitro* digestion models.

System	Abbr.	Digestion compartment	References
Human gastric simulator	HGS	Gastric	Kong and Singh (2010) Ferrua and Singh (2015)
Dynamic gastric simulator	DGM	Gastric	Wickham *et al.* (2012)
Dynamic gastric simulation model	DGSM	Gastric	Do *et al.* (2016)
Near real dynamic *in vitro* human stomach	DIVHS	Gastric	Wang *et al.* (2019)
Human gastric digestion simulator	c-GDS	Gastric	Kobayashi *et al.* (2017)
Artificial gastric digestive system	AGDS	Gastric	W. Liu *et al.* (2019)
In vitro dynamic system	DIDGI®	Gastric Small intestinal (no absorption)	Ménard *et al.* (2014)
Engineered stomach and small intestinal system	ESIN	Gastric Small intestinal	Guerra *et al.* (2016)
TNO gastrointestinal model	TIM	Gastric Small intestinal TIM-2 includes large intestine	Minekus *et al.* (1995)
Simulator of the gastrointestinal tract	SIGMI	Gastric Small intestinal (no absorption) Large intestine	Barroso *et al.* (2015)
Simulator of the human intestinal microbial ecosystem	SHIME®	Gastric Small intestinal (no absorption) Large intestine	García-Villalba *et al.* (2017)
Artificial colon	ARCOL	Large intestine	Deschamps *et al.* (2020)

A large proportion of the dynamic models only simulate the mechanical, dynamic, and chemical conditions of the stomach including the HGS, DGM, DGSM, DIVHS, c-GDS and AGDS (Table 9). These models differ in the mechanical methods used for secreting, mixing, and emptying the stomach contents. For example, the HGS model uses peristaltic pumps for secreting and emptying stomach contents, whilst the c-GDS uses a diaphragm pump for secreting fluids into the simulated stomach and a syringe pump to empty the contents. Only a few models simulate both the stomach and small intestine (DIDGI®, ESIN, TIM, SIGMI, and SHIME®). SIGMI, SHIME® and later models of the TIM also simulate the fermentation of

the large intestine. ARCOL only simulates the fermentation of the large intestine. Table 10 outlines the key features, advantages, and disadvantages of four dynamic *in vitro* digestion models. The HGS model has recently been used with a higher frequently for analysing the digestion of milk proteins (Li, Pan, *et al.*, 2022; Roy *et al.*, 2021; Wang *et al.*, 2018; Ye *et al.*, 2019), is included alongside the DGM, TIM, and DIDGI® models. These four models were selected for detailed analysis out of the twelve available options based on their widespread use, especially in milk digestion studies, and ability to simulate key aspects of digestion, including both biochemical and mechanical processes. Together, they represent a range of approaches and capabilities, offering insights into gastric and intestinal processes, while addressing diverse research needs. This focused selection ensures a clear and concise evaluation while highlighting the most prominent and widely applicable methods in the field.

Table 10. Key characteristics of four dynamic *in vitro* digestion methods.

Method	Description	Advantages	Disadvantages
DGM	Simulates both biochemical and mechanical processes during human gastric digestion. pH affects flow rate of acid secretions.	Simulates gastric mixing, transit, and breakdown forces within the normal physiological range. Enables fed and fasted state comparisons and studies of the impact of different meals/food items on dosage form behaviour. Accurate simulation of gastric dissolution.	Does not account for oral or small intestinal phases of digestion. No way to alter emptying based on gastric phase behaviour. Emptying is based on average properties of the meal.
HGS	Flexible, outer vessel is used to simulate stomach with rollers controlled by a variable-speed motor to simulate peristaltic contractions. Gastric emptying is achieved through distal tube covered by a mesh with 1 mm openings.	Study both physical and chemical breakdown of food. Gastric secretion rate, pH and gastric emptying can be controlled. Large meal volumes of up to 1L can be used.	Mixing and physical property changes of sample meals need to undergo more complete validation with *in vivo* data. Does not account for oral or small intestinal phases of digestion.
TIM	Compartments for stomach, duodenum, jejunum and ileum connected by peristaltic valves. Successive dynamic conditions in the upper GI tract are simulated. Computer software accurately and reproducibly control the system.	Simulate accurately the dynamic physiological GI conditions. Handle specific food ingredients, drugs as well as complete meals. Simulate average GI conditions, biological variation, and disease conditions for different age groups. Can be used for a broad scope of applications. Samples can be collected from the compartments during transit of the chyme analysis.	No feedback on energy density of the food on the GI conditions. No intestinal mucosa, absorption studies limited.

		Highly reproducible.	
DIDGI®	Focuses on upper parts of digestive tract (stomach and small intestine). Computer controlled system reproduces gastric and intestinal transit times, kinetics of gastric and intestinal pH, sequential addition of digestive secretions and stirring of stomach and small intestine contents. Each compartment is surrounded by a glass jacket filled with water pumped using a temperature-controlled water bath.	Robust. Handle foods up to 200g. Transparent compartments enable viewing of real time digestion.	A basic form of Mixing is used and not realistic. Absence of nutrients absorption in the small intestine.

2.4.4 Semi-dynamic *in vitro* digestion

Semi-dynamic *in vitro* digestion protocols bridge the gap between static and dynamic techniques by combining the best aspects of both models. Mulet-Cabero, Egger, *et al.* (2020) developed a standardised semi-dynamic model that builds upon the harmonised INFOGEST static model and includes gradual acidification, fluid and enzyme secretion and emptying. This enables the study of structural changes of foods during simulated gastric digestion, evaluating the effect of the matrix on food disintegration and nutrient delivery to the small intestine. The advantages of this method include the ability to evaluate the changes occurring in the food structure and disintegration. It also allows assessment of the rate and extent of bioaccessibility, does not consume large amounts of expensive enzymes and is easy to operate. Lambers *et al.* (2023) used a semi-dynamic in vitro digestion model to show that experimental formula exhibited higher initial protein digestion during simulated gastric digestion than the intact milk protein control formula, as indicated by a larger proportion of smaller peptides and higher levels of available amino groups. The disadvantages include its variability due to parameters such as the amount of food and number of gastric emptying aliquots, as well as potential losses due to gastric emptying, non-physiologically relevant mechanical forces, and that the oral and intestinal phases are static (Mulet-Cabero, Egger, *et al.*, 2020).

2.4.5 Validation of *in vitro* digestion models

A wide variety of *in vitro* digestion models have been used to analyse milk proteins throughout digestion. To determine the physiological relevance of these *in vitro* models they are typically compared to either human or animal *in vivo* models, with a focus on the gastric and intestinal endpoints. Studies which investigate the effects of *in vitro* and *in vivo* digestion directly within one paper are limited and these studies typically focus on only one component of the food

matrices such as proteins. The INFOGEST static *in vitro* digestion model is the most studied. It has been compared to pigs for both micellar CN and skim milk (Egger *et al.*, 2019; Egger *et al.*, 2017; Miralles *et al.*, 2020) and to human jejunal effluents for whey and CN powders (Sanchón *et al.*, 2018). Validation of dynamic *in vitro* digestion models within a single paper are uncommon. However, some examples do exist for example, Ménard *et al.* (2014) compared the digestion of infant formula in a simple dynamic model and a piglet model. The results show a good correlation of the kinetics of residual immunoreactivity of β-LG and CNs between the two systems. Ye *et al.* (2019) used both the HGS model and rats to analyse the digestion of pasteurised and UHT whole milk. The results illustrated that processing treatment of the milks showed similar trends in both digestion models. Studies have also compared *in vitro* digestion models. For example Sousa *et al.* (2023) found that the digestible indispensable amino acid score (DIAAS) of several food products digested using the INFOGEST model was comparable to *in vivo* results reported in the literature. Similarly, Dupont *et al.* (2019) reviewed the outcomes of eight different dynamic digestion models, employing a similar methodology. They compared the validation of these models to in vivo digestion results from the literature, using parameters such as intragastric pH, mean breaking time, and gastric emptying rates.

Comparisons between *in vitro* digestion models are uncommon. Most studies focus on comparing *in vitro* models with *in vivo* digestion techniques, particularly using animal models for validation. However, a few exceptions exist. For example, a comparison study between static and dynamic digestion by Egger *et al.* (2019) found that protein hydrolysis kinetics were comparable between the first 30 minutes of digestion in the static system and after 60 minutes in the dynamic system (marked by the disappearance of CNs), whilst β-LG was persistent in both systems during the gastric stage but was hydrolysed during the intestinal stage. The gradual peptide generation for both static and dynamic digestion was comparable especially in the gastric phase. Mulet-Cabero, Egger, *et al.* (2020) demonstrated that the standardised semi-dynamic digestion model and the HGS used by Ye *et al.* (2017) had similar coagulation behaviour, timing, consistency of gastric digesta and protein digestion with samples of processed milk.

In vitro methods, even complex dynamic methods, are unable to simulate the complex system of digestion in its entirety (Butts *et al.*, 2012). Gut microbiota and the diverse impacts that antinutritional factors and dietary fibre have on the digestive tract and its processes are difficult to simulate (Butts *et al.*, 2012). Characteristics of *in vitro* digestion such as digestion time,

enzyme contents or enzyme composition must be adjusted to suit the sample being digested regardless of method utilised, for example food samples that contain starch need to have amylase present, a mastication step that is required in solid food samples is not needed for liquid, and different systems need to be implemented for infant versus adult versus elderly versions in regard to meal size, time and pH (Abdel-Aal, 2008).

2.5 Tools to study food-protein digestomics

To investigate how proteins are digested, a range of *in vivo* and *in vitro* methods have been described in section 2.4. However, difficulties arise when analysing the protein "digestome" generated from simulated gastrointestinal digestion of foods due to the complexity of food matrices, the large variety of digestion products generated from the simultaneous action of proteases with diverse cleavage specificity and the interference of endogenous compounds in biological fluids (Picariello *et al.*, 2013). During most digestion stages there is a complex mixture of thousands of protein fragments, polypeptides, oligopeptides, and amino acids released. This mixture is further complicated by a range of post-translational processing such as phosphorylation, glycosylation and disulphide bridges (Mamone *et al.*, 2009). A range of chemical and biochemical techniques have been utilised to investigate these mixtures or components of these mixtures such as chemical assays, liquid chromatography, capillary electrophoresis, mono-dimensional or two-dimensional polyacrylamide gel electrophoresis, ELISA, and MS with direct infusion or coupled with a separation technique (e.g. CE or LC). Figure 2 illustrates how these techniques can be used to study and characterise protein digestomes. They are typically utilised to investigate gastrointestinal digestion kinetics in particular the protein-digestion patterns and peptide generation as well as stability of food allergens. However, one clear disadvantage of many of these techniques is the lack of (gels) or limited (chromatography and immunochemistry) information regarding the identification of the digestion products, especially for uncommon amino acids in the absence of adequate standards (Picariello *et al.*, 2013).

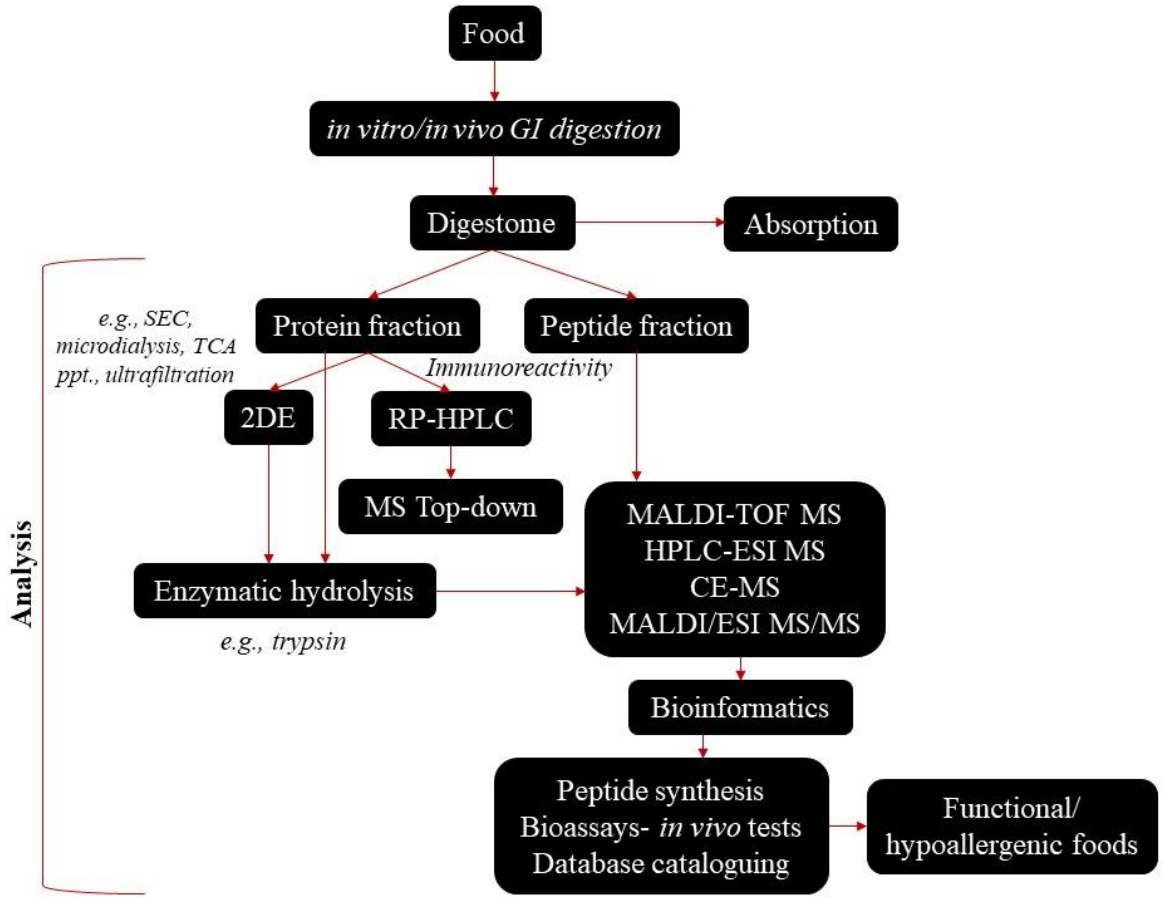

Figure 2. Analytical workflow for the characterization of protein digestomes adapted from Picariello *et al.* (2013).

MS is an analytical tool that is used to measure the mass to charge ratio (m/z) of one or more molecules present in a sample. This enables the identification of unknown compounds via molecular weight determination, quantification of known compounds and the determination of the structure and chemical properties of molecules. MS has increasingly been used in recent years to study protein and peptide digestomics. MS, used independently or in conjunction with other techniques, reduces a lot of the previous limitations. Figure 3 illustrates the increasing use of MS in food protein digestion studies, with an almost 4-fold increase in publications between 2010 and 2020. In contrast to other techniques, MS enables the detection and identification of proteins or peptides independent of antibody specificity, which is especially important when studying ruminant species which have a lack of available antibodies (Picariello *et al.*, 2013). Typically, 'bottom-up' proteomic workflows are utilised prior to MS analysis of milk proteins, which involves fragmenting the proteins into individual peptides then stitching the sequences together to reveal the protein identity and/or characteristics (Gundry *et al.*, 2010). This is also important for digestomics as the proteins are already in a hydrolysed form. This

workflow involves several key steps including preparation of protein sample for digestion, enrichment for any particular peptides of interest and clean up or desalting of the final peptide mixture prior to MS analysis (Gundry *et al.*, 2010).

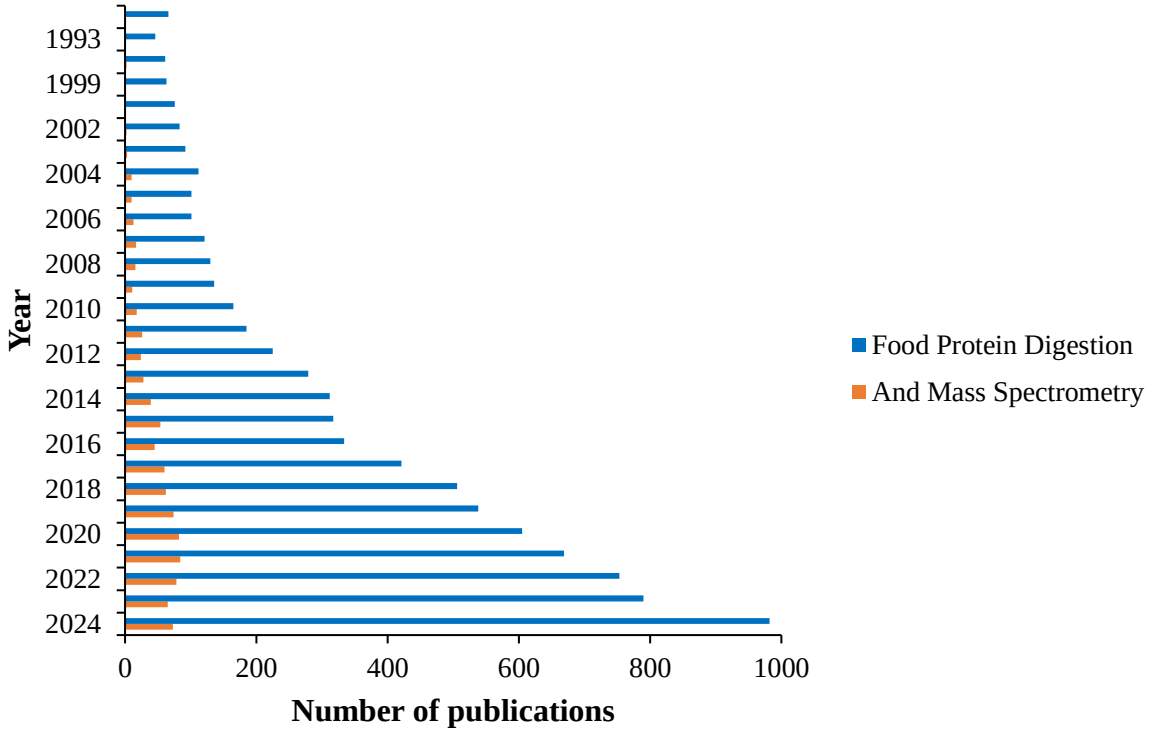

Figure 3. Number of scientific papers retrieved from the PubMed database using the search key "food protein digestion" or "food protein digestion" AND "mass spectrometry" within the period 1991 and 2024.

A wide range of mass spectrometers are available. The three main components that make up a mass spectrometer are the ionisation source, mass analyser and ion detection system. Two ionisation methods that are routinely used for studying proteins and peptides are electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI). These techniques are both sensitive and specific (Picariello *et al.*, 2013; Siuzdak, 2005). ESI produces gaseous ionised molecules directly from a liquid solution using an electric field. It is often paired with high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), whilst MALDI is thought to cause ionisation and transfer of a sample from the condensed phase to the gas phase via laser excitation and vaporisation of the sample matrix (Siuzdak, 2005). Both ESI and MALDI have been utilised in studying milk proteomics and digestions. One study by Mollé *et al.* (2009) compared ESI and MALDI ionisation techniques on cow milk fractions.

Although the presence of basic amino acid residues Lys and Arg and the ratio of Lys/Arg was not significantly different between the two techniques ESI identified more hydrophobic peptides with a wider mass coverage whilst MALDI identified more basic and smaller peptides (Mollé *et al.*, 2009). Thus, it was concluded that a combination of ionisation sources can aid in increasing global proteome coverage.

MS analysers separate ionised molecules according to their m/z ratio. The four most commonly used in peptidomics, and proteomics are quadrupole (Q), ion trap (IT), time-of-flight (TOF) and Fourier-transform ion-cyclotron resonance (FTICR) analysers. These analysers differ in terms of ion separation and analytical performance. Hybrid and multi-stage instruments have been designed to combine the capabilities of different mass analysers e.g., Q-TOF, TOF-TOF and LTQ-Orbitrap.

Other features that can differ between the machines used is the type of dissociation used. Electron transfer dissociation provides increased sequence coverage for small to medium sized peptides and complements conventional collision-induced dissociation (CID) for 'bottom-up' protein identification. Electron capture dissociation produces richer fragmentation of peptide spectra and preserves the labile PTMs. The type of tandem MS data acquisition that is utilised also affects the number of peptides and proteins that are identified. Data dependent acquisition (DDA) is more frequently used and involves fragmenting peptides within a certain mass range. It favours the most intense peptide ions and typically misses low abundance peptides. Data independent acquisition (DIA) is a more recent development in which all peptides within a narrow mass range are fragmented, including low abundance ones. Sun *et al.* (2023) used both DDA and DIA to determine the quantitative differences of goat milk throughout lactation. 311 proteins were identified using DDA whilst 344 proteins were identified using DIA.

2.6 Digestion of milk proteins

2.6.1 Digestion of ruminant milk proteins in humans

After samples have been obtained from any of the digestion models described above, they can be analysed to determine information on protein behaviour during digestion, hydrolysis, peptide formation and absorption. Among the ruminant milks, the most-studied species in terms of digestion is cow due to its high economic value, however, the increased interest in lower-allergenic alternative milks and functional food products means that research regarding

sheep and goat milk digestion has increased. Since the dairy industry for deer milk is relatively new and not widespread there is only limited research regarding deer milk digestibility. Even though there is a disparity in the level of research regarding protein digestibility in ruminant species, differences have been noted regarding gastric clot appearance, protein hydrolysis and peptide kinetics.

During gastric digestion of milk, a clot is formed in the stomach, driven by low pH and enzymatic restructuring of CN micelles by pepsin (Ye *et al.*, 2016a). Wang *et al.* (2018) showed that no clot was formed during *in vitro* digestion even after 220 min digestion of whey protein isolates, whereas skim milk powder and milk protein concentrate formed a clot in approximately 10 min. Coagulation during gastric digestion has been observed for cow, goat, and sheep milk (Roy *et al.*, 2021). However, the physical properties of the clot are thought to differ between species. This is believed to be due to differences in the milk composition between the different species. In particular lower protein content, lower CN-to-whey-protein ratio, and higher β-CN to α_s-CN ratio is thought to cause soft curdling (Roy *et al.*, 2020a). Cow milk is known to form firm curds in the stomach whilst goat milk forms soft curds. This is thought to be due to lower CN content or larger CN micelle size of goat milk compared with cow milk (Claeys *et al.*, 2014; Roy *et al.*, 2020a). Ambrosoli *et al.* (1988) reported that goat milks with high α_{s1}-CN levels produced a firmer curd than those with lower levels. Other factors have also been shown to influence curd formation such as pH and heat treatment (Ye *et al.*, 2019). Most studies investigating curd formation and appearance are related to cheese making rather than to curd formation during digestion (Pazzola, 2019). However, in the last few years several studies have compared the structural and rheological properties of clots formed by different ruminant milks during digestion (Li, Pan, *et al.*, 2022; Roy *et al.*, 2021). At the time of this review, no studies investigating curd formation characteristics of deer milk have been reported. The clot also influences the gastric digestion of both skim and whole milk, as with increasing digestion time the structure of the clot tightens and becomes less permeable to serum and solutes which affect the hydrolysis of CNs and whey proteins by pepsin (Ye *et al.*, 2016a, 2016b)

CNs are thought to have a slower gastric emptying rate compared to whey proteins due to the formation of the clot during the gastric stage (Ye *et al.*, 2016a). This affects the amino acid levels in the plasma after consumption, which in turn affects body protein breakdown and synthesis. Postprandial protein kinetics studies have shown that CNs contribute to a low, slow

and prolonged release of amino acids into the plasma which inhibits protein breakdown whilst whey proteins cause a high, rapid and transient increase in plasma amino acids which promotes protein synthesis (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Mulet-Cabero, Torcello-Gómez, *et al.*, 2020).

Interestingly, studies which investigate protein hydrolysis using SDS-PAGE gels, immunoblotting and LC-MS/MS during digestion rather than plasma amino acid levels suggest that CNs are hydrolysed more rapidly than whey proteins. For example, various studies have shown that CNs are typically digested within the first 30 to 60 min of gastric digestion, depending on whether a static or dynamic *in vitro* digestion system is used (Dupont, Mandalari, Mollé, *et al.*, 2010; Egger *et al.*, 2019; Kopf-Bolanz *et al.*, 2014; Tunick *et al.*, 2016). In contrast, intact β-LG is still present at the end of gastric digestion and some studies report seeing low amounts even after intestinal digestion (Dupont, Mandalari, Mollé, *et al.*, 2010; Egger *et al.*, 2019; Kopf-Bolanz *et al.*, 2014; Tunick *et al.*, 2016), a fact that is attributed to this protein having a structure that is resistant to pepsin hydrolysis. Most studies investigating hydrolysis only study CNs and β-LG, likely due to their higher abundance in milk samples than other proteins. The methods used to analyse digestion are predominantly SDS-PAGE gels and immunoblotting to study protein hydrolysis and MS to study peptide kinetics which favour highly abundant proteins. Studies that report on the abundance of other proteins have less consistency regarding their hydrolysis rate. Both Almaas *et al.* (2006) and Kopf-Bolanz *et al.* (2014) used SDS-PAGE gels to show that various whey proteins including α-LA and serum albumin were rapidly hydrolysed. Conflicting with these results, Hodgkinson *et al.* (2018) and Inglingstad *et al.* (2010) used a combination of SDS-PAGE gels and MS to instead show that α-LA is resistant to hydrolysis.

The efficiency of protein hydrolysis and peptide profiles generated during digestion have been shown to vary between different ruminant species. For example, both Almaas *et al.* (2006) and Hodgkinson *et al.* (2018) have reported that proteins from goat milk are digested more efficiently than proteins from cow milk. In another study it was shown that milk proteins in deer were digested faster by commercial proteolytic enzymes than cow, with a higher number of peptides produced. Although the digestibility of CNs, α-LA and immunoglobulin were higher in deer milk than cow milk, β-LG appeared resistant in both species (N. Vithana *et al.*, 2012; Vithana, 2012). However, these results have not always been conclusive, especially between cow and goat milk, as other studies have shown that more CN from goat milk remained

undigested after 30 minutes of digestion compared to cow milk CN (Inglingstad *et al.*, 2010). Other studies have shown similarities in the protein hydrolysis profile of both the clot and liquid chyme of cow, sheep, and goat milk (Roy *et al.*, 2021). Studies comparing the digestion of milk from the different species mainly only include goat and cow milk. There are fewer studies that include sheep milk or deer milk.

The contradictory observations above are likely due to differences in experimental methodology, design and aims. The studies that investigate plasma amino acid levels tend to be done *in vivo* and measure amino acid absorption, whilst studies that investigate protein hydrolysis tend to be performed *in vitro* and study the digesta released during different stages of gastric and intestinal digestion, which does not take into consideration the proteins still found in the clot. Studies that investigate the proteins from the clot directly via SDS-PAGE gels indicate that it consists almost entirely of CNs and, with the exception of κ-CN which rapidly decreases during the first 20 min, the other CNs (α_s- and β-CN) do not have significant changes in abundance throughout the digestion (Ye *et al.*, 2016b). Protein hydrolysis studies via SDS-PAGE gel, whether from the clot or the released digesta, only investigate the amount of intact protein in a sample not whether it is in the state ready for absorption into the bloodstream.

Experimental methodology differences between papers studying digestion may contribute to differences in results observed for milk protein digestibility. Differences include: starting material (individual proteins, protein powders, milk powders and fresh milk), digestion method (*in vivo* in humans, pigs and rats, static, semi-dynamic and dynamic *in vitro* systems for adults and infants) and even different enzyme sources and concentrations (for example, some papers use human proteolytic enzymes whilst others used pepsin from porcine gastric mucosa) (Almaas *et al.*, 2006; Hodgkinson *et al.*, 2018). Table 10 shows a summary of some of these differences found regarding α-LA hydrolysis.

Table 11. Summary of experimental methods for protein hydrolysis that include α-LA.

Author	Source	Digestion Model	Enzymatic source	Results
Almaas *et al.* (2006)	Skimmed milk	Static *in vitro* digestion using pH drop method	Human digestive enzymes	α-LA rapidly hydrolysed during gastric stage
Hodgkinson *et al.* (2019)	Raw milk	Static *in vitro* digestion with infant conditions	Pepsin from porcine gastric mucosa, 3203 U/mg	No peptides derived from α-LA post digestion
Inglingstad *et al.* (2010)	Pasteurised skim milk	Static *in vitro* digestion using 2-step assay	Human digestive enzymes	After gastric stage α-LA remained almost intact
Kopf-Bolanz *et al.* (2014)	Raw milk	Static *in vitro* digestion using micro scale method	Unspecified source, conc. 2.5 g/L	No intact α-LA after gastric stage
Ye *et al.* (2016b)	Skimmed milk	Dynamic *in vitro* digestion using human gastric simulator	Pepsin from porcine gastric mucosa, 800-2500 U/mg	α-LA remained largely intact in during entire digestion process

2.6.2 Effects of processing on digestion

Processing treatments such as heating and homogenisation have been shown to affect the clot appearance, protein hydrolysis and peptide kinetics of milk during digestion. To date most of the studies investigating the effects of processing have been done on cow milk, with very few on milk of other species such as goat, sheep, and deer.

Processing affects the appearance of milk curds under gastric conditions. Unprocessed milk tends to form a firm curd; however, processed milk tends to form a soft curd clot with a loose structure. This is seen for both heat treatment and a combination of homogenised and heat-treated samples and is seen in both *in vivo* and *in vitro* studies using cow (Ahlborn *et al.*, 2023; Ye *et al.*, 2016b; Ye *et al.*, 2019), goat (Xiangying Li *et al.*, 2020), and sheep milk (Pan *et al.*, 2021). The more highly processed the milk, the softer the curds. For example, Ye *et al.* (2019) showed that this effect was more profound for UHT milk compared to pasteurised milk.

Processing also affects the rate of protein hydrolysis as well as susceptibility of proteins to digestion. Heated milk has been shown to have a faster rate of protein hydrolysis during digestion, potentially due to the looser structure of the clot (Ye *et al.*, 2016b; Ye *et al.*, 2019). Heating is thought to reduce CNs susceptibility to hydrolysis and increase that of β-LG

(Sánchez-Rivera *et al.*, 2015). This is thought to be due to the denaturation and aggregation of these proteins caused by heating, which increases the accessibility of protein cleavage sites to pepsin.

Although only a few papers have investigated the differences in peptides produced between processed and non-processed milks they have indicated that there are differences in the pattern of the peptides released (Sánchez-Rivera *et al.*, 2015). These authors found that the peptide homologies in samples at 4, 50 and 405 min were 34%, 37% and 48%, respectively, between heated and non-heated samples. Certain regions of proteins have also been identified as resistant to digestion, including amino acids 125-135 of β-LG and 76-93, 126-140 and 190-209 of β-CN (Picariello *et al.*, 2010; Sánchez-Rivera *et al.*, 2015).

2.6.3 Other factors affecting digestion

As well as the species the milk is from and processing treatments, various other factors (such as structure) affect digestion. In recent years more researchers have investigated how the structure and type of food influence digestion properties including coagulation, rate of protein hydrolysis and bioactive peptide formation. Nguyen *et al.* (2020) investigated milk and yogurt from cow, goat and sheep and demonstrated that in yogurt β-CN and β-LG were digested faster and released a higher concentration of anti-hypertensive peptides.

2.7 Summary of literature

A multitude of studies have been done investigating the proteomic composition of cow milk during digestion to determine digestibility, typically via SDS-PAGE gels to measure protein abundance during digestion or peptidomics analysis using MS. The effect of different processing treatments of milk has also been investigated using similar means. Fewer studies have been undertaken on other ruminant species, in particular deer which only has a handful of compositional studies performed. Due to the different methodologies being utilised in these studies, such as different *in vitro* or *in vivo* digestion protocols, different milk sources and different MS capabilities, clear distinctions in how these proteins are digested has been difficult to determine. This is especially the case in studies between different species. Therefore, it is important to investigate how processing treatments affect protein structure and digestion of milks from different ruminant species using a consistent protocol. This will enhance the

knowledge of how protein structures differ in these different milks and aid in the development of new nutritional and easily digestible milk products.

Chapter 3 - Materials and Methods

3.1 Introduction

The samples utilised in this study were collected, processed and digested as part of the broader "New Zealand Milk Means More" (NZ3M) study, funded by the Ministry of Business, Innovation and Employment (MBIE). This larger initiative aimed to explore the nutritional and functional properties of milk from various ruminant species, providing a comprehensive understanding of milk digestion and its implications for human health.

To investigate these properties, a series of experiments were conducted to characterise the structural and functional attributes of milk from different species. Compositional analyses revealed that protein content was highest in deer milk (7.2%), followed by sheep (5.4%), cow (3.46%) and goat (3.06%), as reported by Li, Pan, *et al.* (2022) and Li *et al.* (2023). Clot analyses were then performed to compare coagulation behaviour, assessing how differences in protein composition and processing treatments influenced clot formation (Li, Pan, *et al.*, 2022). These findings provided insights into the digestive behaviour and potential impacts on nutrient release and absorption. Beyond clotting properties, protein composition was further examined using SDS-PAGE gels, which enabled comparisons of casein and whey protein distributions across different processing treatments, for sheep (Pan *et al.*, 2021) and goat (Li, Ye, *et al.*, 2022) milk. These analyses highlighted the impact of species-specific variations and processing on protein integrity. Building on these findings, *in vivo* digestion studies using a pig model were conducted to assess the digestion kinetics of processed cow milk (Ahlborn *et al.*, 2023). These experiments provided valuable data on protein breakdown, peptide release and nutrient absorption, bridging the gap between *in vitro* models and physiological responses in a living system.

Expanding on these foundational investigations, the present study utilised these milk samples that were collected and processed under controlled conditions to assess the effects of species-specific differences and processing treatments on protein hydrolysis during digestion. This research advances prior work by integrating advanced analytical techniques, including mass spectrometry and size exclusion chromatography, to achieve a more detailed characterisation of milk protein breakdown.

Milk digestion was systematically evaluated using both *in vitro* and *in vivo* approaches. The *in vitro* digestion experiments were performed using a human gastric simulator (HGS) to replicate physiological gastric and intestinal conditions, while complementary *in vivo* digestion studies were carried out using a pig model. These methodologies provided a robust framework for analysing milk protein hydrolysis, offering new insights into the bioavailability and functional properties of dairy proteins from different sources.

The following sections outline the procedures followed for milk collection, processing, and digestion. Section 3.2 details the sourcing, treatment, and storage of milk from cow, sheep, goat, and deer, as well as the acquisition of commercially processed milk samples. Section 3.3 details the *in vitro* digestion protocol using the HGS model and the *in vivo* digestion approach using a pig model. These methodologies were designed to ensure consistency, reproducibility and relevance to real-world digestive processes, facilitating a robust comparison of milk protein hydrolysis across species and processing conditions.

3.2 Milk supply and initial processing

3.2.1 Milk samples and processing treatments of different ruminant species

Pooled raw whole milk samples from four ruminant species (cow, sheep, goat, and deer) were obtained under chilled (4 ºC) conditions from different farms within New Zealand. All milk samples were collected during mid-lactation. Cow milk was collected at Massey University No. 4 dairy farm (Palmerston North, New Zealand) in the 2020-2021 season. Sheep milk was sampled in Waikato, New Zealand, by two suppliers: Spring Sheep Milk Co. and Maui Milk Co., Ltd. Fresh tank milk from both suppliers was mixed in a 1:1 (wt/wt) ratio on sampling day. Goat milk was provided by Cilantro Cheese Ltd. (Hamilton, New Zealand) and deer milk was provided by Pāmu Farms (Wellington, New Zealand).

Milk from each species was collected from bulk tanks, simulating a factory-like scenario, and thus potentially contained milk from multiple milking times and individuals. The raw milk was kept chilled (4 ºC) immediately after collection and during transport to ensure minimal microbial growth and compositional stability. Processing and *in vitro* digestion of the milk samples occurred at the Riddet Institute, Massey University, New Zealand. The processing treatments were conducted within three days of collection, and the processed milk was stored

in a chill room (4 ºC) until digestion experiments, which were completed within a fortnight of processing.

For each species, three separate batches of raw milk were collected on different days, processed, and used in the digestion experiment. The three batches for each species were processed independently to ensure replicates for each species and processing treatment. The processing treatments were performed on the tubular indirect UHT plant. Five different treatments were applied to the milk samples: (1) raw (unprocessed), (2) pasteurised (75 °C, 15 seconds), (3) pasteurised-homogenised (20/5 MPa, 75 °C, 15 seconds), (4) heated-homogenised (20/5 MPa, heated to 95 °C rapidly and held in an external water bath at 95 °C for 5 minutes) and (5) UHT (140 °C for 5 seconds). The UHT treatment was only performed on cow milk due to the lower heat stability of the other species (Raynal-Ljutovac *et al.*, 2007). After heating, the processed milks were immediately cooled to 20 °C. Sodium azide (0.02% wt/wt) was then added to preserve the milk. For storage between processing and digestion the milks were kept at 4 ºC.

3.2.2 Commercially sourced milk samples

Six batches of two commercially sourced milk samples: Anchor Blue standard milk (pasteurised-homogenised) and Anchor Blue UHT long-life milk (UHT) were obtained from Fonterra, New Zealand. *In vivo* digestion using a pig model was performed on all six batches as described in section 3.3.2 whilst *in vitro* digestion was performed in triplicate on batch 6 as described in section 3.3.1.2.

3.2.3 Collection of deer milk throughout the different lactation stages

Deer milk was provided by Pāmu, sampled from a farm in Gore, New Zealand. The herd consisted of 120 lactating does. Samples of deer milk were collected at eight different dates throughout lactation (Table 12). The initial four timepoints were frozen at the farm at -20ºC from the start of milking whilst the remaining four timepoints were processed according to section 3.2.1 before being frozen. Milk was collected from the bulk tank, ensuring that it was representative of the herd. Different lactation timepoints were collected to investigate the lactational impact of deer milk as, unlike for cow milk, this factor has not been well studied. Once obtained, the frozen samples were transported chilled and stored at -80ºC to maintain the integrity of the milk for further analysis.

Table 12. Timepoints throughout lactation.

Date	Week of lactation
23/11/2020	3
7/12/2020	5
21/12/2020	7
4/01/2021	9
18/01/2021	11
01/02/2021	13
15/02/2021	15
22/02/2021	16

3.3 Digestion of milks

3.3.1 *In vitro* digestion using human gastric simulator

3.3.1.1 *In vitro* digestion of milk from different mammalian species

Milk samples were collected and processed according to section 3.2.1 prior to being digested at Riddet Institute, Massy University as part of the wider NZ3M project. A diagram of how this experiment was designed is outlined in Figure 4. *In vitro* gastric digestion was carried out using a human gastric simulator (HGS) (Kong & Singh, 2010). A protocol as described by Li, Pan, *et al.* (2022) was used and is detailed below.

The electrolyte compositions of the simulated salivary fluid and simulated gastric fluid were prepared as described by Brodkorb *et al.* (2019). The oral phase of digestion was performed by mixing simulated salivary fluid with milk in a 1:1 (wt/wt) ratio to the solids content of the milk (Mulet-Cabero, Egger, *et al.*, 2020). The simulated salivary fluid additions were 28, 35, 24 and 50 g for cow, sheep, goat, and deer milk, respectively, based on the approximate solids content in 200 g of each milk. No amylase was added to the simulated salivary fluid due to the absence of starch in the samples. A 1.25x concentrated electrolyte simulated gastric fluid was prepared and adjusted to pH 1.5. Before the start of the gastric phase, milk was warmed to 37 °C and mixed with 20 mL of simulated gastric fluid (including pepsin) to mimic the basal level in the fasted state, which is in the physiological range (Ulleberg *et al.*, 2011).

During gastric digestion, the electrolyte simulated gastric fluid, pepsin (P7000, determined pepsin activity 432-550 U/mg, Sigma-Aldrich, St. Louis, MO, USA) and calcium chloride

solution (1.5mM) were gradually added to obtain the correct compositions of the final simulated gastric fluid (pepsin activity 2000 U/mL) with a final pH of 2 after 240 mins of digestion. The HGS simulated the peristaltic movement in the stomach chamber at a frequency of 3 times per min. The addition rate of simulated gastric fluid and empty rates varied depending on the species. This accounted for the different buffering capacities of the milks to enable similar pH profiles with a final pH of 2.0. Both goat and cow had an addition rate of 2.5 mL/min and an emptying rate of 3.0 mL/min, sheep had an addition rate of 3.0 mL/min and an emptying rate of 3.6 mL/min and deer had an addition rate of 3.5 mL/min and an emptying rate of 4.2 mL/min (Li, Pan, *et al.*, 2022). The digesta was emptied every 20 mins through a 1 mm sieve to mimic gastric sieving. The digestion was conducted for 240 mins at 37 °C.

Gastric digesta for intestinal digestion were collected at 20, 60, and 180 min, and the pH was immediately adjusted to 7.5 using 6 M sodium hydroxide to inhibit pepsin activity, ensuring a seamless transition between gastric and intestinal phases without delay. Simulated intestinal digestion was performed on a TitroLine® 7000 (SI Analytics, GmbH, Mainz, Germany), a precision titration system that continuously monitors and adjusts pH during enzymatic digestion to maintain stable conditions, mimicking physiological processes. The digestion setup followed the protocol described by Brodkorb *et al.* (2019). Pancreatin from porcine pancreas (8 × USP, P7545) and bovine bile (B3883) purchased from Sigma-Aldrich were used. Sodium bicarbonate was replaced in the simulated intestinal fluid with sodium chloride to avoid pH drift during digestion as recommended by Brodkorb *et al.* (2019). Intestinal digesta were collected at 10 and 60 mins as shown in Figure 4.

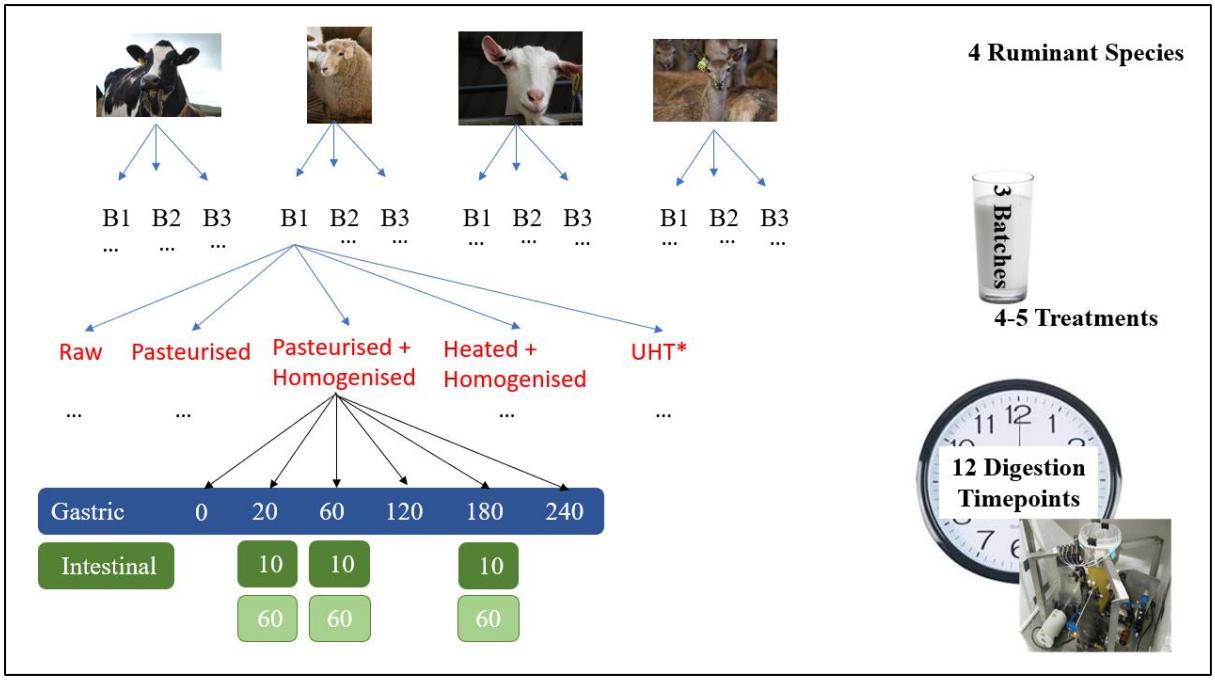

Figure 4. Outline of samples used to compare peptide/protein digestion of milk from four different ruminant species: samples from three different batches (B1-3) of milk were either untreated (raw) or treated by pasteurisation, pasteurisation with homogenisation, heated and homogenisation or UHT followed by simulated *in vitro* digestion using the HGS model. Samples were taken at 0 (milk prior to digestion), 20, 60, 120, 180 and 240 mins during the gastric stage and a subset of these were taken for intestinal digestion for periods of 10 or 60 mins. *UHT treatment only available for cow milk.

3.3.1.2 *In vitro* digestion of commercially processed milk

Commercially processed cow milks (section 3.2.2) were collected and digested at Riddet Institute, Massey University as part of the wider NZ3M project. *In vitro* gastric digestion was carried out using a HGS as described in section 3.3.1.1 using parameters for cow milk. 28 g of simulated salivary fluid was added into 200 g of milk, the addition rate of the simulated gastric fluid was 2.5 mL/min, and the emptying rate was 3.0 mL/min. Gastric digesta for intestinal digestion were collected at 20, 60, and 120 mins. The times taken during the intestinal stage were aligned with the times taken during the *in vivo* digestion study which were 60, 120 and 180 mins. A diagram of how this experiment was designed is provided in Figure 5.

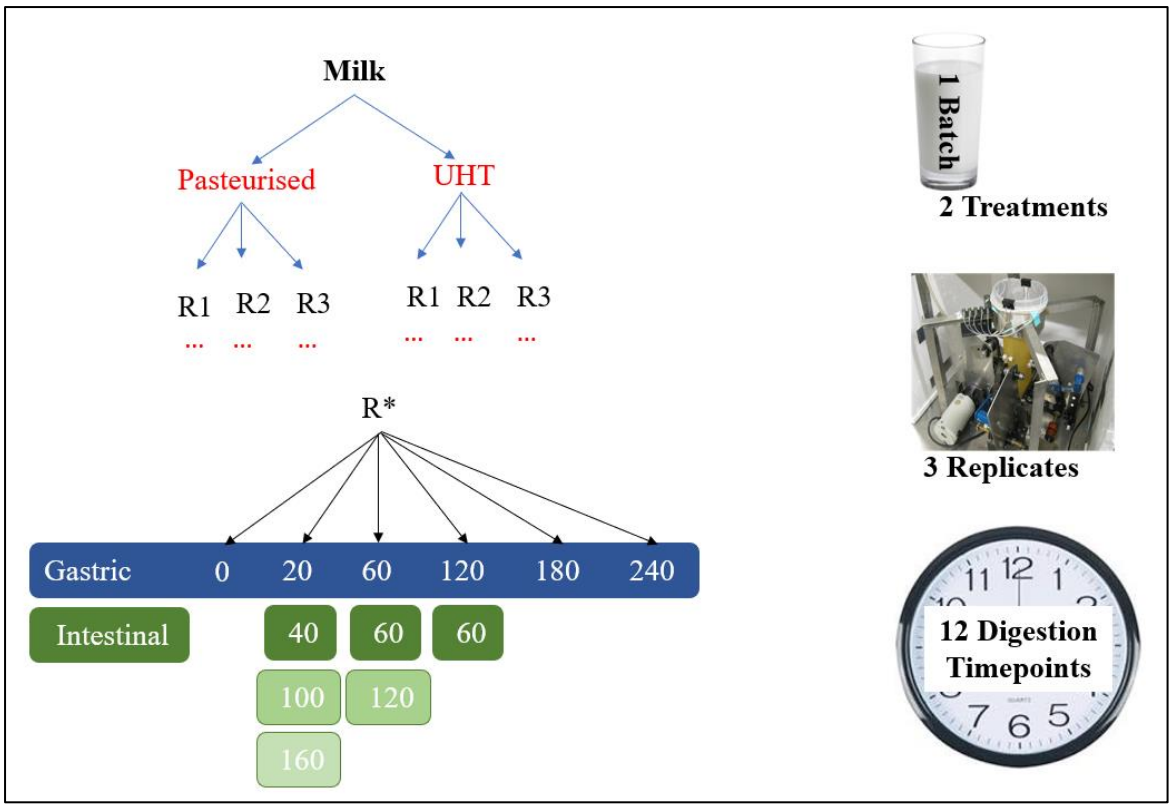

Figure 5. Outline of samples for *in vitro* digestion using HGS: commercially available milk from a single batch, treated by either pasteurisation or UHT was digested *in vitro* using the HGS method, with three replicates (R1-3). Samples were taken at 0, 20, 60, 120, 180 and 240 mins during the gastric stage and a subset of these were taken for intestinal digestion.

3.3.2 *In vivo* digestion using pigs

Freeze-dried stomach contents from pigs fed two different types of commercially produced milk were provided by Massey University as part of the wider NZ3M project. A diagram of how this experiment was designed is shown in Figure 6. *In vivo* digestion was performed using the method described by Ahlborn *et al.* (2023). A summary of the methodology used is outlined below.

The pig study approved by Massey University Animals Ethics Committee (MUAEC no. 19/83) took place between November 2019 and February 2020. Locally sourced male Large White x Landrace pigs weighing approximately 23 kg (9 weeks old) were housed in individual metabolic cages at Massey University, Palmerston North. Pigs (n = 12) were randomly allocated to each dietary treatment and fed the experimental diet for ten days. The commercial diet was gradually substituted by the experimental diets, which consisted of one liquid milk meal (500 mL bovine milk, breakfast) and two solid human-type meals (lunch and dinner). The

human-type meal was formulated based on the USDA food chemical composition data and met the nutrient requirements for growing pigs (NRC, 1998). Meal sizes (lunch and dinner) were calculated to provide 4% of each pig's arrival body weight, on a dry matter basis.

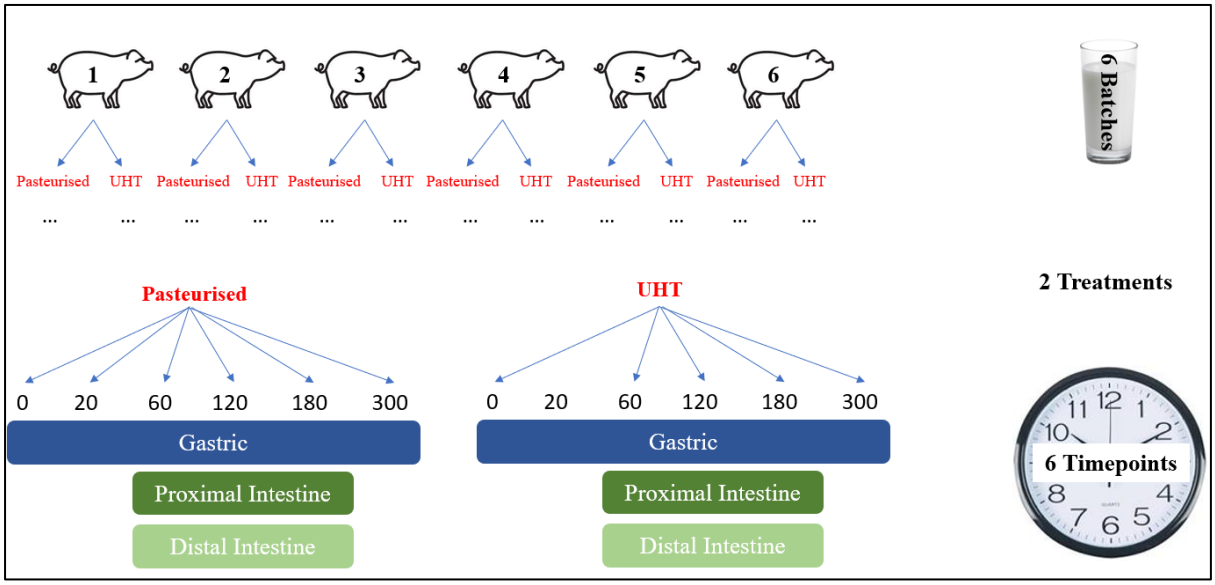

Figure 6. Outline of samples for *in vivo* digestion: 72 pigs were randomly split into 6 groups and fed either pasteurised or UHT milk; after consumption, the pigs were killed at 0, 20, 60, 180 or 300 mins and the stomach contents sampled. Intestinal samples were also taken at the 60, 120 and 180 mins time points.

On day 11, fasted pigs were sacrificed at either 0, 20, 60, 120, 180, or 300 mins after consumption of the breakfast meal (i.e., 500 mL milk or lactose solution supplemented with 1 g of titanium dioxide (TiO_2)). On sampling days, the pigs were sedated using an anaesthetic cocktail and were transported to an on-site surgery room, where they were intravenously administered a second dose of the cocktail (30 µL/kg BW) to induce deep anaesthesia. The abdomen was opened, and portal blood and lymph samples were first collected. Following this, the pigs were euthanised by an intra-cardial injection of sodium pentobarbitone (0.3 mL/kg BW of Pentobarb 300: Provet). Following euthanasia, the stomachs were clamped in the oesophagus and pylorus and carefully removed with minimal directional and orientational change to avoid mixing the stomach liquid and solid phases. The full and empty weights were recorded to determine the content at each postprandial timepoint. A single incision was made from the oesophageal to the pylori on the centre of the superior face of each stomach. The pH was then measured at the proximal and distal stomach region (even if the curd had formed). The stomach contents were poured into a 1 mm sieve, allowing the liquid portion to drain

through into a tared container holding a weighted plastic bag. The curd and liquid portions were then frozen on dry ice, kept at –20 °C, weighed, freeze-dried, weighed again and ground until particle size was a maximum of 1 mm.

Figure 7 outlines what samples were used in each chapter of this thesis, describing the species and processing treatment of the milk as well as the digestion method.

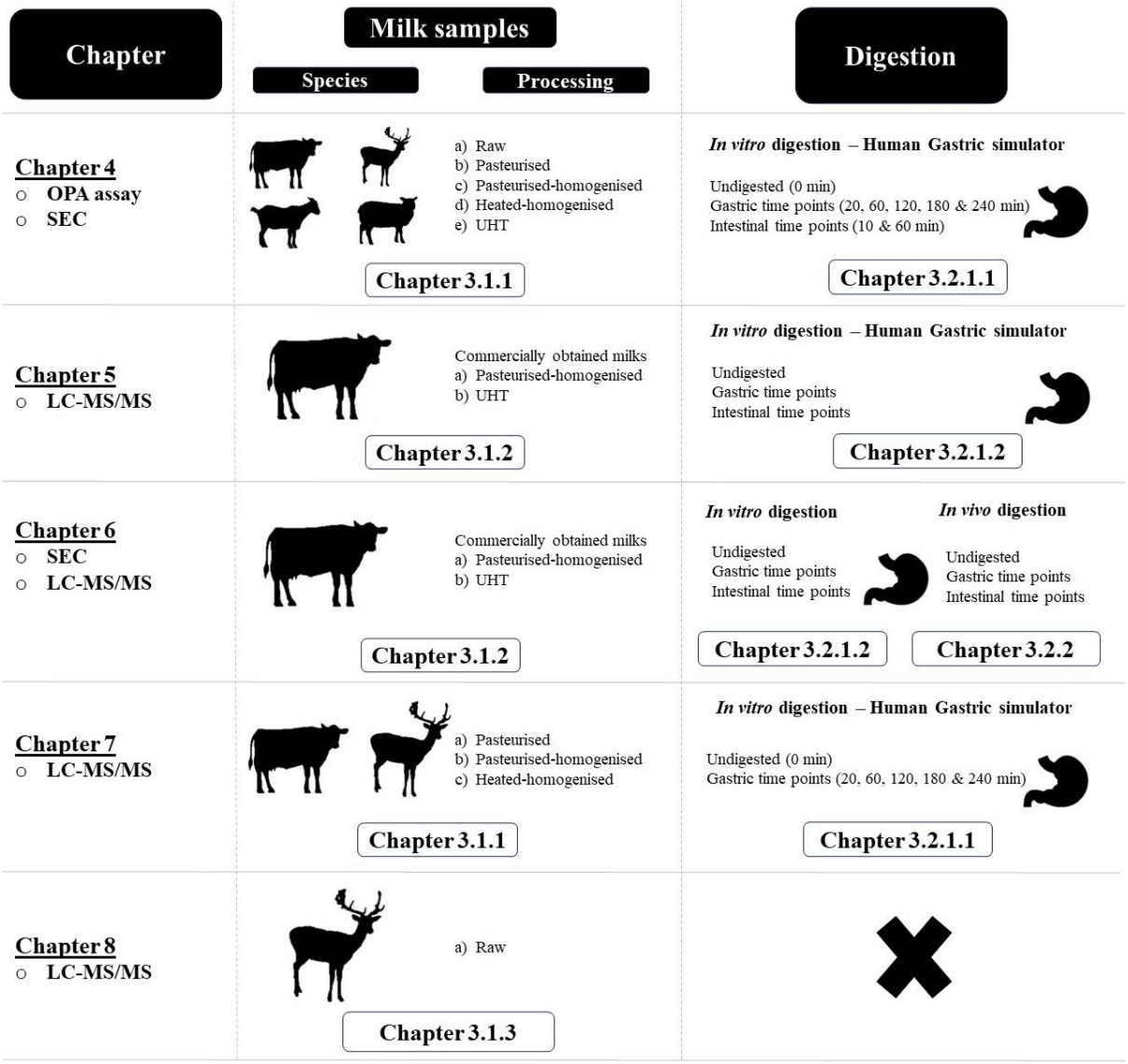

Figure 7. Layout of thesis chapters including which samples and digestion methods were used in each chapter.

Chapter 4 - Comparison of digestibility of proteins in processed cow, goat, sheep, and deer milks

4.1 Introduction

Digestibility is often a term used to assess the quality of a dietary protein. It assesses both the amino acid composition and the proportion of amino acids available for digestion and subsequent absorption (Rieder *et al.*, 2021). A wide range of techniques have been developed to assess the digestibility of dietary proteins. These include the protein efficiency ratio (PER), which is calculated as the gain in body weight divided by the amount of protein consumed (Bender, 1956), protein digestibility corrected amino acid scores (PDCAAS) and digestible indispensable amino acid scores (DIAAS), which use amino acid scoring patterns in combination with protein digestibility based on true faecal nitrogen digestibility or true ileal digestibility of individual amino acids, respectively (Rutherfurd *et al.*, 2015).

Degree of hydrolysis, which is defined as the percentage of cleaved peptide bonds, is commonly used to assess digestibility. Techniques used to measure this include pH stat, trinitrobenzensulphonic acid (TNBS), o-phthalaldehyde (OPA), soluble nitrogen after trichloroacetic acid precipitation (SN-TCA) and formal titration. However, the accuracy of these methods is highly dependent on the type of hydrolytic enzyme, hydrolysed peptide size and reaction temperature (Rutherfurd, 2010). Gel electrophoresis can be used to monitor the degradation of specific proteins, however, peptides generated during digestion are usually too small to be detected. MS is also able to assess digestibility of protein. It can be used to identify peptides released during digestion and is typically used to search for potentially allergenic or bioactive peptides (Mamone *et al.*, 2009; Picariello *et al.*, 2013).

Size exclusion chromatography (SEC) with UV detection is a well-recognised analytical tool for measuring molecular weight (MW) distributions of protein digests (Johns *et al.*, 2011; Wubshet *et al.*, 2017) and has been applied to estimate the proportion of peptides with specific size ranges generated during simulated digestion by partial area integration (Le Roux *et al.*, 2020). SEC is increasingly being used in studies to assess protein digestibility. For example, Yi *et al.* (2021) used it to determine differences in the degree of protein hydrolysis between different protein hydrolysates, Rieder *et al.* (2021) used it to estimate the proportion of small

peptides potentially available for uptake from different food sources including milk, and Sousa *et al.* (2023) used it to assess the comparability between *in vitro* and *in vivo* digestion models.

In order to quantify and compare protein digestibility of different food matrices, appropriate digestion and analytical methods are required. *In vivo* methods are more representative of digestion, however, due to their invasive and expensive nature, *in vitro* methods are typically used. Static and dynamic *in vitro* digestion models such as the harmonised international static INFOGEST model (Brodkorb *et al.*, 2019; Minekus *et al.*, 2014) and the dynamic human gastric simulator (HGS) (Kong & Singh, 2010) have been compared to *in vivo* digestion models. Results have shown that *in vitro* models give a good estimate of protein digestion in rats (Ye *et al.*, 2019), pigs (Egger *et al.*, 2019), and humans (Sanchón *et al.*, 2018). *In vitro* digestion models are often used instead of their *in vivo* counterparts due to their comparatively lower cost, reduced ethical concerns, greater experimental control and reproducibility.

Digestion and health properties of food are influenced by the composition of nutrients, food structure and processing treatments. The nutrient composition of dairy products has been shown to vary between different species, breeds, lactation stage and seasonality (Gantner *et al.*, 2015; Potočnik *et al.*, 2011; Roy *et al.*, 2020a). Processing of dairy products, such as heating, and/or homogenisation, is also known to affect the nutrient composition and structure. Denaturation of whey proteins, aggregation, post-translational modifications (PTMs), decreased size of native fat globule, increased fat surface area and changes in the milk fat globule membrane (MFGM) are some of the effects of processing. Dairy structures and processing treatments have been observed to induce specific effects on digestion rates and physiological responses (Nguyen *et al.*, 2020; van Lieshout *et al.*, 2020).

The aim of this study was to study the *in vitro* digestibility of proteins in unprocessed and processed cow, goat, sheep, and deer milks using the HGS dynamic *in vitro* digestion model. SDS-PAGE and compositional analysis have already been performed on these milk samples, as part of the wider NZ3M project, providing baseline data on protein distribution and composition prior to digestion. Given the limited number of studies utilising dynamic digestion systems – compared to the more commonly used static systems – this study provides new insights into milk protein digestibility under physiologically relevant conditions. The protein digestibility was assessed using both SEC and the OPA assay. Assays such as TNBS or OPA

are much more frequently used to measure the degree of hydrolysis, so the OPA assay was used as a method to compare the validity of the SEC method.

4.2 Materials and Methods

4.2.1 Samples

Milk from four different ruminant species sourced in New Zealand were obtained and processed for this study as described in section 3.2.1. The milks were digested using an *in vitro* HGS digestion model as described in section 3.3.1.1.

4.2.2 Reagents

Potassium dihydrogen orthophosphate was purchased from BDH, Prolabo (Poole, UK). Sodium chloride and sodium dodecyl sulphate (SDS) was purchased from Fisher Scientific (Leics, UK). 37 % hydrochloric acid was purchased from Merck (New Jersey, USA). OPA and di-sodium tetraborate was purchased from Alfa Aesar (Haverhill, MA, USA). Ethanol, di-sodium hydrogen orthophosphate, DL-dithiothreitol (DTT) and L-serine were from Sigma-Aldrich (St. Louis, MO, USA). Unless otherwise stated all chemicals and reagents were of analytical grade.

4.2.3 Size exclusion chromatography (SEC)

4.2.3.1 Sample preparation

The samples were diluted 1:1 with Milli-Q H_2O, then the protein concentrations were estimated at 280 nm using the built-in protein assay on the NanoPhotometer NP80 (Implen, Munich, Germany). An aliquot containing 1 mg of protein was dried for each sample in a CentriVap vacuum centrifuge (Labconco, Kansas City, MO, USA) at 40 ºC. The sample was resuspended in 100 µL mobile phase (0.05 M phosphate buffer / 0.3 M sodium chloride, pH 6.9). Prior to injection the samples were centrifuged in a 2 µm spin filter (PhaseSep Pty Ltd, VIC, Australia) for 2 mins at 10,000 x *g* to remove suspended solids.

4.2.3.2 SEC

SEC was performed on a Dionex Ultimate 3000 HPLC (ThermoFisher Scientific, Waltham, MA, USA) fitted with a Superdex^{TM} 30 Increase column (10 x 300 mm, 9µm particle size) (GE Healthcare, Chicago, IL, USA), which was operated at ambient temperature. 20 µL of each

sample was injected and eluted over 60 mins with SEC buffer at a flow rate of 0.8 mL/min. Detection was performed at 214 nm using a photodiode array detector (ThermoFisher Scientific, Waltham, MA, USA).

4.2.3.3 Data analysis

Protein standards of known MWs: myoglobulin (17 kDa), cytochrome-C (12 kDa), aprotinin (6.5 kDa), Gly-Gly-Gly (189 Da), Gly-Gly (132 Da) and Gly (75 Da) were separated under the same conditions to create a MW calibration curve in Chromeleon version 7.2 chromatography software (ThermoFisher Scientific, Waltham, MA, USA) as seen in Figure 8. In the Chromeleon software, each unknown sample's chromatogram was divided into 250 equal retention time slices, and the MW of each of the slices was calculated from the MW calibration curve. These results were then exported from Chromeleon and the areas and the cumulative % areas of the slices were analysed using an in-house R-script. From the latter data, the script calculated the proportions of peptides falling below designated MW limits (1 kDa and 10 kDa).

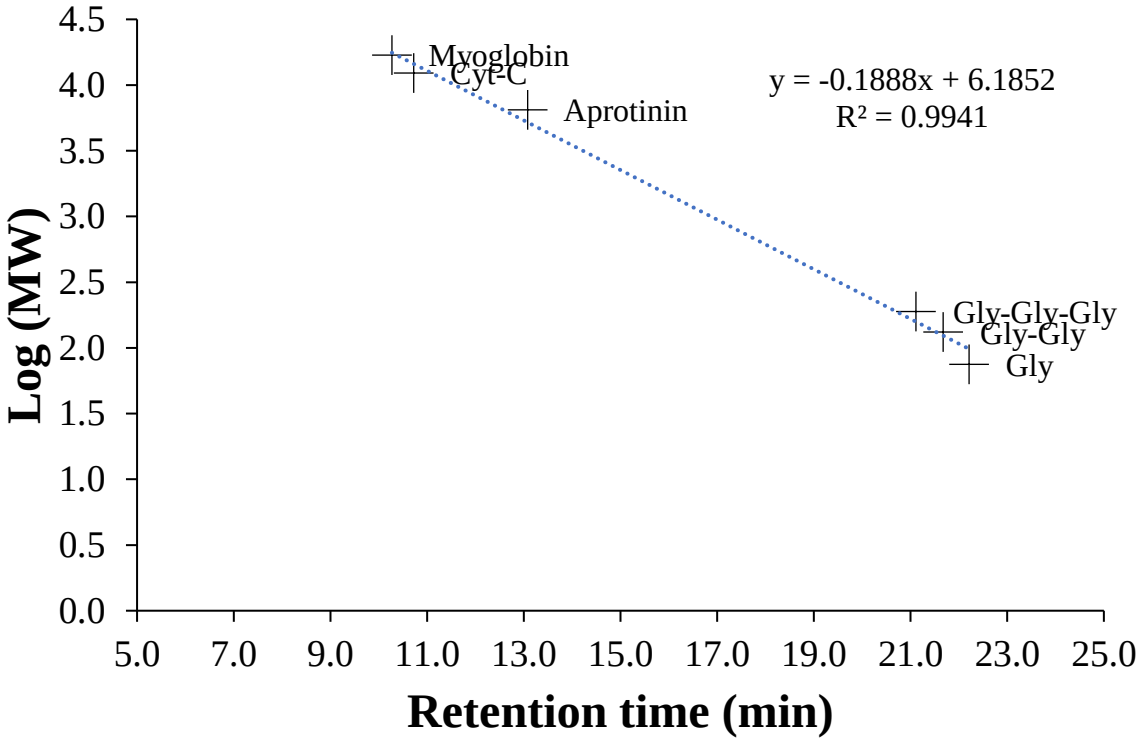

Figure 8. Molecular weight (MW) standard curve generated from SEC showing the retention time (min) on the x-axis and the log of MW on the y-axis.

In this study four metrics were trialled: weight-average MW (Mw), the total area under the curve (AUC) of each sample's cumulative % area plots, the percentage of peptides below 10 kDa (% <10 kDa) and the percentage of peptides below 1 kDa (% <1 kDa). The total AUC gives an indication of whether the overall size distribution of the sample changes and facilitates statistical comparisons of the MW distributions of samples. It must be noted however that two samples with the same total AUC could have very different proteins present, it all depends on whether there are differences in the amount and MW of the proteins present. The % of peptides <1 kDa and <10 kDa provides information on the proportion of small peptides available for uptake in the small intestine. Previous studies have assessed digestibility by the fraction of soluble peptides below 10 kDa (Le Roux *et al.*, 2020), after earlier *in vitro* studies using dialysis or ultrafiltration membranes with 10-12 kDa MW cut offs showed that this MW limit could be used to estimate the apparent digestibility of proteins and gave the best correlation with *in vivo* data in pigs (Cave, 1988; Huang *et al.*, 2000). INFOGEST also suggests that dialysis membranes or centrifugation tubes with a cutoff of 3-10 kDa are able to determine the bioaccessibility of digested nutrients (Brodkorb *et al.*, 2019). However, other studies which used the dynamics of peptide MW distributions during digestion to determine the bioaccessible peptide and protein fraction showed that the region where the intensity decreased (corresponding to hydrolysis of peptides and proteins) and increased (release of peptides) correlated to approximately 1 kDa or 8-10 amino acid residues (Duijsens *et al.*, 2023; Rieder *et al.*, 2021; Sousa *et al.*, 2023). These studies suggest that the bioaccessible peptide fraction is below 1 kDa.

Samples were randomised using Excel and, due to the large number of samples, were run in 18 equal-sized batches. Graphs and statistical analysis (two-way ANOVA) were produced in GraphPad Prism version 10.2.1 (GraphPad Software, Boston, MA, USA) (www.graphpad.com). Statistical significance was determined with p-values less than 0.05.

4.2.4 Quantification of free NH_2 groups with OPA reagent and calculation of degree of hydrolysis

The levels of free NH_2 groups were determined using the standardised OPA spectrophotometric assay in microplates as described by (Mulet-Cabero *et al.*, 2019; Nielsen *et al.*, 2001). Briefly 10 µL of sample/standard was placed in each well and mixed with 200 µL OPA reagent (40 mL of 100 mM di-sodium tetraborate with 4 mM SDS, 40 mg OPA (initially dissolved in 1 mL of

ethanol) and 44 mg DTT, made up to 50 mL with Milli-Q H_2O). The reaction was allowed to proceed for 2 mins, the absorbance was then measured at 340 nm with a Multiskan^{TM} Skyhigh microplate reader (ThermoFisher Scientific, Waltham, MA, USA). A calibration curve was prepared using serine standard solutions as seen in Figure 9. The degree of hydrolysis (DH) was calculated as follows:

$$\% \text{ DH} = 100 \text{ x} \frac{NH_2(t)}{NH_2(t0)}$$

$NH_2(t)$ was the concentration of free amines after t minutes digestion, $NH_2(t0)$ was the concentration of free amines after total acid hydrolysis (HCl 6 N, 110 ºC, 24 hours). All values were expressed as milliequivalents (mEq) of serine. All measurements were carried out in triplicate for each digesta. Graphs and statistical analysis (two-way ANOVA) were produced in GraphPad Prism version 10.2.1 (GraphPad Software (Boston, MA, USA) (www.graphpad.com)). Statistical significance was determined with p-values less than 0.05.

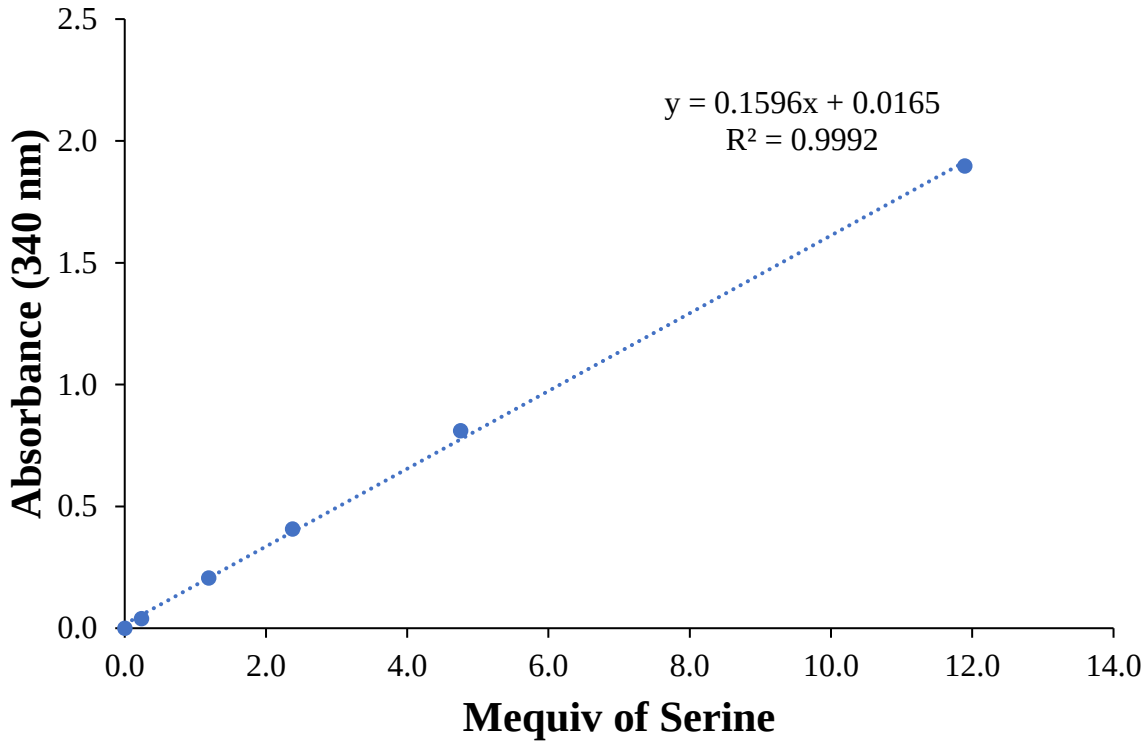

Figure 9. OPA assay standard curve showing absorbance at 340 nm on the y-axis and Mequiv of serine on the x-axis.

4.3 Results and discussion

4.3.1 SEC analysis of milk samples throughout digestion

SEC is a method that separates compounds based on their molecular size and shape. This technique has routinely been used to separate biological compounds such as proteins and peptides and measure the MW distribution of protein digests (Johns *et al.*, 2011; Wubshet *et al.*, 2017). More recently it has been used to assess the protein digestibility of different food matrices (Le Roux *et al.*, 2020; Rieder *et al.*, 2021; Sousa *et al.*, 2023). In this study we used SEC to assess the digestibility of proteins in processed milks from different species. SEC was chosen due to its simplicity, versatility and robustness over a large number of samples (Ó'Fágáin, 2017).

4.3.1.1 Peptide profiles and overall trends throughout digestion

To analyse the protein digestibility using SEC, each unknown sample's chromatogram was divided into 250 equal retention time slices, and the MW of each of the slices was calculated from MW calibration curves using Chromeleon software. An in-house R-script was then used to analyse the raw Chromeleon data and Prism was used to produce figures showing MW distributions (Figure 10) and cumulative percentage areas of the slices (Figure 11) for each of the samples. All samples show a decreasing trend of peaks in the high MW range (over 10 kDa) and an increasing trend of peaks in the low MW range (under 10 kDa) throughout digestion. This indicates that there is an increase in the amount of small peptides and a decrease in amount of proteins and large peptides identified in the emptied digesta.

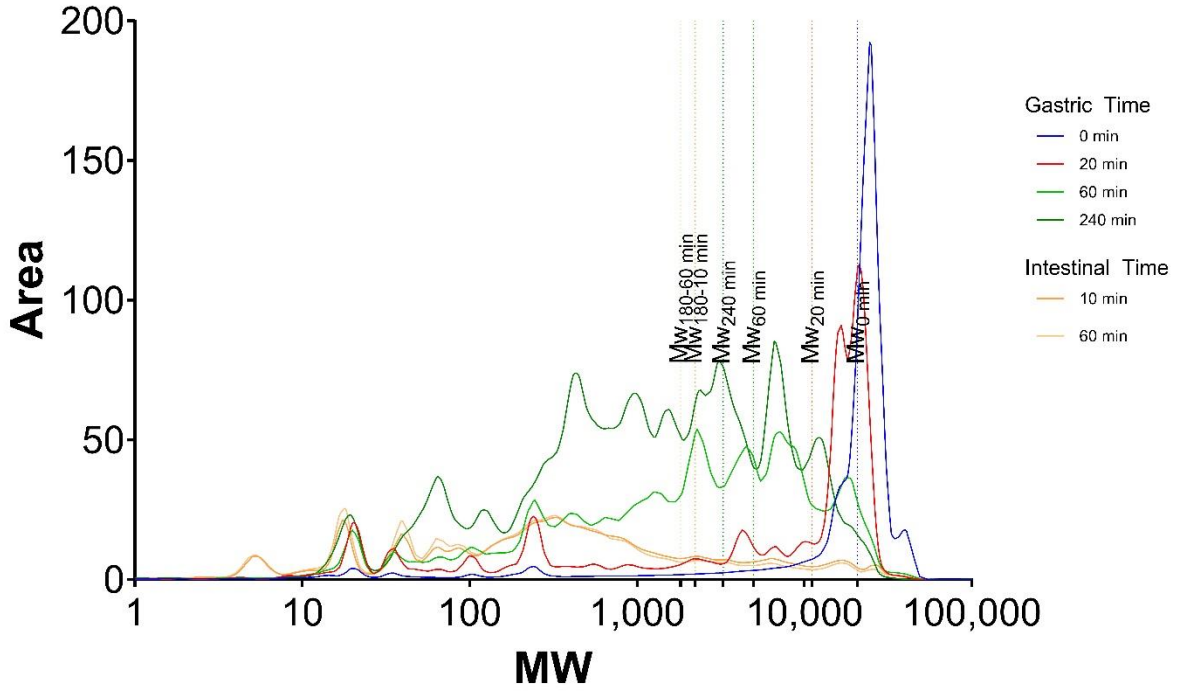

Figure 10. Mean molecular weight (MW) distributions of a representative milk sample throughout digestion. The weight-average MW of each of the digests (Mw) is overlaid on its plot. Gastric time points include 0 min, 20 min, 60 min and 240 min. Intestinal time points include 180 min gastric-10 min intestinal and 180 min gastric-60 min intestinal. The representative milk sample displayed is raw cow.

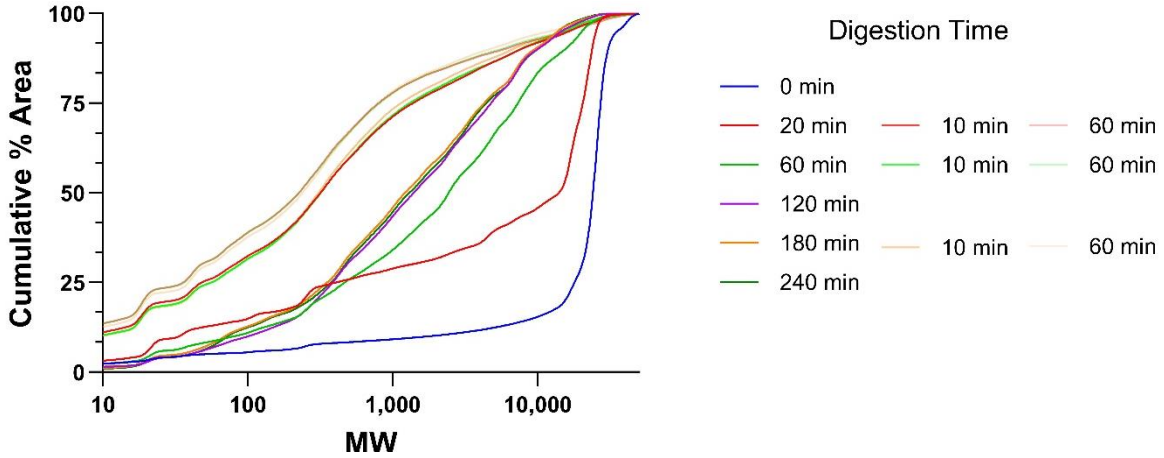

Figure 11. Mean cumulative percentage (%) area against MW for a representative milk sample throughout digestion. Intestinal time points (10 and 60 min) are coloured in a slightly lighter colour for their representative gastric time points. The representative milk sample displayed is raw cow.

The proportion of soluble proteins and peptides for specific MW ranges can then be calculated as area under the curve (AUC), based on the cumulative percentage area for quantitation. Four

parameters were assessed for quantitation of digestibility; the weight-average MW of the sample (Figure 12A), the total AUC of the cumulative percentage area plot for the sample (Figure 12B), the percentage of peptides below 10 kDa (Figure 12C) and the percentage of peptides below 1 kDa (Figure 12D). Due to the large variability in the undigested samples (0 min), likely driven by the column not being suited for the higher MW proteins in the samples, these values were excluded from quantitation. The weight-average MW decreases throughout digestion whilst the total AUC and percentage of peptides below 10 kDa and 1 kDa increase throughout digestion. This illustrates that throughout digestion there is a decrease in proteins and large peptides and an increase in small peptides. During gastric digestion, the largest difference in MW profiles happens early on between 20 min and 60 min. This is likely due to the introduction of the enzyme pepsin, which hydrolyses milk proteins into smaller peptides. As pepsin breaks down CN it promotes the coagulation of proteins, leading to the formation of a clot. This clot formation is further facilitated by the acidic environment of the stomach, which enhances pepsin's proteolytic activity and contributes to the observed changes in MW profiles during the early phase of digestion. Limited differences are observed during late gastric digestion. This trend is also observed in processed milk digested using a semi-dynamic adult *in vitro* model (Mulet-Cabero *et al.*, 2019). Pepsin activity is affected by both pH and protein substrate. Salelles *et al.* (2021) reported that after 2 hours of static *in vitro* digestion the extent of hydrolysis of CN micellar aggregates was almost the same from pH 1 to pH 5. Thus, the plateau during late gastric digestion suggests that although proteins and peptides are still being emptied, the rate of digestion slows, potentially due to pepsin-induced hydrolysis not being as effective.

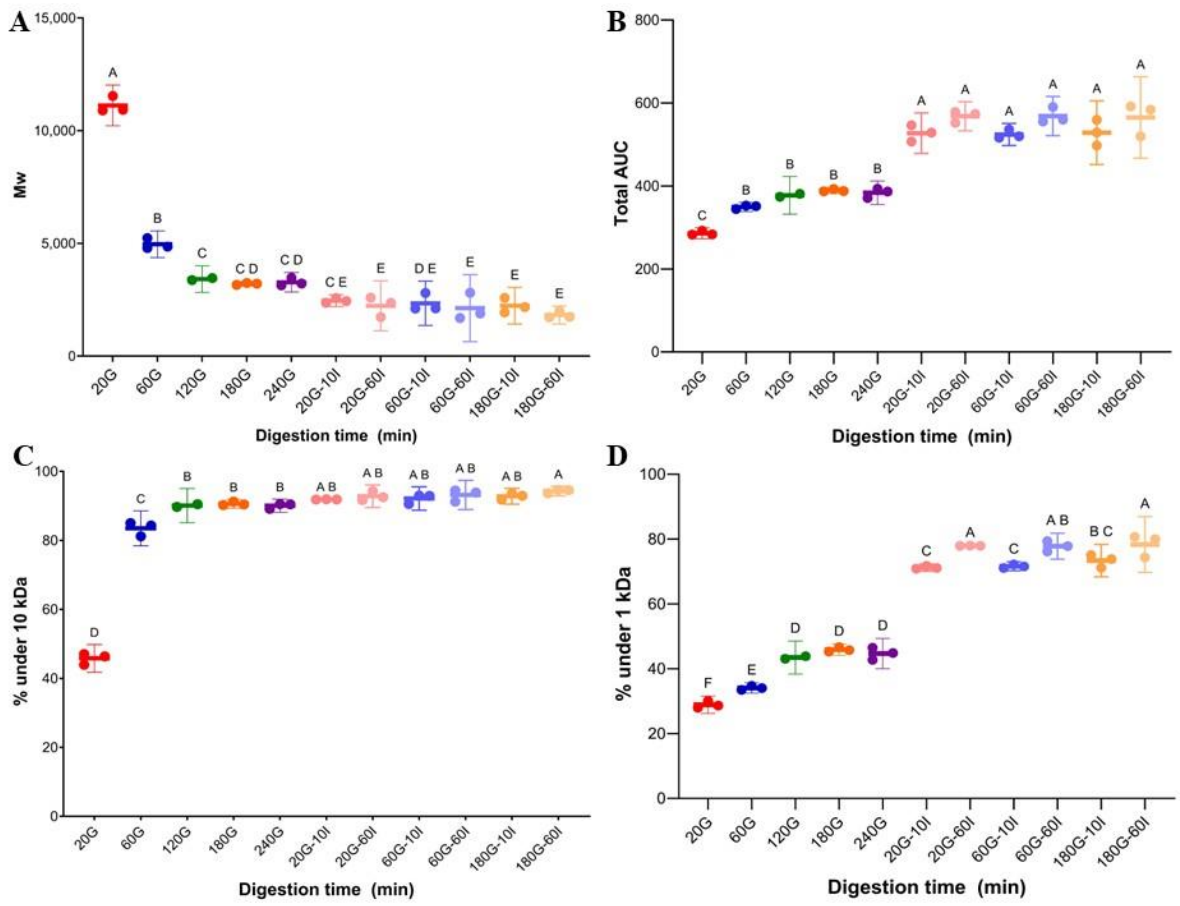

Figure 12. Parameters assessing the quantitation of digestibility: A) Weight-average molecular weight (Mw) throughout digestion. B) Total AUC (based on cumulative % area) throughout digestion. C) Percentage of peptides below 10 kDa throughout digestion. D) Percentage of peptides below 1 kDa throughout digestion. Values are the mean of three independent digestions ± 95 % confidence interval. Different letters indicate significantly different values (P<0.05).

A significant difference in the amount of small peptides (below 10 kDa) is observed between the gastric time points and their subsequent intestinal time points (Figure 12B and D). Rieder *et al.* (2021) also showed that the largest difference in peptide profiles observed throughout digestion was between the 120 min gastric and 10 min intestinal time point. It is believed that this is due to the introduction of new proteases, such as those in pancreatin, in the intestinal phase. While pepsin primarily cleaves peptide bonds near hydrophobic and aromatic amino acids, the main proteases in pancreatin – trypsin and chymotrypsin – target different sites. Trypsin cleaves peptides at the carboxyl side of basic amino acids, whereas chymotrypsin cleaves at the carboxyl side of aromatic amino acids. This leads to further breakdown of proteins resulting in an increased abundance of smaller peptides and free amino acids. Overall, samples digested with the intestinal system for 60 min typically had a greater proportion of

small peptides than samples digested for 10 min. This was only significant when assessing the percentage of peptides below 1 kDa (Figure 12D). This indicates that although peptides are still being further digested after 10 min of intestinal digestion the most significant digestion occurs within the first 10 min. Intestinal digestion was trialled at three different time points throughout gastric digestion 20, 60 and 180 min. No significant difference in peptide amount or elution profile was observed between the three different intestinal time points. A potential reason for this lack of significant difference is the high background of the intestinal samples caused through the introduction of pancreatin and bile. Since pancreatin contains a mixture of proteases and other proteinaceous components, its presence in the intestinal phase could contribute to a high baseline signal. Additionally, bile, may also contain small biomolecules that contribute to the overall signal detected by SEC. An intestinal blank obtained by running water through the intestinal system for 60 min obtained similar chromatogram profiles to the gastric samples run through the intestinal system. The protein concentration of the intestinal blank and intestinal samples was similar and higher than that of the gastric samples suggesting that the background signal is largely influenced by the pancreatin and bile rather than the breakdown of gastric proteins alone. Due to the high background of the blank intestinal sample and the lack of significant differences between the intestinal samples only the gastric samples were further analysed to determine whether different species or processing treatments affected the digestibility of milk.

4.3.1.2 Digestibility of milk from different species during the gastric phase

To assess the digestibility of milk from different species, we evaluated the percentage of bioaccessible peptides below 10 kDa and below 1 kDa during the gastric phase of digestion. Figure 13A and B shows the percentage of peptides below 10 kDa of raw and pasteurised milk during the gastric phase of digestion across four ruminant species. Throughout the digestion process, an increase in the proportion of peptides below 10 kDa is observed in all species, which plateaus after approximately 120 min of gastric digestion. This consistent trend suggests a steady increase in protein hydrolysis as the digestion progresses, reaching a maximum bioaccessible peptide concentration by 120 min.

Notably, cow and deer milk exhibit a significantly higher proportion of small peptides under 10 kDa than goat and sheep milk throughout the digestion process. This trend is evident in both raw and pasteurised milk samples, highlighting the influence of milk species on the protein breakdown process. Deer milk, in particular, shows a significantly higher proportion of small

peptides during the early digestion phases when compared to cow milk. However, this difference diminishes over time, as deer milk reaches a plateau in peptide production at later time points, indicating that the digestibility of deer milk proteins is somewhat slower during the later stages of digestion.

When assessing the percentage of peptides below 1 kDa (Figure 13C and D), similar trends are observed. However, a distinct pattern emerges in deer milk, where the proportion of peptides below 1 kDa does not show the same progressive increase observed in the other species. Instead, deer milk maintains a consistently high level of peptides below 1 kDa throughout digestion. This unique pattern suggests that the protein digestion kinetics in deer milk differ from those in cow, goat, and sheep milk, potentially due to differences in protein composition or the digestive dynamics specific to deer milk.

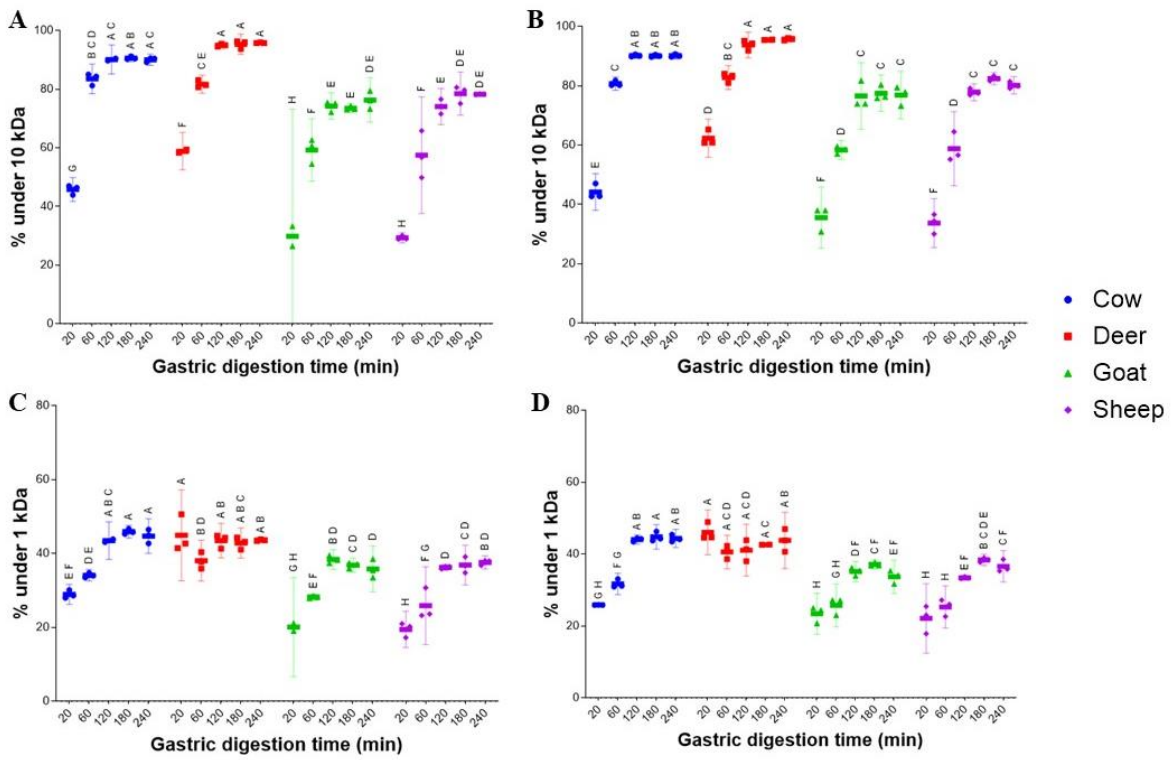

Figure 13. Comparison between the bioaccessible peptides produced during *in vitro* gastric digestion of cow, deer, goat, and sheep milk showing the percentage of peptides below 10 kDa produced for A) raw milk and B) pasteurised milk and percentage of peptides below 1 kDa for C) raw milk and D) pasteurised milk. Values are the mean of three independent digestions ± 95 % confidence interval. Different letters indicate significantly different values (P<0.05).

In unprocessed milk, the digestibility, as assessed by the proportion of peptides below 10 kDa, follows a species-specific order: deer>cow>goat=sheep. This ordering implies that species significantly influences protein digestibility, with deer milk exhibiting the highest bioaccessible peptide proportion. Deer milk's higher protein concentration, known to be greater than that of other species (Claeys *et al.*, 2014; Roy *et al.*, 2020a), likely contributes to this enhanced digestibility, although this effect was minimised by normalising protein concentration prior to SEC analysis in this study.

Differences in milk protein composition, particularly in key proteins such as caseins and whey proteins, further explain these species-specific digestibility patterns. Previous studies have demonstrated that protein structures, including amino acid composition and sequence, vary significantly between species and influence their susceptibility to enzymatic digestion (Claeys *et al.*, 2014; Roy *et al.*, 2020a). Specifically, sheep and goat milk typically form a softer clot with less fused protein networks compared to cow milk, leading to a faster gastric digestion and greater peptide release (Roy *et al.*, 2022; Roy *et al.*, 2021). Such differences in clotting behaviour and protein network formation are crucial for understanding the variations in protein digestibility observed in this study.

Additionally, previous research has consistently shown that the digestibility of milk proteins varies significantly between species. Tagliazucchi *et al.* (2018) found that goat milk releases a significantly higher proportion of free amino groups during static *in vitro* digestion compared to sheep and cow milk, while Roy *et al.* (2021) reported similar protein hydrolysis patterns between cow, goat, and sheep milk using SDS-PAGE analysis during dynamic *in vitro* digestion. These findings further support the idea that the species of milk has a substantial effect of protein digestibility, though discrepancies between studies may stem from differences in experimental models and analytical techniques, as SDS-PAGE is less effective in evaluating peptides generated during digestion. Furthermore, variations in digestion models such as static versus dynamic *in vitro* digestion systems can also contribute to these discrepancies. Static models provide a simplified and controlled environment but may not fully replicate the physiological conditions of digestion, whereas dynamic models better simulate the gastric process, leading to potential differences in protein hydrolysis outcomes.

4.3.1.3 Impact of processing on milk protein digestibility

Figure 14A and B present the percentage of peptides below 10 kDa for pasteurised-homogenised and heated-homogenised milk during the gastric phase of digestion. For all species, an increase in the proportion of peptides below 10 kDa is observed throughout digestion, with the levels plateauing during the later stages of digestion. In the early phases of digestion (20 and 60 min), pasteurised-homogenised deer milk shows a higher percentage of peptides below 10 kDa compared to the other species. However, this difference disappears by the later stages of digestion, suggesting that the impact of milk processing on digestibility diminishes over time. Conversely, heat-homogenised deer and cow milk show a significantly higher proportion of small peptides below 10kDa during the later stages of gastric digestion compared to goat and sheep milk. This indicates that heating and homogenisation treatments can enhance the digestibility of deer and cow milk proteins, particularly in the late digestion phases.

A more nuanced trend is observed when evaluating the percentage of peptides below 1 kDa (Figure 14C and D). For non-cow processed milks (deer, goat and sheep), a decrease in the proportion of peptides below 1 kDa is observed between the 20 min and 60 min time points, with the most notable decrease occurring in deer milk. This decline is not observed in processed cow milk, suggesting that cow milk may undergo a distinct digestive process or be more resistant to peptide breakdown during gastric digestion.

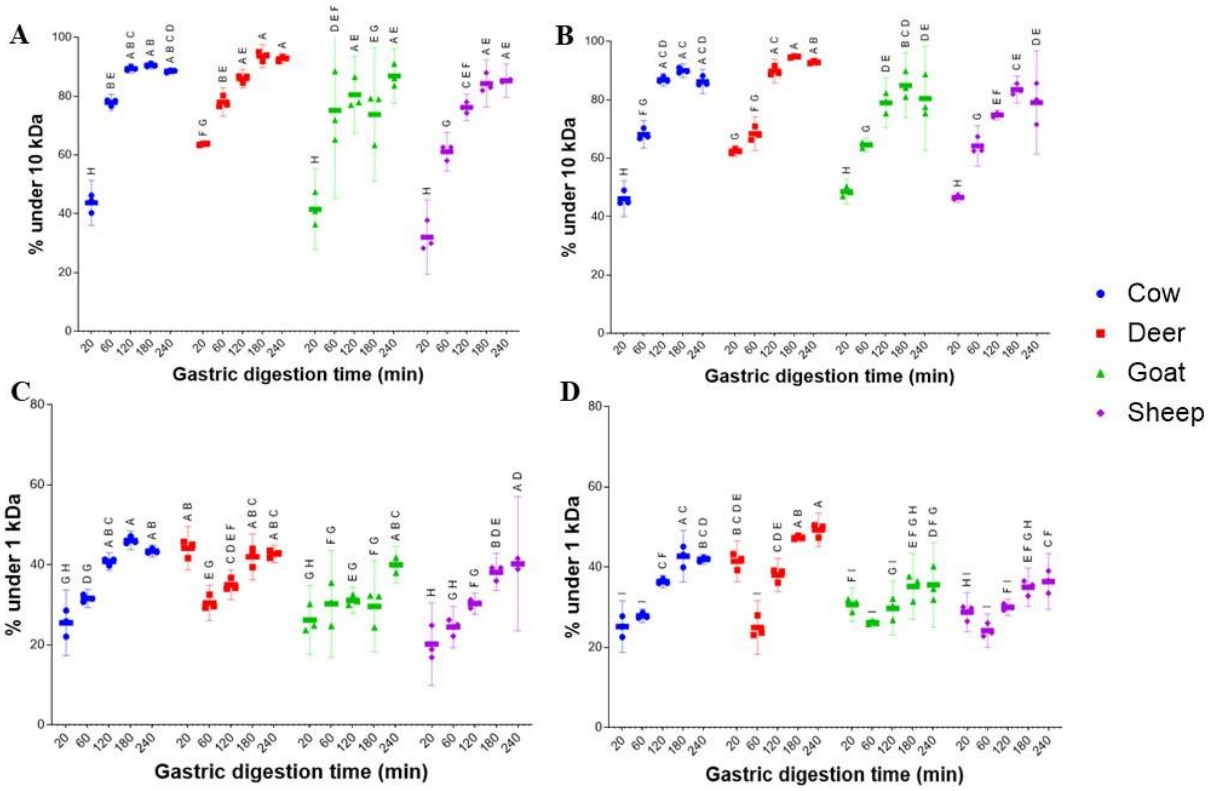

Figure 14. Comparison between the bioaccessible peptides produced during *in vitro* gastric digestion of cow, deer, goat, and sheep milk showing the percentage of peptides below 10 kDa produced for A) pasteurised-homogenised milk and B) heated-homogenised milk and percentage of peptides below 1 kDa for C) pasteurised-homogenised milk and D) heated-homogenised milk. Values are the mean of three independent digestions ± 95 % confidence interval. Different letters indicate significantly different values (P<0.05).

When milk is processed, particularly through homogenisation and pasteurisation, the species-specific differences in digestibility appear to be reduced. For example, the difference in bioaccessible peptides between unprocessed deer and sheep milk (17.5% at 240 min) diminishes significantly in pasteurised-homogenised milk (7.5%), suggesting that homogenisation reduces the impact of species on digestion. This reduction in digestibility differences is also observed in heated-homogenised milk, where the peptide profile of deer and sheep milk becomes more similar. The more processed milks exhibit a lower proportion of peptides below 10 kDa during early digestion, particularly in cow and deer milk, supporting the idea that processing treatments, such as heat and homogenisation, may alter the digestibility of milk proteins, especially in the early stages of digestion.

One explanation for this phenomenon is the formation of looser clots in heated and homogenised milk. Li, Pan, *et al.* (2022) observed that heat-treated and homogenised milks form less compact protein aggregates during gastric digestion, which may result in quicker enzymatic hydrolysis and peptide production. However, the structural changes in the milk proteins due to processing may lead to less efficient peptide release int eh later stages of digestion, as observed in the decrease in peptides below 1 kDa between 20 and 60 min in non-cow processed milks.

Figure 15 provides a species-specific breakdown of peptides below 10 kDa for processed milks. No significant differences are observed between processing treatments during late gastric digestion. However, early digestion (60 min) shows that more processed cow and deer milks (heat-homogenised and UHT for cow) produce fewer peptides than 10 kDa than less processed counterparts, indicating lower digestibility. This trend reverses in goat and sheep milk, where highly processed samples exhibit higher peptide proportions below 10 kDa, suggesting that processing enhances digestibility in these species.

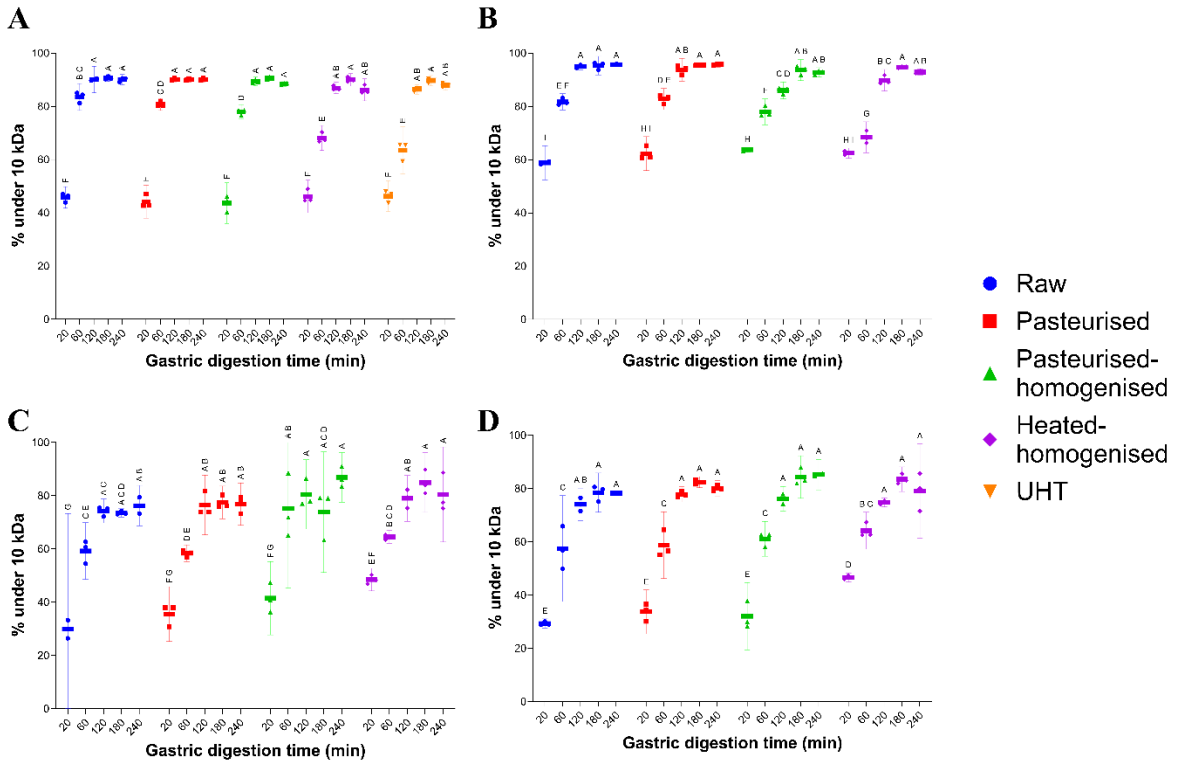

Figure 15. Percentage of peptides below 10 kDa for processed milks from different species: A) cow milk, B) deer milk, C) goat milk and D) sheep milk. Values are the mean

of three independent digestions ± 95 % confidence interval. Different letters indicate significantly different values (P<0.05).

These findings align with previous studies. Barbé *et al.* (2013) reported that heat treatment increased milk retention time in the stomach, affecting protein hydrolysis patterns. Sánchez-Rivera *et al.* (2015) observed similar trends in an *in vitro* model, while Islam *et al.* (2017) found that homogenisation enhanced β-LG hydrolysis. Structural changes in processed milk, such as softer coagula, may accelerate digestion kinetics as noted in both *in vivo* (Lacroix *et al.*, 2008) and *in vitro* (Ye *et al.*, 2017) studies.

For deer milk, limited research exists on processing effects, but recent studies suggest that heat treatment increases CN micelle size, affecting digestibility (Li *et al.*, 2023). Deer milk's high protein and fat content may lead to greater aggregation and changes in the MFGM during processing, influencing digestion. For goat and sheep milk, findings indicate that heat and homogenisation improve pepsin digestibility. Li, Ye*, et al.* (2022) found that processing led to highly fragmented clots, accelerating hydrolysis and protein emptying. Ren *et al.* (2023) observed that heating goat milk (≥80 ºC) formed extensive gastric clots with faster protein digestion, though whey protein aggregation persisted. Studies on sheep milk primarily focus on cheese and yoghurt production, but Pan *et al.* (2021) and Li, Pan*, et al.* (2022) reported that heating and homogenisation resulted in looser, more fragmented curds, promoting faster breakdown and digestion.

Overall, the impact of milk processing on digestibility is species dependent. Cow and deer milk exhibit reduced early digestion efficiency with increased processing while goat and sheep milk show enhanced digestibility. These differences likely stem from variations in clot structure, protein aggregation and processing-induced changes in milk composition.

4.3.2 Comparison of digestibility quantification methods

Protein digestibility estimated as percentage of small peptides (<10 kDa or <1 kDa) based on SEC was compared with the amount of free amine groups determined as DH% (OPA) which is frequently used to estimate protein digestibility. Figure 16 shows the DH% of raw and heated-homogenised cow milk throughout digestion. The same trend is observed in both the SEC and %DH results with an overall increase in the hydrolysed products during early gastric digestion that plateaus during late gastric digestion. A significant difference in the hydrolysed products

is observed between the gastric time points and their subsequent intestinal time points, as was observed in the SEC results.

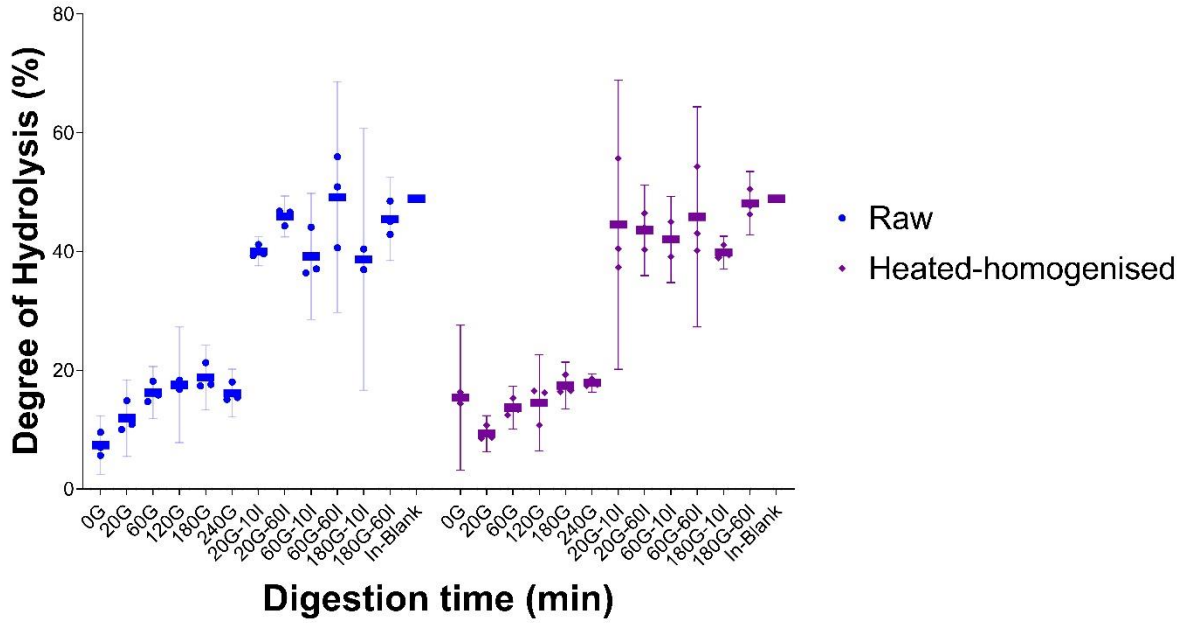

Figure 16. Degree of hydrolysis throughout digestion of cow milk (raw and heated-homogenised. Values are the mean of three independent digestions ± 95 % confidence interval.

Figure 17 shows the same trend between species as was previously observed for the SEC analysis with deer and cow milk having a higher degree of hydrolysis than sheep and goat for both unprocessed and processed milk. No significant difference was observed between the processing treatments in any of the species. A potential reason for the difference between the OPA and SEC analysis is due to the larger amount of variation in the OPA assay.

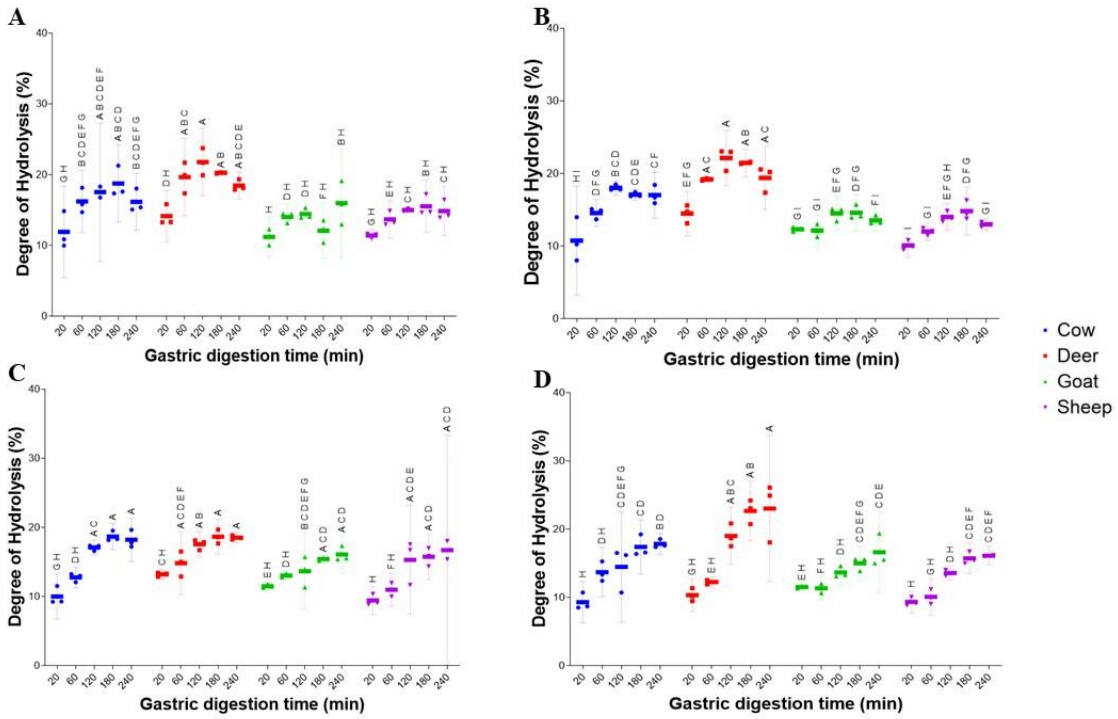

Figure 17. Degree of hydrolysis (%) for A) raw, B) pasteurised, C) pasteurised-homogenised and D) heated-homogenised milk from different species. Values are the mean of three independent digestions ± 95 % confidence interval. Different letters indicate significantly different values (P<0.05).

4.4 Conclusions

This study investigated the impact of milk species and processing treatments on the bioaccessibility of peptides during digestion. Results from SEC indicate that, regardless of milk origin or processing, the proportion of small peptides available for absorption increases throughout digestion, with the most significant changes occurring during early gastric and intestinal phases. This is due to clot restructuring and enzyme activity.

The findings highlight that milk species influence the proportion of small peptides available for absorption, particularly during gastric digestion. Deer milk consistently exhibited a higher proportion of small peptides throughout gastric digestion, followed by cow milk, with sheep and goat milk showing the lowest proportions. The effect of processing treatments was found to be species dependent. Homogenisation generally reduced species-specific differences, with its impact varying based on heat treatment. When combined with pasteurisation, homogenisation had a stronger effect during late digestion, whereas pairing it with high-heat treatment (95°C, 5 min) had a more pronounced impact during early digestion. Additionally,

high-heat treatment altered small peptide proportions across all species, decreasing them in deer and cow milk while increasing them in goat and sheep milk. This variation is likely due to changes in coagulum properties, with the most noticeable reduction in low molecular weight peptides (<1 kDa) occurring in non-cow milks, particularly deer milk, at 60 min of digestion. SEC trends were supported by OPA assay results, though high variability in OPA measurements reduced statistical significance.

These findings contribute to understanding species- and processing-dependent differences in milk protein digestion. However, this study does not provide information on specific peptide sequences or resistant protein regions. Future work utilising LC-MS/MS is recommended to further characterise the digestion patterns of different milk proteins and their potential bioactivities. Understanding the bioaccessibility of peptides from different milk sources can inform the development of functional dairy products tailored for specific nutritional needs. This research may have implications for infant formula, sports nutrition, and medical nutrition, where optimised peptide profiles could enhance protein absorption and bioavailability. Additionally, these findings could help improve dairy processing techniques to maintain or enhance the nutritional quality of milk-based products.

Chapter 5 - Method optimisation for peptidomics analysis of cow milk throughout gastrointestinal digestion

5.1 Introduction

Proteomics and peptidomics require methods that are reliable and efficient to maximise the quantity and quality of information obtained. A wide range of methods have been described in the literature for the analysis of both milk proteins and peptides using liquid chromatography tandem mass spectrometry (LC-MS/MS). Methods vary according to the starting material used, extra fractionation steps to remove fat or separate whey and CN proteins, differences in protein and peptide extraction and separation methods, as well as differences in the instruments used and subsequently the settings of these instruments.

Various studies have compared different aspects of the proteomic or peptidomics workflow on milk such as peptide extraction methods (Dingess *et al.*, 2019), protein extraction methods (Vincent, Ezernieks*, et al.*, 2016) and fractionation methods (Nissen *et al.*, 2012, 2013). However, overall, the lack of consistency in methodology used to study milk proteins and peptides indicates that there is not one method that is universally superior and that the methodology changes to reflect the different aims and objectives of the individual studies. Thus, it is important to optimise the sample preparation steps to best address the research aims and objectives.

During this study we aimed to improve the peptidomics workflow for milk digestomics samples focusing on extraction methods, MS methods and database search strategies. The optimised peptidomics workflow was selected based on the number of peptides identified as well as ease and length of set-up.

5.2 Materials and methods

5.2.1 Reagents

Water, acetonitrile, methanol, formic acid (FA) and trifluoracetic acid (TFA), all LC-MS grade, and urea were sourced from ThermoFisher Scientific (Waltham, MA, USA). Thiourea was from Acros organics (China). Chloroform and ammonium bicarbonate were purchased from BDH Prolabo (Poole, UK). DL-Dithiothreitol (DTT) and iodoacetamide (IAM) were from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Unless otherwise specified, all chemicals were of analytical grade.

5.2.2 Samples

Three different commercially processed bovine milk samples (3.1.2) were chosen using the lucky dip method to ensure a range of digestion timepoints (3.2.1.2). The samples selected were 0 mins gastric, 180 mins gastric and 60 mins gastric-120 mins intestinal.

5.2.3 Extraction of peptides

Three different methods were trialled to extract peptides from digested milk samples: liquid-liquid extraction and ultrafiltration using two different molecular weight cut-off membranes (3 kDa and 10 kDa). The different extraction methods were performed in duplicate for each sample. Prior to peptide extraction all samples were adjusted to 5% acetonitrile by adding 30 µL of acetonitrile to 570 µL sample. The samples were then heated to 40 ºC for 15 mins on a thermomixer (Eppendorf Thermomixer R Mizer 5355, Eppendorf AG, Hamburg, Germany) at 500 rpm and centrifuged (Hettich Mikro 200 R bench top Centrifuge, Tuttlingen, Germany) for 10 mins at 20, 000 x *g* (4 ºC).

Filter-aided

250 µL of sample was added to a 3 or 10 kDa NanoSep^{TM} centrifugal device (Pall, NY, USA) and then centrifuged for 90 mins at 10,000 x *g* at 4 ºC. The concentration of the ultrafiltrate was estimated at 280 nm using the built-in protein assay on the NanoPhotometer NP80 (Implen, Munich, Germany). An aliquot containing 250 µg of peptides was then dried for each sample in a CentriVap vacuum centrifuge (Labconco, Kansas City, MO, USA) operated at 40 ºC. Prior

to running on the LC-MS the samples were resuspended in 50 µL of 0.1 % TFA and diluted 5x with 0.1 % FA.

Liquid-Liquid extraction (chloroform-methanol extraction (CME)))

This method was based upon previous methods described by Wessel and Flügge (1984) and Dingess *et al.* (2019) with some modifications. 400 µL of methanol was added to 100 µL of sample followed by 100 µL of chloroform followed by 300 µL of water. Between each addition the sample was vortexed briefly. The samples were then centrifuged for 1 min at 14, 000 x *g* (Eppendorf benchtop centrifuge 5424, Eppendorf AG, Hamburg, Germany) before the aqueous layer containing peptides was transferred into a clean Eppendorf tube. 400 µL of methanol was then added to the precipitate (protein fraction), vortexed and centrifuged for 2 mins at 14, 000 x *g*. Again, the supernatant of the aqueous layer was removed and then the precipitate (protein fraction) was air dried. The concentration of the supernatant containing peptides was estimated at 280 nm using the built-in protein assay on the NanoPhotometer NP80 (Implen, Munich, Germany). An aliquot containing 250 µg of peptides was then dried for each sample in a CentriVap vacuum centrifuge (Labconco, Kansas City, MO, USA) operated at 40 ºC. Prior to running on the LC-MS the peptides were resuspended in 50 µL of 0.1 % TFA and diluted 5x with 0.1 % FA.

5.2.4 Protein preparation

Protein extraction

The protein concentrations from both the filter-aided extraction method (retentate) and liquid-liquid extraction method (precipitate) were estimated at 280 nm using the built-in protein assay on the NanoPhotometer NP80 (Implen, Munich, Germany). An aliquot containing 250 µg of protein of each sample was dried in a CentriVap vacuum centrifuge (Labconco, Kansas City, MO, USA) operated at 40 ºC. 100 µL of urea buffer (7 M urea, 2 M thiourea and 50 mM DTT) was added to each ample and then incubated at 25 °C overnight. Chloroform-methanol extraction was performed based on previous methods described by Wessel and Flügge (1984) as outlined in section 5.2.3. The dried precipitate was then resuspended in 50 µL of 50 mM ammonium bicarbonate by sonicating for 5 mins.

Protein reduction, alkylation, and digestion

The extracted protein samples were reduced by addition of 10 mM DTT and incubation at 56 ºC for 45 min and then alkylated using 20 mM iodoacetamide for 30 min in the dark at room temperature (21 °C). Trypsin digestion was achieved by adding 2 µg of trypsin (1:50 enzyme:substrate) and mixing at 37 °C overnight. Desalting was performed using Pierce C18 pipette tips (ThermoScientific, Waltham, MA, USA) following the manufacturer's instructions and eluted peptides were dried in a CentriVap vacuum centrifuge. Prior to running on the LC-MS the samples were resuspended in 50 µL of 0.1 % TFA and diluted 5x with 0.1 % FA.

5.2.5 Chromatography and mass spectrometry

Nanoflow LC-MS and LC-MS/MS were performed on an Ultimate 3000 HPLC system (ThermoScientific, Waltham, MA, USA) directly interfaced with a CaptiveSpray ion source to an Impact HD Q-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The CaptiveSpray was fitted with a nanoBooster device (Bruker Daltonik, Bremen, Germany), which infused acetonitrile into the mass spectrometer's nitrogen gas supply to improve sensitivity. For each sample, 1 µL was injected onto a PepMap100 C18 Nano-Trap column (ThermoScientific) to trap peptides, which were then eluted onto a ProntoSIL C18AQ analytical column (150 mm x 100 µm i.d., 3 µm particle size, 200 Å pore size; nanoLCM Solutions, Oroville, CA, USA). Both columns were maintained at 50 ºC in a column oven. Separation of peptides was carried out at a flow rate of 1 µL/min using a multistep linear gradient of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). Initially 2 % B was used for 2 min, this increased to 20 % B over 43 min, and then to 45 % B over a further 16 min. The column was cleaned by increasing to 95 % B over 4 min and holding at that level for 7 min. After that, the column was re-equilibrated by reducing back to 2 % B over 2 min and holding at that for 10 min. The total run time was 84 min.

Mass spectrometry settings

There are a variety of different ways to adjust the mass spectrometry settings. It was deemed impractical to trial all available combinations. During this experiment three different mass spectrometry setups were trialled. In all three LC-MS/MS methods the mass spectrometer was operated using collision-induced dissociation (CID) and automated data dependent acquisition (DDA).

MS Method 1:

Each full scan MS spectrum (50–1800 m/z, 2 Hz sampling rate) was followed by a fixed cycle time of 3 seconds for acquisition of MS/MS spectra of precursors in the range m/z 150-1800, at a sampling rate of 2–32 Hz (depending upon precursor intensity). A preference for selection of charged (1+ to 8+) precursor ions was set, and precursors were excluded from reacquisition for 1 min unless their intensity increased at least 3-fold during that period.

MS Method 2:

Each full scan MS spectrum (50–1800 m/z, 1 Hz sampling rate) was followed by acquisition of MS/MS spectra of a maximum of 10 precursors in the range m/z 150-1800, at a sampling rate of 2–32 Hz (depending upon precursor intensity). A preference for selection of charged (1+ to 8+) precursor ions was set, and precursors were excluded from reacquisition for 1 min unless their intensity increased at least 3-fold during that period.

Method 3:

Each full scan MS spectrum (50–2200 m/z, 2 Hz sampling rate) was followed by acquisition of MS/MS spectra of a maximum of 10 precursors in the range m/z 150-2200, at a sampling rate of 2–32 Hz (depending upon precursor intensity). A preference for selection of charged (1+ to 8+) precursor ions was set, and precursors were excluded from reacquisition for 1 min unless their intensity increased at least 3-fold during that period.

5.2.6 Software analysis

Peptides and proteins were identified using PEAKS Studio version 10.6 (Bioinformatics Solutions Inc., Toronto, Canada) (Ma *et al.*, 2003). Searches against three different databases were performed, including the UniProt Bos Taurus database (taxonomy id 9913, 10-10-2022, 6,034 sequences), the bovine milk proteome database (BoMiProt 2.0, 10,314 sequences (Das *et al.*, 2022)) and a custom protein database (13,769 sequence) that included the sequences from UniProt (taxonomy id 9913, 10-10-2022, 6,034 sequences) and the bovine milk proteome database (BoMiProt 2.0, 10,314 sequences (Das *et al.*, 2022)) dereplicated with an in-house Python script (make_nr_fasta.py version 1.0.7).

The following parameters were kept fixed for all searches: precursor ion mass error tolerance of 10 ppm, and fragment mass error tolerance of 0.05 Da; maximum allowed variable PTM per peptide was set at 2; peptide false discovery rate (FDR) was set at 1%; proteins were accepted if their -10lgP values were above 20 and at least 1 unique peptide and additional significant supporting peptides were observed. For the exploratory PEAKS PTM search all 312 post translational modifications (PTMs) in the UniMod database (https://www.unimod.org/modifications_list.php) were included, while for the optimised search only relevant PTMs found during the exploratory search were selected. Four different enzyme selections were trialled: no enzyme (unspecific), pepsin pH 1.3 (semi-specific), pepsin pH >2 (semi-specific) and trypsin (semi-specific). A maximum of 2 missed cleavages was selected for all except the no enzyme searches. Two online tools were utilised to analyse the results: Venny (Oliveros, 2007) and Peptigram (Manguy *et al.*, 2017).

5.3 Results and discussion

5.3.1 Mass spectrometry settings

Different mass spectrometry settings were trialled to optimise the peptides identified from the digesta samples. Three different mass spectrometry acquisition methods were tested these included differences in the m/z ratio range as well as changes in the cycle time and collision-induced dissociation settings. Figure 18 illustrates an example of the (A) chromatograms and (B) number of peptides identified for these different settings. Overall, the patterns of the chromatograms looked very similar however, method 1 (297) identified slightly more peptides than method 2 (287), with method 3 (158) identifying the least. Method 1 was chosen due to the increased number of peptides identified.

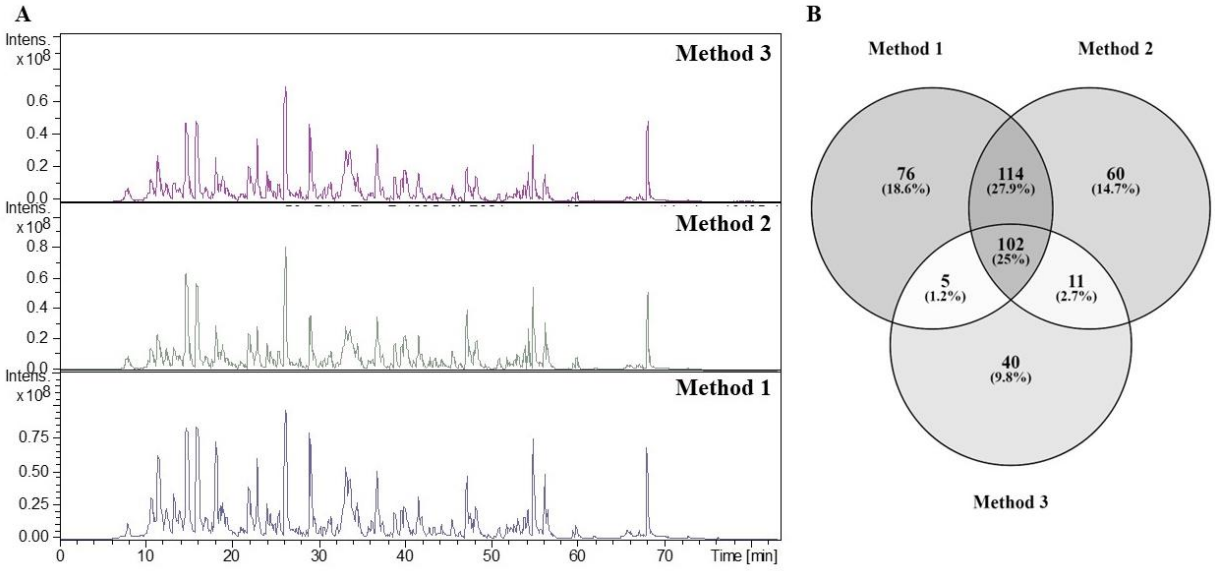

Figure 18. Mass spectrometry settings effect on A) chromatogram and B) number of peptides identified for 180 mins gastric sample

5.3.2 Peptide fraction

5.3.2.1 Protein database used in PEAKS

The most frequently used repositories from which protein databases are obtained are the freely accessible UniProt (https://www.uniprot.org/) and NCBI (https://www.ncbi.nlm.nih.gov/). Table 13 illustrates the number of proteins found in commonly used databases for bovine milk studies (Consortium, 2022; Das *et al.*, 2022; Delosière *et al.*, 2020; Stastna, 2023). To identify milk proteins both the bovine milk proteome 2.0 compiled by Das *et al.* (2022) and the bovine milk protein dataset compiled by Delosière *et al.* (2020) used approximately 20 peer-reviewed publications to compile known bovine milk proteins. They also include additional features such as search criteria for primary and secondary information of proteins. All proteins identified in the milk specific databases have been cross-referenced with the literature.

Table 13. Number of proteins identified in different databases and datasets specifically investigating Bos Taurus

Database	Number of proteins
UniProt/Swiss-Prot	6,036
UniProt/TrEMBL	41,094
NCBI	145,978
Bovine Milk Proteome 2.0	10,642
Bovine milk protein dataset (Atlas)	4,654

During this study three different bovine databases were trialled to determine which one provided the most peptide identifications: Swiss-Prot with reviewed *Bos taurus* taxonomy (dereplicated to make non-redundant) produced in 2022 (S05), bovine milk proteome 2.0 (BoMiProt) and a customised database including the proteins identified in both the Swiss-Prot and BoMiProt database (Swiss-Prot (S05) + BoMiProt). Figure 19 shows the number of peptides identified using the three databases for all samples. Approximately 40% of the peptides identified were found in all three databases. The highest number of peptides were identified using the combined database of the Swiss-Prot (S05) + BoMiProt, followed by BoMiProt and then Swiss-Prot (S05). This is likely due to the greater number of milk-relevant proteins present in the combined database than the individual databases (Table 13). The peptides identified in both the Swiss-Prot and BoMiProt databases came from 30 proteins respectively, though only 13 of these were identified in both databases. The Swiss-Prot + BoMiProt database identified 48 proteins with 16 not being found using the other two databases. Although the combined Swiss-Prot + BoMiProt database identified the greatest number of peptides, the BoMiProt database was chosen as the preferred database to analyse cow milk peptides due to the proteins present in the database only being found in milk.

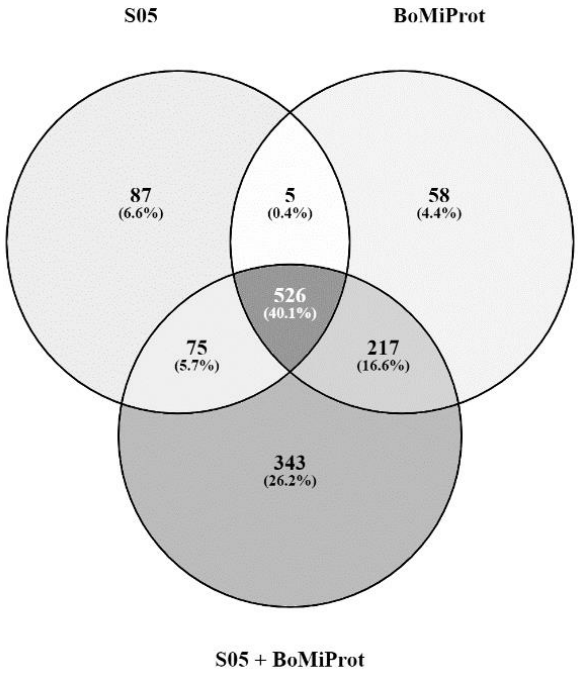

Figure 19. Number of peptides identified in three different databases: Swiss-Prot (S05), BoMiProt and a custom database that included both Swiss-Prot (S05) + BoMiProt databases.

5.3.2.2 Enzyme selected in PEAKS

PEAKS performs *de novo* sequencing and searches the protein database *in silico* to generate peptide candidates. It is necessary to specify the enzyme for protein digestion, whether non-specific cleavages are allowed, as well as the number of missed cleavage sites allowed in a peptide. Typically, in protein samples the enzyme used to digest the proteins is selected, for example trypsin. However, in gastrointestinal digestion samples, digestive enzymes are used to generate the peptides, and these vary depending on the digestion stage. In this study there are undigested samples whose peptides are typically generated by enzymes present in the milk such as plasmin, as well as gastrointestinal digestion samples whose peptides are caused by the introduction of pepsin in the case of gastric digesta and intestinal digesta whose peptides are generated by the further introduction of a mixture of enzymes including trypsin and chymotrypsin. Three different enzyme options were trialled in PEAKS, including pepsin (semi-specific), trypsin (semi-specific) and no enzyme (unspecific). Figure 20 shows that the no enzyme selection identified a far greater number of peptides (806) than either the pepsin (341) or trypsin (85) selection. This is likely due to the high degree of non-specificity between the different samples with a range of enzymes generating the peptides. The no enzyme option can cut at every residue generating peptides with lengths up to 65 amino acids. Meanwhile trypsin

can only identify peptides that are cleaved after arginine and lysine and not before proline. Two different pepsin options are found in PEAKS, pepsin (pH 1.3) and pepsin (pH >2). Both options identify peptides that are cleaved after phenylalanine and leucine and before any amino acid residue but the option for pH >2 also identifies peptides that are cleaved after tryptophan, tyrosine, alanine, glutamic acid, and glutamine. During the gastric phase in the human gastric simulator the pH is gradually acidified to pH 2, which is why both the pH 1.3 and pH >2 options were selected. Interestingly the pH 1.3 setting identified a greater number of peptides. The setting for both the trypsin and pepsin selected enzymes were semi-specific which means that it allows non-specific cleavage at one end of the peptide. Both the pepsin and trypsin selected enzyme showed a lower number of peptides than the no enzyme selection, thus going forward for peptidomics no enzyme was selected.

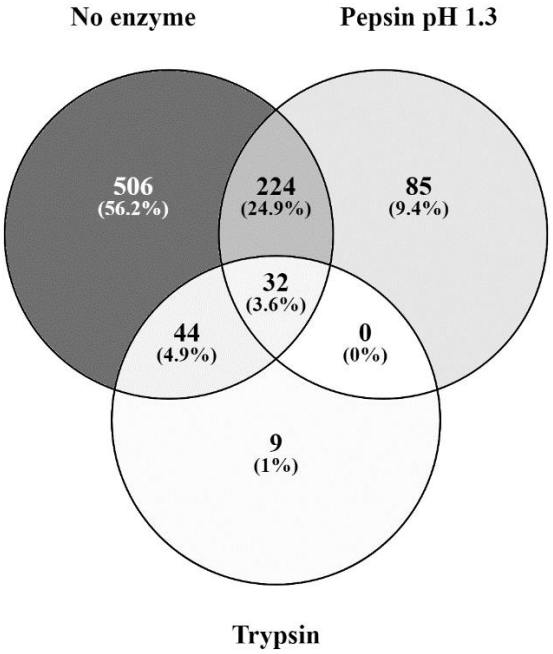

Figure 20. Number of peptides identified using three different enzymes in Peaks – no enzyme (unspecific), pepsin pH 1.3 (semi-specific) and trypsin (semi-specific).

5.3.2.3 Post translational modifications in PEAKS

PEAKS provides the option to both search for all post translational modifications (PTMs) in the database or to select specific PTMs to be considered. Initially all 312 PTMs as identified by Unimod were selected for the searches. However, to improve the statistics of the peptide-spectrum match, owing to reduced search space, only a select number of PTMs with a PEAKS

peptide score with -10lgP values above 20 were selected to re-run. Figure 21 illustrates how optimising the PTM search increased the number of peptides identified by 5 %.

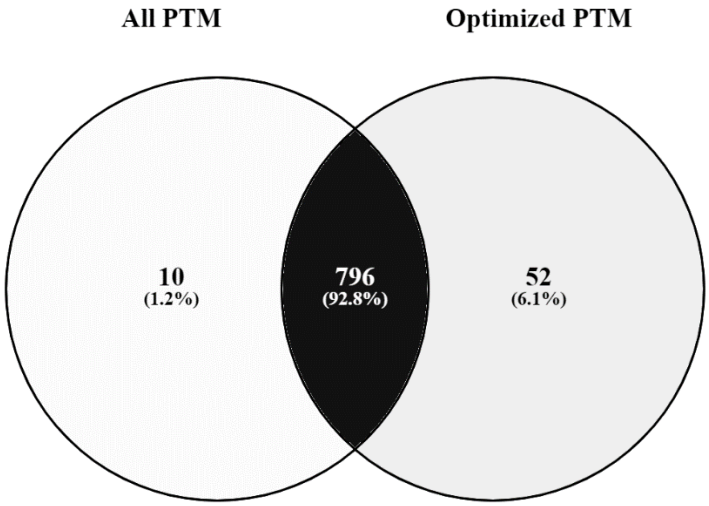

Figure 21. Number of peptides identified with two different PTM parameters (one includes all 312 modifications whilst the other only includes the relevant PTMs).

5.3.2.4 Peptide extraction method

Overall, a greater number of peptides were identified using the CME method compared to filter aided extraction. The CME method identified 671 peptides whilst the 10 kDa and 3 kDa filtration method identified 442 and 321 respectively (Figure 22). 24.2% or 205 peptides were identified in all three methods whilst 37.1%, 9.9% and 8% are unique to the CME, 10 kDa and 3 kDa methods respectively. This trend was present in all three samples tested.

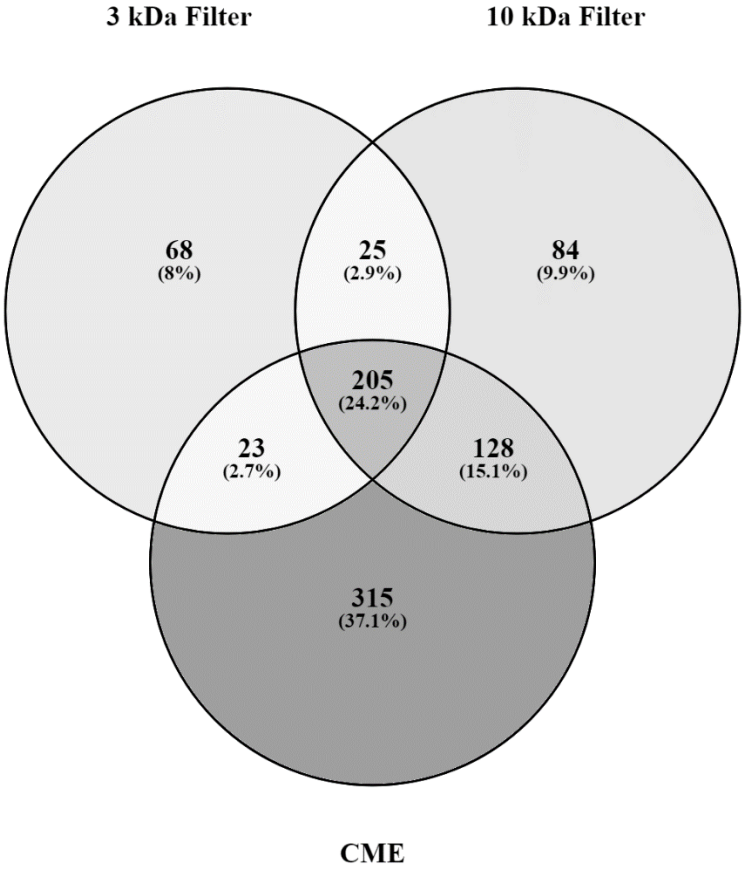

Figure 22. Number of peptides identified for the different extraction methods for all samples using the combined (Swiss-Prot and BoMiProt) database.

A potential reason for the greater number of peptides identified in the CME method is the lack of a MW cut-off. Although all three methods had a greater abundance of low MW peptides (below 20 amino acids in length) with 68.2%, 59.3 % and 52.5 % for method 3 kDa, 10 kDa and CME respectively (Figure 23). It was observed that the CME method had a greater abundance of high MW peptides with approximately 5.6 % being above 30 amino acids in length. In contrast the 3 kDa method did not identify any peptides above 30 amino acids in length whilst the 10 kDa method only identified 0.5 % of peptides above 30 amino acids in length. Another reason, that could contribute to the lower amount of peptides identified in the filter-aided method is potential loss of peptides on the ultrafiltrates.

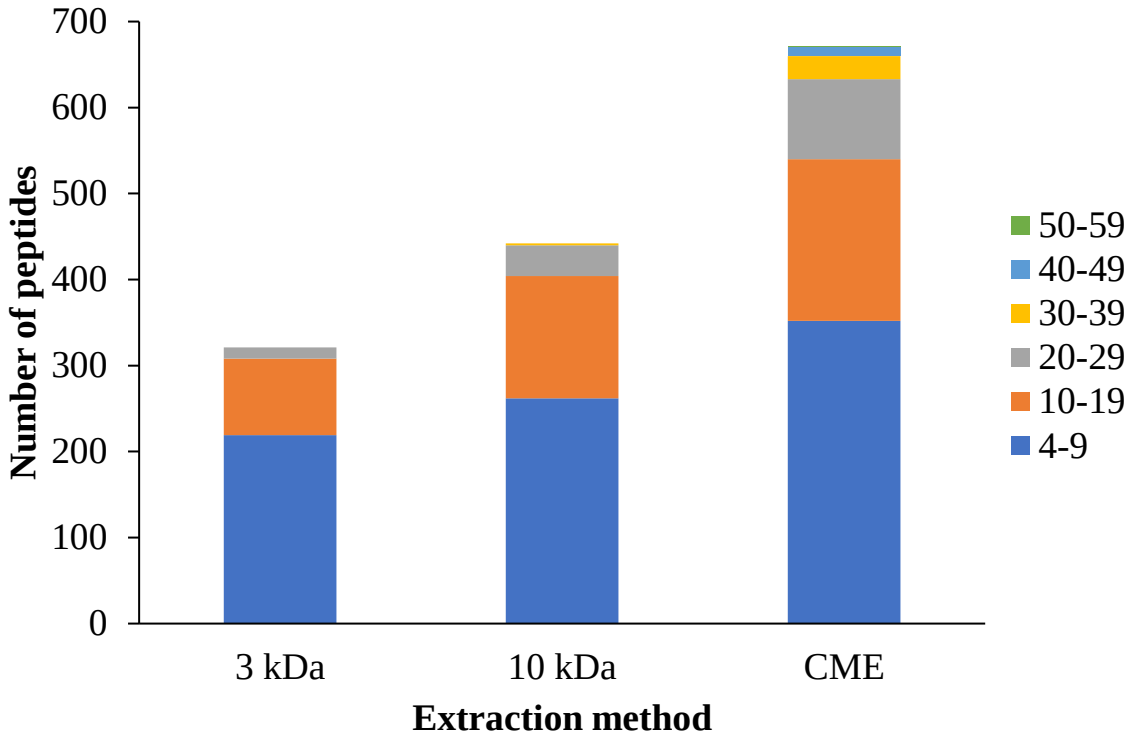

Figure 23. Number of peptides identified at different length ranges for the three different extraction methods: 3 kDa, 10 kDa and CME.

Figure 24 shows the coverage and reproducibility of the peptides identified in the top two most abundant proteins β-CN and α_{S1}-CN in the different extraction methods and samples. The undigested sample shows the best coverage in the CME method (Ext3) for the undigested sample and the least in 3 kDa filtration (Ext1) for both β-CN and α_{s1}-CN. Whilst both the gastric and intestinal samples show a similar coverage in all three extraction methods. The results from α_{S2}-CN and κ-CN are present in the supplementary material (Appendix Figure 1) and show similar trends.

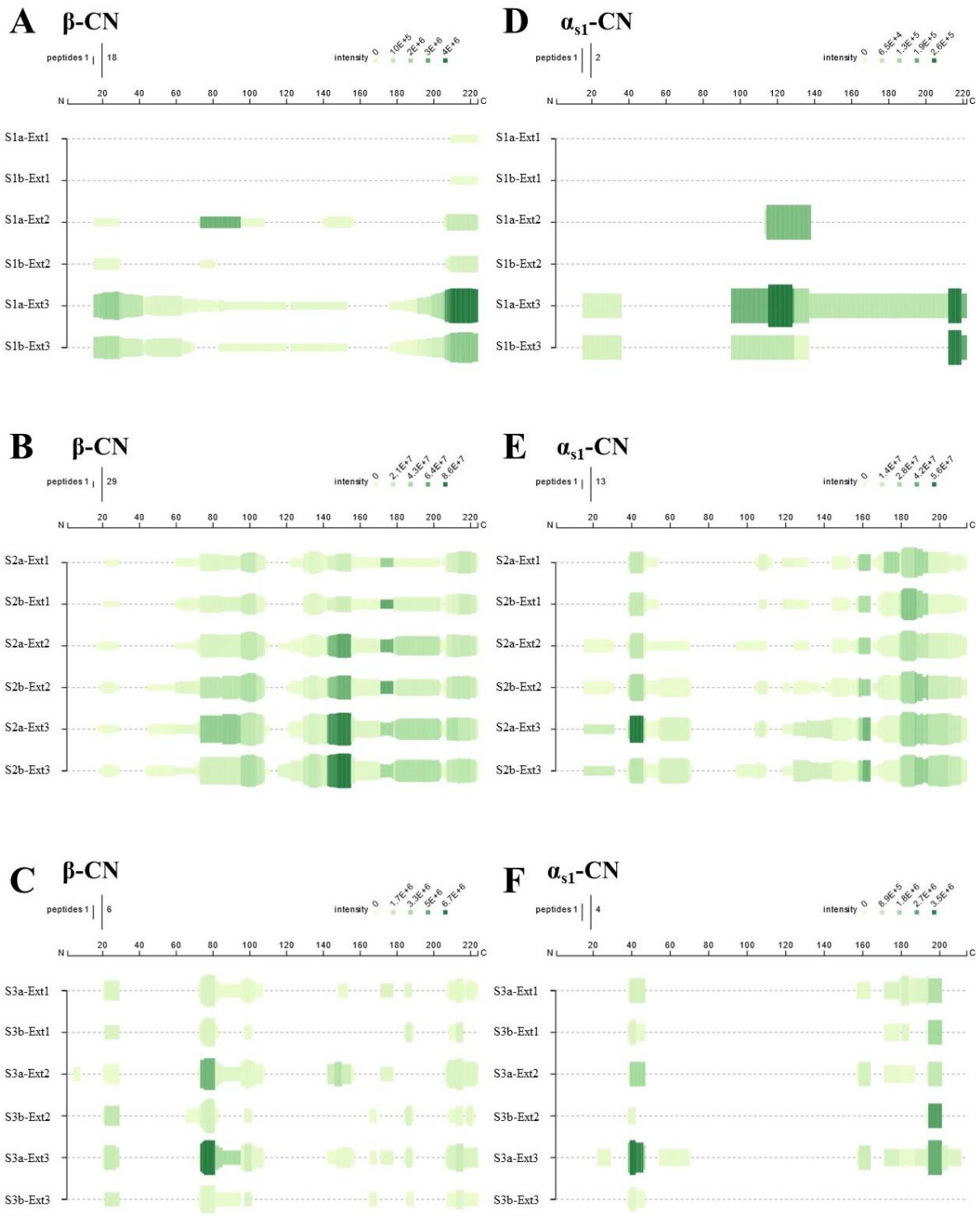

Figure 24. Peptigram images showing coverage and reproducibility of the different extraction methods for β-CN in A) no digestion (S1), B) gastric (S2) and C) intestinal samples (S3) and α_{s1}-CN in D) no digestion (S1), E) gastric (S2) and F) intestinal samples (S3). The different extraction methods are 3 kDa filtration (Ext1), 10 kDa filtration (Ext2) and CME (Ext3). Each sample is duplicated and labelled A and B. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

Although the CME extraction method identified the most peptides and showed the most coverage it had some undeniable flaws that made the filter-aided method the preferable option. Multiple steps were required during the CME method which increased the risk of peptide loss as well as decreasing the ease of workflow. Also, the lack of filtration step means that it is essential to desalt the peptide samples prior to running on the MS to prevent blocking the column which increases the length of workflow. Using the 10 kDa filtration method also matches with experimental data obtained using SEC.

5.3.3 Protein fraction

5.3.3.1 Protein fraction versus peptide fraction

The protein fraction of milk was also investigated to determine the proteins remaining after digestion. Figure 25A illustrates the number of proteins identified in both the protein and peptide fractions. 13 proteins were identified in both fractions, these included the main milk proteins such as the CNs, β-LG, and α-LA. Just over 30% were unique to either the peptide or protein fraction. The full list of proteins and the fraction in which they were identified is given in the Appendices (Appendix Table 1). Figure 25B illustrates the number of peptides identified in both the protein and peptide fraction. A greater number of peptides were identified in the peptide fraction, with only 2% of the peptides overlapping between the different fractions. This is likely due to the different enzymes being used to produce the peptides in the two fractions, with the peptide fraction being produced using enzymes that cleave at multiple sites such as pepsin whilst the protein fraction was produced using trypsin which is highly selective for cleavage at lysine and arginine. Two of the three samples selected for this trial were digesta samples which are expected to have the proteins broken down into peptides.

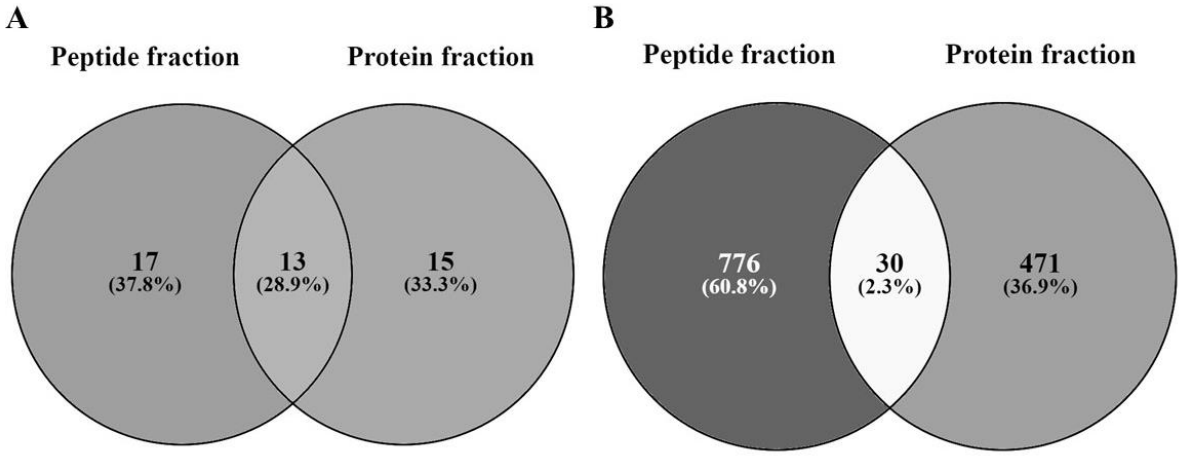

Figure 25. Venn diagrams showing the number of A) proteins identified in the protein and peptide fractions and B) peptides identified in the protein and peptide fractions.

Peptigram was used to compare the regions of the protein that were digested for both the protein and peptide fractions for CNs and the two major whey proteins, β-LG, and α-LA. All samples showed a distinct difference in the coverage and number of peptides found in the protein and peptide fraction. The undigested samples showed more peptides in the protein fraction, especially for whey proteins (Table 14). Interestingly many CN peptides, were found in the peptide fraction, especially peptides from β-CN. This is likely due to CN being especially susceptible to hydrolysis by endogenous enzymes such as plasmin (Boland & Singh, 2019).

Table 14. Coverage and number of peptides for the four CN proteins in the undigested sample (combining all extraction methods).

Protein	Peptide fraction	Protein fraction
β-CN	82% (53 peptides)	73% (55 peptides)
α_{s1}-CN	50% (21 peptides)	66% (43 peptides)
α_{s2}-CN	32% (9 peptides)	50% (30 peptides)
κ-CN	24% (10 peptides)	74% (33 peptides)
β-LG	11% (1 peptides)	81% (49 peptides)
α-LA	0% (0 peptides)	66% (20 peptides)

Gastric samples showed a higher number of peptides in the peptide fraction for the CNs (Table 15). However, the whey proteins still had a larger number of peptides and higher coverage in the protein fraction. This suggests that whilst the CNs are rapidly broken down during gastric digestion the whey proteins especially β-LG do not. This agrees with previous studies that suggest that β-LG is resistant to gastric digestion (Boland & Singh, 2019).

Table 15. Coverage and number of peptides for the six major milk proteins in the gastric sample (combining all extraction methods).

Protein	Peptide fraction	Protein fraction
β-CN	83% (177 peptides)	58% (30 peptides)
α_{s1}-CN	75% (92 peptides)	63% (32 peptides)
α_{s2}-CN	46% (34 peptides)	49% (40 peptides)
κ-CN	67% (59 peptides)	24% (5 peptides)
β-LG	41% (15 peptides)	79% (51 peptides)
α-LA	24% (6 peptides)	35% (7 peptides)

Comparably fewer peptides (and lower coverage) are found in the intestinal samples in both the peptide and protein fraction (Table 16). This is potentially due to the proteins being highly broken down in this stage to amino acids or peptides less than 4 amino acids in length. The protocol used in this analysis can only detect peptides greater than or equal to 4 amino acids long. For all 6 of the major proteins investigated more peptides were identified in the peptide fraction than the protein fraction.

Table 16. Coverage and number of peptides for the six major milk proteins in the intestinal sample (combining all extraction methods).

Protein	Peptide fraction	Protein fraction
β-CN	43% (32 peptides)	16% (4 peptides)
α_{s1}-CN	34% (17 peptides)	12% (5 peptides)
α_{s2}-CN	14% (4 peptides)	8% (2 peptides)
κ-CN	17% (6 peptides)	5% (1 peptides)
β-LG	9% (15 peptides)	5% (8 peptides)
α-LA	6% (4 peptides)	0% (0 peptides)

Figure 26 shows the peptigram profile of β-LG and κ-CN for the gastric sample. This further illustrates that the gastric samples β-LG showed more coverage in the protein fraction compared to the peptide fraction, supporting the evidence that it is resistant to digestion (Boland & Singh, 2019) whilst κ-CN showed more peptides in the peptide fraction compared to the protein fraction, showing that it is easier to digest. The remaining figures for the different samples and proteins are shown in the Appendices (Appendix Figure 2-6). The coverage was fairly reproducible between replicates and extraction methods. These results indicate that investigating the protein fraction alongside the peptide fraction gives further information into how the proteins are broken down.

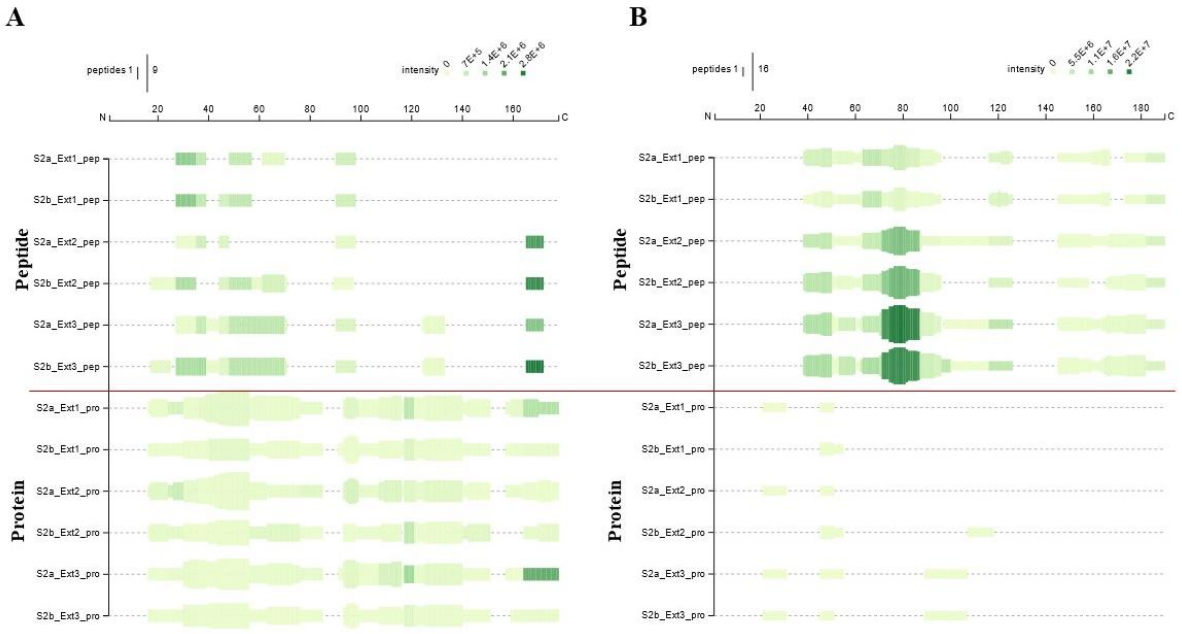

Figure 26. Peptigram plots showing the profile for two proteins A) β-LG and B) κ-CN for the peptide and protein fractions of an *in vitro* gastric digestion sample (S2). A and B are duplicates. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

5.3.3.2 Effect of extraction method on protein fraction

Over 85% of the proteins identified were found in all three extraction methods and approximately 50% of the peptides leading to their identification were identical in the three different extraction methods (Figure 27). This indicated that regardless of the method used to extract the peptides it did not greatly influence identifications in the protein fraction.

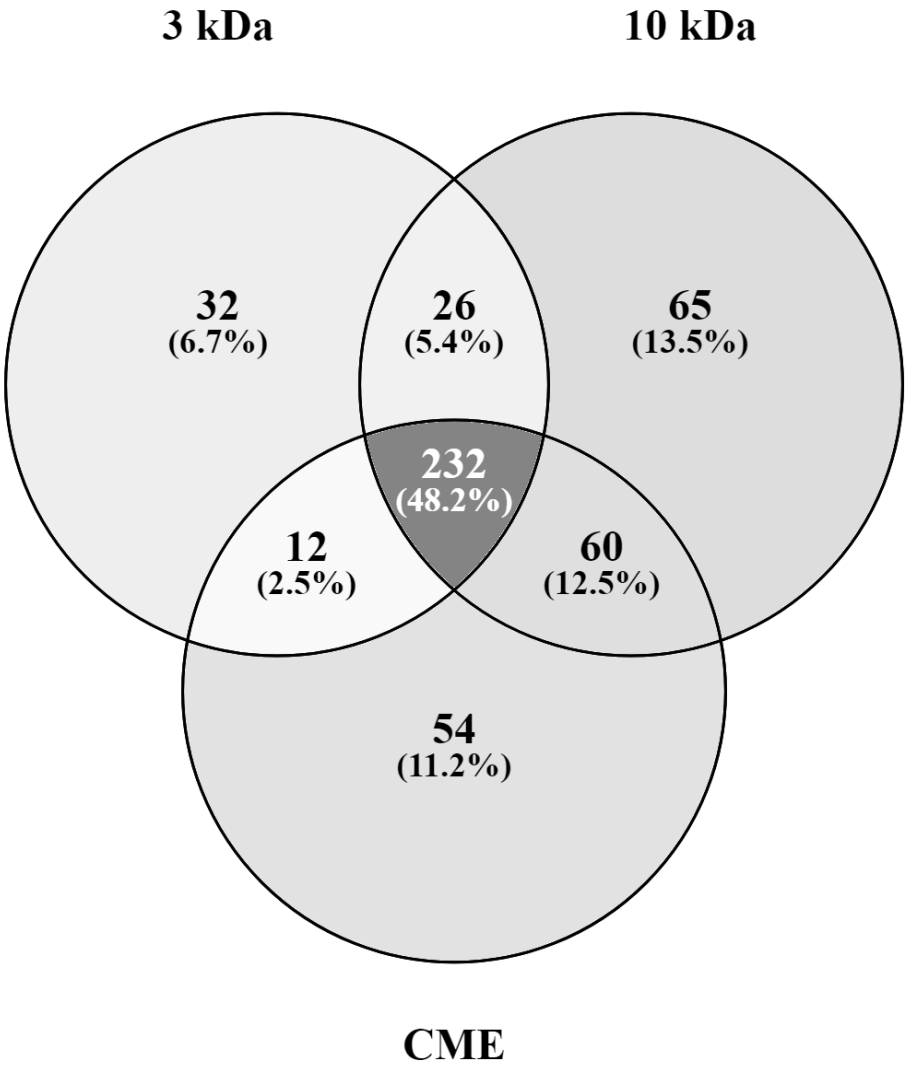

Figure 27. Venn diagram showing the number of peptides identified in the protein fraction for the three different extraction methods.

5.4 Conclusion

The optimised peptidomics method selected involved the following parameters:

The 10 kDa filter-aided extraction method was utilised to separate the protein and peptide fraction. Although this method did not result in the greatest number of peptide identifications it was a simpler method, with fewer steps for loss of material and enabled a fixed size of peptides to be analysed which matched with the MW size distribution analysed using SEC in Chapter 4.

Option 1 of the mass spectrometry settings (full scan MS spectrum (50–1800 m/z, 2 Hz sampling rate) followed by a fixed cycle time of 3 seconds for acquisition of MS/MS spectra of precursors in the range m/z 150-1800, at a sampling rate of 2–32 Hz) was selected as it

resulted in the identification of the greatest number of peptides. The preferred settings when analysing the samples with PEAKS was the BoMiProt database with no enzyme selected as the proteins in this database have been proven to be found in milk.

The peptide extraction method did not have a significant influence on the protein fraction of milk. This study suggested that analysing the protein fraction of milk alongside the peptide fraction gives further insight into how the proteins are broken down. However, due to the extra time required to analyse the protein fraction it was not further utilised in this study.

Chapter 6 - Peptidomics analysis of processed cow and deer milks during gastric digestion

6.1 Introduction

Gastrointestinal digestion of dietary proteins is a crucial step, both to supply the organism with amino acids which are necessary for maintenance and growth of body protein and for the release of peptides with potential biological activity. Peptidomics has been shown to be a suitable method to monitor the hydrolysis of food proteins during digestion (De Cicco *et al.*, 2019; Nguyen *et al.*, 2020; Tagliazucchi *et al.*, 2018). It enables the comprehensive qualitative and quantitative analysis of peptides in a biological sample (Schulz-Knappe *et al.*, 2001). Since 2008 it has been increasingly used to investigate peptides that are found in food products or generated during food processing, storage, or digestion (Martini *et al.*, 2021). The most common technique used to monitor peptides is high resolution MS. It enables the identification of many, though not all, of the peptides released during digestion, though it is limited in its ability to identify very short peptides, large-sized polypeptides and disulfide cross-linked hetero-oligomers (Martini *et al.*, 2021). In recent years peptidomics is being used more frequently to investigate how digestion of different dairy products influences the protein breakdown, release of bioactive peptides and potential allergenic determinants.

Peptidomics has been employed in digestion studies to investigate the release of bioactive peptides during *in vitro* gastrointestinal digestion of cow, goat, and sheep milk and yoghurt using the static INFOGEST digestion model (Nguyen *et al.*, 2020; Tagliazucchi *et al.*, 2018). These studies identified a wide range of bioactive peptides with angiotensin-converting enzyme (ACE)-inhibitory, antioxidant and dipeptidyl peptidase IV (DDP-IV)-inhibitory activity. Tagliazucchi *et al.* (2018) indicated that cow milk was the best source of DPP-IV-inhibitory peptides and antioxidant peptides, whilst sheep milk was the best source of ACE-inhibitory peptides. Nguyen *et al.* (2020) showed that regardless of species yoghurt samples released a higher concentration of peptides with antihypertensive activity than their milk counterparts. These results suggest that the types of bioactive peptides released during digestion is dependent on both species and processing treatment.

Likewise, Kopf-Bolanz *et al.* (2014), Sánchez-Rivera *et al.* (2015) and Barbé *et al.* (2014) applied peptidomics approaches to study differences in the protein hydrolysis and release of bioactive peptides in unprocessed and processed cow milk. Different digestion models were used in these studies including a static *in vitro* digestion model (micro scale method), a dynamic *in vitro* digestion model (DIDGI®) and an *in vivo* digestion model (pig). These studies observed that processing treatment affected the kinetics of milk protein digestion, regardless of digestion model.

Peptidomics has also been used to compare the digestion of milk proteins in different digestion models. For example, peptides released during the static INFOGEST digestion model has been compared to the peptides released in cannulated pigs for micellar CN (Miralles *et al.*, 2020), human jejunal effluents for whey and CN powders (Sanchón *et al.*, 2018) and both pigs and the dynamic DIDGI® model for skim milk (Egger *et al.*, 2019; Egger *et al.*, 2017). These studies have shown similar peptide profiles at the end points of gastric and intestinal digestion between the two models being compared.

To date, the majority of peptidomics studies have investigated the digestion of cow milk, with limited studies investigating other species. Recently the commercial production of red deer milk for human consumption has been investigated due to its high nutritional value and unique characteristics, including a higher content of protein, fat, calcium, zinc, iodine, branched-chain fatty acids and α-linolenic acid than cow milk (Gathercole, 2022; Li *et al.*, 2023; Wang *et al.*, 2017). The high protein content of deer milk offers a unique potential for generating high amounts of protein hydrolysates with unique peptides that may have potential medicinal benefits. Although no studies have used peptidomics to analyse the digestion of red deer milk, some studies have indicated that deer milk proteins are more digestible and produced more peptides than cow milk (O. N. L. Vithana *et al.*, 2012).

Given these differences in composition and digestibility, we hypothesise that the peptide profiles generated during *in vitro* gastric digestion will differ between processed cow and deer milk due to variations in protein composition and processing methods. Furthermore, we expect the processing will influence the extent of protein breakdown in both types of milk, but the effect may vary between species. To test this hypothesis, the objective of this study was to investigate the peptide profiles generated by processed cow and deer milk during *in vitro*

gastric digestion using the HGS model. This will enable an understanding of how different processing treatments affect protein breakdown during digestion and whether this processing effect differs between deer milk and cow milk.

6.2 Materials and Methods

6.2.1 Reagents

Water, acetonitrile, methanol, formic acid (FA) and trifluoracetic acid (TFA), all LC-MS grade, were sourced from ThermoFisher Scientific (Waltham, MA, USA).

6.2.2 Samples

Processed cow and deer milk samples were collected and digested as described in section 3.1.2 and 3.2.2 respectively. During this study only the gastric time points were selected from the pasteurised, pasteurised-homogenised, and heated-homogenised samples.

6.2.3 LC-MS/MS

6.2.3.1 Peptidomic sample preparation

Prior to LC-MS/MS analysis, samples were adjusted to 5 % acetonitrile by adding 30 µL of acetonitrile to 570 µL of each digested milk sample. The samples were then heated at 40 °C for 15 mins prior to centrifugation at 20,000 x *g* for 10 mins at 4 °C (Hettich Mikro 200 R bench top Centrifuge, Tuttlingen, Germany). To isolate the peptide fraction, 200 µL of each sample was added to a 10 kDa NanoSep centrifugal device (Pall, NY, USA). After centrifugation at 10,000 x *g* for 90 mins at 20 °C, the ultrafiltrate was kept for LC-MS/MS analysis. The high temperature (20 °C) was used to improve peptide retention in the ultrafiltrate (Harper *et al.*, 2004). The peptide concentration of the ultrafiltrate was determined using the Thermo Scientific™ Pierce™ quantitative colorimetric peptide assay. Each sample was measured in triplicate and results were accepted if they had a coefficient of variation (CV) under 5 %. A volume containing 100 µg of peptides based on the peptide assay results was then dried for each sample in a CentriVap vacuum centrifuge (Labconco, Kansas City, MO, USA) operated at 40 ºC.

To ensure reproducibility a quality control sample containing equal amounts of 24 samples, selected randomly, were run throughout the batch as well as 6 pools separately including cow

or deer early gastric digestion (0 and 20 mins), mid gastric digestion (60 and 120 mins), and late gastric digestion (180 and 240 mins).

6.2.3.2 Liquid chromatography tandem mass spectrometry

Prior to running on the LC-MS, the samples were resuspended in 50 µL of 0.1 % TFA and diluted 10x with 0.1 % FA.

Nanoflow LC-MS and LC-MS/MS were performed on an Ultimate 3000 HPLC system (ThermoScientific, Waltham, MA, USA) directly interfaced with a CaptiveSpray ion source to an Impact HD Q-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The CaptiveSpray was fitted with a nanoBooster device (Bruker Daltonik, Bremen, Germany), which infused acetonitrile into the mass spectrometer's nitrogen gas supply to improve sensitivity. For each pooled sample, 1 µL was injected onto a PepMap100 C18 Nano-Trap column (ThermoScientific) to trap peptides, which were then eluted onto a C18 Thermo Acclaim PepMap analytical column (250 mm x 75 µm i.d., 3 µm particle size, 100 Å pore size; ThermoScientific). Both columns were maintained at 35 ºC in a column oven. Separation of peptides was carried out at a flow rate of 0.3 µL/min using a multistep linear gradient of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). Initially 2 % B was used for 2 min, then to 20 % over 45 min, and then to 45 % B over a further 15 min. The column was cleaned by increasing to 95 % B over 4 min and holding at that level for 7 min. After that, the column was re-equilibrated by reducing back to 2 % B over 2 min and holding at that for 13 min. The total run time was 88 min.

LC-MS/MS: During LC-MS/MS runs the mass spectrometer was operated using collision-induced dissociation (CID) and automated data dependent acquisition (DDA). Two acquisition methods were utilised to optimise the number of peptides identified. One method involved a full scan MS spectrum (50–2200 m/z, at 1 Hz scan rate) followed by a maximum of 10 MS/MS of precursors in the range m/z 150-1200, at a sampling rate of 1–20 Hz (depending upon precursor intensity). A preference for selection of multiply charged (2+ to 5+) precursor ions was set, and precursors were excluded from reacquisition for 1 min unless their intensity increased at least 4-fold during that period. The other method involved a full scan MS spectrum (50–2200 m/z, at 2 Hz scan rate) followed by a maximum of 10 MS/MS of precursors in the range m/z 150-2200, at a sampling rate of 1–20 Hz (depending upon precursor intensity). A

preference for selection of multiply charged (1+ to 8+) precursor ions was set, and precursors were excluded from reacquisition for 1 min unless their intensity increased at least 5-fold during that period.

Scheduled precursor lists (SPLs): SPLs were generated for each pooled sample using ProteinScape™ version 4.0 (Bruker, Billerica, MA, USA) allowing for time tolerances of ±2 min and size tolerances of ±100 mDa. A SPL was generated for the pooled cow and deer milk samples separately. Each pooled sample was then run with their respective exclusion SPL which contained m/z values and retention times of precursor ions of confidently identified peptides in the initial LC-MS/MS runs, to try and identify lower abundance peptides. This was done using both MS/MS acquisition methods outlined above.

LC-MS: For milk samples and a QC mixture that contained equal amounts of all samples, a MS acquisition method with a full scan MS spectrum (150-2200 m/z, at 1 Hz) in positive mode was used.

6.2.3.3 Sequence database searching

Peptides and proteins were identified using PEAKS Studio version 10.6 (Bioinformatics Solutions Inc., Toronto, Canada) (Ma *et al.*, 2003), with a minimum peptide length set to 4. Peptides were identified with no specific enzyme using the bovine milk proteome database (BoMiProt 2.0, 10,314 sequences) for cow milk samples (Das *et al.*, 2022). Peptides from deer milk were identified with no specific enzyme using an AgResearch in-house deer FASTA database comprised of 337,834 non-redundant sequences that were derived from three sources: (A) the longest open reading frames (ORFs) from red deer lymph node mRNA (43,654 sequences); (B) the mCerELa1.1_genome and the identical protein group of the *Cervu*s organism from NCBI (13,722 and 103,698 sequences, respectively, downloaded 12.10.2022); and (C) all the UniProt *Cervus* database (49,309 sequences, downloaded 12.10.2022). Dereplication was performed using an in-house Python script (make_nr_fasta.py, version 1.0.07).

The following parameters were kept fixed for all searches: precursor ion mass error tolerance of 10 ppm and fragment mass error tolerance of 0.05 Da; maximum allowed variable PTM per peptide was set at 3; peptide false discovery rate (FDR) was set at 1%; proteins were accepted

if their -10lgP values were above 20 and at least 1 unique peptide and additional significant supporting peptides were observed. Variable modifications of oxidation (M), deamidation (NQ), phosphorylation (STY) and pyro-Glu from Q were used for the PEAKS DB search. A PEAKS spider search was performed after the PTM search to identify amino acid substitutions.

6.2.3.4 Label free quantitation (LFQ)

The PEAKS LFQ search used MS data with peptide identifications transferred to MS features from the PTM search (for cow milk) and from the Spider search (for deer milk) with a mass error tolerance of 20 ppm and a retention shift tolerance of 2 min. The FDR threshold was set at 1% and the reference training samples were autodetected. An in-house R-script was used to normalise the intensities of the identified peptides using the total ion chromatograms (TIC) over the time it took to get to 50% B.

6.2.3.5 Statistical analysis

To assess the differences and similarities between the different species and processing treatments during *in vitro* digestion both Venn diagrams (Oliveros, 2007) and principal component analysis (PCA) were used.

To investigate the effect of digestion on the peptide length for milks from processed cow and deer milk the peptides were classified into five groups according to their length, <10 AA, 11–15 AA, 16–20 AA, 21–30 AA, and >30 AA. The relative mean abundances of each peptide and peptide groups were calculated from the three replicates. Graphs were produced in GraphPad Prism version 10.2.1 for Windows, GraphPad Software (Boston, MA, USA) (www.graphpad.com).

6.2.3.6 Protein degradation plots

PrintMap-R (Weaver *et al.*, 2023) was used to visualise the progressive release of peptides from major milk proteins (β-CN, α_{s1}-CN, α_{s2}-CN, κ-CN, and β-LG) throughout gastric digestion, and to illustrate the influence of processing treatment on the degradation of the proteins. The data were exported into the PrintMap-R software along with the respective FASTA file. The proteins in the FASTA file were modified to remove the signal peptides. In the generated plots, the x-axis is the amino acid position within the protein and the y-axis presents the cumulative intensities of identified peptides covering each amino acid position.

Regions of the proteins that were particularly susceptible to digestion by pepsin may be seen as broad peaks in these plots.

6.2.3.7 Identification of bioactive peptides

Identified peptides after gastric digestion of processed cow and deer milk with previously reported bioactivity were investigated using the Milk Bioactive Peptide Database (Nielsen *et al.*, 2023; Nielsen *et al.*, 2017). A species-specific search was carried out for peptides identified from cow milk, whilst for deer milk the search was widened to include all available species.

6.3 Results and discussion

Peptides extracted from dynamic *in vitro* digestion samples were identified using LC-MS/MS. 1,561 peptides, with a minimal length of four amino acids, were identified throughout gastric digestion. Of these, 961 peptides were identified from digested cow milk and 714 were identified from digested deer milk (Figure 28A). The differences and similarities between the two species were evaluated using principal component analysis (PCA) shown in Figure 28B. The results from this study show noticeable differences in the peptides identified in cow and deer milk regardless of processing treatment with only 7.4 % of the sequences being identified in both cow and deer milk. The difference in the peptide products identified during digestion from the two species is likely due to the structural differences in the proteins prior to digestion. Although cow and deer have a similar protein composition (Claeys *et al.*, 2014; Roy *et al.*, 2020a), the amino acid sequences of the proteins differ with Uniprot showing a 70-99% difference between the two species depending on the protein (Consortium, 2022). This difference will likely result in differences in which sites are readily available for enzymes such as pepsin to cleave. The amino acid differences will also result in different peptides being identified using this analysis regardless of whether they are from the same region of the protein.

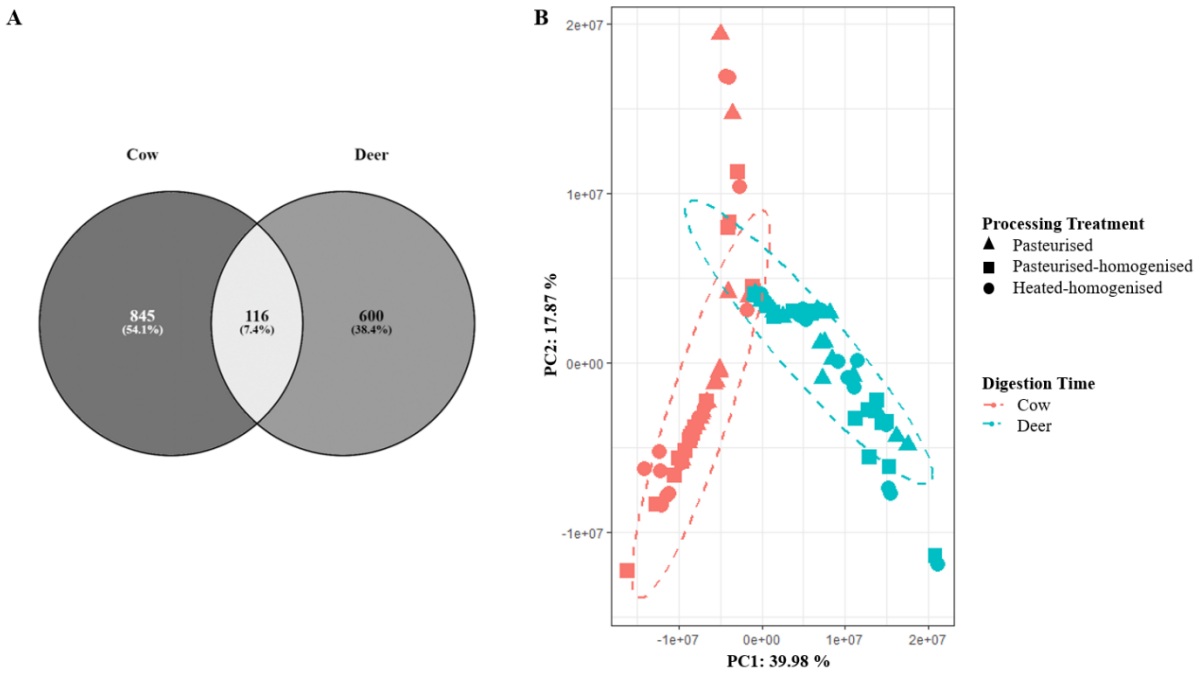

Figure 28. Comparison of peptides between digested cow and deer milk samples. A) Venn diagram of peptides identified from cow and deer milk samples. B) PCA plot of peptides throughout *in vitro* digestion of cow and deer milk samples.

The differences and similarities in the peptide profiles between the three processing treatments at each stage of digestion were evaluated by PCA for both cow and deer milk (Figure 29). The most significant difference was between the different digestion time points in both cow and deer milk. In cow milk the most noticeable difference was observed between early-mid gastric time points (0 min, 20 mins, 60 mins and 120 mins) with the least difference observed during late gastric time points (180 mins and 240 mins). In deer milk more overlap was observed between the different time points especially during early gastric digestion (0 min and 20 mins) and mid-late gastric time points (120 mins, 180 mins and 240 mins). The difference observed during early gastric digestion time points can likely be attributed to changes in the proteins due to formation and restructuring of the clot. Although protein coagulation has been observed as early as 8 mins into gastric digestion (Ye *et al.*, 2016b), structural rearrangement of the clot is still occurring during the first 20-60 mins of digestion (Li, Pan, *et al.*, 2022). This could contribute to changes in proteins available for digestion. Previous studies have also shown via SDS-PAGE gels that intact CNs disappeared after 60 mins of dynamic digestion (Egger *et al.*, 2019), further indicating that a high degree of structural rearrangement is happening during early digestion. The PCA of both cow and deer milk showed limited differences between

processing treatment during most time points except during mid-gastric digestion (60 to 120 mins). These results are consistent with SEC observations found in section 4.3.1.2.

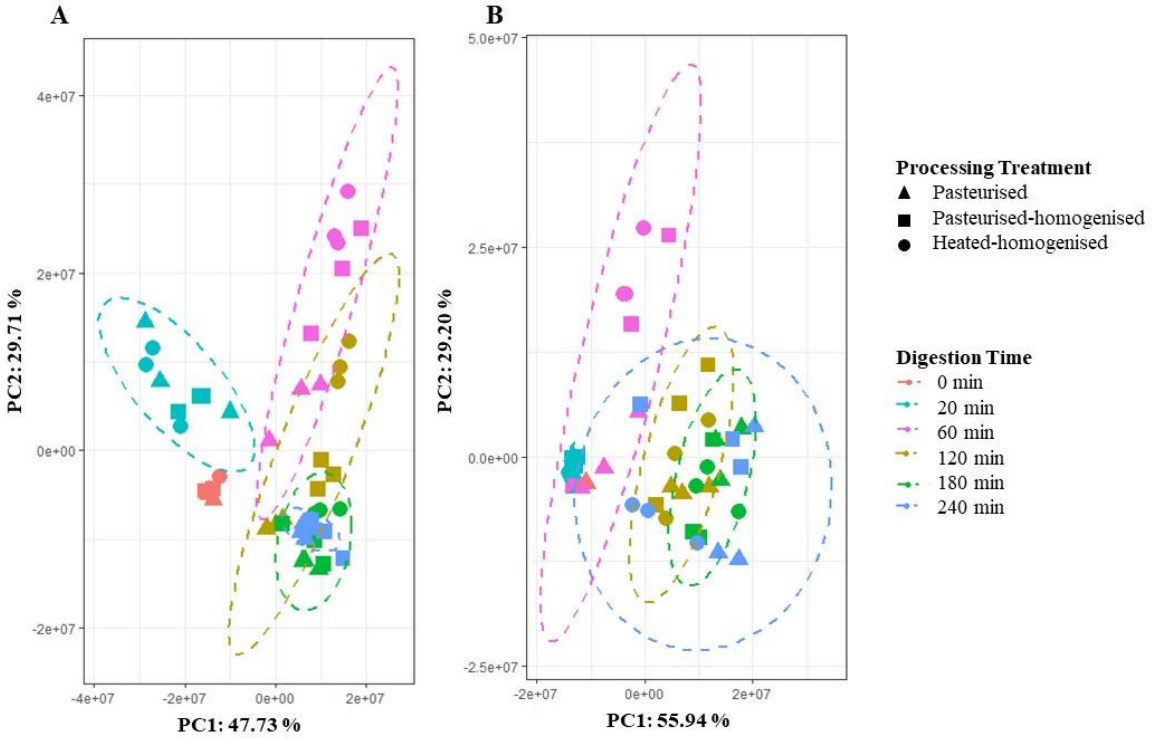

Figure 29. PCA plots of peptides <10 kDa analysed by LC-MS after *in vitro* digestion. A) illustrates samples from cow and B) illustrates samples from deer.

This study identified a similar number of peptides derived from CN in cow (480 peptides) and deer (485 peptides) milk. The lower percentage of peptides derived from CN in cow milk (49.9 %) than in deer milk (67.9 %) is likely due to the higher number of whey proteins identified in the cow milk samples compared to deer milk (95 total proteins for cow milk and 18 total proteins for deer milk). The top three whey proteins identified during this study were β-LG, α-LA, and glycosylation-dependent cell adhesion molecule 1, regardless of species selected. To assess the degradation of proteins throughout digestion in processed cow and deer milk the peptides were sorted into five different groups according to their length which ranged from 4 amino acids (AA) to 39 AAs. The peptide degradation profiles showed that the percentage of relative abundance of peptides for small peptides (<10 AA) gradually increased over time, whilst medium sized peptides (11-15 AA) showed an initial increase from 20 to 60 mins however remained stable for the rest of digestion, and larger peptides (>16 AA) gradually decreased over time (Figure 30). This trend was observed in all processed cow and deer milks. Interestingly, amino acids between positions 4 and 10 were found to be higher in undigested

cow milk compared to those after 20 min of gastric digestion. This may be attributed to the proteolytic activity of digestive enzymes, such as pepsin, which can break down specific peptide bonds, leading to the degradation of these amino acids.

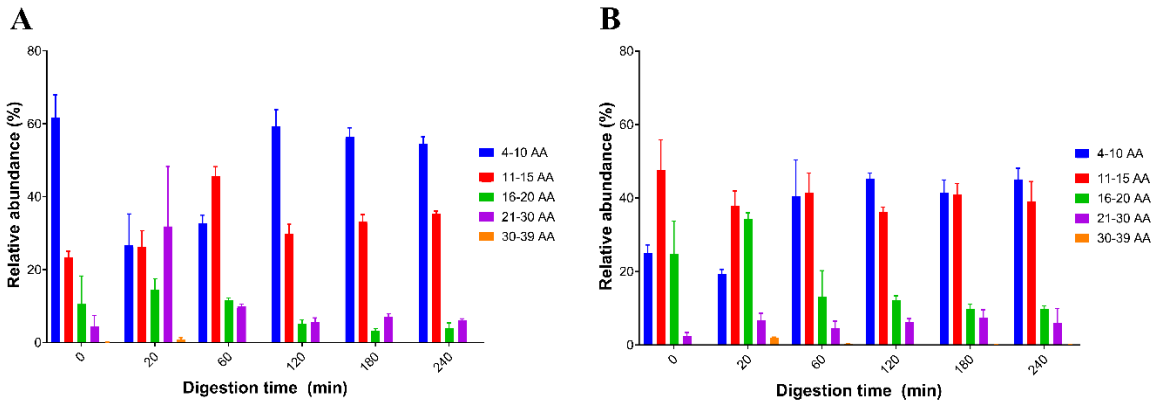

Figure 30. Degradation profiles of peptides in processed A) cow and B) deer milk during 240 min gastric digestion using the HGS dynamic *in vitro* digestion model. The plots show percentage relative mean abundance of the peptides. Each column is the average of three independent replicates and the error bars represent the standard deviation.

6.3.1 Degradation of major milk proteins

For all identified peptides, PrintMap-R was used to visualise the progressive release of peptides from major milk proteins as described in the Materials and Methods section (6.2.3.6), where the y-axis represents the peptide intensity, and the x-axis represents the AA sequence of the protein. Figure 31-40, illustrates the peptide intensity obtained, following the different phases of gastric digestion for β-CN, α_{s1}-CN, α_{s2}-CN, κ-CN, and β-LG. High peptide intensity at a given location on the protein backbone signifies that the concerned protein regions are susceptible to enzyme action during the respective digestion phase.

6.3.1.1 Evolution of β-CN peptides throughout gastric digestion in processed cow and deer milk

Figure 31 shows the intensities of peptides identified from β-CN in pasteurised, pasteurised-homogenised, and heated-homogenised cow milk throughout gastric digestion mapped to AA position in the protein. Four distinct areas along the parent protein had a high intensity of peptides: 59-70, 128-140, 164-190 and 199-209 in all three processing treatments. The abundance of peptides belonging to regions 59-70, 128-140 and 199-209 increased throughout gastric digestion whilst the abundance of peptides belonging to region 164-190 decreased

throughout digestion (Table 17). Highly abundant areas of the protein during early digestion could suggest that these regions are susceptible to hydrolysis with pepsin or that they are soluble in the liquid phase and thus emptied early. Meanwhile highly abundant areas of the protein during late digestion could suggest that these regions are resistant to digestion, or that other factors such as being associated with the coagulum are resulting in peptides from these areas being emptied late. Processing treatment did not appear to have a significant effect on the regions of CN that are resistant to digestion.

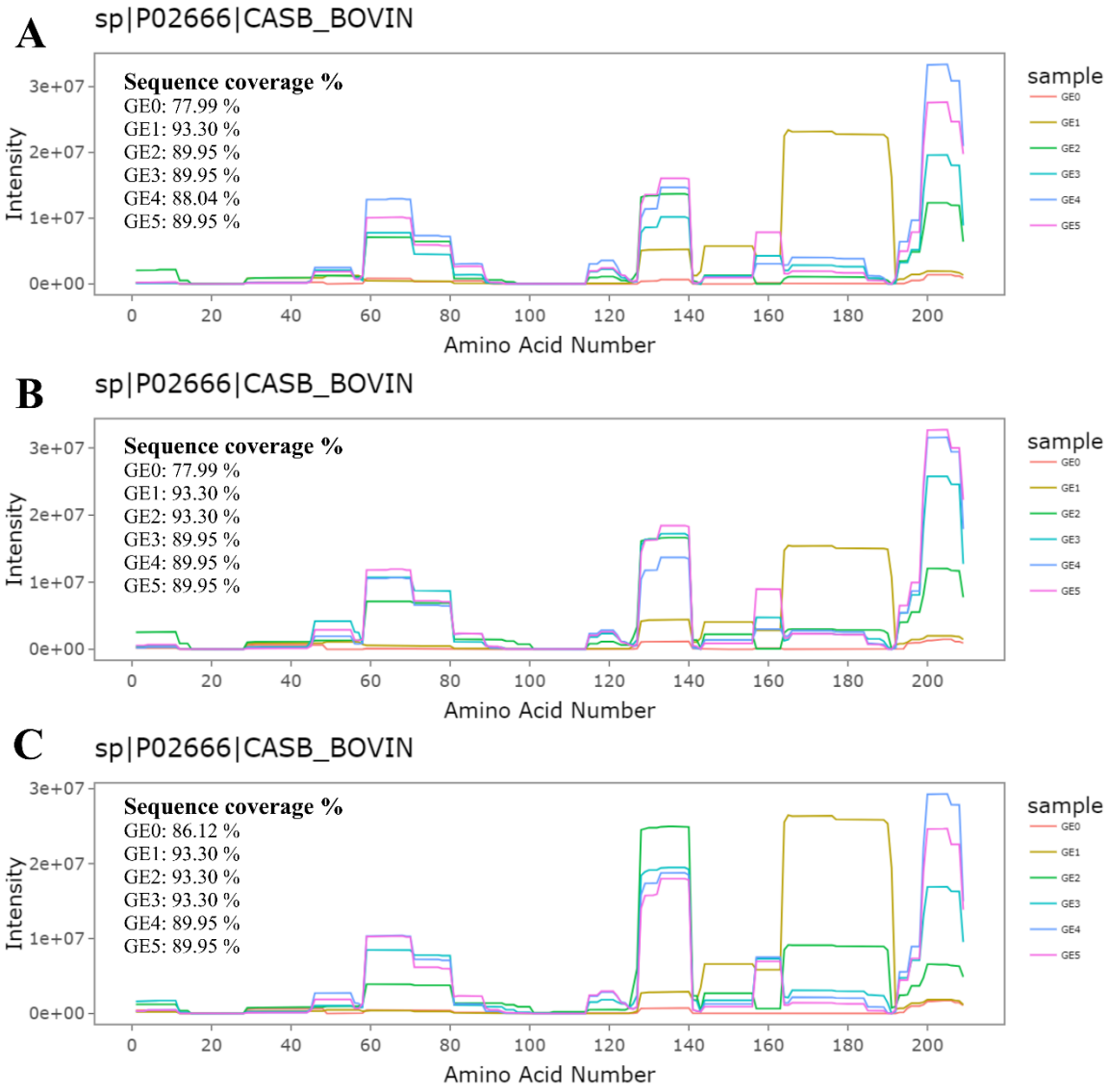

Figure 31. Distribution of peptides from β-CN throughout gastric digestion from A) pasteurised, B) pasteurised-homogenised, and C) high heat-homogenised cow milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min),

GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Table 17. Protein regions of β-CN with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised cow milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
59-70	180 min	240 min	240 min
128-140	240 min	240 min	60 min
164-190	20 min	20 min	20 min
199-209	180 min	240 min	180 min

Figure 32 shows the intensities of peptides from β-CN identified in pasteurised, pasteurised-homogenised, and heated-homogenised deer milk throughout gastric digestion mapped to AA position in the protein. Peptides originating from β-CN in deer milk showed alternating patterns of high and low intensity across the sequence. In particular, region 127-139 showed the highest intensity. In all three processing treatments the intensity of peptides found in this region increased throughout digestion.

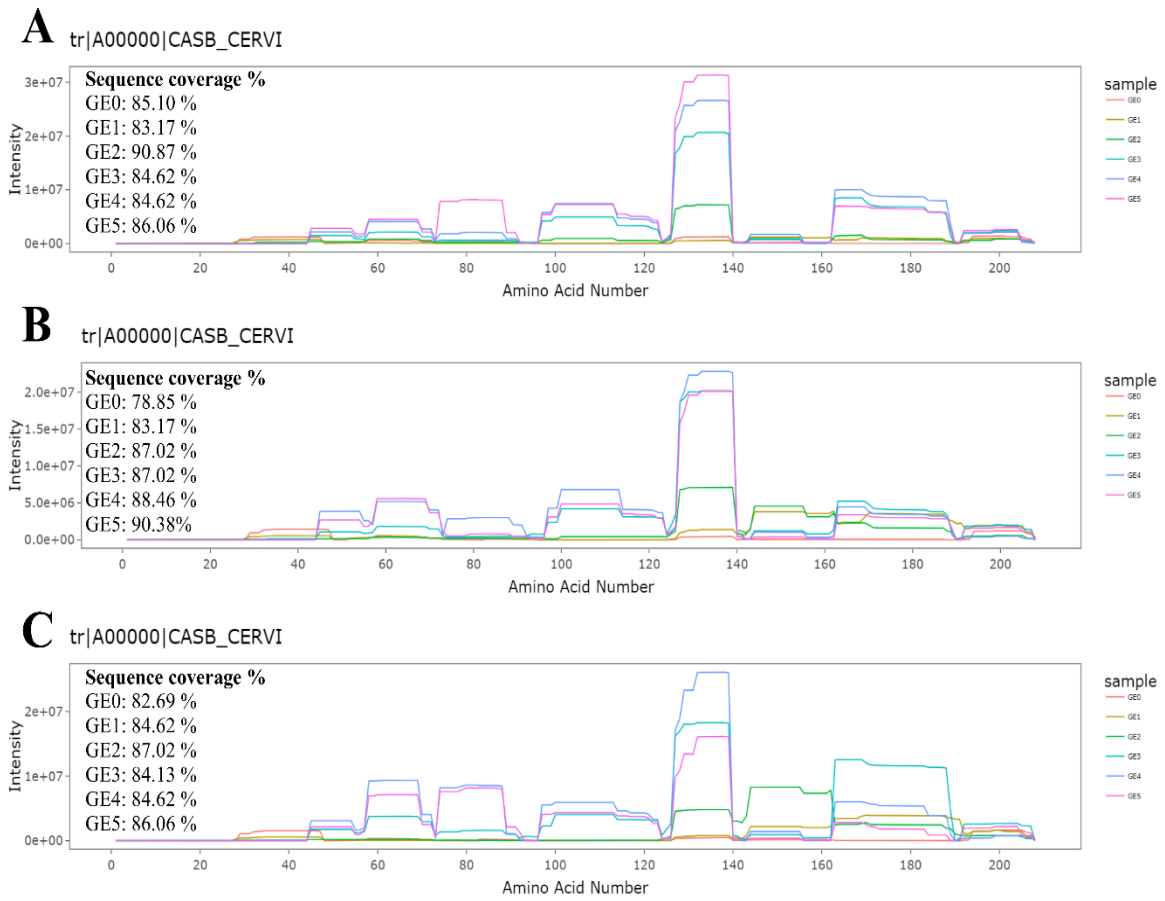

Figure 32. Distribution of peptides from β-CN throughout gastric digestion from A) pasteurised, B) pasteurised-homogenised, and C) high heat-homogenised deer milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Similarities and differences were observed in the degradation of β-CN of processed cow and deer milk. In both cow and deer milk region 127-140 had a high relative abundance of peptides during late digestion. The sequence in this region is conserved between the two species. Previous studies have found this region resistant to digestion (Dupont, Mandalari, Molle, *et al.*, 2010; Sánchez-Rivera *et al.*, 2015), which is consistent with the findings in this study. This region has a high content of proline residues and is hydrophobic at pH 3 which is thought to protect the region from pepsinolysis (Dupont, Mandalari, Molle, *et al.*, 2010). The C-terminal region of β-CN in cow milk had a 3x higher intensity than the equivalent region in deer milk, although the number of peptides generated from this area were similar (18 in cow and 15 in deer). Previous studies have shown that although pepsin digestion of β-CN begins at the C-

terminal region this region is resistant to digestion (Picariello *et al.*, 2010; Sánchez-Rivera *et al.*, 2015). The decreased abundance observed in this region for the deer milk samples suggest that this region is less resistant to digestion in deer milk compared to cow milk. A difference is observed in the AA sequence of β-CN from cow and deer milk in the C-terminal region. The sequence in cow is PVRGPFPIIV, whilst for deer it is PVRGPFPIIN (last 10 AAs, the difference is highlighted in red). This suggests that even minor changes in AA sequence can lead to changes in resistance.

Processing treatment does not appear to have a significant effect on the regions of β-CN that had high intensity. However, slight differences in the sequence coverage were observed. The highest sequence coverage of β-CN in cow milk was 93.3 %, for all processing treatments at the 20 min time point. The sequence coverage decreased throughout digestion at different rates. In pasteurised milk the sequence coverage decreased at 60 mins, for pasteurised-homogenised milk it decreased at 120 mins and for heated-homogenised milk it decreased at 180 mins. The decrease in sequence coverage throughout digestion could be due to well-hydrolysed regions having peptides that were unable to be detected using the method used in this study, *e.g.* the peptides were below 4 AAs. These results suggest that high heat treatment may make β-CN more resistant to hydrolysis. In contrast no clear pattern was observed for the sequence coverage for deer milk. The highest sequence coverage of β-CN for pasteurised and pasteurised-homogenised deer milk throughout gastric digestion was 90.97 % in the 60 mins and 240 mins digesta respectively whilst for heated-homogenised milk it was 87.02 % in the 60 mins digesta.

6.3.1.2 Evolution of α_{s1}-CN peptides throughout gastric digestion in processed cow and deer milk

Figure 33 shows the intensities of peptides from α_{s1}-CN identified in pasteurised, pasteurised-homogenised, and heated-homogenised cow milk throughout gastric digestion mapped to AA position in the protein. The highest sequence coverage of α_{s1}-CN throughout digestion was 90.95% for pasteurised-homogenised and heated-homogenised milk and 89.45% for pasteurised cow milk. Throughout digestion four areas of the parent protein contribute to the highest intensity, regardless of processing treatment. These are between residues 24-39, 110-120, 144-149 and 165-179. Throughout digestion the most abundant peptides belonged to the region comprised between residues 24-39. Table 18 illustrates the time points with the highest intensity of peptides within these four regions for the three processing treatments. These results

indicate that processing treatment affects the kinetics of protein hydrolysis especially for protein regions 144-149 and 165-169. In pasteurised milk the highest observed intensity of these regions is 240 mins whilst for heated-homogenised milk it is 60 mins. This could suggest that these regions are less resistant to digestion in milk that has been homogenised and exposed to high heat. Homogenisation and intense heat treatment is known to affect the coagulum structure making it more loose and crumbly (Li *et al.*, 2021), which could promote hydrolysis in these regions.

Table 18. Protein regions of α_{s1}-CN with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised cow milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
24-39	60 min	60 min	60 min
110-120	240 min	180 min	240 min
144-149	240 min	240 min	60 min
165-179	240 min	60 min	60 min

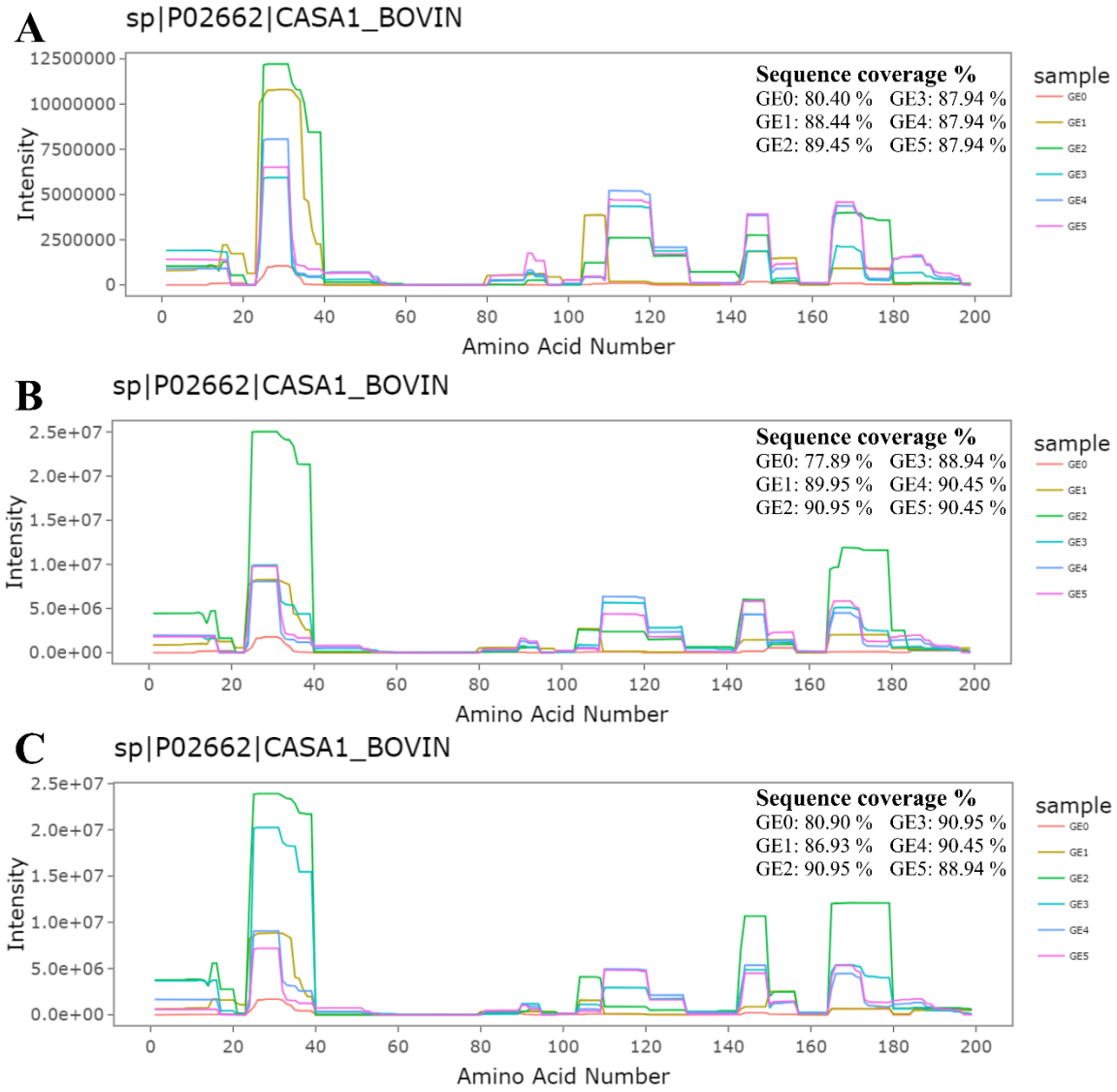

Figure 33. Distribution of peptides from α_{s1}-CN throughout gastric digestion from A) pasteurised, B) pasteurised-homogenised, and C) high heat-homogenised cow milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Figure 34 shows the intensities of peptides from α_{s1}-CN identified in pasteurised, pasteurised-homogenised, and heated-homogenised deer milk throughout gastric digestion mapped to AA position in the protein. The highest sequence coverage of α_{s1}-CN was 71.2 % for pasteurised and heated-homogenised milk and 69.11 % for pasteurised-homogenised milk during mid-digestion (60-120 min). Throughout digestion four areas of the parent protein contribute to the highest intensity, regardless of processing treatment. These are between residues 24-39, 99-

109, 136-144 and 166-172. Table 19 illustrates that processing treatment affects the kinetics of protein hydrolysis. As observed for cow milk the time points with the highest intensity of peptides are earlier in samples that have been homogenised and exposed to a higher heat treatment. This is observed for all four regions suggesting that deer milk is more susceptible to the changes caused by different processing treatments.

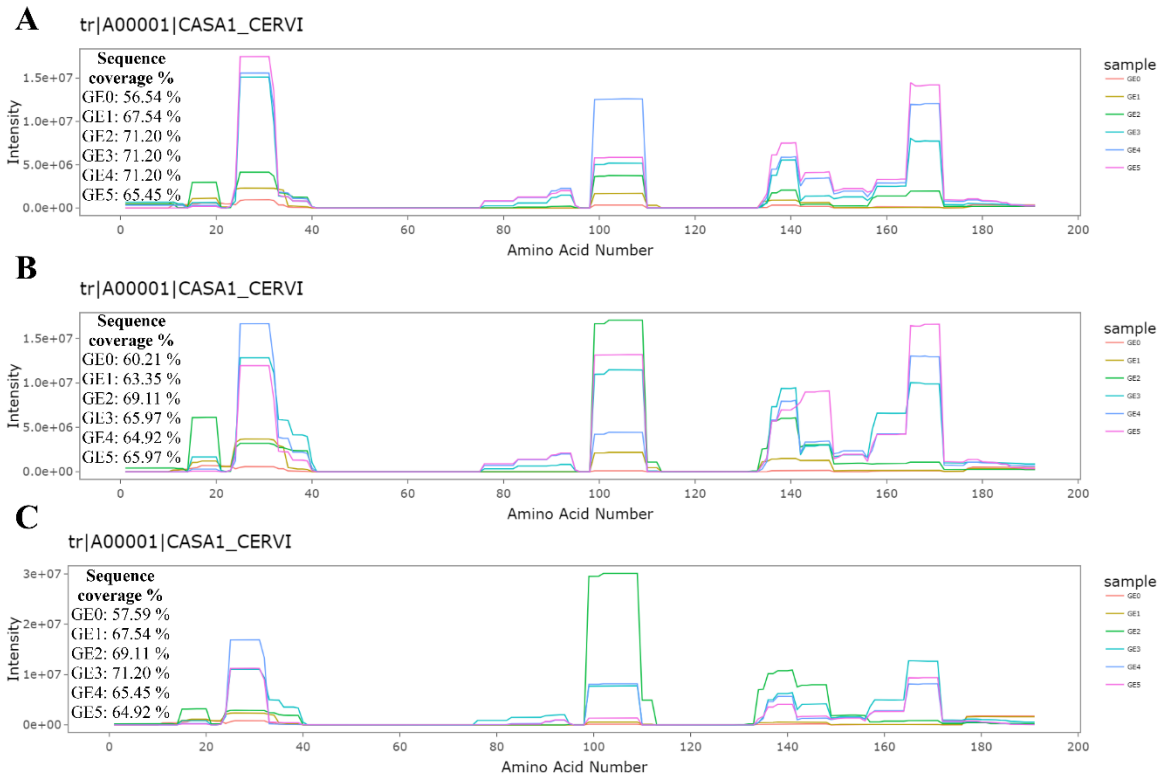

Figure 34. Distribution of peptides from α_{s1}-CN throughout digestion from A) pasteurised, B) pasteurised-homogenised, and C) high heat-homogenised deer milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Table 19. Protein regions of α_{s1}-CN with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised deer milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
24-39	240 min	180 min	180 min
99-109	180 min	60 min	60 min
136-148	240 min	240 min	60 min
166-172	240 min	240 min	120 min

Both cow and deer milk show a similar pattern of high and low intensity across the parent protein of α_{s1}-CN that corresponds to roughly the same regions. Hydrolysis of α_{s1}-CN starts at the N-terminal region. The bond between Phe^{23}-Phe^{24} has been shown to have a high susceptibility to hydrolysis in cow milk (Sánchez-Rivera *et al.*, 2015) which gives rise to peptides between 1-23 and 24-39. This bond is not found in deer milk and instead the bond Leu^{23}-Phe^{24} is found. During this study the intensity of residues between 24-39 was 2.5x higher than those from residue 1-23 in cow milk. A similar trend is observed in deer milk with the intensity of residues between 24-39 being 3.5x higher than those from residue 1-23. The time point with the highest intensity of region 24-39 is 60 mins in cow milk and 240 mins in deer milk. At 60 mins the intensity of this region is 1.8x higher in pasteurised cow milk than deer milk and 3x higher in pasteurised-homogenised and heated-homogenised cow milk than deer milk. However, at 240 mins no difference in this region's intensity was observed between the species. This suggests that whilst the AA substitution in α_{s1}-CN for deer milk does not affect the cleavage site, it may mean that this region is less susceptible to hydrolysis during early digestion than cow milk.

6.3.1.3 Evolution of κ-CN peptides throughout gastric digestion in processed cow and deer milk

Figure 35 shows the intensities of peptides from κ-CN identified in processed cow milk throughout gastric digestion mapped to AA position in the protein. The sequence coverage of κ-CN varies depending on digestion time and processing treatment. The highest sequence coverage for pasteurised milk was 88.17 % at 180 mins, for pasteurised-homogenised milk it was 94.8 % at 20 mins and for heated-homogenised milk it was 95.86 % at 60 mins. Five distinct areas along the parent protein had high intensity: 18-29, 42-50, 56-66, 106-124 and 141-161 in all three processing treatments. The intensity pattern of these regions varied

depending on both the digestion time and processing treatment. Table 20 illustrates the time points with the highest intensity of peptides within these five regions for the three processing treatments. These results indicate that the time point with the highest intensity is earlier for milks that have been homogenised and exposed to more intense heat treatment in most of these regions except region 141-161. Although this region showed a higher intensity at the 120 mins time point in heated-homogenised milk it had a lower intensity than the 120 mins time point in pasteurised milk.

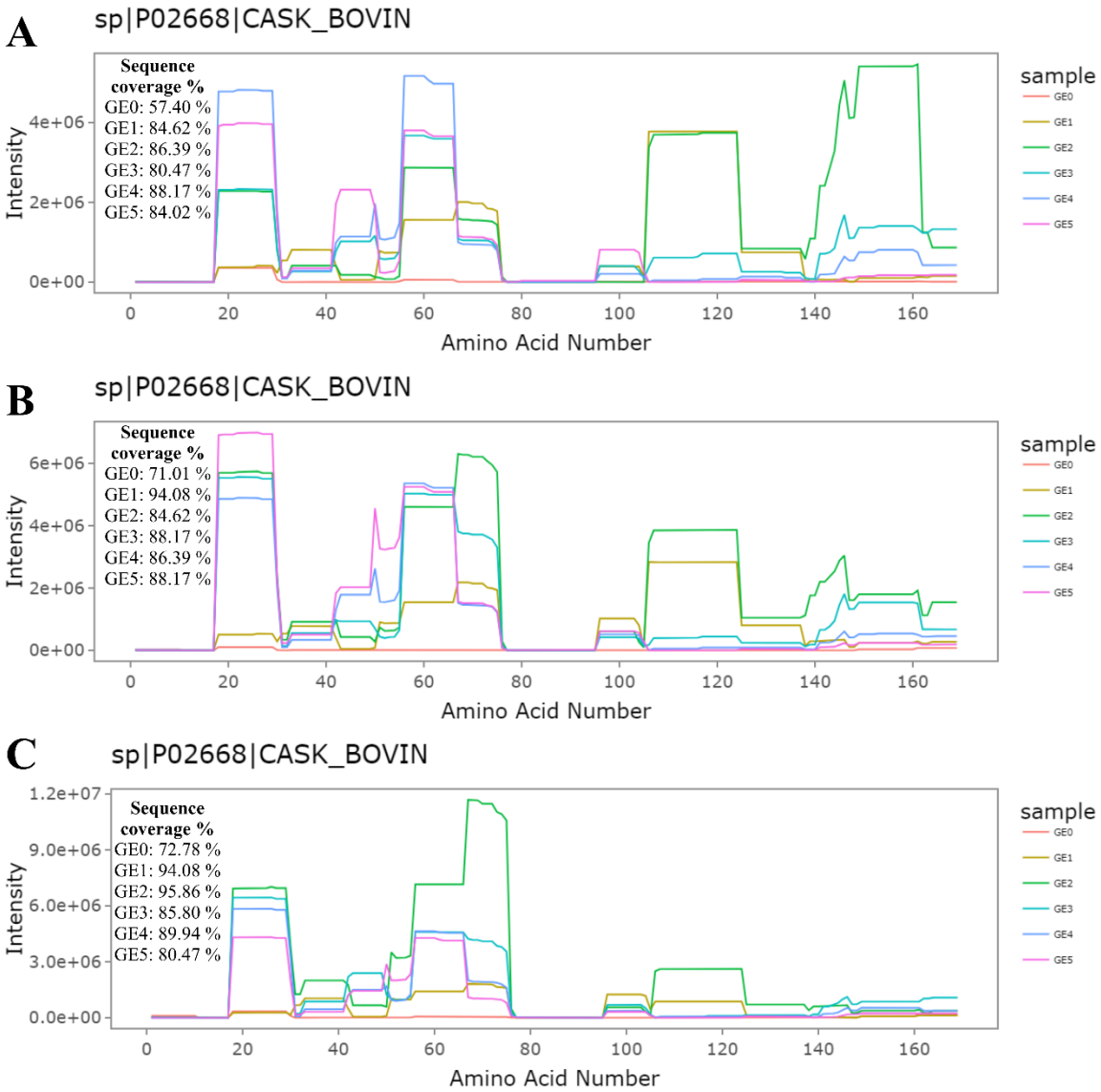

Figure 35. Distribution of peptides from κ-CN throughout digestion from A) pasteurised, B) pasteurised-homogenised, and C) high heat-homogenised cow milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3

(120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Table 20. Protein regions of κ-CN with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised cow milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
18-29	180 min	240 min	60 min
42-50	240 min	240 min	120 min
56-66	180 min	240 min	60 min
106-124	60 min	60 min	60 min
141-161	60 min	60 min	120 min

Figure 36 shows the intensities of peptides from κ-CN identified in pasteurised, pasteurised-homogenised, and heated-homogenised deer milk throughout gastric digestion mapped to AA position in the protein. Peptides covering the entire sequence of κ-CN are observed throughout digestion in deer milk irrespective of processing treatment. Five distinct areas along the parent protein had a high intensity of peptides: 18-29, 42-50, 56-75, 106-124 and 141-161 in all three processing treatments. The intensity pattern of these regions varied depending on both the digestion time and processing treatment. Table 21 illustrates the time points with the highest intensity of peptides within these five regions for the three processing treatments. These results indicate that the effect of processing treatment differs depending on the region of the protein. For example, region 106-124 and 127-137 has a higher intensity during early digestion regardless of processing treatment, however, the highest intensity is slightly later in heated-homogenised milk (60 and 120 mins respectively) compared to pasteurised milk (20 mins and 60 mins respectively). Whereas the region between 18-29, 33-41 and 56-75 have a higher intensity during late digestion with the highest intensity being slightly earlier in heated-homogenised milk (60 mins-120 mins) than pasteurised milk (180 mins-240 mins).

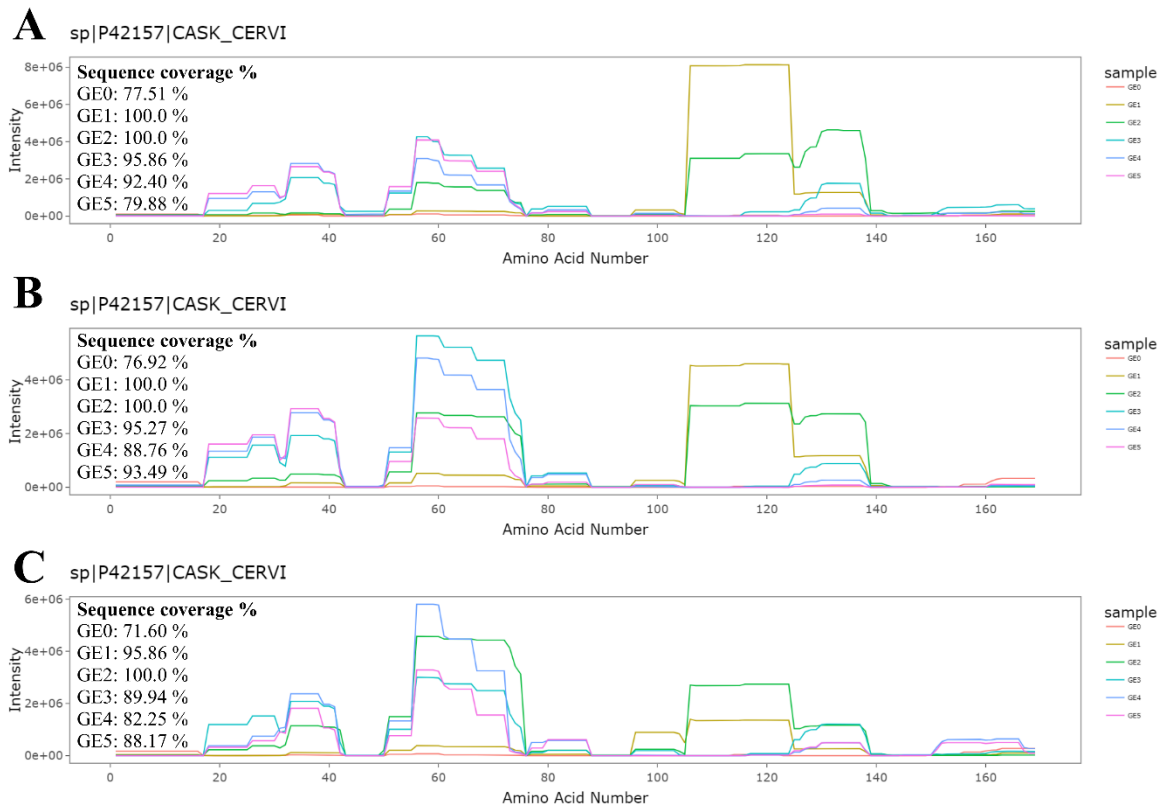

Figure 36. Distribution of peptides from κ-CN throughout digestion from A) pasteurised, B) pasteurised-homogenised, and C) high heat-homogenised deer milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Table 21. Protein regions of κ-CN with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised deer milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
18-29	240 min	240 min	120 min
33-41	240 min	240 min	180 min
56-60	240 min	120 min	180 min
61-75	240 min	120 min	60 min
106-124	20 min	20 min	60 min
127-137	60 min	60 min	120 min

It is well known that during early digestion pepsin cleaves κ-CN at the 105-106 site to produce two macropeptides, a hydrophobic N-terminal extreme named para- κ-CN that is associated with protein coagulum and a soluble and hydrophilic C-terminal moiety called caseinomacropeptide which can be emptied during early digestion (Carlson *et al.*, 1987; Yang *et al.*, 2022). This is consistent with this study's findings, which show that during early digestion both cow and deer have a high intensity of peptides contributing to AA occurrence of κ-CN of 106+ whilst 1-105 peptides were emptied during late digestion. The intensity of peptides contributing to AA occurrence of κ-CN differed between cow and deer milk, with certain regions of the protein having a higher intensity in cow milk and certain regions having a higher intensity in deer milk. During digestion, three regions of κ-CN contributed to peptides with high intensity suggesting that they are resistant to digestion. These included regions 18-29, 56-66 and 106-124. Differences in the intensity between cow and deer milk were investigated for these regions. In all three processing treatments the intensity of peptides originating from the region 18-29 was 2-6-fold greater in cow milk than deer milk. The difference in intensity was dependent on digestion time and was higher during early digestion than during late digestion. Similar intensities were observed between residues 56-60 in cow and deer milk. The differences observed in peptides found between residues 106-124 were dependent on digestion time. During early digestion (20 mins and 60 mins) no difference was found between cow and deer milk, however, after 120 mins cow milk showed a 3-fold greater intensity compared to deer milk. Although no difference in AA was observed for the cleavage site in these three regions, differences in the surrounding AAs were observed.

6.3.1.4 Evolution of α_{s2}-CN peptides throughout gastric digestion in processed cow and deer milk

Figure 37 shows the intensities of peptides from α_{s2}-CN identified in pasteurised, pasteurised-homogenised, and heated-homogenised cow milk throughout gastric digestion mapped to AA position in the protein. The sequence coverage of α_{s2}-CN is lower than the other CNs, this may be due to α_{s2}-CN having a higher resistance to *in vitro* digestion compared to other CNs (Dupont, Mandalari, Molle, *et al.*, 2010). Throughout digestion peptides from pasteurised milk covered 50.24-56.52 % of the parent protein, pasteurised-homogenised milk covered 49.76-55.56% of the parent protein and heated-homogenised milk covered 51.69-57.00% of the parent protein depending on the digestion time. Regardless of the comparably lower coverage, six distinct areas along the parent protein had a high intensity of peptides: 36-49, 89-95, 100-

123, 154-163, 175-182 and 199-207. The intensity pattern of these regions varied depending on both the digestion time and processing treatment. Table 22 illustrates the time points with the highest intensity of peptides within these six regions for the three processing treatments. These results indicate that the C-terminal region of α_{s2}-CN is hydrolysed early. They also indicate that the effect of processing treatment differs depending on the region of the protein. For example, the region between 199-207 has the highest intensity at 60 mins for heated-homogenised samples whilst for pasteurised samples the highest intensity is at 20 mins and the region between 100-123 has the highest intensity at 60 mins for heated-homogenised samples whilst for pasteurised samples the highest intensity is at 180 mins. In all three processing treatments region 89-95 had the highest intensity after 240 mins of gastric digestion. This suggests that this region is either resistant to digestion or is emptied during late digestion due to interaction with the coagulum.

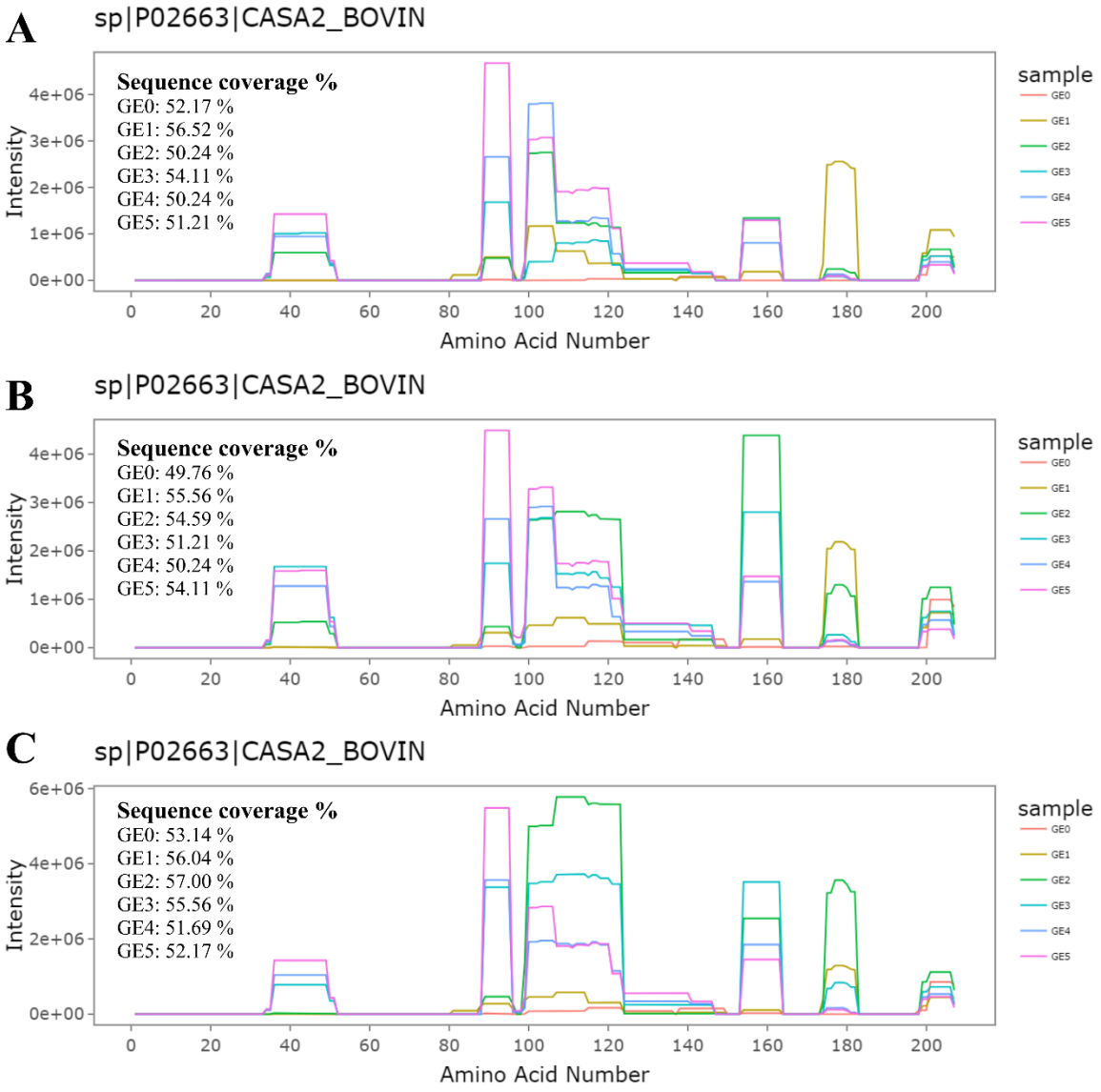

Figure 37. Distribution of peptides from α_{s2}-CN throughout digestion from A) pasteurised, B) pasteurised-homogenised, and C) high heat-homogenised cow milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Table 22. Protein regions of α_{s2}-CN with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised cow milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
36-49	240 min	120 min	240 min
89-95	240 min	240 min	240 min
100-123	180 min	60 min	60 min
154-163	240 min	60 min	120 min
175-182	20 min	20 min	60 min
199-207	20 min	60 min	60 min

Figure 38 shows the intensities of peptides from α_{s2}-CN identified in pasteurised, pasteurised-homogenised, and heated-homogenised deer milk throughout gastric digestion mapped to AA position in the protein. The sequence coverage of α_{s2}-CN was also lower than the other CNs in deer milk. Throughout digestion peptides from pasteurised milk covered 25.12-47.83 % of the parent protein, pasteurised-homogenised milk covered 19.32-48.79 % of the parent protein and heated-homogenised milk covered 22.71-48.31 % of the parent protein depending on the digestion time. Regardless of the comparably lower coverage, four distinct areas along the parent protein had high intensity: 85-97, 100-106, 170-179 and 186-206. The intensity pattern of these regions varied depending on both the digestion time and processing treatment. Table 23 illustrates the time points with the highest intensity of peptides within these four regions for the three processing treatments. The time point with highest intensity varies depending on processing treatment with pasteurised milks having the highest intensity at 120-240 mins depending on region whilst heated-homogenised milks having the highest intensity at 180-60 mins.

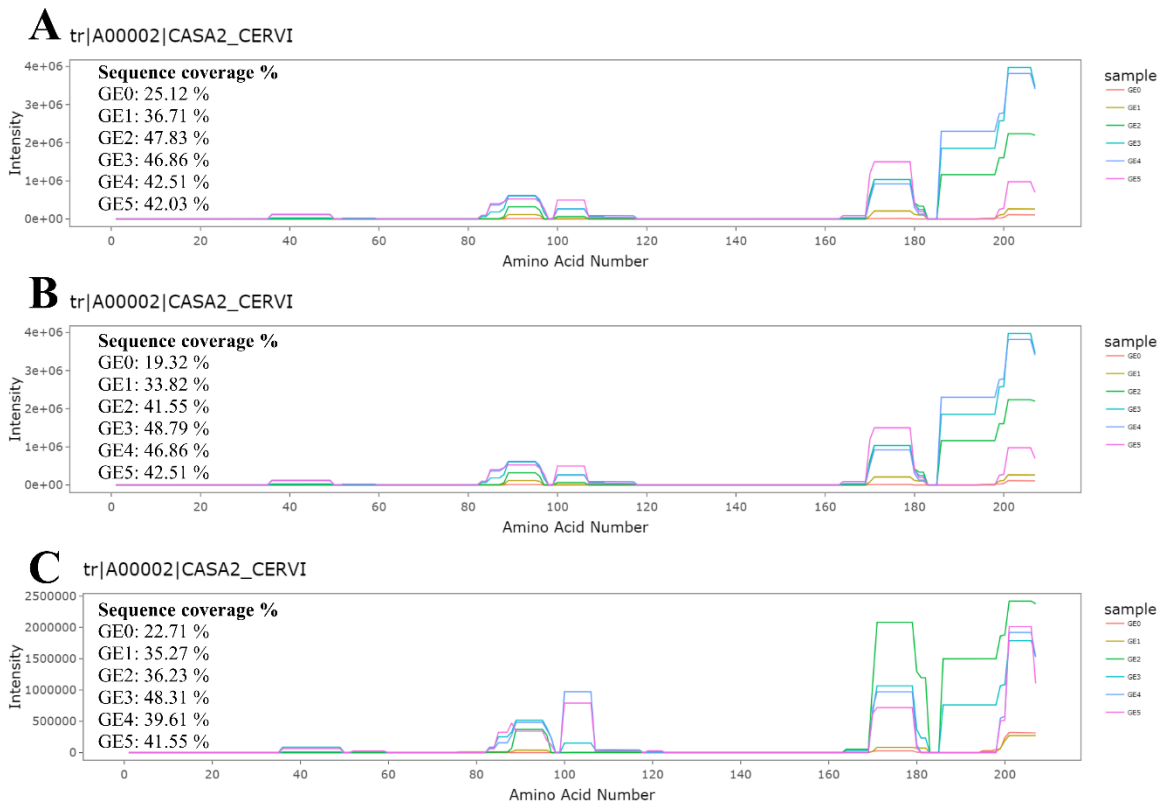

Figure 38. Distribution of peptides from α_{s2}-CN throughout gastric digestion from A) pasteurised, B) pasteurised-homogenised, and C) high heat-homogenised deer milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Table 23. Protein regions of α_{s2}-CN with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised deer milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
85-97	240 min	240 min	180 min
100-106	240 min	240 min	180 min
170-179	240 min	240 min	60 min
186-206	120 min	180 min	60 min

The regions of high intensity of the peptides from α_{s2}-CN differed in cow and deer milk. This is likely due to differences in the sequence of AAs resulting in a different structure of α_{s2}-CN

resulting in different regions of the protein being more exposed or susceptible to pepsin during digestion. The sequence similarity between cow and deer α_{s2}-CN is 87.96 %.

6.3.1.5 Evolution of β-LG peptides throughout gastric digestion in processed cow and deer milk

Figure 39 shows the intensities of peptides from β-LG identified in pasteurised, pasteurised-homogenised, and heated-homogenised cow milk throughout gastric digestion mapped to AA position in the protein. The sequence coverage of β-LG fluctuated throughout digestion in cow milk. Peptides covered 54.32-73.46 % of the parent protein in pasteurised milk, 48.15-73.56% in pasteurised-homogenised milk and 62.96-72.22 % in heated-homogenised milk. Three distinct areas along the parent protein had high intensity: 1-54, 96-104, and 123-130. Table 24 illustrates the time points with the highest intensity of peptides within these three regions for the three processing treatments.

All three regions show a higher intensity during late digestion (240 mins) in heated-homogenised milk compared to pasteurised milk which peaks at 120 mins. This could indicate that these regions are resistant to digestion or that there is a delay in emptying peptides from heated-homogenised samples. Previous studies have indicated that heat treatment of milk makes β-LG more susceptible to hydrolysis. This trend has been observed between non-heated and heated (90 ºC, 10 mins) milks using a dynamic DIDGI® *in vitro* digestion model (Sánchez-Rivera *et al.*, 2015) and between pasteurised (72 ºC, 15 sec) and UHT milk (140 ºC, 2 sec) using a static *in vitro* digestion model (Wada & Lönnerdal, 2014). A potential reason for the contradictory results is the processing treatments used in this study, as different heating times are known to influence the degree of protein denaturation which could affect regions which are more accessible to pepsin (Oldfield *et al.*, 1998). Processing treatments, such as high heat treatment are also known to affect PTMs of proteins. Wada and Lönnerdal (2014) showed that lactulosyllysine, a PTM which reflects a decrease in protein digestibility in milk proteins including β-LG, was higher in UHT milk than pasteurised milk. PTMs caused by heat treatment including lactosylation and glycosylation represent an additional difficulty for peptide sequencing by MS and thus were not specifically searched for in this study. Processing treatments, such as high heat treatment are also known to cause denaturation and aggregation of whey proteins (Wada & Lönnerdal, 2014), which can lead to their incorporation into the curds, this could explain why its hydrolysis is delayed during late digestion. Additionally, pepsin activity increases throughout gastric digestion as the pH decreases reaching peaks at

both 120 mins (70 % activity) when the pH is ~4 and another peak around 240 mins (100 % activity) when the pH is ~2 (Salelles *et al.*, 2021)which could explain why the intensities peak at these time points.

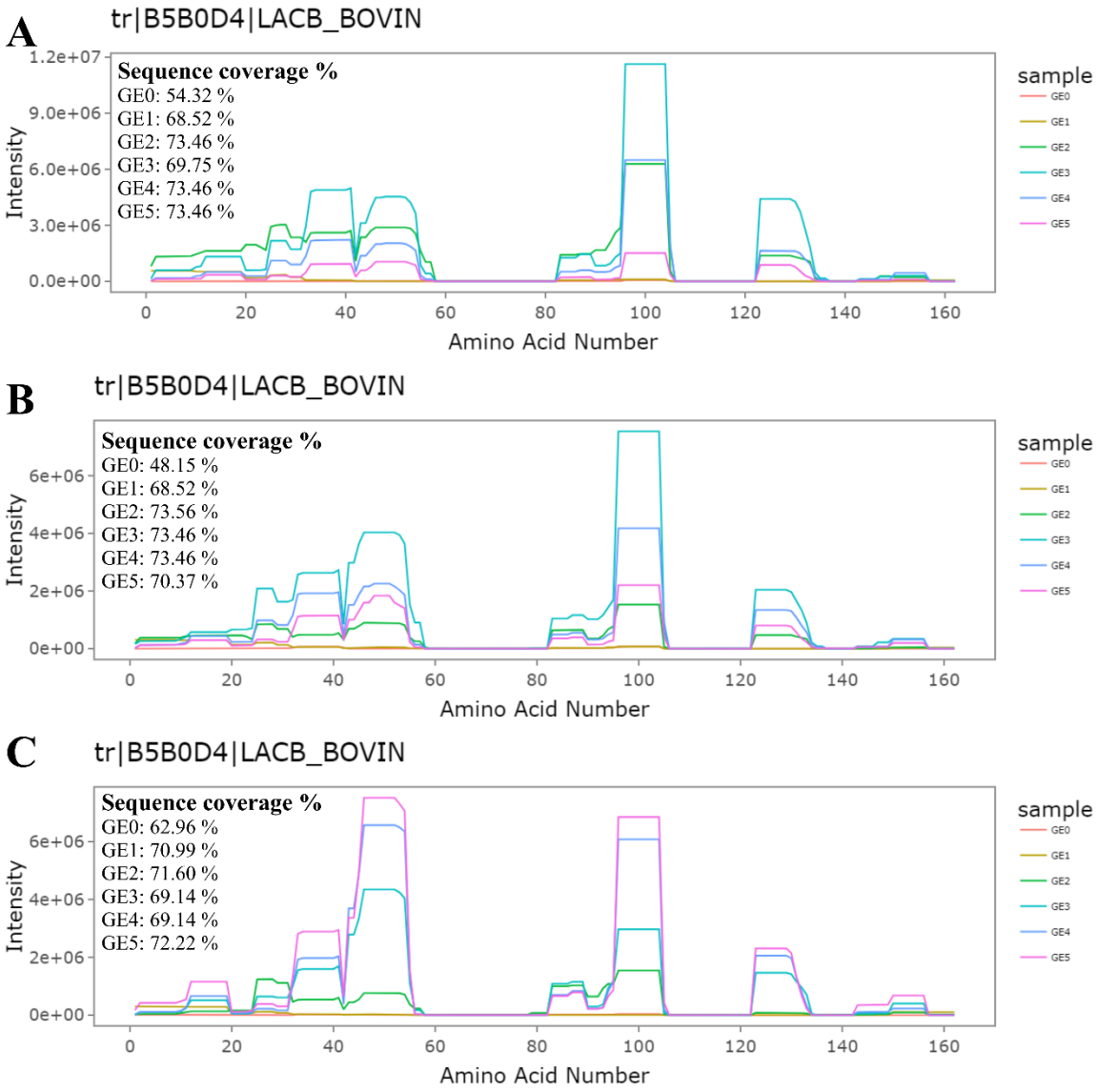

Figure 39. Distribution of peptides from β-LG throughout digestion from A) pasteurised, B) pasteurised-homogenised and C) high heat-homogenised cow milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Table 24. Protein regions of β-LG with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised cow milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
1-54	120 min	120 min	240 min
96-104	120 min	120 min	240 min
123-130	120 min	120 min	240 min

Figure 40 shows the intensities of peptides from β-LG identified in processed deer milk throughout gastric digestion mapped to AA position in the protein. The sequence coverage of β-LG fluctuated throughout digestion in deer milk. Peptides covered 45.06-74.69 % of the parent protein in pasteurised milk, 58.64-74.69% in pasteurised-homogenised milk and 58.02-74.69 % in heated-homogenised milk. Four distinct areas along the parent protein had high intensity: 33-54, 96-104, 123-131, and 150-162. Table 25 illustrates the time points with the highest intensity of peptides within these four regions for the three processing treatments. The abundance of peptides belonging to region 150-162 decreased throughout gastric digestion, with the highest observed intensity in the 20 min digesta, regardless of processing treatment. This suggests that this region is emptied early and/or is susceptible to hydrolysis. Meanwhile regions 33-54, 96-104, and 123-131 increased throughout digestion, with the highest observed intensity being 120 mins for pasteurised samples and 180 mins for pasteurised-homogenised and heated-homogenised samples. This suggests that these regions are either more resistant to digestion or emptied later during digestion due to incorporation into the clot.

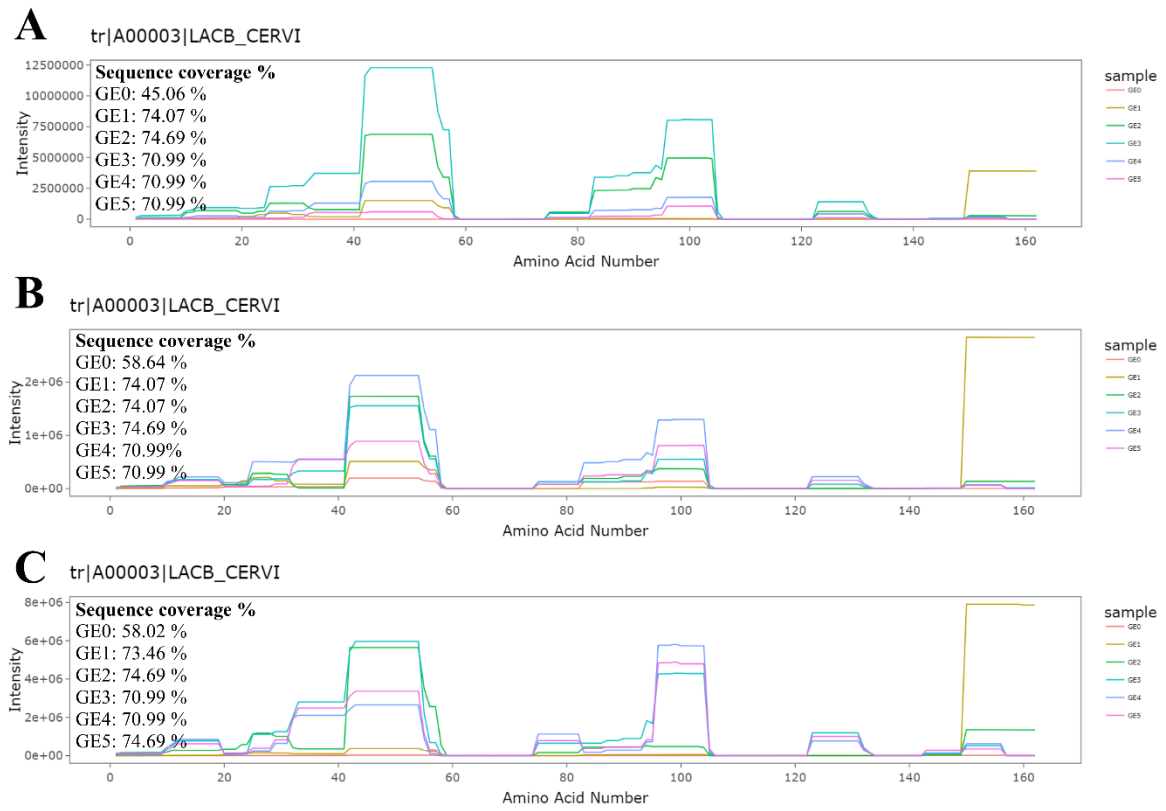

Figure 40. Distribution of peptides from β-LG throughout digestion from A) pasteurised, B) pasteurised-homogenised and C) high heat-homogenised deer milk. The y-axis presents the intensity of peptides per amino acid position of the protein. Gastric emptying (GE) times include GE0 (0 min – undigested milk), GE1 (20 min), GE2 (60 min), GE3 (120 min), GE4 (180 min), and GE5 (240 min). Sequence coverage (%) for each time point is overlaid on the plots.

Table 25. Protein regions of β-LG with highest intensity observed throughout digestion and the corresponding time point with the highest intensity observed for pasteurised, pasteurised-homogenised and heated-homogenised deer milk.

Protein region	Digestion time point with highest observed intensity		
	Pasteurised	Pasteurised-homogenised	Heated-homogenised
33-54	120 min	180 min	120 min
96-104	120 min	180 min	180 min
123-131	120 min	180 min	180 min
150-162	20 min	20 min	20 min

During digestion of both cow and deer milk peptides originating from similar regions of β-LG showed high intensity at time points 60 mins to 240 mins. However, at 20 mins of digestion

differences were observed. In cow milk the intensity of peptides is highest at the N-terminal end of the peptide from residue 1-19, whilst in deer milk the intensity of peptides is highest at the C-terminal end from residue 150-162. Differences were also observed within the regions that had the most intensity for example at the 60 mins time point the highest intensity for processed cow milk was region 96-104, whilst for processed deer milk it was region 42-54. A high intensity of peptides was observed between residues 123-131 in both cow and deer milk. This region overlaps with 125-135 which has previously been found to be resistant to proteolysis (Picariello *et al.*, 2010).

6.3.2 Bioactive peptides identified through gastric digestion of processed cow and deer milk

This study identified 51 bioactive peptides in the milk digesta samples (Table 26). In digested cow milk samples 40 bioactive peptides were with 12 from β-CN, 10 from α_{s1}-CN, 4 from α_{s2}-CN, 7 from κ-CN, 4 from β-LG and 3 from α-LA. In digested deer milk samples only 14 bioactive peptides were identified when the search was limited to bioactive peptides from cow milk, this increased to 20 bioactive peptides when the search was broadened to include all available species. Of these 20 bioactive peptides, 5 were identified from β-CN, 7 from α_{s1}-CN, 2 from α_{s2}-CN, 2 from κ-CN, 3 from β-LG and 1 from α-LA. For both deer and cow milk the top bioactive function was angiotensin-converting enzyme (ACE)-inhibitory. ACE raises blood pressure by converting angiotensin I released from angiotensinogen by renin into the potent vasoconstrictor angiotensin II (Pihlanto-Leppälä, 2000). It can also degrade vasodilative bradykinin and stimulate the release of aldosterone in the adrenal cortex (Pihlanto-Leppälä, 2000). Bioactive peptides with ACE-inhibitory activity may help to inhibit these functions. Only 9 bioactive peptides were identified in both the cow and deer milk samples. Differences were observed in the digestion fraction they were identified from, and the relative abundance of the peptides. For example, bioactive peptide DAYPSGAW from α_{s1}-CN was found at every time point during digestion of cow and deer milk, however, in both species it was more abundant during late digestion than early digestion, which was more apparent in deer milk (Figure 41). Further investigation into the bioactive properties of digested deer and cow milk would be beneficial to further understand their nutritional value and potential health benefits. This could assist in the development of innovative dairy products with improved health functional properties.

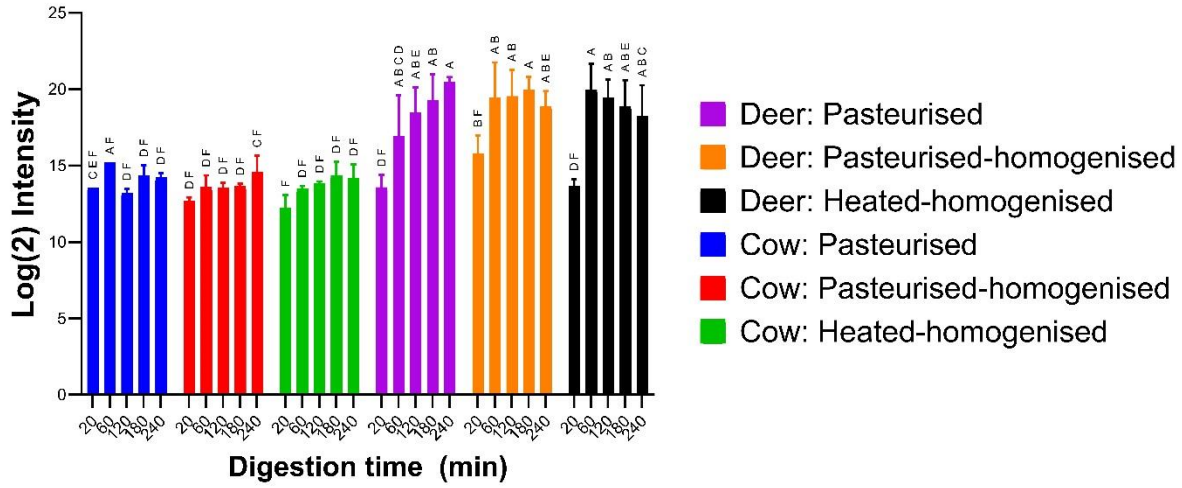

Figure 41. Abundance of bioactive peptide DAYPSGAW (log2 intensity) from α_{s1}-CN throughout digestion for processed cow and deer milk.

Table 26. Identified peptides after gastric digestion of processed cow and deer milk with previously reported bioactivity, and the species in which they are identified in each sample.

Search peptide	Protein ID	Fragment	Function	Digesta	References
Alpha-S1-CN					
AYFYPEL	P02662	158-164	ACE-inhibitory Antihypertensive Antioxidant Increase mucin secretion	Cow	del Mar Contreras *et al.* (2013)
DAYPSGAW	P02662	172-179	ACE-inhibitory	Cow & Deer	Pihlanto-Leppälä *et al.* (1998)
FVAPFPEVFG	P02662	39-48	ACE-inhibitory	Cow & Deer	Robert *et al.* (2004)
LAYFYPEL	P02662	157-164	Immunomodulatory	Cow	Totsuka *et al.* (1998)
LRLKKYKVPQL	P02662	114-124	Antimicrobial	Cow	McCann *et al.* (2006)
RYLGY	P02662	105-109	ACE-inhibitory Antihypertensive Antioxidant Opioid	Cow & Deer	Sánchez-Rivera *et al.* (2019)
RYLGYL	P02662	105-110	Anticancer Opioid	Deer	Hatzoglou *et al.* (1996)
SDIPNPIGSENSEK	P02662	195-208	Antimicrobial	Cow	Hayes *et al.* (2006)
TTMPLW	P02662	209-214	ACE-inhibitory Antimicrobial	Deer	Pihlanto-Leppälä *et al.* (1998)
YFYPEL	P02662	159-164	ACE-inhibitory Antioxidant Increase mucin secretion Opioid	Cow	Amigo *et al.* (2020)
YFYPQL	O62823	159-164	Antioxidant Immunomodulatory	Deer	Kandukuri *et al.* (2019)

YKVPQL	P02662	119-124	ACE-inhibitory	Cow	Maeno *et al.* (1996)
YKVPQLEIVPNSAEER	P02662	119-134	Increase calcium uptake	Cow	Cao *et al.* (2017)
YLGY	P02662	106-109	ACE-inhibitory Antioxidant	Deer	del Mar Contreras *et al.* (2013)
Alpha-S2-CN					
FALPQY	P02663	189-194	ACE-inhibitory	Cow	Tauzin *et al.* (2002)
FALPQYLK	P02663	189-196	ACE-inhibitory Antioxidant	Cow	Tauzin *et al.* (2002)
TKVIPYVRYL	P02663	213-222	Antimicrobial	Cow	Alvarez-Ordóñez *et al.* (2013)
YQKFPQY	P33049	105-111	ACE-inhibitory Antioxidant	Deer	del Mar Contreras *et al.* (2013)
YQKFPQYLQY	P02663	104-113	ACE-inhibitory	Cow & Deer	Xue *et al.* (2018)
Beta-CN					
ENLHLPLPLL	P02666	146-155	ACE-inhibitory	Cow & Deer	Robert *et al.* (2004)
EPVLGPVRGPFP	P02666	210-221	ACE-inhibitory	Cow	Hayes *et al.* (2007)
LLYQEPVLGPVRGPFPIIV	P02666	206-224	ACE-inhibitory	Cow	Yamamoto *et al.* (1994)
LNVPGEIVE	P02666	21-29	ACE-inhibitory	Cow	Gobbetti *et al.* (2000)
LPLP	P05814	141-144	ACE-inhibitory	Deer	
LYQEPVLGPVRGPFPIIV	P02666	207-224	Immunomodulatory	Cow	Coste *et al.* (1992)
NLHLPLPLL	P02666	147-155	ACE-inhibitory	Cow & Deer	Robert *et al.* (2004)
QEPVLGPVRGPFPIIV	P02666	209-224	ACE-inhibitory	Cow	Lu *et al.* (2016)
TDVEN	P02666	143-147	Cholesterol regulation	Cow	Jiang *et al.* (2020)
VENLHLPLPLL	P02666	145-155	ACE-inhibitory	Cow & Deer	Robert *et al.* (2004)
VYPFPGPIPN	P02666	74-83	ACE-inhibitory Antioxidant	Cow	Eisele *et al.* (2013)
YQEPVL	P02666	208-213	ACE-inhibitory	Cow	Pihlanto-Leppälä *et al.* (1998)
YQEPVLGPVRGPFPIIV	P02666	208-224	ACE-inhibitory Anticancer Antimicrobial Antithrombotic Immunomodulatory	Cow	Yamamoto *et al.* (1994)
PLPLL	L8I8G5	151-155	ACE-inhibitory	Deer	Mao *et al.* (2007)
Kappa-CN					
ARHPHPHLSFM	P02668	117-127	Antioxidant	Cow	Tonolo *et al.* (2020)
INNQFLPYPY	P02670	72-81	DPP-IV Inhibitory	Deer	Y. Zhang *et al.* (2015)
MAIPPKKNQDKTEIPTINT	P02668	127-145	Antimicrobial	Cow	Robitaille *et al.* (2012)
SRYPSY	P02668	54-59	Opioid	Cow & Deer	Sienkiewicz-Szłapka *et al.* (2009)

STVATL	P02668	162-167	Antimicrobial	Cow	López-Expósito *et al.* (2006)
VESTVATL	P02668	160-167	Antimicrobial	Cow	López-Expósito *et al.* (2006)
VQVTSTAV	P02668	183-190	Antimicrobial	Cow	López-Expósito *et al.* (2006)
YYQQKPVA	P02668	63-70	Antimicrobial	Cow	López-Expósito *et al.* (2006)
Beta-lactoglobulin					
DAQSAPLRVY	P02754	49-58	ACE-inhibitory	Cow	Tavares *et al.* (2011)
IQKVAGTW	P02754	28-35	ACE-inhibitory DPP-IV Inhibitory	Cow & Deer	Lacroix *et al.* (2016)
LDIQKVAGTW	P02755	28-37	ACE-inhibitory	Deer	Lacroix *et al.* (2016)
LKPTPEGDL	P02754	62-70	DPP-IV Inhibitory	Cow	Lacroix and Li-Chan (2014)
LKPTPEGDLE	P02754	62-71	DPP-IV Inhibitory	Cow	Lacroix and Li-Chan (2014)
VLDTDY	P02754	110-115	ACE-inhibitory DPP-IV Inhibitory	Deer	Lacroix *et al.* (2016)
Alpha-lactalbumin					
DKVGINY	P00711	116-122	ACE-inhibitory	Cow	Tavares *et al.* (2011)
DKVGINYW	P00711	116-123	ACE-inhibitory	Cow	Tavares *et al.* (2011)
IVQNNDSTEYGLF	P00711	60-72	DPP-IV Inhibitory	Cow	Lacroix and Li-Chan (2014)
YGLF	P00711	69-72	ACE-inhibitory Antioxidant DPP-IV Inhibitory Opioid Increase mucin secretion	Deer	Pihlanto-Leppälä (2000) Martinez-Maqueda *et al.* (2012)

6.4 Conclusions

The present study investigated the protein degradation of key milk proteins identified by LC-MS/MS in processed cow and deer milk after application of the HGS *in vitro* digestion protocol. All samples had peptides contributing to areas of high and low intensity along the parent sequence. Although these areas were conserved between processing treatments, differences were observed between species. For example, the peptides generated from the C-terminal end of β-CN were more abundant in cow milk than in deer milk. The AA sequence of milk proteins from cow and deer are known to differ which is likely driving the differences in these high intensity regions, due to changes in easily accessible pepsin cleavage sites or a change to/from a residue for which pepsin has selectivity to/from one that it does not. However, for some regions where the sequence is conserved similar areas of high intensity are observed. Both cow and deer had a high intensity of residues between 127/8-139/140 an area of β-CN that is known to be resistant to digestion. Differences in the types and intensities of the bioactive peptides was also observed between species.

In both cow and deer milk the largest source of variation observed throughout digestion was between the different time points, especially during early digestion. This suggests that digestion of proteins occurs more rapidly during early digestion which can be attributed to digestion of proteins that are not associated with the clot and/or restructuring of the clot meaning that proteins are more accessible to pepsin. Although processing treatment did not show a significant effect on protein degradation it was observed that the kinetics of degradation for specific regions varied and was affected by species. For example, in α_{s1}-CN intensity for region 165-179 was highest at 240 mins in pasteurised milk for both cow and deer milk whilst it was highest at 60 mins for heated-homogenised cow milk and at 120 mins for heated-homogenised deer milk. These results could indicate that peptides are emptied faster in heated-homogenised samples or are less resistant to digestion, potentially due to the looser structure of the curd in heated-homogenised milk enabling more access to pepsin cleavage sites.

This study indicated that species and processing treatment affected the digestibility and biological activity of peptides derived from gastric digestion of milk.

Chapter 7 - Alignment study between *in vitro* and *in vivo* digestion of commercially processed cow milk

7.1 Introduction

Commercially produced cow milk is typically processed by heat treatment and/or homogenisation. This improves consumer acceptance, ensures biological safety, and prolongs shelf life (Xiong *et al.*, 2020). However, these processes are known to affect the nutrient composition and structure of milk. Commonly applied heat treatments are pasteurisation, which involves heating milk at 72 ºC for approximately 15 sec, and UHT sterilisation which exposes milk to higher temperatures for a shorter period, for example, 140 ºC for around 2 sec. Thermal treatment of milk causes denaturation of whey proteins, especially β-LG, aggregation of proteins, and PTMs of milk proteins such as glycation (van Lieshout *et al.*, 2020; Wijayanti *et al.*, 2014). Homogenisation of milk reduces the average size of the native fat globule, initially surrounded by the MFGM, from 4 µm to below 1 µm (Lopez, 2005). Homogenisation also leads to partial disruption of the MFGM and changes the interface composition (Lopez, 2005; Michalski & Januel, 2006). An increase in the proteins (particularly CNs and β-LG) interacting with or adsorbed to the milk fat globules occurs when milk is either solely homogenised or homogenised and heated (Lee & Sherbon, 2002; Sandra & Dalgleish, 2005).

Digestion and absorption of nutrients are influenced by the structure of food. Due to the different digestion models applied in these studies conflicting results have often been obtained. Limited studies are available on the *in vivo* digestion of processed milk. Lacroix *et al.* (2008) found that UHT milk had a significantly higher transfer of dietary nitrogen to serum amino acids and protein and to body urea than pasteurised or microfiltered milk in healthy humans. These results have been supported by Bach *et al.* (2017) who showed that urinary nitrogen secretion was greater following consumption of UHT-milk than pasteurised or raw milk using young dairy calves as a model. Both Ahlborn *et al.* (2023) and Ye *et al.* (2019) reported faster emptying rates of dry matter, protein and lipids of UHT milk compared to other processed milks in growing pigs and rats, respectively. It is postulated that a potential reason for the difference in digestive kinetics is UHT milk forming a softer coagulum in the gastric compartment, resulting in higher enzyme accessibility. This contrasts to other work where heat treatment of milk products (90 ºC, 10 mins) increased the mean retention time in the stomach of mini-pigs compared to no heat treatment (Barbé *et al.*, 2013).

Whey proteins, in particular β-LG, has been found to be more susceptible to protein hydrolysis after heating in both *in vivo* digestion models of suckling rats (Wada & Lönnerdal, 2014), static *in vitro* digestion models (Tunick *et al.*, 2016) and dynamic *in vitro* digestion models (Sánchez-Rivera *et al.*, 2015). This observation was observed for pasteurised (72 ºC, 15 sec), UHT (140 ºC, 2 sec) and longer heat treated (90 ºC, 10 mins) milks. This is thought to be due to the increased denaturation of proteins when subjected to heat, increasing the accessibility of protein cleavage sites to pepsin. However, opposing observations have been made for the hydrolysis of CNs. Sánchez-Rivera *et al.* (2015) and Dupont, Mandalari, Molle, *et al.* (2010) have reported that heat treatment of milk resulted in resistance of CNs to hydrolysis in both an *in vitro* dynamic model and an *in vitro* infant static model respectively. This is believed to be due to aggregation of CNs and whey proteins and heat-derived modifications such as lactosylation and glycosylation causing a decrease in protein digestibility (Wada & Lönnerdal, 2014). In contrast, Tunick *et al.* (2016) reported a rapid digestion of CNs in both processed (pasteurised, homogenised and UHT) and non-processed samples using a static digestion model.

To date, few studies have compared the trends between *in vitro* and *in vivo* digestion models for milk, especially regarding protein digestion. These studies have used various *in vitro* and *in vivo* digestion models as well as starting materials. The most common *in vitro* digestion model used is the static INFOGEST harmonised protocol (Egger *et al.*, 2016). This has been compared to digestion in cannulated pigs for micellar CN (Miralles *et al.*, 2020), human jejunal effluents for whey and CN powders (Sanchón *et al.*, 2018) and both pigs and the dynamic DIDGI® model for skim milk (Egger *et al.*, 2019; Egger *et al.*, 2017). These studies have shown similar peptide profiles at the end points of gastric and intestinal digestion between the two models being compared. Comparisons investigating the digestion of milk proteins in dynamic *in vitro* models and *in vivo* models are more sparse. Ménard *et al.* (2014) showed that infant formula showed similar kinetics of protein digestion in both a simple dynamic digestion model and piglets, whilst Ye *et al.* (2019) showed similar trends in coagulation of processed whole milks in the dynamic HGS model and *in vivo* rat model.

The objective of this study was to: (a) study the peptides produced throughout digestion using the HGS as a dynamic *in vitro* digestion model and (B) to compare the peptides produced upon

the application of the dynamic *in vitro* digestion model to those in *in vivo* in growing pigs to evaluate the HGS digestion model.

7.2 Materials and methods

7.2.1 Reagents

Water, acetonitrile, methanol, formic acid (FA) and trifluoracetic acid (TFA), all LC-MS grade, and urea were sourced from ThermoFisher Scientific (Waltham, MA, USA). Chloroform, ammonium bicarbonate and potassium dihydrogen orthophosphate were purchased from BDH, Prolabo (Poole, UK). Sodium chloride was purchased from Fisher Scientific (Liecs, UK). Di-sodium hydrogen orthophosphate, DL-dithiothreitol (DTT) and iodoacetamide (IAM) were from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Unless otherwise stated all chemicals and reagents are of analytical grade.

7.2.2 Samples

Two commercially processed cow milks were collected as described in section 3.1.2. The milks were digested using either an *in vivo* pig digestion model as described in section 3.2.1.2, or an *in vitro* HGS digestion model as described in section 3.2.2. The samples were stored at -80 ºC prior to analysis.

7.2.3 Size exclusion chromatography

Molecular weight (MW) distributions of the digesta were determined by size exclusion chromatography (SEC). These data were used to determine which samples should be subjected to LC-MS/MS analysis.

7.2.3.1 Sample preparation

Digesta samples from the *in vivo* pig model were received lyophilised, whilst milk samples digested using the HGS model arrived as a frozen liquid. Thus, their preparation for SEC was slightly different: 20 mg of lyophilised digesta sample were solubilised in 500 µL MS grade H_2O whilst liquid samples were diluted 1:1 with H_2O after thawing. Protein concentrations of all samples were estimated at 280 nm using the built-in protein assay on the Nanophotometer NP80 (Implen, Munich, Germany). An aliquot containing 1 mg of protein of each sample was dried in a CentriVap vacuum centrifuge (Labconco, Kansas City, MO, USA) operated at 40 ºC. The dried samples were each resuspended in 100 µL of SEC buffer (0.05 M phosphate

buffer containing 0.3 M sodium chloride, pH 6.9). Prior to injection the samples were centrifuged in a 2 µm spin filter (PhaseSep Pty Ltd, VIC, Australia) for 2 min at 10,000 x *g* to remove suspended solids.

7.2.3.2 SEC

SEC was performed on a Dionex Ultimate 3000 HPLC (ThermoFisher Scientific, Waltham, MA, USA) fitted with a Yarra SEC-2000 column (300 x 7.8 mm, 3µm particle size, 145 Å pore size) (Phenomenex, Auckland, NZ), which was operated at ambient temperature. 20 µL of each sample was injected and eluted over 25 min with SEC buffer at a flow rate of 1 mL/min. Detection was performed at both 214 and 280 nm using a photodiode array detector (ThermoFisher Scientific, Waltham, MA, USA).

7.2.3.3 Data analysis

Protein standards of known MWs: thyroglobulin (669 kDa), immunoglobulin G (160 kDa), bovine serum albumin (66.7 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), cytochrome-C (12.4 kDa) and aprotinin (6.5 kDa) were separated under the same conditions to create a MW calibration curve in Chromeleon version 7.2 chromatography software (ThermoFisher Scientific, Waltham, MA, USA) as seen in Figure 42. In the Chromeleon software, each unknown sample's chromatogram was divided into 250 equal retention time slices, and the MW of each of the slices was calculated from the MW calibration curve. These results were then exported from Chromeleon and the areas and the cumulative % areas of the slices were analysed using an in-house R-script. From the latter data, the script calculated the proportions of peptides falling below designated MW limits (1 kDa and 10 kDa).

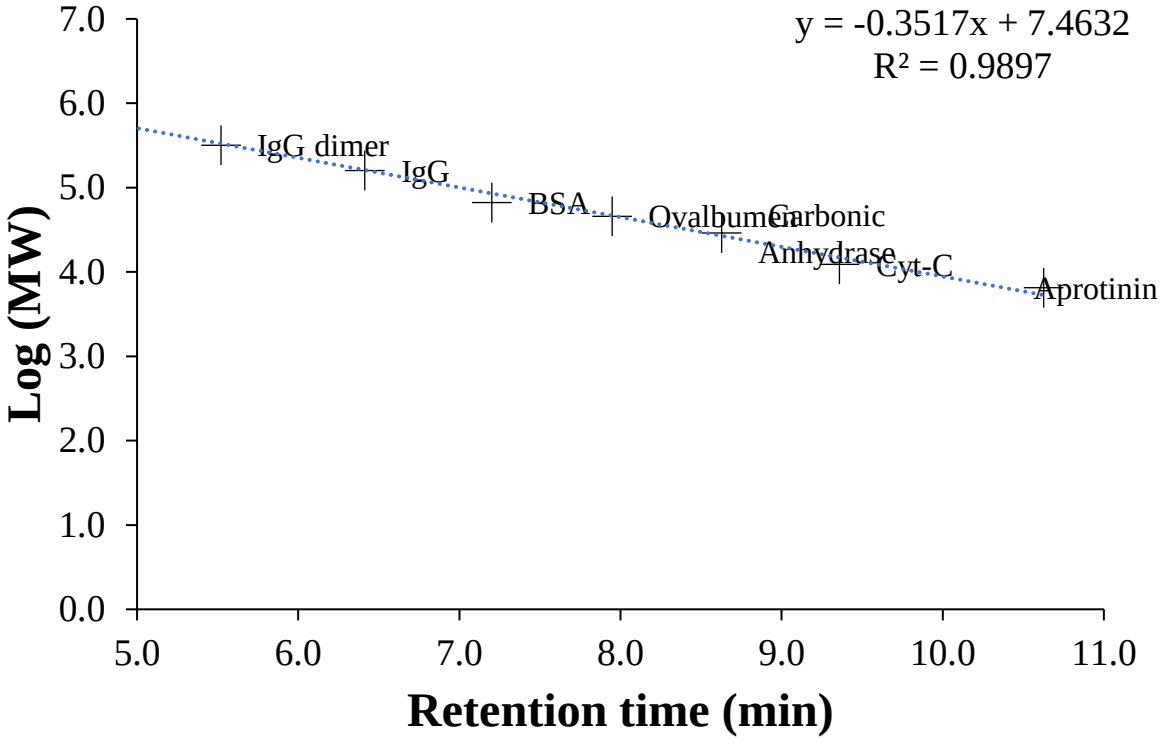

Figure 42. Molecular weight (MW) standard curve generated from SEC showing the retention time (min) on the y-axis and the log of MW on the x-axis.

7.2.4 LC-MS/MS

7.2.4.1 Peptidomic sample preparation

Prior to LC-MS/MS analysis, samples were adjusted to 5 % acetonitrile by adding 30 µL of acetonitrile to 570 µL of digested milk sample. The samples were then heated at 40 °C for 15 mins prior to centrifugation at 20, 000 x *g* for 10 mins at 4 °C. To isolate the peptide fraction, 200 µL of each sample was added to a 10 kDa NanoSep centrifugal device (Pall, NY, USA). After centrifugation at 10,000 x *g* for 90 mins at 4 °C the ultrafiltrate was kept for LC-MS/MS analysis. Peptide concentrations were estimated at 280 nm in the ultrafiltrate samples using the built-in protein assay on the Nanophotometer NP80 (Implen, Munich, Germany). A volume containing approximately 250 µg of peptides was then dried for each sample in a CentriVap vacuum centrifuge (Labconco, Kansas City, MO, USA) operated at 40 ºC.

7.2.4.2 Chromatography and mass spectrometry

Prior to running on the LC-MS, the samples were resuspended in 50 µL of 0.1% TFA and diluted 10x with 0.1% FA.

Nanoflow LC-MS and LC-MS/MS were performed on an Ultimate 3000 HPLC system (ThermoScientific, Waltham, MA, USA) directly interfaced with a CaptiveSpray ion source to an Impact HD Q-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The CaptiveSpray was fitted with a nanoBooster device (Bruker Daltonik, Bremen, Germany), which infused acetonitrile into the mass spectrometer's nitrogen gas supply to improve sensitivity. For each pool sample, 1 µL was injected onto a PepMap100 C18 Nano-Trap column (ThermoScientific) to trap peptides, which were then eluted onto a ProntoSIL C18AQ analytical column (150 mm x 100 µm i.d., 3 µm particle size, 200 Å pore size; nanoLCM Solutions, Oroville, CA, USA). Both columns were maintained at 50 ºC in a column oven. Separation of peptides was carried out at a flow rate of 1 µL/min using a multistep linear gradient of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). Initially 2 % B was used for 2 mins, this increased to 20 % B over 43 mins, and then to 45 % B over a further 16 mins. The column was cleaned by increasing to 95 % B over 4 mins and holding at that level for 7 mins. After that, the column was re-equilibrated by reducing back to 2 % B over 2 mins and holding at that for 10 mins. The total run time was 84 mins.

MS/MS data were acquired in a data-dependent acquisition (DDA) mode where each full scan MS spectrum (50–1800 m/z, 2 Hz sampling rate) was followed by a fixed cycle time of 3 seconds for acquisition of MS/MS spectra of precursors in the range m/z 150-1800, at a sampling rate of 2–32 Hz (depending upon precursor intensity). A preference for selection of charged (1+ to 8+) precursor ions was set, and precursors were excluded from reacquisition for 1 min unless their intensity increased at least 3-fold during that period.

7.2.4.3 Data analysis

Peptides and proteins were identified using PEAKS Studio version 10.6 (Bioinformatics Solutions Inc., Toronto, Canada) (Ma *et al.*, 2003), with minimum peptide length set to 4. Database searches utilised the bovine milk proteome database (BoMiProt 2.0, 10,314 sequences, (Das *et al.*, 2022)).

The following parameters were kept fixed for all searches; precursor ion mass error tolerance of 10 ppm, fragment mass error tolerance of 0.05 Da. The maximum allowed variable PTM per peptide was set at 2. The peptide false discovery rate (FDR) was set at 1%. Proteins were accepted if their -10lgP values were above 20 and at least 1 unique peptide and additional

significant supporting peptides were observed. Two online tools were utilised to analyse the results: Venny (Oliveros, 2007) and Peptigram (Manguy *et al.*, 2017)

7.3 Results and discussion

7.3.1 SEC analysis of commercially processed cow milks digested using *in vitro* and *in vivo* digestion models

SEC is a method that separates compounds based on their molecular size and shape. This technique has routinely been used to separate biological compounds such as proteins and peptides and measure the MW distribution of protein digests (Johns *et al.*, 2011; Wubshet *et al.*, 2017). More recently it has been used to assess the protein digestibility of different food matrices (Le Roux *et al.*, 2020; Rieder *et al.*, 2021; Sousa *et al.*, 2023). In this study we used SEC to determine which *in vitro* and *in vivo* digestion time points would be subjected to LC-MS/MS analysis to get a clear overview of how different digestion models affect protein breakdown of commercially processed milks. SEC was chosen due to its simplicity, versatility and robustness over a large number of samples (Ó'Fágáin, 2017). Analysis of SEC, as described in section 4.2.3 was performed including areas under the curves (AUCs) of cumulative percentage area plots and percentage of peptides below 10 kDa.

Figure 43 illustrates the cumulative percentage areas for the commercial milks in both the A) *in vitro* (HGS) and B) *in vivo* (pig) model. Throughout gastric digestion shifts to the left (*i.e.* to lower MWs) are observed in the cumulative area percentages plots indicating that there are progressive increases in the proportions of small peptides and accompanying progressive decreases in proportion of proteins and large peptides identified in the emptied digesta. These shifts are more apparent during early digestion whilst during late digestion the plots become overlaid. During *in vitro* digestion the plateau is observed after 120 mins, and this trend is also observed in section 4.3.1.1. However, during *in vivo* digestion the plateau is observed earlier with limited differences being observed after 60 mins of gastric digestion. A potential reason for the plateau during late gastric digestion is the dynamic nature of both the *in vitro* and *in vivo* models being used. In dynamic digestion experiments, solutions are continually being secreted and emptied from the stomach and the formation of the curd will limit the amounts of proteins and peptides being emptied as well.

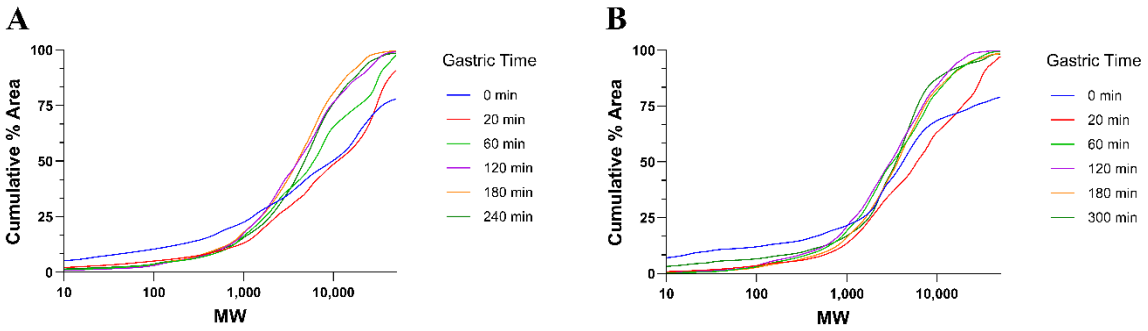

Figure 43. Mean cumulative percentage (%) areas against MW for gastric milk samples using A) the *in vitro* (HGS) model and B) the *in vivo* (pig) model.

The early plateau time for the *in vivo* model paired with the fact that a greater proportion of high MW proteins and peptides are found in the *in vitro* model at 60 min of gastric digestion (Figure 44) indicate that the breakdown of proteins may occur more rapidly *in vivo* than using the *in vitro* model.

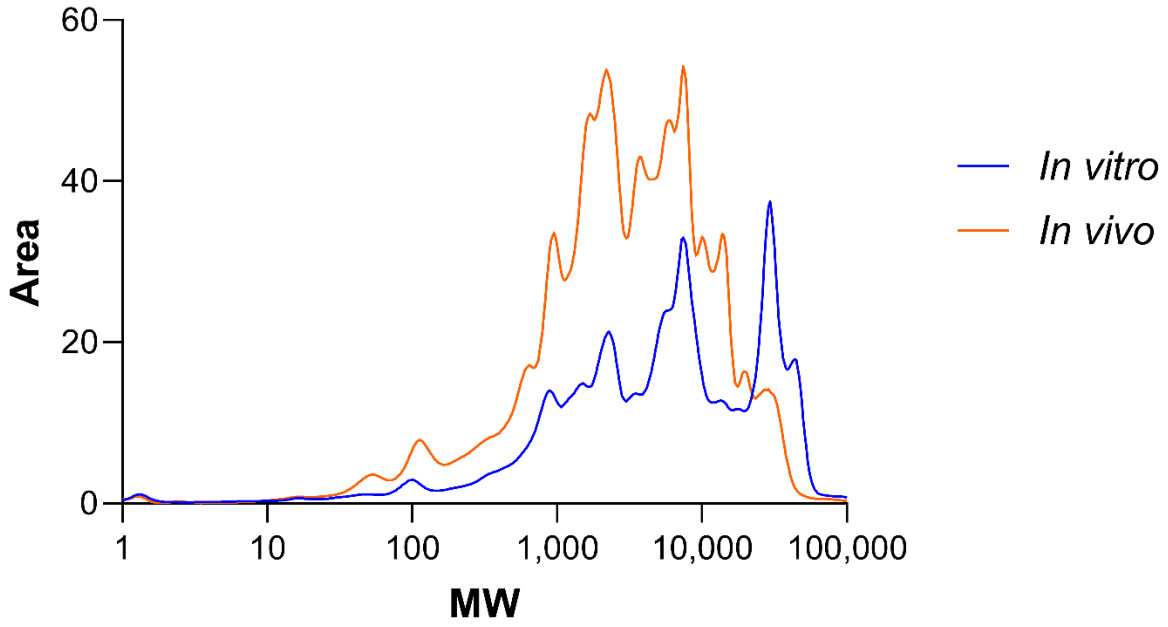

Figure 44. Overlaid mean molecular weight (MW) distributions of 60 min gastric digested milk using the *in vitro* (HGS) and *in vivo* (pig) model.

As observed in section 4.3.1.1 limited differences were observed between the intestinal samples for the *in vitro* digestion model (Figure 45A). This was also observed for the *in vivo* samples, although more variation is observed, and there are no consistent trends between the samples

(Figure 45B). In the *in vitro* samples the limited differences between time points are attributed to the high background of the intestinal samples caused through the introduction of pancreatin and bile. The variation within the *in vivo* intestinal samples is indicative of the differences in the natural protein and peptide content of individual pigs.

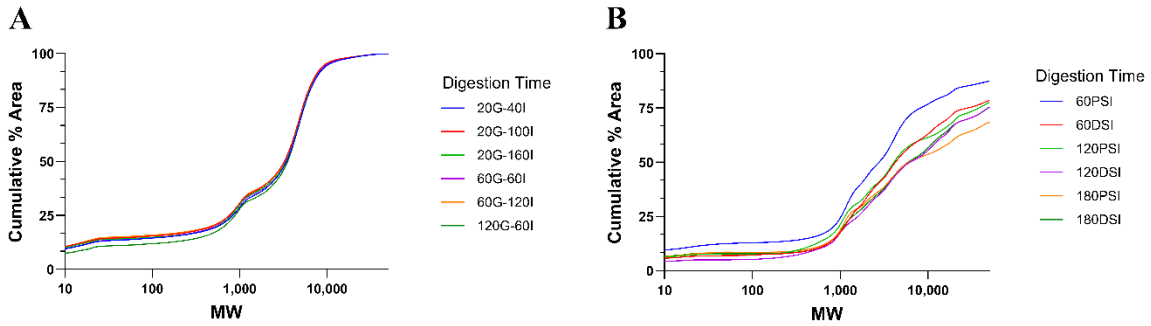

Figure 45. Mean area of cumulative percentage (%) against the MW for intestinal milk samples using A) the *in vitro* (HGS) model and B) the *in vivo* (pig) model.

7.3.1.1 Comparing the SEC results for *in vitro* and *in vivo* digestion of commercially processed milks

A similar pattern of gastric digestion is observed between *in vitro* (HGS) and *in vivo* (pigs) digestion models for both pasteurised-homogenised and UHT milk (Figure 46A and B). Both digestion models show a similar increase in percentage of peptides below 10 kDa throughout digestion, however this is more pronounced for the pasteurised-homogenised milk than for UHT milk. More variation is observed for the *in vivo* samples especially the 0 min time point (which for the *in vivo* samples is the stomach contents prior to consumption of milk) and late digestion time points (180 mins and 300 mins).

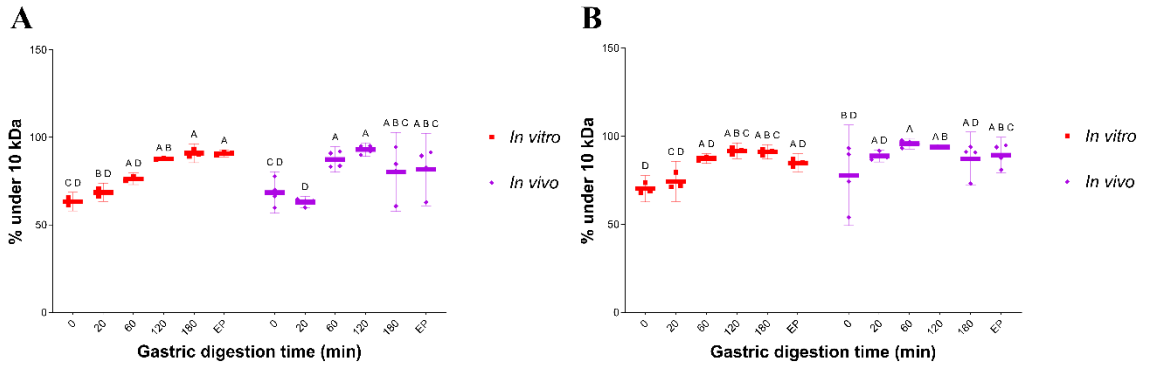

Figure 46. Effect of digestion model on digestion of A) pasteurised-homogenised milk and B) UHT milk. The endpoint (EP) of gastric digestion for the *in vitro* digestion model

was 240 min whilst for the *in vivo* digestion model was 300 min. Values are the means of at least three independent digestions ± 95 % confidence interval. Different letters indicate significantly different values (P<0.05).

To date there is limited literature available comparing digestion of milk proteins using the human gastric simulator (HGS) to *in vivo* models, although there have been a few studies comparing digestion of other food materials such as apple and rice mixtures (Kong & Singh, 2010) and konjac glucomannan powders mixed with glucose solution and rice porridge (Guo *et al.*, 2022). These studies show comparable results between the two models. Ye *et al.* (2019) analysed curd formation in processed milks using both the HGS and rat *in vivo* model. The results showed that the curd appearance was consistent between the two models for the heated and unheated milk with consistent trends in protein hydrolysis as observed by SDS-PAGE gels.

7.3.1.2 Effect of heat treatment on digestion of commercially processed milks

In this study UHT milk had a greater proportion of peptides less than 10 kDa compared to pasteurised-homogenised milk during the early stage of gastric digestion in both the *in vitro* (Figure 47A) and *in vivo* (Figure 47B) digestion models. This was more pronounced in samples digested using the *in vitro* digestion model, with the UHT milk producing a significantly higher percentage of peptides below 10 kDa at the 20-, 60- and 120-min time points. An exception to this was the endpoint of gastric digestion. In the *in vitro* digestion model the endpoint was 240 mins and showed a lower percentage of peptides below 10 kDa for the UHT milk compared to the pasteurised-homogenised milk; this was not observed for the equivalent sample during in vivo digestion (300 mins).

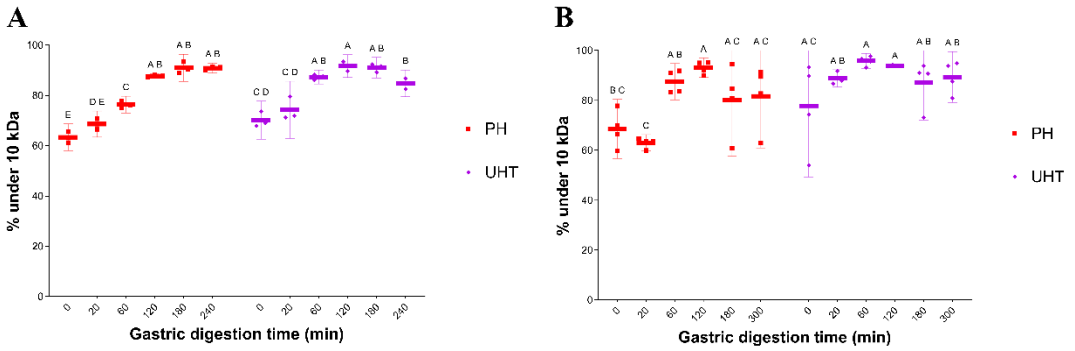

Figure 47. Effect of heat treatment on digestion of commercially sourced milks for A) *in vitro* digestion and B) *in vivo* digestion. Values are the means of at least three independent digestions ± 95 % confidence interval. Different letters indicate significantly different values (P<0.05).

The greater percentage of low MW peptides and proteins observed especially during early to mid-gastric digestion suggests that UHT milk is digested more rapidly than pasteurised-homogenised milk. In this study it was also observed that the undigested UHT milk contained a higher percentage of low MW peptides compared to the pasteurised-homogenised milk. This suggests that partial hydrolysis of proteins may occur during UHT processing which could contribute to the greater amount of low MW peptides being generated during digestion.

Previous studies have also observed that UHT milk is digested more rapidly than pasteurised-homogenised milk. Ahlborn *et al.* (2023) reported that UHT milk had faster emptying rates of protein in growing pigs compared to other processed milks whilst Lacroix *et al.* (2008) found that UHT milk had a higher transfer of dietary nitrogen to serum amino acids and protein and body urea than pasteurised milk in humans. Ye *et al.* (2019) showed that UHT milk formed curds with fragmented and crumbled structures whilst unheated and pasteurised milk had more cohesive curds. This was correlated with a faster rate of protein hydrolysis in the UHT milk. The more fragmented and crumbled structure in the heated milk may make the pepsin cleavage sites within proteins more accessible.

7.3.1.3 Decision on samples to use for LC-MS/MS

Table 27 outlines the samples that were selected from the SEC results to analyse using LC-MS/MS. Undigested commercially processed milk samples were selected as controls. Gastric time points 20 min, 60 min and 180 mins were selected for each sample. The most significant changes observed in the SEC results were during early gastric digestion. The 180 min time

point was chosen to represent the endpoint of gastric digestion. This time point aligned between the *in vitro* and *in vivo* samples. SEC results showed a plateau in the proportion of small peptides produced during late gastric digestion. The SEC analysis did not identify any clear differences between the intestinal time points. However, it was determined to trial a select number of intestinal samples using LC-MS/MS as this is a more sensitive technique.

Table 27. Samples selected from SEC results for analysis by LC-MS/MS. PSI refers to the proximal small intestine and DSI refers to the distal small intestine. Samples selected for LC-MS/MS that include more than one time point were pooled prior to analysis.

***In vitro* Milk Samples**	**Samples selected for LC-MS/MS**	***In vivo* Milk Samples**	**Samples selected for LC-MS/MS**
Gastric time points (min)			
Undigested (0)	☑	Undigested (0)	☑
20	☑	20	☑
60	☑	60	☑
120		120	
180	☑	180	☑
240		300	
Intestinal time points (min)			
20G-40I	☑	60 (PSI)	☑
60G-60I	☑	60 (DSI)	
20G-100I	☑	120 (PSI)	☑
20G-160I	☑	120 (DSI)	
60G-120I		180 (PSI)	☑
120G-60I		180 (DSI)	

7.3.2 Qualitative peptidomics analysis for in vitro and in vivo digestion of commercially processed milks

Studying the peptides released throughout the digestion process enables an understanding of the nutrients that are first available to the body. This study analysed 117 individual samples from 2 commercially processed milks, sampled at 3 gastric timepoints and 6 intestinal

timepoints. *In vitro* digestion had one milk batch and three replicates, *in vivo* digestion had six milk batches and each time point belonged to a different pig. More variation was likely to be found in the *in vivo* system due to this. The data sets acquired for peptides in the commercially processed milks throughout digestion were processed using PEAKS Studio software to obtain confident identification of peptides that were observed in at least 50 % of the replicates of each sample. Overall, 993 unique peptides were identified from 103 proteins. Of these 993 peptides, over 60 % were derived from major milk proteins with 27.2 % from β-CN, 17.3 % from α_{s1}-CN, 9.2 % from κ-CN, 6.1 % from α_{s2}-CN, 5.7 % from β-LG and 1.9 % from α-LA.

7.3.2.1 Peptides identified in undigested pasteurised-homogenised and UHT milk

Prior to digestion, 226 peptides from 17 proteins were identified in commercially processed milk using LC-MS/MS. The peptide length ranged from 4 to 35 residues with an average length of 14.7 residues. Over 80 % of the identified peptides came from CNs with 42.5 % from β-CN, 25.2 % from α_{s1}-CN, 11.1 % from α_{s2}-CN, and 6.2 % from κ-CN. Endogenous peptides are cleaved from the proteins by proteases naturally present in milk (Boland & Singh, 2019). The most abundant protease in cow milk is plasmin which shows a high specificity for β-, α_{s1}-, and α_{s2}-CN with only low or no activity towards κ-CN, β-LG, and α-LA (Baum *et al.*, 2013). Other important proteases include cathepsin D, cathepsin B and elastase. The number of endogenous peptides identified are similar to those found in the literature. Dallas *et al.* (2014) identified 159 peptides from six individual healthy cows and Baum *et al.* (2013) identified 248 peptides from raw pooled milk. More recently an increased number of endogenous peptides are being identified in milk, with 612 peptides identified in raw milk by Wölk *et al.* (2020). This increase can likely be attributed to more sophisticated extraction methods and mass spectrometers than used in this study.

Processing treatment influenced the endogenous peptidome (Figure 48). More peptides were identified in UHT treated milk compared to pasteurised-homogenised milk, and of these peptides 35.8 % were the same between the two milks. The average length of peptides identified in UHT milk was shorter than those identified in pasteurised-homogenised milk with 14.0 and 15.4 residues, respectively. This data supports the SEC findings which show that undigested UHT milk had a greater percentage of peptides below 10 kDa compared to undigested pasteurised-homogenised milk. Wölk *et al.* (2020) also detected more endogenous peptides in processed milk products than in raw milk. Processing treatments such as heat

treatment not only causes denaturation of whey proteins, aggregation of proteins and PTMs (Wada & Lönnerdal, 2014) but it also influences endogenous enzyme activity (Lu *et al.*, 2009) which is likely to affect the amount and types of peptides produced during digestion.

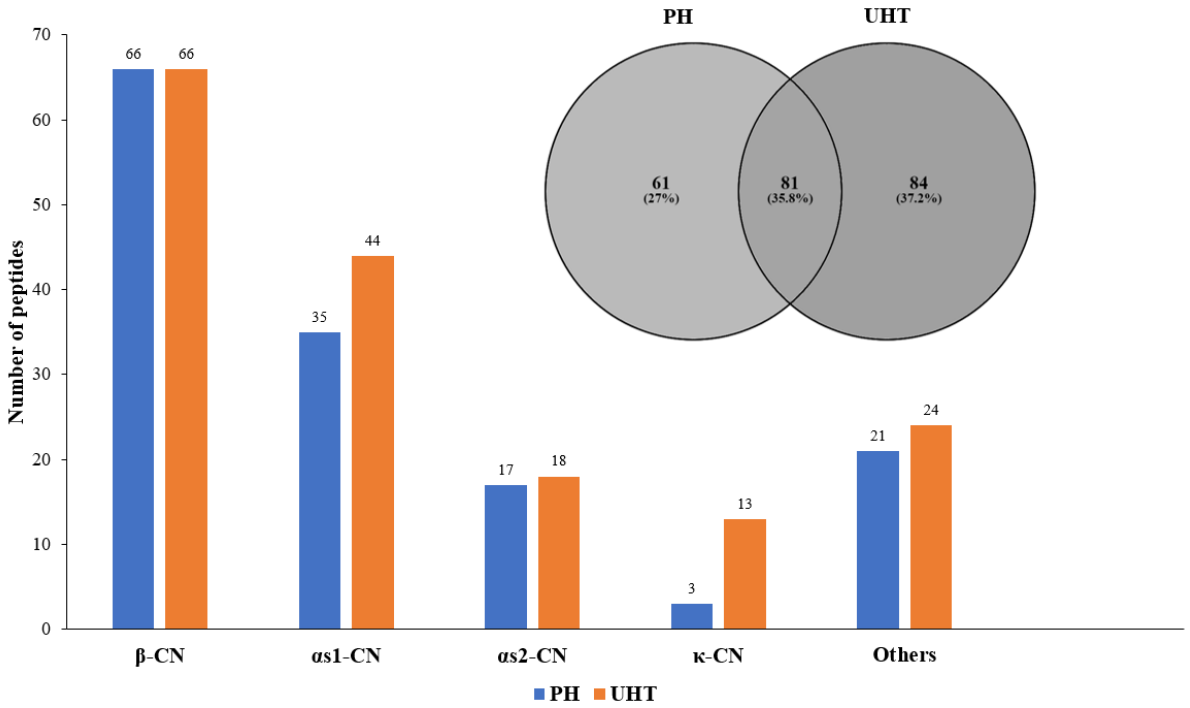

Figure 48. Number of peptides identified for different proteins in undigested pasteurised-homogenised (PH, blue) and UHT (orange) treated milk. The insert contains a Venn diagram showing the number of identified unique and common peptides for pasteurised-homogenised and UHT milk.

β-CN was the dominant source of free peptides. Previous studies have observed a greater number of peptides from α_{s1}-CN, which is believed to be due to its higher concentration in cow milk (Baum *et al.*, 2013; Wölk *et al.*, 2020). The higher number of peptides from β-CN in this study, and also reported by Leite *et al.* (2021) for both ovine and bovine milks and by Barbé *et al.* (2014) for bovine milk digested via mini-pigs, may be due to the structure (primary to quaternary) of β-CN making it more susceptible to proteolysis. Although equal numbers of peptides were identified in the two different processing treatments only 37.5 % had the same sequence between the two types of milk. The peptides in pasteurised-homogenised milk covered the full mature protein sequence of β-CN, whereas the sequences 109-122, 158-176, and 192-205 were missing in UHT milk (Figure 49A).

α_{s1}-CN was the second most abundant source of peptides. A greater number of peptides were identified in the UHT treated milk compared to the pasteurised-homogenised milk with 38.6 % of these peptides having the same sequence between the two treatments. Irrespective of processing treatment most peptides originated from the region between 187-214 with lesser amounts between 39-47 and 121-138 and no coverage between 139-179 (Figure 49B). Previous research has shown more peptides from α_{s1}-CN in UHT milk compared to raw milk (Wölk *et al.*, 2020).

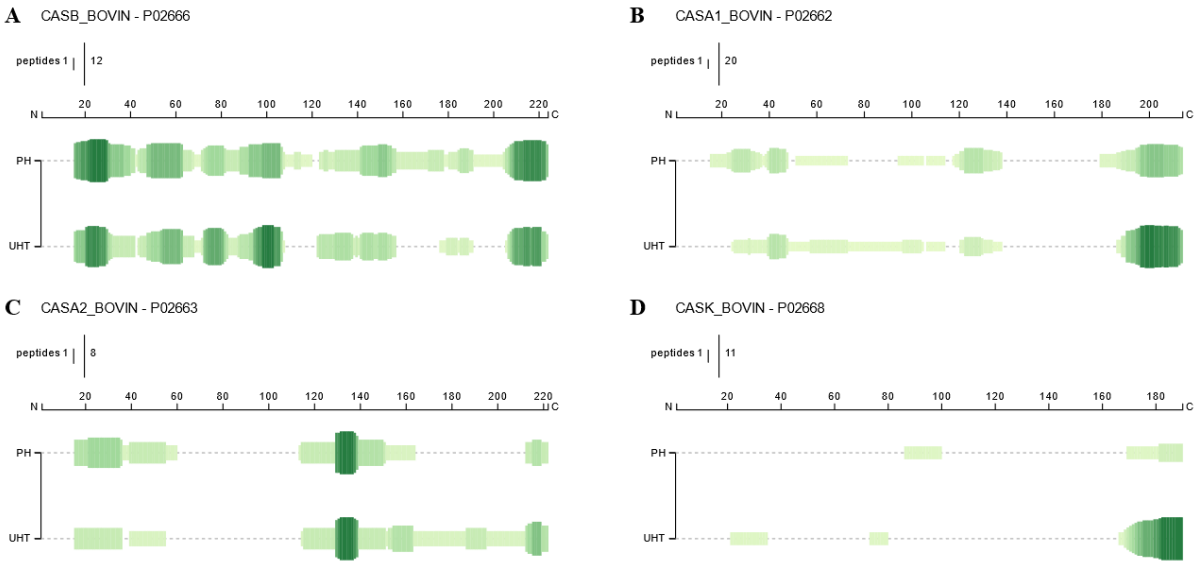

Figure 49. Peptide profile of endogenous peptides from pasteurised-homogenised (PH) and UHT milk for A) β-CN, B) α_{s1}-CN, C) α_{s2}-CN, and D) κ-CN. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

40 % of the 25 peptides derived from α_{s2}-CN were present in both processing treatments (Figure 49C). The comparatively lower number of peptides identified from α_{s2}-CN maybe due to the lower concentration of this CN in cow milk, and has also been found in similar studies (Wölk *et al.*, 2020). Independent of sample type, most peptides originated from three protein regions, 16-55, 115-163 and 213-222. A relatively long sequence from 61-113 was not covered by any detected peptide in either type of milk, whilst the sequence 165-212 was not detected in pasteurised-homogenised milk but was seen in UHT milk.

Very few peptides were identified from κ-CN, which is likely due to it being resistant to plasmin proteolysis (Baum *et al.*, 2013). Only 13.3 % of the peptides identified were detected in both processing treatments, with a greater amount being found in the UHT processed milk.

Most of the detected peptides originated from the C-terminal end of κ-CN (from 170-190). The higher number of κ-CN peptides in UHT milk maybe due to dissociation of κ-CN from the CN micelle during heating making it more accessible to enzymes (Anema, 2008).

Peptides originating from 13 non-CN proteins were also detected in undigested samples as shown in Table 28. Most non-CN protein derived peptides originated from glycosylation-dependent cell adhesion molecule 1. Although only 8 peptides were identified these covered 35 % of the parent protein and originated from region 72-93 in both processing treatments and region 19-40 and 62-71 for UHT treatment milk only. These results agree with research by Dallas *et al.* (2014) and Wölk *et al.* (2020), who also showed that peptides from glycosylation-dependent cell adhesion molecule 1 were the most abundant among non-CN proteins.

Table 28. Peptides originating from whey proteins identified in undigested milk samples.

Whey proteins	Number of peptides
α-LA	1
β-LG	1
BRD4 interacting chromatin remodelling complex associated protein	1
Butyrophilin	3
Glycoprotein 2	1
Glycosylation-dependent cell adhesion molecule 1	8
Histone lysine methyltransferase	5
Lactoperoxidase	2
Osteopontin	2
Perilipin	2
Plection	1
Polymeric immunoglobulin receptor	5
Sodium-dependent phosphate transport protein 2B	2

7.3.2.2 Peptide composition in commercially processed milks digested using an *in vitro* digestion model (HGS)

LC-MS/MS identified 560 peptides from 38 proteins in commercially processed milk digested using the HGS *in vitro* model. The peptide lengths ranged between 4 and 35 amino acid residues with an average length of 11.8 residues. 62.4 % of the identified peptides came from

CNs with 30.7 % from β-CN, 16.9 % from α_{s1}-CN, 5.0 % from α_{s2}-CN, and 9.8 % from κ-CN. The highest number of non-CN peptides were derived from β-LG (8.8 %). Throughout gastric digestion the number of peptides identified increased from 145 peptides identified after 20 mins of digestion to 258 peptides identified after 180 mins of digestion. As expected, the average length of the peptides decreased throughout gastric digestion, with these values being 15.0, 13.1, and 12.1 amino acid residues per peptide after 20, 60 and 180 mins, respectively.

Intestinal digestion was static and performed on a subset of the samples obtained from the HGS. Overall, the numbers of peptides identified in the intestinal samples were lower than in the gastric samples. 378 peptides were identified in the gastric samples, whilst only 216 were identified in the intestinal samples. Only 7.6 % of identified peptides were found in both the gastric and intestinal samples. The average peptide length decreased from 12.6 amino acid residues in gastric samples to 10.7 amino acid residues in intestinal samples. The lower number of peptides identified in the intestinal samples was potentially due to those samples containing an increased number of peptides having fewer than 4 amino acid residues, which was the lower limit for the database searches. The effect of gastric time on intestinal digestion was also studied. Starting material was obtained from both the 20 mins gastric and 60 mins gastric samples and subjected to a total digestion time of 120 mins by varying the intestinal digestion time, corresponding to the 20G-100I and 60G-60I samples, respectively. Both samples had approximately the same number of peptides identified, with a sequence similarity of 58.2% suggesting that the starting material does influence the peptidome.

The peptides in a blank intestinal digestion sample were used to investigate whether cow milk peptides were present prior to introduction of the gastric sample due to the introduction of intestinal enzymes and bile salts. LC-MS/MS identified 53 peptides in the intestinal blank, over 80 % of these peptides were also present in the intestinal samples. These peptides originated from 15 different proteins, with the largest amount originating from α-amylase (16). However, a few CN peptides were present which suggests that contamination of the sample due to bleeding off the column may have occurred.

Processing treatment influenced the peptidome released throughout digestion. Overall, more peptides were identified in UHT treated milk compared to pasteurised-homogenised milk (Figure 50). The average length of peptides identified in UHT milk (11.8 AA) was slightly

shorter than those identified in pasteurised-homogenised milk (12.1 AA). The distribution of peptides was similar between the two processing treatments for CNs, however a greater number of peptides from β-LG were identified in the UHT samples. This is especially apparent during the 60- and 180-min gastric time points (Figure 51). This suggests that β-LG is digested more readily in UHT milk than pasteurised-homogenised milk. This supports previous studies which show that β-LG is more susceptible to hydrolysis by pepsin after heating (Sánchez-Rivera *et al.*, 2015; Tunick *et al.*, 2016; Wada & Lönnerdal, 2014). In both pasteurised-homogenised milk and UHT milk, peptides derived from β-LG are present in the intestinal digestion time points.

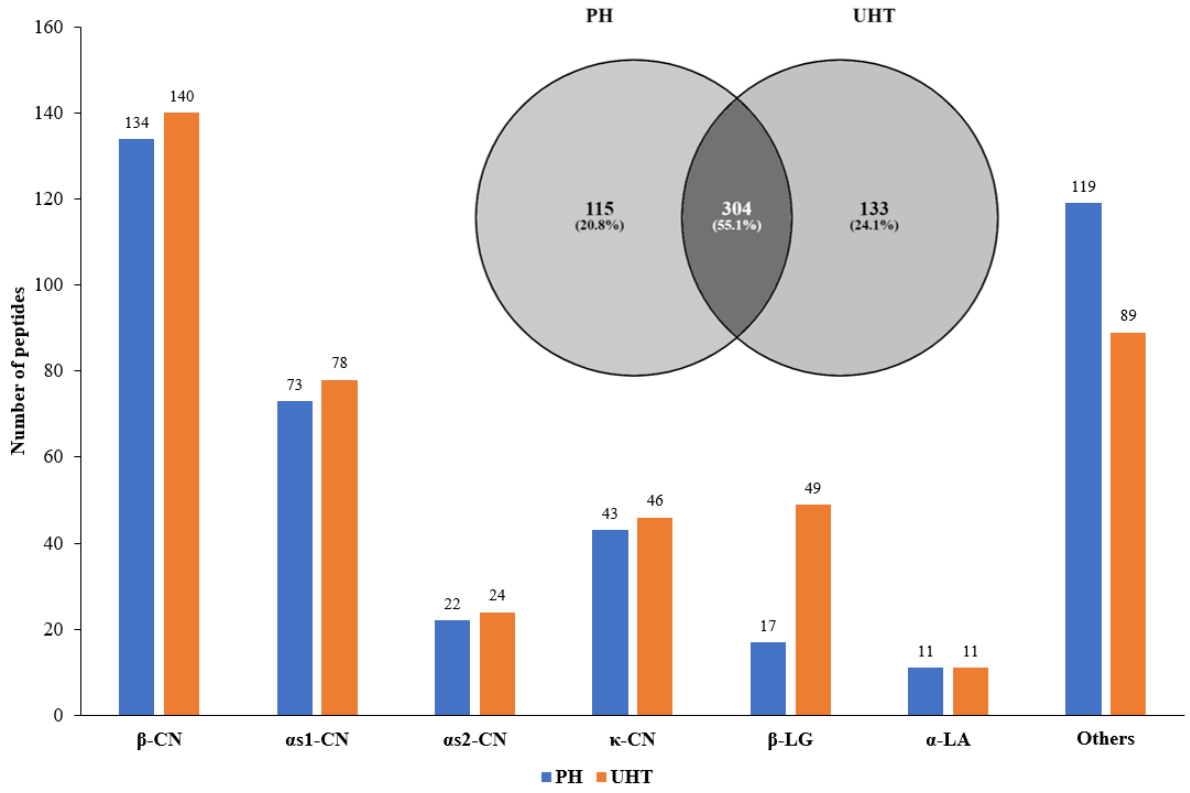

Figure 50. Numbers of peptides identified for different proteins in pasteurised-homogenised (PH, blue) and UHT (orange) treated milk during *in vitro* digestion. The insert shows a Venn diagram showing the total number of identified peptides for pasteurised-homogenised and UHT milk during *in vitro* digestion.

The sequence similarity, assessed by the number of peptides with identical sequences, between the two processing treatments was assessed at different time points. The proportions of peptides with identical sequences between pasteurised-homogenised and UHT samples at 20, 60 and 180 minutes of gastric digestion were 45.5 %, 43.3 %, and 57.4 %, respectively. For intestinal

time points at 20G-40I, 20G-100I, 60G-60I and pooled 180I the proportions of peptides with identical sequences were 34.7 %, 29.6 %, 43.1 %, and 47.1 %, respectively. Overall, throughout *in vitro* digestion the proportion of peptides with identical peptide sequences was only 55.1 % suggesting that processing treatment may influence peptide release. However, due to the commercial nature of the samples it is unclear whether the initial protein content and composition prior to processing would be identical. Sánchez-Rivera *et al.* (2015) showed that peptide homology between heated and non-heated milk was 48 % at the end of gastric digestion, the higher homology in this study is likely because both milk samples were heated prior to digestion.

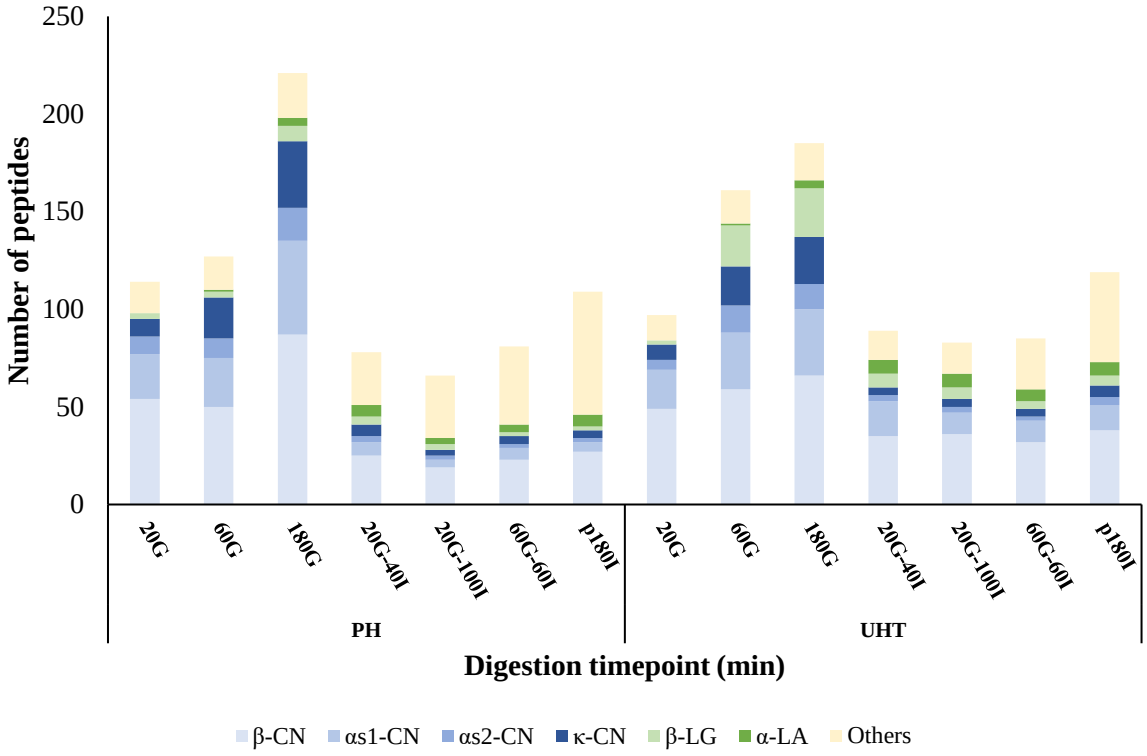

Figure 51. Numbers of peptides identified for different proteins in pasteurised-homogenised (PH) and UHT treated milk at different timepoints during *in vitro* digestion.

The Peptigram tool was used to map and align identified peptides to their parent protein sequence (Manguy *et al.*, 2017). Figure 52 shows the peptides derived from β-CN and α_{s1}-CN identified in pasteurised-homogenised and UHT milk throughout *in vitro* digestion. Peptides from α_{s1}-CN and κ-CN are shown in Figure 53 and peptides from β-LG and α-LA are shown in Figure 54.

All four CNs show a similar pattern of peptides irrespective of digestion time. This trend has also been observed during *in vivo* digestion of micellar CN and could be due to similarities in the material emptied from the stomach (Miralles *et al.*, 2020). The peptide coverage throughout *in vitro* digestion is similar for pasteurised-homogenised and UHT milk for β-CN (86 % and 84 % respectively) though is slightly lower for α_{s1}-CN (69 % and 60 % respectively). A high number of peptides derived from β-CN and α_{s1}-CN originated from the C-terminal region for both pasteurised-homogenised and UHT treated milk. This observation has also been made in previous studies (Barbé *et al.*, 2014; Miralles *et al.*, 2020; Sánchez-Rivera *et al.*, 2015) and could be a result of hydrolysis of these proteins starting in this region, peptide abundance and/or ionisation capacity of the peptides from this region. In addition, peptides from regions 21-29, 72-104 and 141-155 of β-CN were consistently present throughout digestion. Very few peptides derived from region 112-129 of β-CN were identified, peptides from this region were only identified in a few intestinal samples. Peptides from region 39-47 from α_{s1}-CN were present throughout all digestion timepoints, whilst regions from 58-67 were only present during late gastric and intestinal samples. This suggests that these regions could be resistant to digestion and/or that these regions are incorporated into the clot contributing to the late emptying time.

Figure 52. Peptides derived from β-CN and α_{s1}-CN during gastrointestinal digestion using the HGS *in vitro* model. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

Comparative to the other CNs, α_{s2}-CN had a very low sequence coverage, 37 % for pasteurised-homogenised milk and 27 % for UHT milk. The greatest number of peptides derived from α_{s2}-CN were found in the region 114-138 throughout all digestion time points. Gastric samples also contained peptides from the C terminal region of the protein which were absent in the intestinal samples. The coverage for κ-CN was 65 % for both pasteurised-homogenised and UHT milk. The peptides derived from κ-CN differed throughout digestion. During gastric digestion a large number of peptides were found between residue 39-96 and the C-terminal region. Intestinal digestion mainly only had peptides from region 173-183.

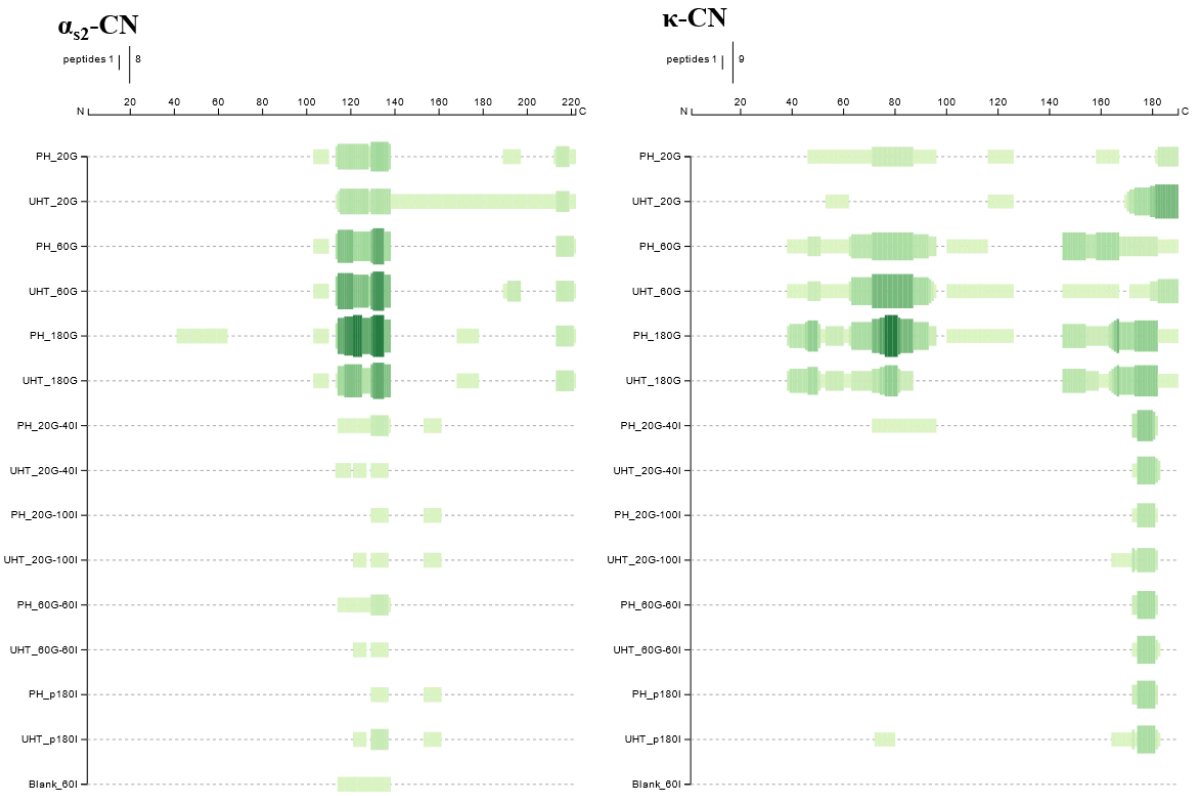

Figure 53. Peptides derived from α_{s2}-CN and κ-CN during gastrointestinal digestion using the HGS *in vitro* model. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

Peptides from β-LG were present throughout *in vitro* gastrointestinal digestion. An increase in the coverage is observed for β-LG between UHT milk and pasteurised-homogenised milk with UHT milk peptides covering 57 % of the parent protein whilst peptides from pasteurised-homogenised milk only covers 39 %. This is particularly apparent in region 58-73 throughout digestion and region 141-150 during intestinal digestion. During gastric digestion the most peptides from β-LG are found between residue 17-74. Very few peptides were present from α-LA and the coverage was only 26 %. Peptides were only identified during late gastric and intestinal digestion from regions 38-46 and 99-109.

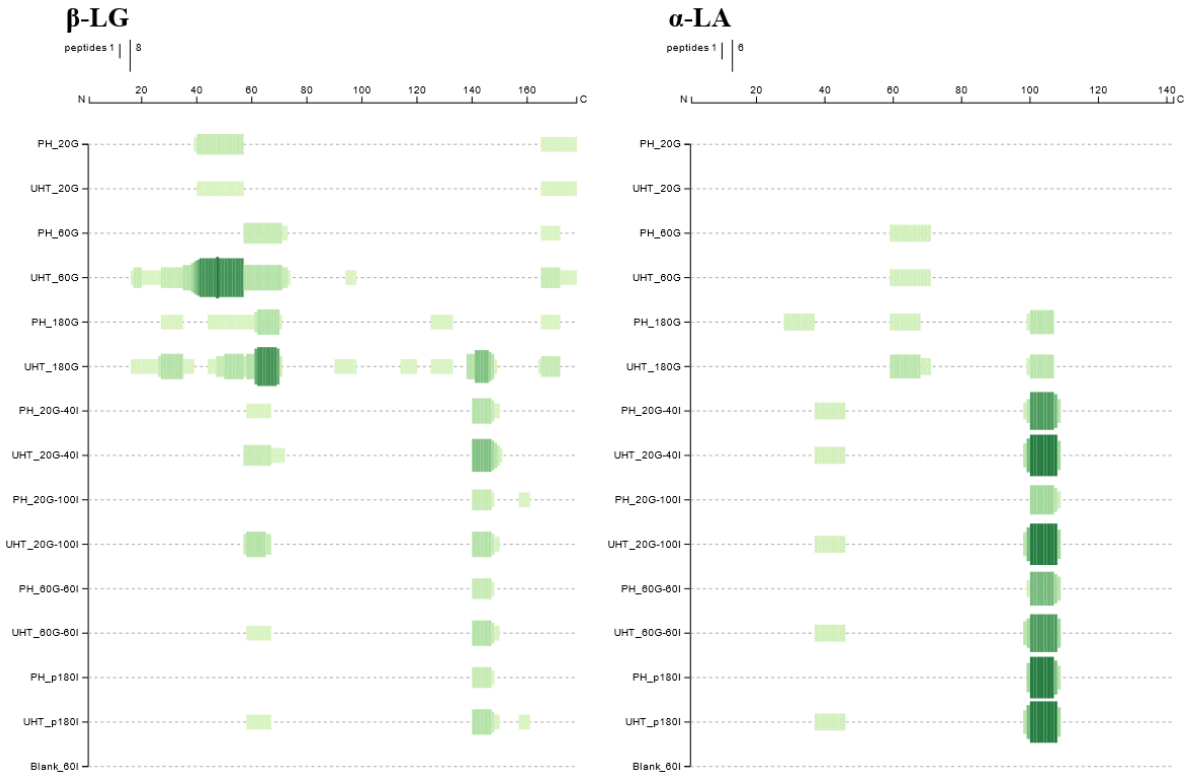

Figure 54. Peptides derived from β-LG and α-LA during gastrointestinal digestion using the HGS *in vitro* model. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

7.3.2.3 Peptides composition in commercially processed milks digested using an *in vivo* digestion model (pigs)

LC-MS/MS identified 627 peptides from 82 proteins in commercially processed milk digested using an *in vivo* model. The peptide length ranged between 4 and 35 AA residues with an average length of 11.5 residues. 65.1 % of the identified peptides came from CNs with 30.9 % from β-CN, 17.4 % from α_{s1}-CN, 5.4 % from α_{s2}-CN, and 11.3 % from κ-CN. The whey protein with the most identified peptides was β-LG (5.4 %). Throughout gastric digestion the number of peptides identified decreased from 226 peptides identified after 20 mins of digestion to 179 peptides identified after 180 mins of digestion. The average length of the peptides also decreased throughout gastric digestion with 13.4, 12.6, and 10.9 amino acid residues being identified after 20, 60 and 180 mins respectively. During intestinal digestion the number of peptides also decreased from 158 peptides identified after 60 mins of digestion to 79 peptides identified after 180 mins of digestion. The average length of peptides during intestinal digestion was 10.7.

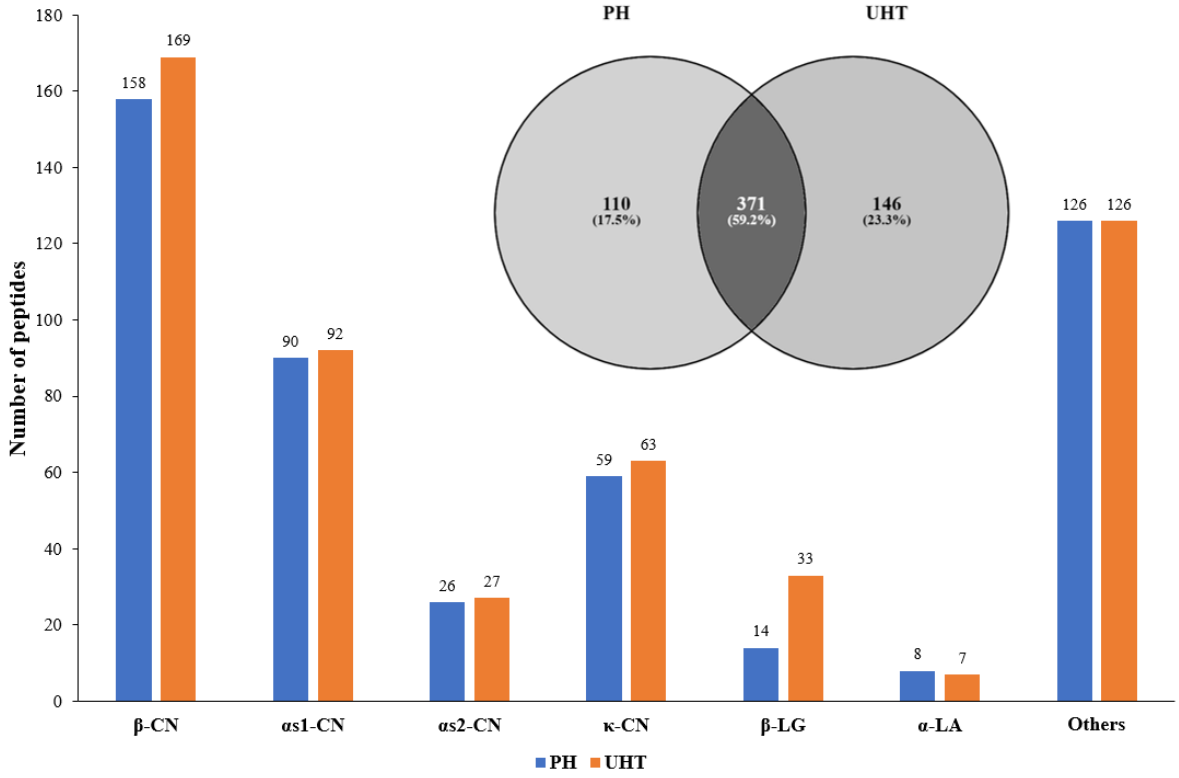

Figure 55. Numbers of peptides identified for different proteins in pasteurised-homogenised (PH, blue) and UHT (orange) treated milk during *in vivo* digestion. The insert shows a Venn diagram showing the total number of identified peptides for pasteurised-homogenised and UHT milk during *in vivo* digestion.

Processing treatment influenced the peptidome released throughout digestion (Figure 55). Similarly, to *in vitro* digestion more peptides were identified in UHT treated milk compared to pasteurised-homogenised milk. No difference was observed between the average length of peptides between UHT and pasteurised-homogenised milk. The distribution of peptides was similar between the two processing treatments for CNs, however UHT treated milk identified a greater amount of β-LG. This trend was also observed during *in vitro* digestion. However, during *in vivo* digestion although the β-LG derived peptides were higher during gastric digestion for UHT treated milk, after 60 mins of intestinal digestion no peptides derived from β-LG were present for either processing treatment (Figure 56).

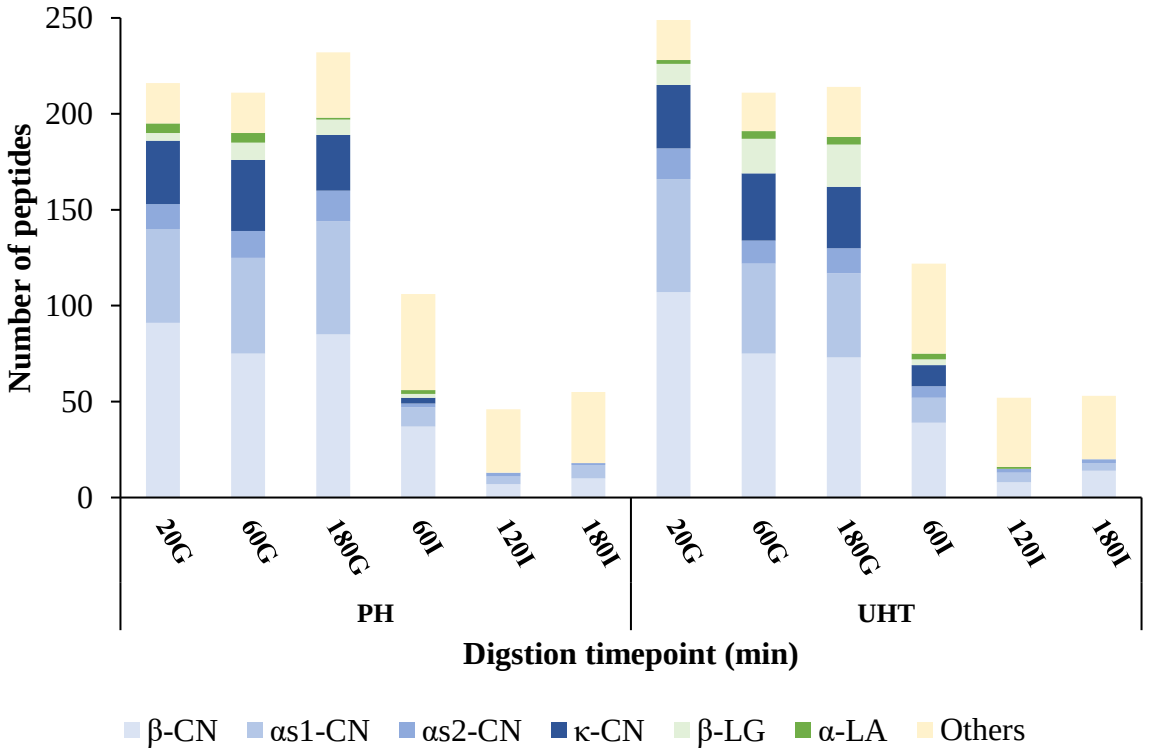

Figure 56. Numbers of peptides identified for different proteins in pasteurised-homogenised (PH) and UHT treated milk at different timepoints during *in vivo* digestion.

Assessment of sequence similarities of the peptides generated throughout *in vivo* digestion showed that samples at 20, 60 and 180 mins of gastric digestion had 60.6 %, 63.7 %, and 52 % identical peptides respectively between pasteurised-homogenised and UHT milk. The sequence similarities observed in samples at 60, 120 and 180 mins of intestinal digestion was 40.5 %, 34.2 % and 36.7 % respectively. Overall, throughout *in vivo* digestion the sequence similarity was 59.2 %. This was slightly higher than during *in vitro* digestion, however, still suggests that processing treatment could result in differences in peptide release during digestion.

Peptigram was also used to investigate how peptides identified during *in vivo* digestion aligned to their parent protein sequence. Figure 57 shows the peptides derived from β-CN and α_{s1}-CN identified in pasteurised-homogenised and UHT milk throughout *in vivo* digestion. Peptides from α_{s1}-CN and κ-CN are shown in Figure 58 and peptides from β-LG and α-LA are shown in Figure 59. The protein coverage was approximately 87 % for β-CN throughout digestion for both pasteurised-homogenised milk and UHT milk. For α_{s1}-CN it was 72 % for pasteurised-homogenised milk and 65 % for UHT milk. Both β-CN and α_{s1}-CN showed a large number of peptides originating from the C-terminal region throughout digestion. In addition, peptides

were consistently found in region 21-31, 74-107 and 141-155. As well as peptides derived from the C-terminal region α_{s1}-CN also shows a great many peptides from region 39-76 during gastric time points and region 77-89 during intestinal time points.

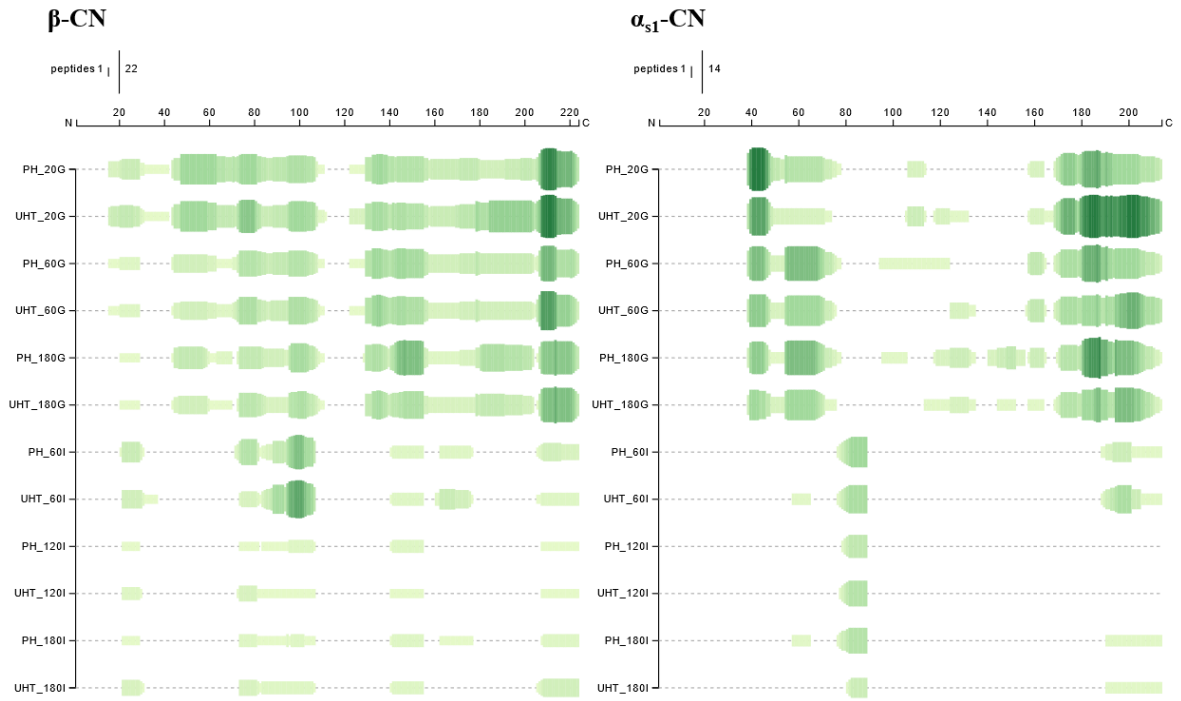

Figure 57. Peptides derived from β-CN and α_{s1}-CN during gastrointestinal digestion using the pig *in vivo* model. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

The coverage for α_{s2}-CN was lower than the other CNs ranging from 36 to 38 % for UHT and pasteurised milk respectively. The coverage of κ-CN was 67 % for pasteurised-homogenised milk and 64 % for UHT milk. Peptides during gastric digestion were mainly found from region 114-139 of α_{s2}-CN and during intestinal digestion from region 68-80. Very few peptides from κ-CN were found during intestinal samples the peptides derived from κ-CN in gastric samples were either concentrated in the C-terminal region or between region 39-96.

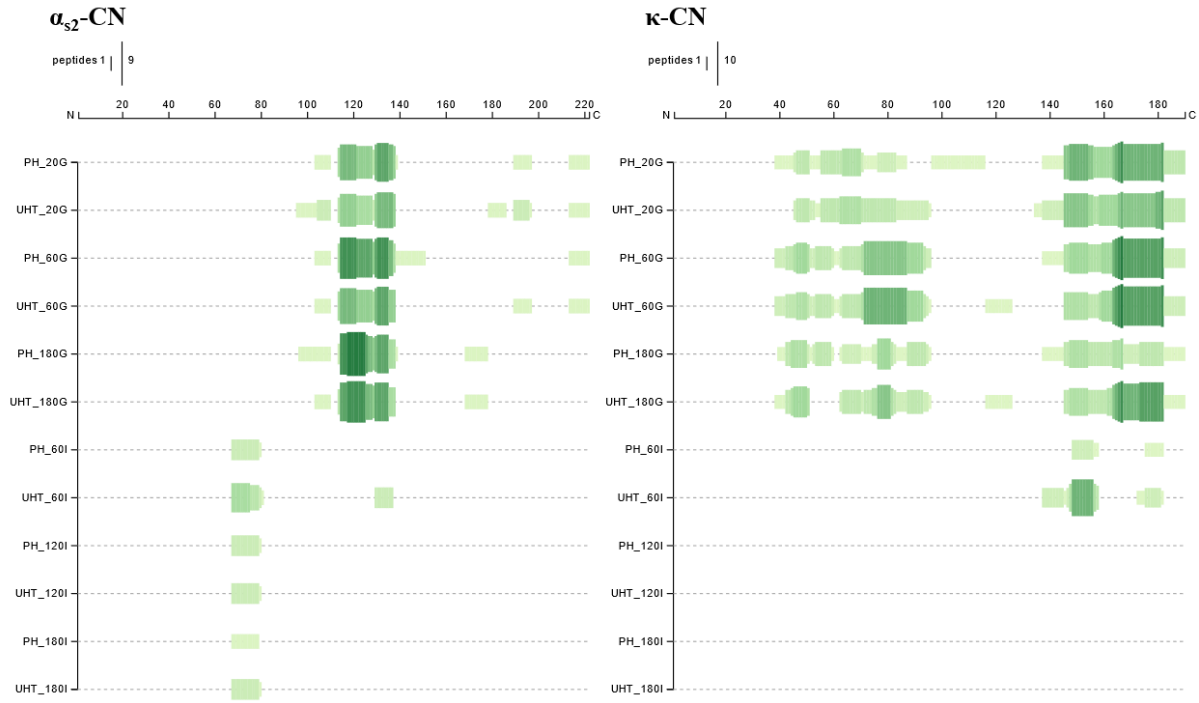

Figure 58. Peptides derived from α_{s2}-CN and κ-CN during gastrointestinal digestion using the pig *in vivo* model. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

Peptides from β-LG were only identified during gastric digestion and early intestinal digestion (60 mins). An increase in the coverage is observed for β-LG between UHT milk and pasteurised-homogenised milk with UHT milk peptides covering 53 % of the parent protein whilst peptides from pasteurised-homogenised milk only covers 30 %. This is particularly apparent in region 58-73 throughout digestion. During gastric digestion the most peptides from β-LG are found between residue 18-73. Very few peptides were present from α-LA and the coverage was only 20 %. Peptides were mainly identified during gastric and early intestinal digestion from regions 38-46, 60-71, and 99-109.

Figure 59. Peptides derived from β-LG and α-LA during gastrointestinal digestion using the pig *in vivo* model. Each vertical bar corresponds to an amino acid identified as part of a peptide sequence.

7.3.2.4 Comparisons of peptides released during *in vitro* and *in vivo* digestion

A greater number of peptides are identified during *in vivo* digestion (627 peptides) compared to *in vitro* digestion (560 peptides). Although the amount of proteins these peptides are derived from is larger in the *in vivo* samples (82 proteins) compared to the *in vitro* samples (38 proteins) the distribution of CNs is similar at approximately 60 %. This difference is likely due to the increased interspecies variation of *in vivo* digestion and is consistent with other studies (Sanchón *et al.*, 2018). The sequence similarity of the peptides identified between the two digestion methods is 34.6 %, this increases to 49.5 % when just studying peptides derived from CN.

Both digestion methods identify a greater number of peptides in UHT treated milk compared to pasteurised-homogenised milk. The sequence similarity between processing treatments is 55.1 % for *in vitro* digestion and 59.2 % for *in vivo* digestion. These results agree with Sánchez-Rivera *et al.* (2015) and Dupont, Mandalari, Mollé, *et al.* (2010) who show that processing treatment increased the number of peptides found in digested samples and results in different peptide patterns for both dynamic DIDGI® and static infant *in vitro* methods. Both models

suggest that processing treatment influences peptide release during digestion. In both digestion methods the number of peptides derived from CNs stays constant regardless of digestion method, however the number of peptides derived from β-LG are greater in UHT milk compared to pasteurised-homogenised milk. During *in vitro* digestion β-LG peptides are still present at 180 min of intestinal digestion whereas during *in vivo* digestion no β-LG peptides are present after 60 min of intestinal digestion. This is likely due to absorption of peptides occurring during the intestinal stage of *in vivo* digestion which is lacking during *in vitro* digestion. This study suggests an increased susceptibility of β-LG to hydrolysis when exposed to UHT treatment. This is consistent with other studies (Sánchez-Rivera *et al.*, 2015; Wada & Lönnerdal, 2014). It has been shown that high heating temperatures over 90 ºC induce conformational changes in β-LG that result in the exposure of hydrophobic regions which cause this increased susceptibility to hydrolysis by pepsin (Peram *et al.*, 2013).

Peptigram was used to map peptides identified with their parent proteins over time. Similarities were observed between the two different digestion models. During gastric digestion irrespective of time point, both models consistently showed peptides in the same regions for all CN proteins and β-LG. β-CN consistently had peptides in the following region: 21-29, 72-104, 141-155 and the C-terminal region. Peptides were more frequently found from the C-terminal region and between residues 39-47 of α_{s1}-CN. For α_{s2}-CN they were situated between residues 114-138 and in κ-CN between residues 39-96 and the C-terminal region. β-LG consistently had peptides between residues 18-73. During intestinal digestion differences were observed in the location of peptides identified between *in vitro* and *in vivo* digestion models. This is likely due to no absorption occurring during intestinal digestion of the *in vitro* model. No peptides were found during late intestinal digestion for κ-CN and β-LG during *in vivo* digestion. However, during *in vitro* digestion peptides were found from these proteins at all stages of gastric and intestinal digestion. The consistent regions found throughout digestion of the CN proteins could be due to these regions being resistant to digestion, some but not all of these overlap with previously found resistant regions such as residue 79-93 and 190-209 from β-CN (Sánchez-Rivera *et al.*, 2015).

The coverage of CNs ranged from 84-88 % for β-CN, 60-72 % for α_{s1}-CN, 27-38 % for α_{s2}-CN and 64-67 % for κ-CN. The coverage of major whey proteins was 30-57 % for β-LG and 20-26 % for α-LA. In general, the coverage of CN proteins was higher during *in vivo* digestion

and lower for whey proteins compared to *in vitro* digestion. The absence of identified peptides in a given part of the protein sequence could be explained by extensive hydrolysis of the area leading to peptides that are too small for PEAKS to confidently identify, resistance to digestion leading to protein fragments that are too large for this method to detect, PTMs, or low abundance of peptides leading to no MS/MS being acquired during DDA (Barbé *et al.*, 2014; Dupont, Mandalari, Mollé, *et al.*, 2010).

7.4 Conclusion

In the present work, a comparison of the dynamic *in vitro* model (HGS) with the *in vivo* digestion data from pigs is shown for two different types of commercially processed milks. Although some differences are found, the gastric digestion data from both the *in vitro* and *in vivo* model showed similarities in the peptides released throughout digestion with peptides consistently being from the same region of the parent protein. Intestinal digestion using the *in vitro* and *in vivo* models showed differences in the type and distribution of the peptides released. This is likely due to the intestinal phase of the *in vitro* model not capturing the dynamic nature of *in vivo* intestinal digestion. This suggests that although the HGS model is suitable for the study of gastric digestion of protein-rich food due to its similarity with *in vivo* pig digestion more work is needed to improve the intestinal step so that it mimics the dynamic nature of *in vivo* digestion.

Both *in vitro* and *in vivo* digestion showed that more excessive heat treatment resulted in β-LG becoming more susceptible to hydrolysis. The homology values between the different treatments (55.1 % for *in vitro* digestion and 59.2 % for *in vivo* digestion) indicate that processing affects the identity of peptides generated throughout digestion. Processing treatment also influenced the number of peptides produced throughout digestion. A higher proportion of low molecular weight peptides was identified in UHT milk compared to pasteurised-homogenised milk throughout digestion. The different peptide profiles generated by the two processing treatments could have implications for health. Further analysis of the bioactive properties of these peptides could give further clarity.

Chapter 8 – Comparative analysis of red deer milk proteins throughout lactation using quantitative proteomics

8.1 Introduction

Milk is a rich source of nutrients and bioactive compounds, enhancing the functional diversity of dairy products (Qin *et al.*, 2021). Notably, red deer milk is characterized by higher levels of protein, fat, calcium, zinc, iodine, branched-chain fatty acids and α-linolenic acid compared to other ruminant milks (Li *et al.*, 2023). Research on deer milk has predominantly addressed overall compositional features (Landete-Castillejos *et al.*, 2000; Malacarne *et al.*, 2015; Wang *et al.*, 2017), with limited focus on specific protein characteristics or their variations throughout lactation (Ha *et al.*, 2014; Li *et al.*, 2023; Wang *et al.*, 2021).

Protein is a key quality indicator for milk. Red deer milk contains approximately twice the total protein content (8.8%) of cow milk (4.1%). Although casein (CN) content is higher in red deer milk (8.7%) compared to cow milk (4.0%), whey protein levels are comparable (O. N. L. Vithana *et al.*, 2012). The predominant CN in red deer milk is thought to be β-CN (Vithana, 2012), differing from cow milk, where α_{s1}-CN is predominant (Park, 2004). Ha *et al.* (2015) reported that α_{s1}-CN is also the predominant CN found in deer milk; however, they were unable to determine the concentration of β-CN due to a shift in elution profiles during RP-HPLC analysis. Overall, red deer milk is reported to have significantly higher amounts of β-CN and lower amounts of κ-CN and αs1-CN than cow milk (Vithana, 2012). While whey proteins are less abundant than CNs, they possess a wide range of biological activities. The dominant whey protein in red deer is β-LG, similar to cow milk, but is found in significantly higher amounts (9.8 g/L in red deer vs. 3.2-3.3 g/L in cow milk). Other differences include a lower α-LA and a higher lactoferrin content in red deer milk (Wang *et al.*, 2017).

The proportions of nutritive constituents, especially fat and protein, in milk vary throughout lactation (Boland & Singh, 2019). The lactation period of red deer is relatively short, lasting approximately 4-5 months. Studies have shown that over this period, milk volume decreases while protein and fat content increase in both red deer and roe deer (Landete-Castillejos *et al.*, 2000; Malacarne *et al.*, 2015). Variations in lactose levels throughout lactation has been reported, with some studies indicating stability (Berruga *et al.*, 2021; Landete-Castillejos *et al.*, 2000) and others showing a decrease (Li *et al.*, 2023). Similar trends in protein and fat

increases, along with lactose decreases, have also been observed in other ruminant species such as cows, sheep, and goats (Li, Delger, *et al.*, 2022; Li *et al.*, 2019).

Limited information is available on how individual proteins in red deer milk vary throughout lactation. Conflicting results have been reported regarding CN changes: some studies have reported an increase in CN concentration during lactation (Berruga *et al.*, 2021; Malacarne *et al.*, 2015), while other suggest stability (Li *et al.*, 2023), or variability depending on the individual deer (Arman *et al.*, 1974). A previous study conducted by our group used SDS-PAGE and HPLC to investigate protein composition changes in red deer milk across lactation stages (Li *et al.*, 2023). While this work revealed trends such as stability in β-CN, increases in κ-CN and decreases in α_{s2}-CN, along with some unknown whey proteins throughout lactation, HPLC's limited ability to detect low-abundance proteins or resolve complex mixtures highlighted the need for more advanced analytical methods.

To address these limitations, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed. This technique offers higher sensitivity and specificity, enabling the identification of less abundant proteins and providing detailed structural insights into complex protein profiles (Cunsolo, Muccilli, Saletti, *et al.*, 2011). LC-MS/MS has been used to characterize milk proteins from different species such as cow (Nissen *et al.*, 2012; Vincent, Elkins, *et al.*, 2016), sheep (Ha *et al.*, 2015), and goat (Chen *et al.*, 2019; Sun *et al.*, 2023). It has also been used to study how individual proteins vary throughout lactation in cow (Delosière *et al.*, 2020), goat, (Qin *et al.*, 2021; Sun *et al.*, 2023) and sheep milk (Zhang *et al.*, 2020). These protein changes affect the digestibility and overall quality of the milk products consumed. For instance, Sun *et al.* (2019) identified differences in protein hydrolysis and peptide composition between colostrum and mature goat milk during digestion, particularly in the duodenum.

The objective of this study was to identify the proteins in red deer milk using LC-MS/MS and characterize their profiles throughout lactation (3 and 16 weeks). These insights will enhance understanding of the production, processing, and commercialization of red deer milk across lactation stages.

8.2 Materials and Methods

8.2.1 Reagents

Water, acetonitrile, methanol, formic acid (FA) and trifluoracetic acid (TFA), all LC-MS grade, and urea were sourced from ThermoFisher Scientific (Waltham, MA, USA). Thiourea was from Acros organics (China). Chloroform and ammonium bicarbonate were purchased from BDH Prolabo (Poole, UK). DL-Dithiothreitol (DTT), iodoacetamide (IAM) and 37 % hydrochloric acid (HCl) were from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Unless otherwise specified, all chemicals were of analytical grade.

8.2.2 Sample collection

The samples were collected from a Pāmu deer milk supply partner farm in Gore, New Zealand. The herd consisted of 120 lactating does. Deer milk was collected at eight different time points during the lactation period between 23/8/2020 and 22/02/2021 as described in section 3.2.3. Aliquots of samples from all time points were stored at -80 ºC prior to analysis.

8.2.3 LC-MS/MS

8.2.3.1 Fractionation of whey and CN proteins

The cream fraction was removed by centrifuging 1 mL of the sample at 11,500 x *g* for 45 min at 4 ºC, then scraping off with a small spatula. 500 µL of the skimmed milk fraction was aliquoted for separation of the whey and CN fractions. To precipitate out the CN fraction, the pH of the samples was reduced to ~4.5 using 0.1 M hydrochloric acid. The pH-adjusted samples were left to incubate at room temperature for 1 hour prior to centrifugation at 14,000 x *g* for 25 min at 4 °C. The whey fraction (supernatant) was transferred to a clean Eppendorf tube. A spatula was used to transfer a small amount of the precipitate (CN fraction) to an Eppendorf tube prior to it being dissolved in 100 µL of 50 mM ammonium bicarbonate. The protein concentrations of both the whey and CN fractions were estimated at 280 nm using the built-in protein assay on the Nanophotometer NP80 (Implen, Munich, Germany).

8.2.3.2 Protein extraction

An aliquot containing 250 µg of protein of each sample was dried in a CentriVap vacuum centrifuge (Labconco, Kansas City, MO, USA) operated at 40 ºC. 100 µL of urea buffer (7 M urea, 2 M thiourea and 50 mM DTT) was added to each sample and then incubated at 25 °C overnight. Chloroform/methanol extraction was performed based on previous methods

described by Wessel and Flügge (1984) as outlined in section 5.2.3. The supernatant was removed and discarded before the precipitated protein was air dried. The dried precipitate was then resuspended in 50 µL of 50 mM ammonium bicarbonate by sonicating for 5 mins.

8.2.3.3 Protein reduction, alkylation, and digestion

The extracted protein samples were reduced by addition of 10 mM DTT and incubation at 56 ºC for 45 mins and then alkylated using 20 mM iodoacetamide for 30 mins in the dark at room temperature (21 °C). Trypsin digestion was achieved by adding 2 µg of trypsin (1:50 enzyme:substrate) and mixing at 37 °C overnight. Desalting was performed using Pierce C18 pipette tips (ThermoScientific, Waltham, MA, USA) following the manufacturer's instructions and eluted peptides were dried in a CentriVap vacuum.

To ensure reproducibility a quality control containing equal amounts of all samples were run throughout the batch as well as 2 pools including all the whey samples and all the CN samples.

8.2.3.4 Chromatography and mass spectrometry

Prior to running on the LC-MS, the samples were resuspended in 50 µL of 0.1% TFA and diluted 10x with 0.1% FA.

Nanoflow LC-MS and LC-MS/MS were performed on an Ultimate 3000 HPLC system (ThermoScientific, Waltham, MA, USA) directly interfaced with a CaptiveSpray ion source to an Impact HD Q-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The CaptiveSpray was fitted with a nanoBooster device (Bruker Daltonik, Bremen, Germany), which infused acetonitrile into the mass spectrometer's nitrogen gas supply to improve sensitivity. For each sample, 1 µL was injected onto a PepMap100 C18 Nano-Trap column (ThermoScientific) to trap peptides, which were then eluted onto a ProntoSIL C18AQ analytical column (150 mm x 100 µm i.d., 3 µm particle size, 200 Å pore size; nanoLCM Solutions, Oroville, CA, USA). Both columns were maintained at 50 ºC in a column oven. Separation of peptides was carried out at a flow rate of 1 µL/min using a multistep linear gradient of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). Initially 2 % B was used for 4 mins, this increased to 20 % B over 45 mins, and then to 45 % B over a further 15 mins. The column was cleaned by increasing to 95 % B over 4 mins and holding at that level for 7 mins. After that, the column was re-

equilibrated by reducing back to 2 % B over 2 mins and holding at that for 8 mins. The total run time was 85 mins.

LC-MS/MS: During LC-MS/MS runs the mass spectrometer was operated using collision-induced dissociation (CID) and automated data dependent acquisition (DDA). Two acquisition methods were utilised to optimise the number of peptides identified. One method involved a full scan MS spectrum (50–2200 m/z, at 1 Hz scan rate) followed by a maximum of 10 MS/MS of precursors in the range m/z 150-1200, at a sampling rate of 1–20 Hz (depending upon precursor intensity). A preference for selection of multiply charged (2+ to 5+) precursor ions was set, and precursors were excluded from reacquisition for 1 min unless their intensity increased at least 4-fold during that period. The other method involved a full scan MS spectrum (150–2200 m/z, at 2 Hz scan rate) followed by a maximum of 10 MS/MS of precursors in the range m/z 350-2200, at a sampling rate of 8–32 Hz (depending upon precursor intensity). A preference for selection of multiply charged (2+ to 3+) precursor ions was set, and precursors were excluded from reacquisition for 1 min unless their intensity increased at least 5-fold during that period.

SPL: SPL were generated for each pooled sample using ProteinScape^{TM} version 4.0 (Bruker, Billerica, MA, USA) allowing for time tolerances of ±120 sec and size tolerances of ±100 mDa. The SPL list for all pools were combined. Each pooled sample were then re-run with their respective SPL which contained m/z values and retention times of precursor ions of confidently identified peptides in the initial LC-MS/MS runs, to try and identify lower abundance peptides. This was done using both LC-MS/MS methods outlined above.

LC-MS: To quantify the protein abundance, the milk samples and a QC mixture that contained equal amounts of all samples, were run with a MS acquisition method with a full scan MS spectrum (150-2200 m/z, at 1 Hz) in positive mode was used.

8.2.3.5 Protein identification and quantification

Peptides and proteins were identified using PEAKS Studio version 10.6 (Bioinformatics Solutions Inc., Toronto, Canada) (Ma *et al.*, 2003). Database searches utilised an AgResearch in-house deer FASTA database comprised of 278,740 non-redundant sequences that were derived from four sources: (A) two red deer and elk nucleotide databases containing 92,918 and 13,287 expressed sequence tag (EST) contig sequences (63,454,351 and 6,875,407

nucleotide residues respectively), which had been annotated by BLAST searches of the NCBI nr protein database; (B) the longest open reading frames (ORFs) from red deer lymph node mRNA (43,654 sequences); (C) the mCerELa1.1_genome and the identical protein group of the *Cervu*s organism from NCBI (13,722 and 103,698 sequences, respectively, downloaded 07.03.2024); and (D) all the UniProt *Cervus* database (49,309 sequences, downloaded 06.03.2024). Dereplication was performed using an in-house Python script (make_nr_fasta.py, version 1.0.11).

The following parameters were kept fixed for all searches; precursor ion mass error tolerance of 10 ppm, fragment mass error tolerance of 0.05 Da; semi-specific trypsin as enzyme with a maximum of 2 missed cleavages; the peptide false discovery rate (FDR) was set at 1%; proteins were accepted if their -10lgP values were above 20 and at least 1 unique peptide and additional significant supporting peptides were observed. Fixed modifications of carbamidomethyl (C) and variable modifications of Oxidation (M), deamidation (NQ), phosphorylation (STY) and carbamidomethyl (DHKE, @N) were used for the PEAKS search. A PEAKS spider search was performed after the PTM search to identify amino acid substitutions.

8.2.3.6 Label free quantitation (LFQ)

The LFQ search used MS data with peptide identifications transferred to MS features from the PTM search (for cow milk) and from the Spider search (for deer milk) with a mass error tolerance of 20 ppm and a retention shift tolerance of 2 min. The FDR threshold was set at 1% and the reference training samples were autodetected. An in-house R-script was used to normalise the intensity of the identified peptides by the total ion chromatogram (TIC) till the acetonitrile gradient reached 50% B.

8.2.3.7 Bioinformatics and statistical analysis

Gene ontology (GO) enrichment analysis of the proteins identified in this study were analysed using two different programmes; DAVID Bioinformatics Resources 6.8 software (david.ncifcrf.gov/summary.jsp), in which *P*-value <0.05 was considered to be significantly enriched (Huang *et al.*, 2009b; Sherman *et al.*, 2022) and GO enrichment analysis powered by Panther (geneontology.org), which used Fisher's exact test to determine which terms were significantly enriched (Ashburner *et al.*, 2000; Central *et al.*, 2023).

Quantified proteins were analysed for difference throughout lactation using two different tests. For each protein, a regression line was fitted on the protein concentration measured at each lactation time point from week 3 to week 16. To reliably estimate a regression line, only proteins that were observed in each of the 8 time points were selected. The slope was used to determine whether the protein concentration increased or decreased between the time points. To determine whether the decrease or increase in concentration was significant p-value < 0.05. As well as the regression line the fold change and t-test were also calculated between week 3 and week 16 of lactation for each protein, significance was based on p-values below 0.05. Prism version 10.2.1 (GraphPad Software, Boston, MA) was utilised to make heatmaps to visualise the quantitative changes throughout lactation.

8.3 Results and discussion

8.3.1 Identification and quantification of red deer milk proteins

In the current study, 73 milk proteins were identified in deer milk using an LC-MS/MS approach; the full list is provided in Table 29. Among the 73 identified proteins, 9 components were well known deer milk proteins (CNs, α-LA, β-LG, serum albumin, lactotransferrin and immunoglobulins) which had already been identified in deer milk (Ha *et al.*, 2014; Li *et al.*, 2023; Wang *et al.*, 2017). Whereas the others (88%) had not previously been described in deer milk proteomic studies, however, has been identified in cow milk (Das *et al.*, 2022; Delosière *et al.*, 2020).

Table 29. Proteins identified in deer milk during LC-MS/MS.

UNIPROT ID	SPECIES	PROTEIN	GENE
A0A6J0VYA4	Odocoileus virginianus texanus Muntiacus reevesi	Actin	ACTB
Q4TU70	Cervus canadensis canadensis	Alpha hemoglobin chain	HBA
A0A6J0WBI5	Odocoileus virginianus texanus	Alpha-2-HS-glycoprotein	AHSG
A0A212DFY0	Cervus elaphus hippelaphus Cervus elaphus xanthopygus	Alpha-lactalbumin	LALBA
A0A6J0VVP2	Cervus elaphus hippelaphus Muntiacus muntjak Odocoileus virginianus texanus	Alpha-S1-CN	CSN1S1
A0A212D6E7	Odocoileus virginianus texanus Cervus elaphus hippelaphus	Alpha-S2-CN	CSN1S2
A0A6J0XJ59	Muntiacus muntjak Muntiacus reevesi Odocoileus virginianus texanus Cervus nippon hortulorum	Annexin	
Q2Q1M6	Cervus elaphus	Annexin A2	ANXA2
A0A212DI82	Cervus elaphus hippelaphus Odocoileus virginianus texanus	Apolipoprotein AI	APOA1

UNIPROT ID	SPECIES	PROTEIN	GENE
A0A6J0VYG	Odocoileus virginianus texanus Alces alces Muntiacus reevesi	Beta-CN	CSN2
Q00P86	Rangifer tarandus tarandus	Beta-lactoglobulin	LGB
A0A212D5D9	Cervus elaphus hippelaphus	Butyrophilin subfamily 1 member A1	BTN1A1
A0A6J0WL20	Cervus elaphus hippelaphus	Collagen alpha-1(I) chain-like	COL1A1
A0A6J0Z0K5	Odocoileus virginianus texanus	Collectin-46	CL46
A0A5J5MNL6	Muntiacus reevesi Muntiacus muntjak	C-type lectin domain-containing protein	CLEC
A0A6J0Y3C4	Odocoileus virginianus texanus Muntiacus muntjak	Cytoplasmic dynein 2 heavy chain 1	DYNC2H1
A0A212CAL2	Cervus elaphus hippelaphus Muntiacus reevesi Muntiacus muntjak Odocoileus virginianus texanus	Elongation factor 1-alpha	EEF1A1
A0A6J0WPP2	Odocoileus virginianus texanus	Fatty acid-binding protein	FABP3
A0A220IGB8	Cervus elaphus	Fetal beta-globin	
A0A5N3VSU4	Muntiacus muntjak Muntiacus reevesi	Folate receptor-like domain-containing protein	
A0A5N3UKR3	Muntiacus reevesi	Globin domain-containing protein	HBA1
A0A212DFU4	Cervus elaphus hippelaphus	Glycosylation-dependent cell adhesion molecule 1	GLYCAM1
A0A5N3W0N5	Muntiacus reevesi Muntiacus muntjak	H15 domain-containing protein	H1-5
B6D985	Cervus elaphus	Haptoglobin	HP
A0A6J0YGS2	Odocoileus virginianus texanus	Hemoglobin fetal subunit beta	
A0A6J0Y2T5	Rangifer tarandus Odocoileus virginianus texanus	Hemoglobin subunit alpha	HBA
A0A6J0Y2J9	Odocoileus virginianus texanus	Hemoglobin subunit alpha-like	HBA
A0A6J0WWZ2	Odocoileus virginianus texanus	Histone H1.3 isoform X1	H1-3
A0A6J0WX52	Odocoileus virginianus texanus	Histone H1.3 isoform X2	H1-4
A0A833SCJ5	Cervus hanglu yarkandensis Muntiacus reevesi Muntiacus muntjak Odocoileus virginianus texanus	Histone H2A	H2A
A0A6J0WXB3	Odocoileus virginianus texanus	Histone H3.1-like	H3-1
A0A6J0WX68	Odocoileus virginianus texanus	Histone H4	H4
A0A212CM59	Cervus elaphus hippelaphus Muntiacus reevesi	Ig-like domain-containing protein	
A0A212D5R7	Cervus elaphus hippelaphus	Immunoglobulin J	JCHAIN
A0A5N3V8L0	Muntiacus muntjak Muntiacus reevesi	Joining chain of multimeric IgA and IgM	JCHAIN
Q95149	Cervus elaphus Cervus nippon	Kappa-CN	CSN3
A0A212CT53	Cervus elaphus hippelaphus	Lactadherin	MFGE8
A0A212C9V5	Cervus elaphus hippelaphus Odocoileus virginianus texanus Muntiacus muntjak	Lactotransferrin	LTF
A0A5N3WTM7	Muntiacus muntjak	Lipocalin/cytosolic fatty-acid binding domain-containing protein	

UNIPROT ID	SPECIES	PROTEIN	GENE
A0A212C9X7	Cervus elaphus hippelaphus Muntiacus reevesi Muntiacus muntjak Odocoileus virginianus texanus	L-lactate dehydrogenase	LDHB
A0A5N3XIV9	Muntiacus reevesi	L-lactate dehydrogenase A chain	LDHA
A0A212CR41	Cervus elaphus hippelaphus	Msx2-interacting protein	SPEN
A0A212CF87	Cervus elaphus hippelaphus	Mucin 1	MUC1
A0A5N3V0T7	Muntiacus muntjak	Non-specific serine/threonine protein kinase	PINK1
A0A212CLU0	Cervus elaphus hippelaphus Cervus nippon	Osteopontin	SPP1
A0A212DE23	Cervus elaphus hippelaphus	Peptidoglycan-recognition protein	PGRP
A0A212CKA1	Cervus elaphus hippelaphus Muntiacus reevesi Muntiacus muntjak Odocoileus virginianus texanus	Peptidyl-prolyl cis-trans isomerase	PPIA
A0A212CQF6	Cervus elaphus hippelaphus Muntiacus reevesi Muntiacus muntjak Odocoileus virginianus texanus	Phosphopyruvate hydratase	ENO1
A0A212CL42	Cervus elaphus hippelaphus	Platelet glycoprotein 4	CD36
A0A212CRH2	Cervus elaphus hippelaphus Odocoileus virginianus texanus	Polymeric immunoglobulin receptor	PIGR
A0A6J0XNE6	Odocoileus virginianus texanus	Polyubiquitin	
A0A6J0WMU2	Odocoileus virginianus texanus	Polyubiquitin-B	UBB
A0A6J0XG22	Odocoileus virginianus texanus	Polyubiquitin-C	UBC
A0A5N3WBS0	Muntiacus muntjak Muntiacus reevesi	Protein S100	S100
A0A6J0X6C4	Odocoileus virginianus texanus	Putative elongation factor 1-alpha-like 3	
A0A6J0XJA7	Odocoileus virginianus texanus	Pyruvate kinase	PKM
A0A6J0Z7G8	Odocoileus virginianus texanus	Ribosomal protein S6 kinase	RPS6KB1
A0A5J5MZ62	Muntiacus muntjak Muntiacus reevesi	SEA domain-containing protein	
A0A5N3XVI2	Muntiacus muntjak Muntiacus reevesi	Secretoglobin family 1A member 1	SCGB1A1
A0A6J0ZFK	Odocoileus virginianus texanus	Secretoglobin family 1D member-like	SCGB1D
A0A6J0ZDI0	Odocoileus virginianus texanus	Serotransferrin	TF
A0A6J0XZZ8	Odocoileus virginianus texanus	Serpin A3-3 isoform X1	SERPINA3-3
A0A6J0XZL1	Odocoileus virginianus texanus	Serpin A3-3 isoform X2	SERPINA3-4
A0A5N3XDE4	Muntiacus reevesi	Serpin domain-containing protein	
A0A212D5P0	Cervus elaphus hippelaphus Cervus nippon	Serum albumin	ALB
A0A5N3VKC1	Muntiacus muntjak	Tr-type G domain-containing protein	
A0A5N3WWM1	Muntiacus muntjak Odocoileus virginianus texanus	Ubiquitin-ribosomal eS31 fusion protein	RPS27A
A0A6J0WU44	Muntiacus muntjak Odocoileus virginianus texanus Cervus hanglu yarkandensis	Ubiquitin-ribosomal protein eL40 fusion protein	UBA52
A0A5N3WF64	Muntiacus muntjak Muntiacus reevesi	Ubiquitin-like domain-containing protein	
A0A5N3WQE0	Muntiacus muntjak	Ubiquitinyl hydrolase 1	TNFAIP3

UNIPROT ID	SPECIES	PROTEIN	GENE
A0A5N3WFI1	Muntiacus muntjak Muntiacus reevesi	Uncharacterised protein	
A0A5N3V3L3	Muntiacus muntjak Muntiacus reevesi Odocoileus virginianus texanus	Vitamin D-binding protein	GC
A0A212CXS3	Cervus elaphus hippelaphus Odocoileus virginianus texanus	Xanthine dehydrogenase/oxidase	XDH

The number of proteins identified in this study is lower compared to similar studies of milk from different species. In donkey milk, 106 unique gene products were identified using capillary RP-HPLC/nESI-MS/MS analysis of in-gel enzymatic digests (Cunsolo, Muccilli, Fasoli, *et al.*, 2011) and 216 proteins were identified using LC-MS/MS (Zhang *et al.*, 2019). In goat milk 331, 314 and 524 proteins were identified in whey, colostrum and mature milk respectively using quantitative proteomics (Sun *et al.*, 2023; Sun *et al.*, 2020). An in depth proteomic study of whey from sheep milk identified 669 proteins (Ha *et al.*, 2015). A recent study investigating the milk whey proteome in Indian Zebu cow identified over 6,000 non-redundant proteins using LC-MS/MS methods (Chopra *et al.*, 2020).

A potential reason for the lower number of proteins identified in this study could be the poor separation of whey and CN proteins. Other proteomic studies from red deer investigating their antlers or meat have identified 259 and 320 number of proteins respectively, suggesting that the low numbers are due to the methodology used rather than the deer database (López-Pedrouso *et al.*, 2019; López-Pedrouso *et al.*, 2021). Even though acidification was used to separate the whey and CN fraction, CN was still one of the most dominant proteins in the whey fraction, with α_{s1}-CN being the 5th most abundant protein in the PEAKS PTM search. This will have resulted in fewer of the low abundant proteins being identified, since MS/MS spectra of low abundance peptides will not have been acquired during the relatively long duty cycle of the Q-TOF mass spectrometer used. Deer has a higher CN to whey ratio than other ruminant species like cow and goat (O. N. L. Vithana *et al.*, 2012) suggesting that a more rigorous fractionation process is required. Studies that have a substantial number of proteins identified typically use multiple methods to extract proteins, including in-gel and in-solution, different extraction methods, multiple fractionation methods and more sensitive MS instruments such as Orbitrap that are known to have higher resolution (Chopra *et al.*, 2020; Ha *et al.*, 2015). To the best of our knowledge this is the first study analysing the proteome of red deer milk using LC-MS/MS. Previous studies have analysed the proteome using techniques such as SDS-

PAGE and HPLC and consequently identified less than 10 proteins within deer milk (Ha *et al.*, 2014; Li *et al.*, 2023). Thus, although this study did not identify a great number of proteins, it is still to date the most comprehensive list of proteins identified in red deer milk.

8.3.2 GO analysis of proteins in deer milk

All 73 proteins were analysed using Gene Ontology (GO) functional annotation using two different online programmes, GO ontology enrichment and DAVID, and classified according to biological process, molecular function, and cellular process. Although the genome for red deer has been sequenced (Bana *et al.*, 2018), it is not considered a model organism and limited information regarding genes and annotation is publicly available. Due to this it was only possible to classify the genes against *Cervus elaphus* using the DAVID database, and only a subset of the proteins had information available. Biological processes related to metabolic processes such as carboxylic acid metabolic process and carbohydrate metabolic processes and immune based processes such as defence response to bacterium were annotated. Molecular functions related to binding (GTP binding) and activity (L-lactate dehydrogenase activity, translational elongation factor activity and GTPase activity). Extracellular region was annotated for the cellular component.

To improve the information available, the closest related species to deer that was available in both packages and contained more complete information, *Bos taurus*, was selected. The top functional annotations from these categories are outlined in Figure 60 and Figure 61 for the GO ontology enrichment and DAVID analysis, respectively.

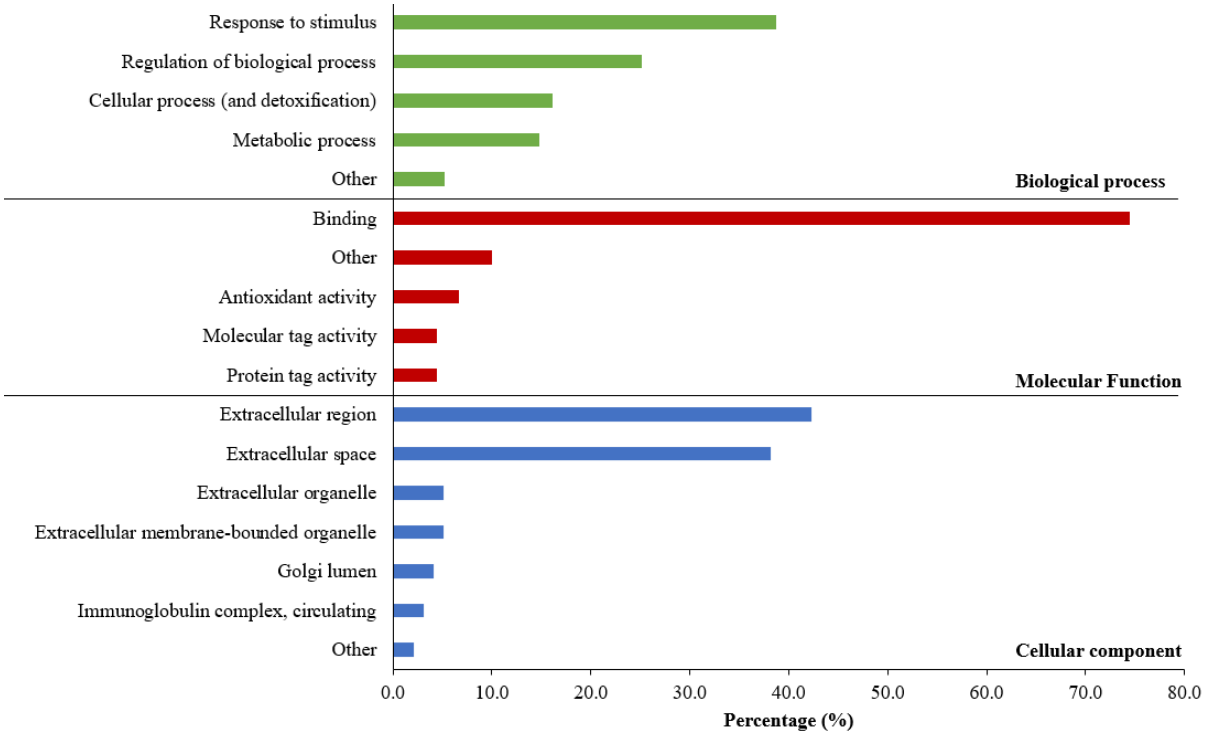

Figure 60. GO annotation of proteins in red deer milk via GO enrichment analysis powered by PANTHER using *Bos taurus* as the species selected.

Similarities were observed in both packages. The top three biological processes in both analyses were response to stimulus, regulation of biological processes and cellular processes (and detoxification). The main molecular functions were binding and various activities. The GO ontology enrichment analysis identified the top three cellular components as being extracellular region, extracellular space, and extracellular organelle, whilst for the DAVID analysis the top three cellular components were nucleus, nucleosome, and extracellular space. GO ontology tools are split into different classes based on their enrichment algorithms (Huang *et al.*, 2009a). The GO ontology enrichment tool belongs to class I (Singular enrichment analysis) whilst the DAVID tool belongs to class III (modular enrichment analysis). The difference in class likely explains the differences observed in the annotations. The annotations obtained from the DAVID database when searching against the organism *Cervus elaphus* and *Bos taurus* for the identified proteins showed an overlap.

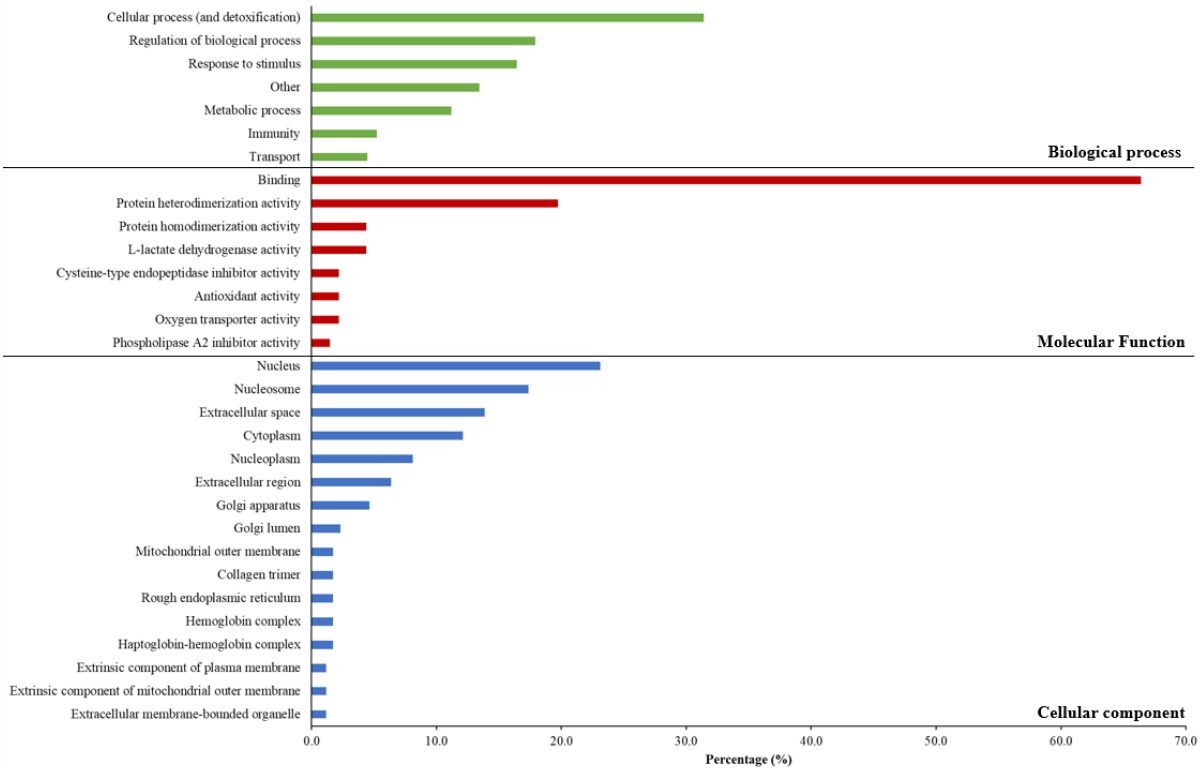

Figure 61. GO annotation of proteins in red deer milk via DAVID analysis using *Bos taurus* as the species selected.

8.3.3 Quantitative changes of whey proteins in red deer milk over lactation

Of the quantified proteins, 51 proteins were consistently present in at least 70 % of the samples regardless of time point (8 time points, 3 replicates). Only the whey fraction was analysed to investigate differences throughout lactation. The method used to resolubilise the proteins in the CN fraction after acidification means that it is not suitable for quantification and the presence of these proteins in deer milk is well known (Ha *et al.*, 2014; Li *et al.*, 2023; Wang *et al.*, 2017). Figure 62 illustrates the quantitative changes in the whey proteins throughout lactation. To determine which proteins significantly differ throughout lactation two different tests were utilised. Simple linear regression calculations were performed for each of the 51 proteins. A total of 27 proteins showed a significant increase or decrease, and they are listed in Appendix Table 2. The differences between the samples at week 3 and week 16 were analysed using log fold change. A total of 21 proteins showed a significant increase or decrease. These proteins are outlined in Appendix Table 3. To improve the accuracy of the study proteins were only deemed significantly different if they met the criteria of both tests. 17 proteins met this criterion and are outlined in Table 30, with 6 proteins being upregulated and 11 proteins being downregulated.

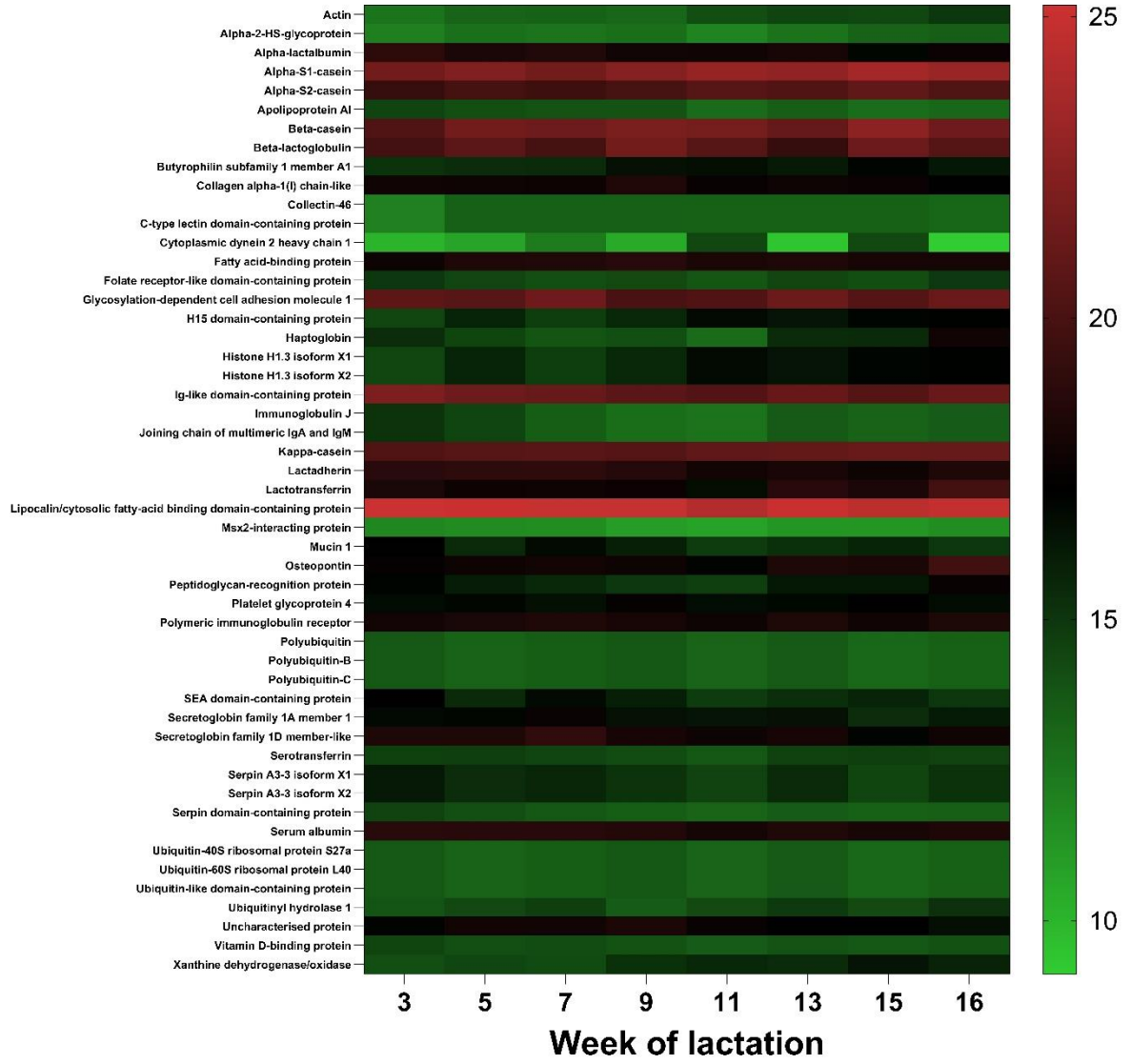

Figure 62. Quantitative changes (log_2) of whey proteins in deer milk from week 3 to week 16 of lactation. The colour of the scale bar indicates the abundance (log_2) of the protein with red being the most abundant and green being the least abundant.

The most prevalent biological function of the 17 proteins that significantly differed over lactation annotated in the UniProt knowledgebase was immune-related functionality (35.3 %). Other differentially expressed proteins were related to transport (17.6%), protease inhibition (17.6 %) and enzyme activity (5.9 %). Analysis of the proteins that changed throughout lactation using GO annotation illustrated that the top biological processes were response to stimulus and regulation of biological processes, the top molecular functions were binding, and enzyme inhibitor activity and the top cellular component was extracellular space and organelle

(Figure 63). The UniProt annotations are descriptors of the proteins functionality whilst the GO annotation further describes the biological, molecular, and cellular functions of the protein.

Table 30. Red deer milk proteins from the whey fraction that significantly differed over lactation.

Protein name	Uniprot ID	Species	Gene	Biological function
Upregulated throughout lactation				
Actin	A0A6J0VYA4	*Odocoileus virginianus texanus* *Muntiacus reevesi*	ACTB	Cell
Alpha-2-HS-glycoprotein	A0A6J0WBI5	*Odocoileus virginianus texanus*	AHSG	Immunity
κ-CN	Q95149	*Cervus elaphus* *Cervus nippon*	CSN3	Transport
Lactotransferrin	A0A212C9V5	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus* *Muntiacus muntjak*	LTF	Immunity
Osteopontin	A0A212CLU0	*Cervus elaphus hippelaphus* *Cervus nippon*	SPP1	Immunity
Ubiquitinyl hydrolase 1	A0A5N3WQE0	*Muntiacus muntjak*	TNFAIP3	Other
Downregulated throughout lactation				
α-LA	A0A212DFY0	*Cervus elaphus hippelaphus* *Cervus elaphus xanthopygus*	LALBA	Milk component
Apolipoprotein AI	A0A212DI82	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	APOA1	Transport
Ig-like domain-containing protein	A0A212CM59	*Cervus elaphus hippelaphus* *Muntiacus reevesi*		Immunity
Immunoglobulin J	A0A212D5R7	*Cervus elaphus hippelaphus*	JCHAIN	Immunity
Joining chain of multimeric IgA and IgM	A0A5N3V8L0	*Muntiacus muntjak* *Muntiacus reevesi*	JCHAIN	Immunity
Secretoglobin family 1A member 1	A0A5N3XVI2	*Muntiacus muntjak* *Muntiacus reevesi*	SCGB1A1	Other
Serpin A3-3 isoform X1	A0A6J0XZZ8	*Odocoileus virginianus texanus*	SERPINA3-3	Protease Inhibitor
Serpin A3-3 isoform X2	A0A6J0XZL1	*Odocoileus virginianus texanus*	SERPINA3-4	Protease Inhibitor
Serpin domain-containing protein	A0A5N3XDE4	*Muntiacus reevesi*		Protease Inhibitor
Serum albumin	A0A212D5P0	*Cervus elaphus hippelaphus* *Cervus nippon*	ALB	Other
Vitamin D-binding protein	A0A5N3V3L3	*Muntiacus muntjak* *Muntiacus reevesi* *Odocoileus virginianus texanus*	GC	Transport

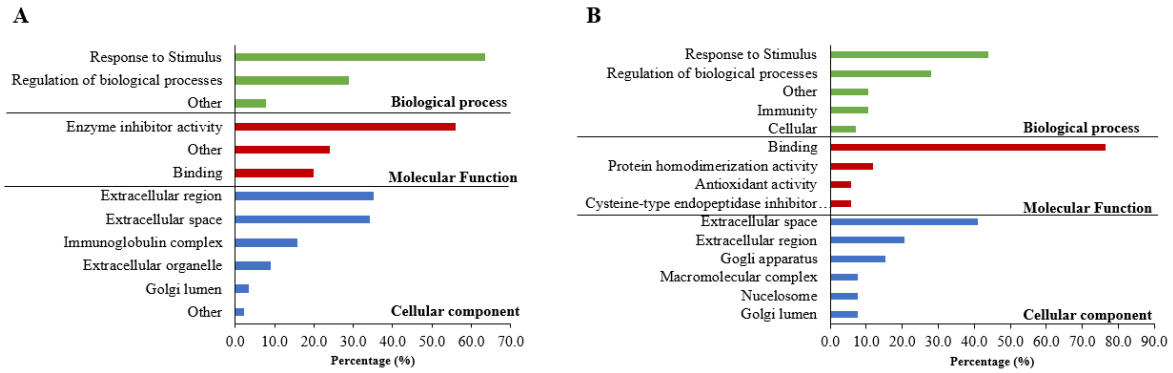

Figure 63. GO annotation of differentially expressed proteins in red deer milk throughout lactation via A) GO enrichment analysis or B) DAVID analysis using *Bos taurus* as the species selected.

Differentially expressed proteins associated with immune function

This study observed an increase in proteins associated with immune system processes, such as osteopontin, lactotransferrin, and alpha-2-HS-glycoprotein as lactation progressed (Figure 64). Osteopontin is a secreted phosphorylated glycoprotein that is involved in diverse biological functions such as immune activation, wound healing, angiogenesis, bone remodelling, cell migration and invasion of mammary epithelial cells (Hubbard *et al.*, 2013; Lund *et al.*, 2009). It has been observed to increase throughout lactation in both cow milk (L. Zhang *et al.*, 2015b) and goat milk (Sun *et al.*, 2023). Lactotransferrin is an iron-binding glycoprotein with antimicrobial activity (Riley *et al.*, 2008). The increase in lactotransferrin in this study, particularly towards the end of lactation, is also observed in cow milk (Riley *et al.*, 2008; L. Zhang *et al.*, 2015b) and goat milk (Sun *et al.*, 2023). It is thought that lactotransferrin has a regulatory role during early involution of the mammary gland, decreasing CN expression and reducing bovine mammary epithelial cell viability (Riley *et al.*, 2008). Alpha-2-HS-glycoprotein, also known as fetuin-A, is a free fatty acid transporter and an acute-phase protein that enhances cellular lipid uptake and lipogenesis (Ning *et al.*, 2023; Strieder-Barboza *et al.*, 2018). Prior studies have shown that although alpha-2-HS-glycoprotein is higher in bovine colostrum than mature milk (Ning *et al.*, 2023), an overall increase is observed throughout lactation (L. Zhang *et al.*, 2015b).

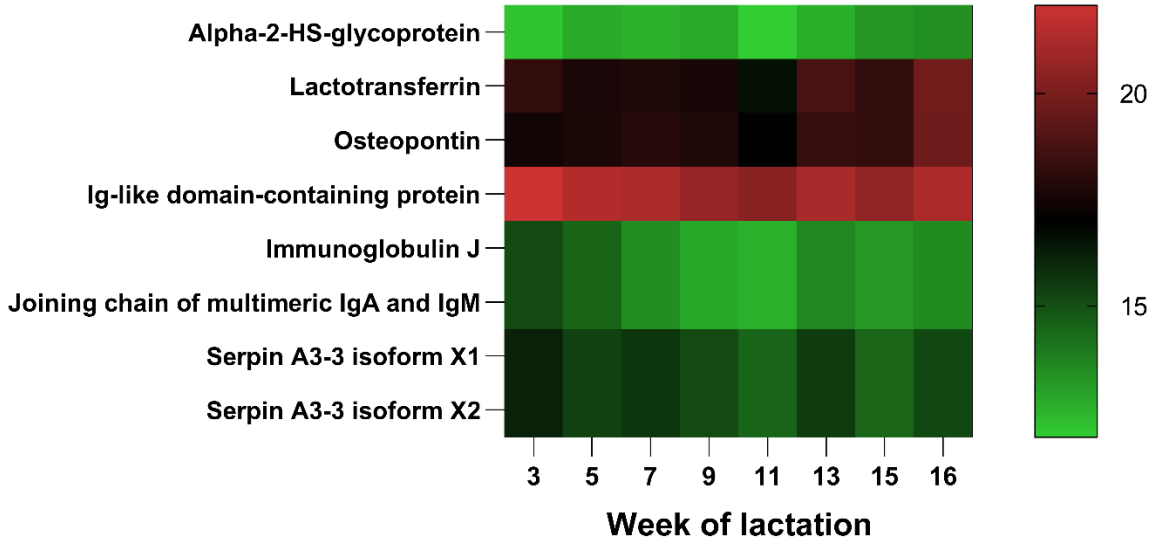

Figure 64. Quantitative changes (log_2) of immunity-related proteins in deer milk from week 3 to week 16 of lactation. The colour of the scale bar indicates the abundance (log_2) of the protein with red being the most abundant and green being the least abundant.

Meanwhile, immunoglobulin-related proteins such as immunoglobulin J (IgJ) decreased throughout lactation (Figure 64). IgJ is a joining protein that links monomers of antibodies IgM and IgA to form polymeric antibodies capable of secretion (Johansen *et al.*, 2000). Although immunoglobulins such as IgM and IgA were not detected in this study, the decrease in IgJ suggests that they are present in deer milk and that they decrease throughout lactation. A decrease in immunoglobulins throughout lactation has been reported in both goat and cow milk (Sun *et al.*, 2023; L. Zhang *et al.*, 2015b). The decrease in immunoglobulins throughout lactation, is thought to be due a decreased ability to transfer immune related proteins from mother to child (L. Zhang *et al.*, 2015a).

This study also observed a decrease in the serine protease inhibitor serpin A3-3 (Figure 64). Previous studies have also observed a decrease in serpins throughout lactation in cow milk which is strongly correlated with the decrease in immunoglobulins (L. Zhang *et al.*, 2015b). Thus it is thought that serine protease inhibitors have a role in protecting the immunoglobulins and promoting the maturation of the newborns immune system (L. Zhang *et al.*, 2015a).

Overall, the differences in immune-related proteins throughout lactation is likely driven by the needs of the fawn (particularly during early to mid-lactation) and protection of the mammary gland during involution (towards the end of lactation) (L. Zhang *et al.*, 2015b).

Differentially abundant proteins associated with transport

This study observed a decrease in transport-related proteins, such as apolipoprotein E and vitamin D-binding protein throughout the lactation period of week 3 to 16 (Figure 65). Apolipoprotein E is responsible for transporting cholesterol. Cholesterol plays an important role in the synthesis of vitamin D and the steroid hormones, which are critical to the development of the newborn (Berg *et al.*, 2002). In cow milk apolipoprotein E decreased from two weeks to the middle of the lactation cycle (L. Zhang *et al.*, 2015b) and in goat milk it is decreased from day 1 to 240 of lactation (Sun *et al.*, 2023). Vitamin D-binding protein is primarily responsible for preventing vitamin D from biodegradation (Bouillon *et al.*, 2020). Sun *et al.* (2023) found a decrease in vitamin D-binding protein between day 1 and 240 of lactation in goat milk.

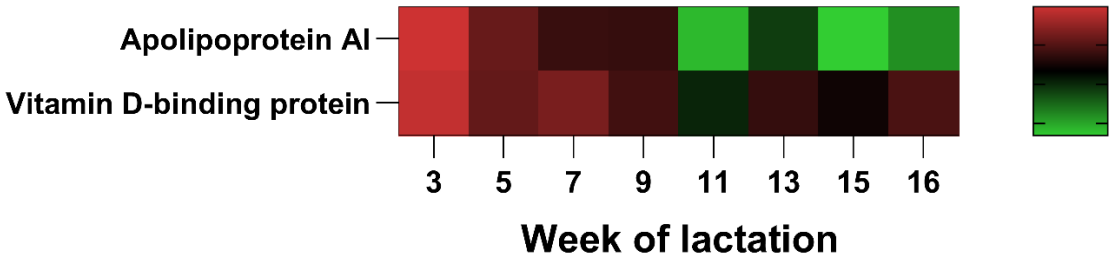

Figure 65. Quantitative changes (log_2) of transport-related proteins in deer milk from week 3 to week 16 of lactation. The colour of the scale bar indicates the abundance (log_2) of the protein with red being the most abundant and green being the least abundant.

Other differentially expressed proteins

A decrease in α-LA was observed from week 3 to week 16 of lactation (Figure 66). α-LA is a regulatory component of the lactose synthase heterodimer and plays a role in the synthesis of lactose (Boland & Singh, 2019). This coincides with the decrease in lactose found in deer milk throughout lactation observed by Li *et al.* (2023). However, other studies have shown that lactose remains constant throughout lactation (Berruga *et al.*, 2021; Landete-Castillejos *et al.*, 2000). In cow milk studies an increase in α-LA has been observed between two weeks and mid

lactation, which coincides with a higher synthesis of lactose over that period (L. Zhang *et al.*, 2015b). The same study shows a decrease in α-LA between 9 months and the last time point of late lactation. Due to this it is believed that the decrease of α-LA may aid or even accelerate mammary gland involution (L. Zhang *et al.*, 2015b).

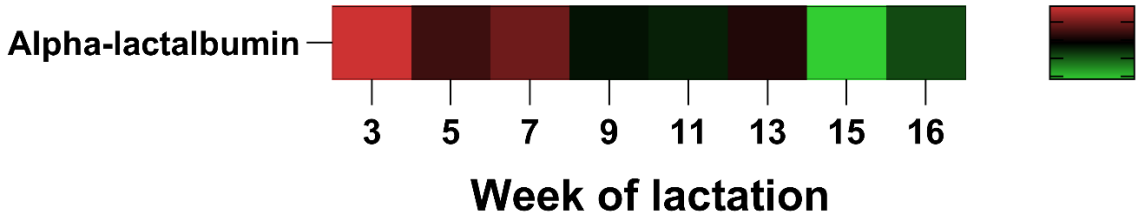

Figure 66. Quantitative changes (log_2) of proteins with enzyme activity in deer milk from week 3 to week 16 of lactation. The colour of the scale bar indicates the abundance (log_2) of the protein with red being the most abundant and green being the least abundant.

8.4 Conclusion

This study used LC-MS/MS to identify proteins in red deer milk and investigate the quantitative changes of these proteins throughout lactation. Although a lower number of proteins are identified compared to similar studies in ruminant species such as cow and goat, it still shows a significant increase in the number of proteins identified in red deer milk in the literature to date. An attempt to categorise the proteins based on GO annotation was hindered by the lack of readily available information on deer proteins as well as the low number of identified proteins.

This study observed quantitative differences in the milk proteome throughout lactation. In particular proteins related to immunity such as osteopontin, lactotransferrin, alpha-2-HS-glycoprotein and immunoglobulins, transport such as apolipoprotein E and vitamin D-binding protein and enzyme activity such as α-LA are key proteins that change throughout lactation and reflect not only the changing needs of the newborn but also the changing needs of the mammary gland. These results suggest that lactation stage has an effect on individual proteins and that these proteins change according to biological function to reflect the development and protection of the mammary gland over lactation.

Chapter 9 – Conclusions and Future Work

Dairy is an important source of dietary protein providing essential amino acids necessary for the maintenance and growth of body proteins. Additionally, dairy proteins possess a range of bioactive properties released during digestion. Various factors such as species, processing treatment and lactation stage have been reported to affect the digestibility of milk proteins due to differences in protein structure and composition. This could have important implications for developing dairy products that deliver superior digestive and nutritional outcomes to targeted consumer groups.

This PhD thesis has successfully investigated the complex interactions between milk species, processing treatments and protein digestion, providing valuable insights into the bioaccessibility of peptides derived from various milk sources. The overarching hypothesis, that milk from different species will exhibit distinct protein digestion patterns and that processing treatments will differentially affect these patterns, has been supported through experiments and analyses, including SEC and LC-MS/MS.

The study's findings demonstrate that milk species significantly influence the profile of small peptides available for absorption during digestion, particularly in the gastric phase. Notably, deer milk consistently yielded a higher proportion of small peptides compared to cow, sheep and goat milk. This highlights the unique protein composition and digestion dynamics of deer milk, suggesting its potential as a source of bioactive peptides with health benefits. Additionally, the observation that processing treatments, such as homogenisation and heat treatment, had varying effects on protein digestibility across species emphasises the need for tailored processing techniques to preserve or enhance the nutritional quality of milk-based products.

Furthermore, the application of SEC showed that the availability of small peptides increases throughout digestion, with critical changes occurring during the early gastric and intestinal phases due to clot restructuring and enzymatic activity. Further peptidomics analysis revealed that peptides generated from the C-terminal end of β-CN were more abundant in cow milk than in deer milk, highlighting differences in peptide release patterns influenced by species-specific protein structures. These insights underscore the importance of understanding digestion

kinetics to optimise peptide profiles for improved bioavailability in functional dairy product development.

However, limitations in methodology used may influence these results. As one limitation of SEC include its inability to separate very small peptides from free amino acids, which may lead to an incomplete profile of the peptide pool. Similarly, the use of LC-MS/MS, while effective for analysing peptide profiles, primarily identified major proteins, as many minor proteins were either not detected or only fragments were identified. This limitation restricts the understanding of the full range of bioactive peptides that may be present. To improve these analyses, combining SEC with complementary techniques, such as ultra-high performance liquid chromatography (UHPLC) could enhance the separation of smaller peptides and free amino acids. Additionally, implementing more sensitive MS methods or employing fractionation techniques prior to LC-MS/MS could increase the detection of minor peptides, providing a more comprehensive view of the peptide landscape in different milk types.

The peptidomics analysis was only performed on cow and deer milk, expanding this analysis to include goat and sheep milk would help to provide a more comprehensive understanding of species-specific peptide profiles and their potential bioactivity. Furthermore, it is important to note that this analysis was conducted solely on the emptied digesta and primarily focused on small peptides below 10 kDa. To gain a fuller picture of the digestion process, future studies should also compare the peptide profiles with the corresponding protein fractions and assess the protein breakdown of the milk clot. This would allow for a more detailed evaluation of how different proteins contribute to peptide formation during digestion and how processing treatments may influence these dynamics across various milk types.

The comparison between the *in vitro* digestion (HGS) and *in vivo* digestion in pigs revealed some insightful findings, particularly regarding the gastric digestion dynamics, which appeared similar across both models. However, notable differences in the patterns of intestinal digestion were observed, emphasising the limitations of current *in vitro* models. These discrepancies indicate that existing *in vitro* methods may not fully capture the complexities of *in vivo* digestion, particularly during the intestinal phase.

In this study, LC-MS/MS was primarily employed to compare the peptide profiles between the two digestion models. This approach primarily identified major proteins such as CNs, as many

minor proteins were either not detected or only fragments of the proteins were identified. This limitation in the detection of minor proteins could further contribute to the differences observed in intestinal digestion patterns and it is unclear whether they behave the same way in the gastric phase of the models.

Moreover, it is important to note that this study was conducted exclusively with cow milk. Consequently, it remains unclear whether similar trends would be observed with other species milk. Future research should aim to refine in vitro digestion models to enhance their predictive capabilities and expand their applicability to a wider range of milk sources. Additionally, exploring the digestive dynamics of different species could provide valuable insights into species-specific digestion mechanisms and contribute to the development of more robust *in vitro* models.

Additionally, the investigation into the quantitative changes of protein composition in deer milk throughout lactation provided new knowledge on how the nutritional and bioactive properties of milk evolve in response to the developmental needs to the offspring. The identification of key proteins related to immunity, transport and enzymatic activity emphasises the significance of lactation stage in determining the functional properties of milk, which could have implications for neonatal nutrition and health. One limitation of this study was the limited number of proteins identified. This constraint may restrict the depth of understanding regarding the complex interactions of milk proteins and their roles in supporting neonatal development. A more comprehensive identification of proteins could enhance the applicability of the findings to dairy production and improve strategies for optimising nutrition. The pooling of milk from 120 does means that individual animal variation throughout lactation was not addressed. Previous studies have shown significant differences in the concentration of proteins in the milk of individual cows at various lactation stages, indicating that individual variability may play an important role in protein composition. Furthermore, this study only included time points from week 3 of lactation onwards, meaning that no colostrum samples were taken. Investigating the differences between colostrum and mature milk could provide valuable insights into the effects that individual proteins have on the development of the newborn and the protection of the mammary gland.

To enhance the experimental design and increase the number of proteins identified in future studies, more comprehensive sampling protocols could be implemented, including the

examination of milk from individual does at multiple points throughout lactation. Additionally, determining whether the differences in proteins throughout lactation influence the digestion of milk proteins would further aid in the production of specialised dairy products tailored to specific nutritional needs. Such investigations could significantly contribute to our understanding of deer milk's unique properties and its potential applications in neonatal nutrition.

In conclusion, this study contributes significantly to the scientific literature on milk digestion and peptide bioaccessibility, providing a foundation for future endeavours. It opens avenues for the development of functional dairy products tailored to specific nutritional needs, including infant formula, sports nutrition and medical nutrition, where optimised peptide profiles could enhance protein absorption and bioavailability. Additionally, understanding the unique protein characteristics of deer milk could inspire innovative processing techniques aimed at preserving its nutritional integrity.

Future research should focus on using advanced techniques, such as LC-MS/MS to characterise specific peptide sequences and their bioactivities in greater detail. Investigating the health benefits associated with different peptide profiles generated through various processing methods will be crucial for the advancement of the dairy industry. Overall, the findings of this thesis not only enhance our understanding of milk protein digestion but also have the potential to inform he development of new dairy products that align with consumer health trends and nutritional requirements.

The aim of this PhD thesis was to investigate how factors such as milk species and processing treatment affect the digestion of proteins using a dynamic *in vitro* gastric digestion model paired with a static *in vitro* intestinal digestion model. It also investigated whether the peptide profile of digesta produced in the *in vitro* digestion model aligned with an *in vivo* digestion model. In addition, due to the comparatively fewer studies on deer milk this study investigated the protein composition of deer milk throughout different lactation stages.

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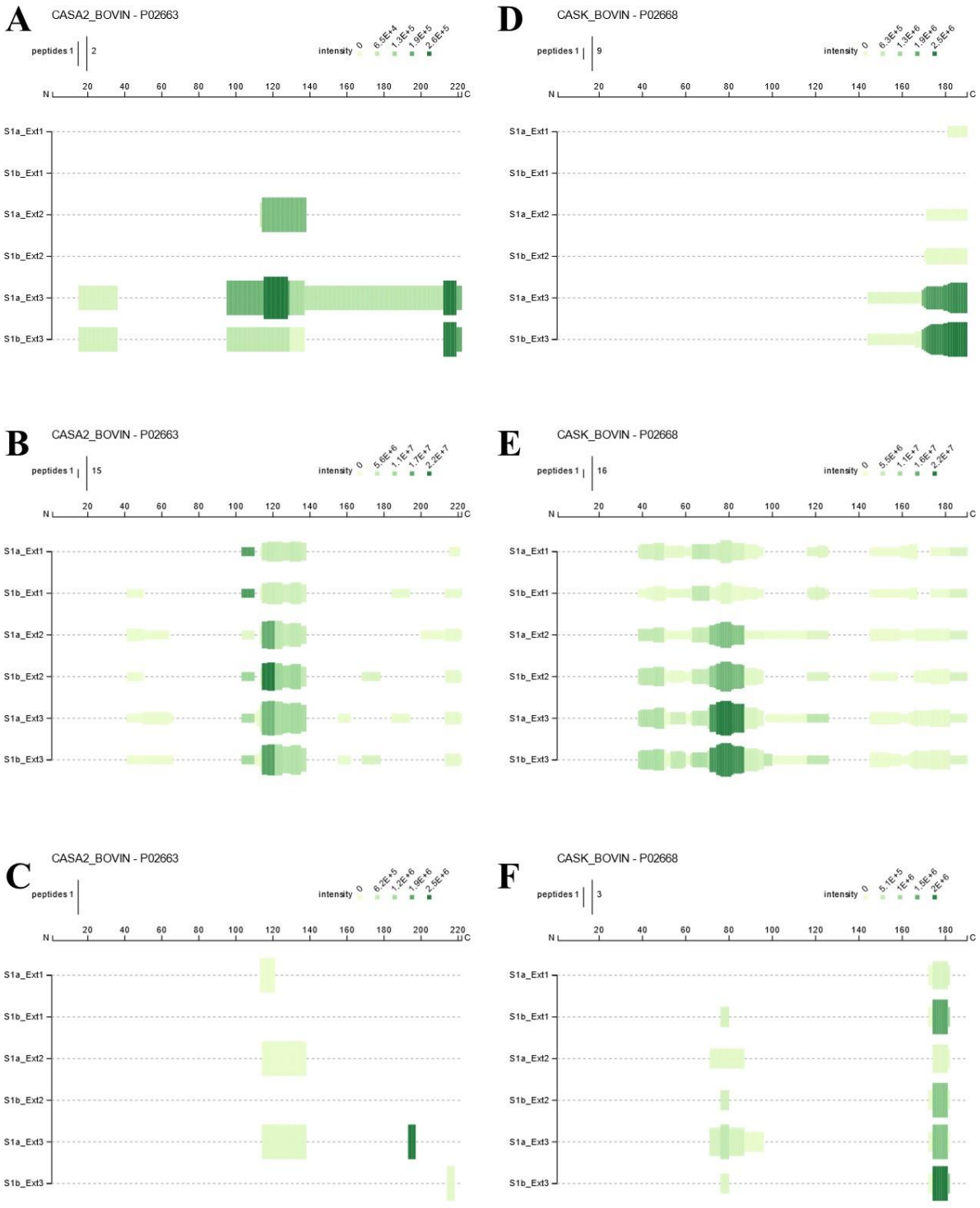
Appendices

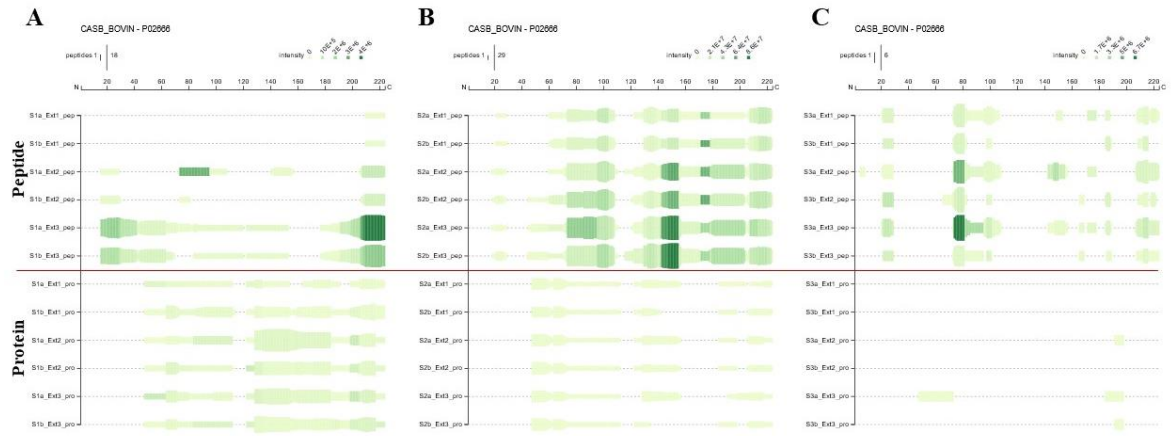


Appendix Figure 1. Peptigram images showing coverage and reproducibility of the different extraction methods for α_{s2}-CN in a) no digestion, b) gastric and c) intestinal samples and κ-CN in d) no digestion, e) gastric and f) intestinal samples. The different extraction methods are Ext1 (3 kDa filtration), Ext2 (10 kDa filtration) and Ext3 (CME).

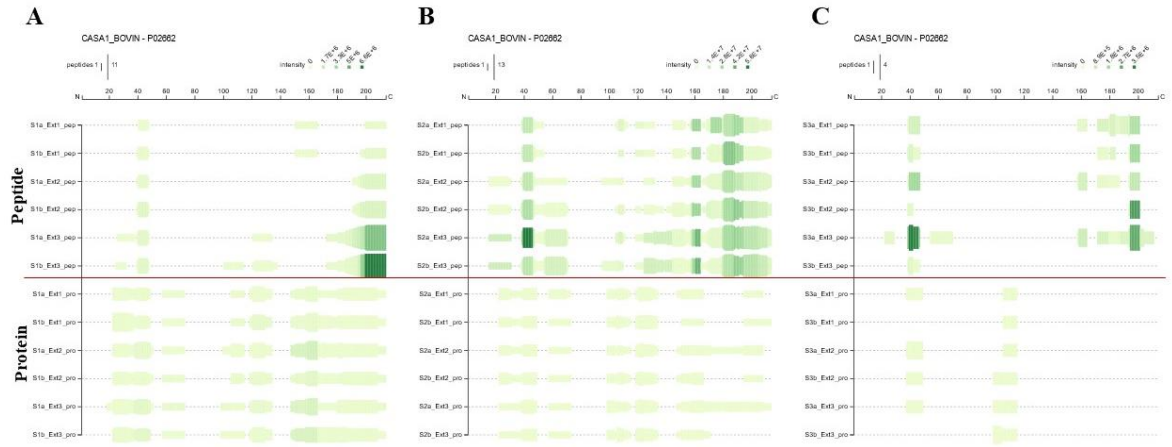
Appendix Table 1. Proteins identified during the optimisation trial in the peptide and protein fraction.

No.	Accession No.	Protein Description	Fraction
1	P02662	Alpha-S1-Casein	Both
2	P02663	Alpha-S2-Casein	Both
3	P02666	Beta-Casein	Both
4	P02668	Kappa-Casein	Both
5	P80457	Xanthine dehydrogenase/oxidase	Both
6	P80195	Glycosylation-dependent cell adhesion molecule 1	Both
7	F1MR22	Polymeric immunoglobulin receptor	Both
8	P18892	Butyrophilin subfamily 1 member A1	Both
9	G3N0V0	Ig-like domain-containing protein	Both
10	Q3T101	Ig-like	Both
11	A0A140T897	Albumin	Both
12	P00711	Alpha-lactalbumin	Both
13	B5B0D4	Major allergen beta-lactoglobulin	Both
14	F1MJQ3	Alpha-amylase	Peptide
15	F1MZU6	Collagen type IV alpha 3 chain	Peptide
16	F1MYZ3	Histone H3-lysine (4) N-methyltransferase	Peptide
17	A0A3Q1MI55	Signal-induced proliferation-associated 1	Peptide
18	F1N2K3	Ubiquitin specific peptidase 53	Peptide
19	A0A3Q1MG98	Solute carrier family 34 member 2	Peptide
20	F1MWH8	Tyrosine-protein kinase	Peptide
21	E1BJV5	Zinc finger protein 638	Peptide
22	F1MM57	Myosin heavy chain 14	Peptide
23	E1BCZ2	RB binding protein 6, ubiquitin ligase	Peptide
24	P02453	Collagen alpha-1(I) chain	Peptide
25	P02465	Collagen alpha-2(I) chain	Peptide
26	A0A3Q1MR22	Desmoplakin	Peptide
27	Q29461	Chymotrypsin-like elastase family member 2A	Peptide
28	E1BP50	Tankyrase 1 binding protein 1	Peptide
29	A0A3Q1MSK9	Espin 2	Peptide
30	F1MYR5	Nitric oxide synthase	Peptide
31	P02754	Beta-lactoglobulin	Protein
32	P00593	Phospholipase A2	Protein
33	Q3T160	Nucleophosmin	Protein
34	A6QNZ7	Keratin 10	Protein
35	P06394	Keratin, type I cytoskeletal 10	Protein
36	G3N0V2	Keratin, type II cytoskeletal 1	Protein
37	B3VTM3	Lactotransferrin	Protein
38	Q3T0K7	MFGE8 protein	Protein
39	A0A3Q1LYX2	Lipocalin/cytosolic fatty-acid binding domain-containing protein	Protein

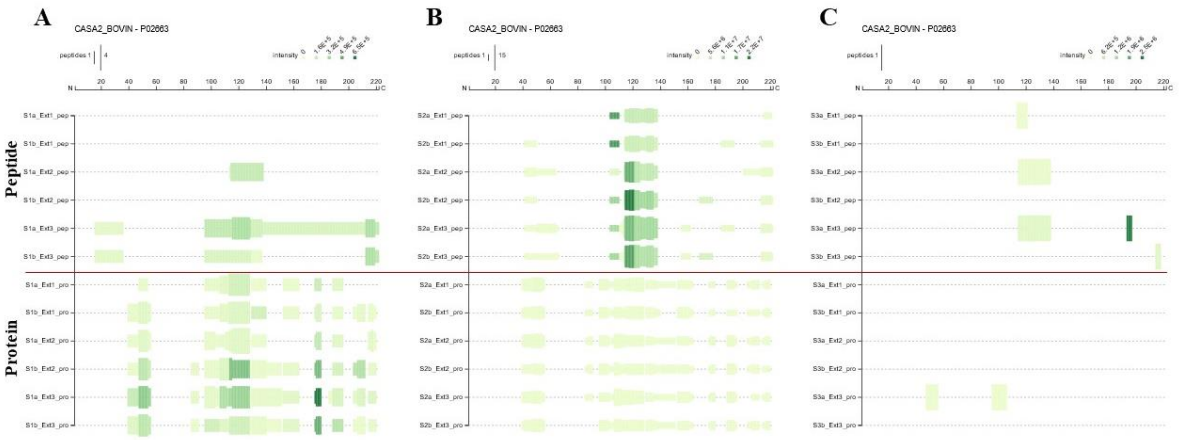
40	P00794	Chymosin	Protein
41	P10790	Fatty acid-binding protein	Protein
42	Q2UVX4	Complement C3	Protein
43	Q3ZCL0	Cysteine-rich secretory protein 2	Protein
44	P11151	Lipoprotein lipase	Protein
45	Q148I8	Keratin 31	Protein



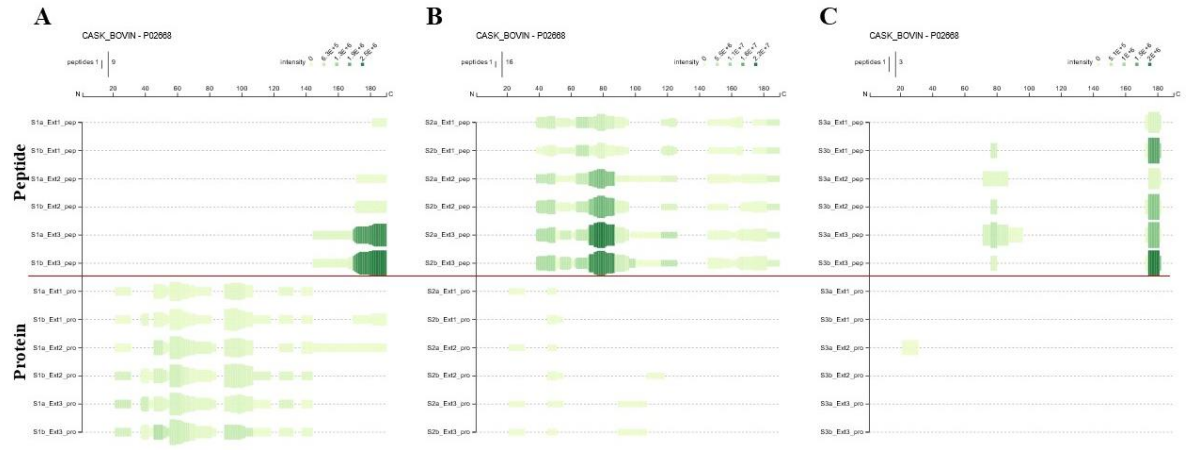
Appendix Figure 2. Peptigram images showing coverage and reproducibility of the different extraction methods for β-CN in A) no digestion, B) gastric and C) intestinal samples. Both the peptide and protein fraction of the milk are included. The different extraction methods are Ext1 (3 kDa filtration), Ext2 (10 kDa filtration) and Ext3 (CME).



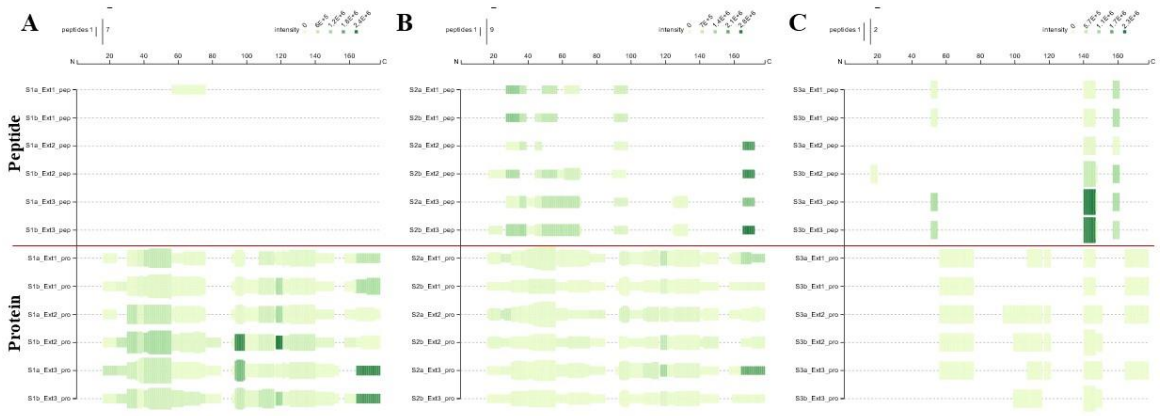
Appendix Figure 3. Peptigram images showing coverage and reproducibility of the different extraction methods for α_{s1}-CN in A) no digestion, B) gastric and C) intestinal samples. Both the peptide and protein fraction of the milk are included. The different extraction methods are Ext1 (3 kDa filtration), Ext2 (10 kDa filtration) and Ext3 (CME).



Appendix Figure 4. Peptigram images showing coverage and reproducibility of the different extraction methods for α_{s2}-CN in A) no digestion, B) gastric and C) intestinal samples. Both the peptide and protein fraction of the milk are included. The different extraction methods are Ext1 (3 kDa filtration), Ext2 (10 kDa filtration) and Ext3 (CME).



Appendix Figure 5. Peptigram images showing coverage and reproducibility of the different extraction methods for κ-CN in A) no digestion, B) gastric and C) intestinal samples. Both the peptide and protein fraction of the milk are included. The different extraction methods are Ext1 (3 kDa filtration), Ext2 (10 kDa filtration) and Ext3 (CME).



Appendix Figure 6. Peptigram images showing coverage and reproducibility of the different extraction methods for β-LG in A) no digestion, B) gastric and C) intestinal samples. Both the peptide and protein fraction of the milk are included. The different extraction methods are Ext1 (3 kDa filtration), Ext2 (10 kDa filtration) and Ext3 (CME).

Appendix Table 2. Significantly different red deer milk proteins from the whey fraction over lactation based on linear regression values.

Protein name	Uniprot ID	Species	Gene	Slope	P-value
Upregulated					
Actin	A0A6J0VYA4	*Odocoileus virginianus texanus* *Muntiacus reevesi*	ACTB	3.7	5.79E-06
Alpha-2-HS-glycoprotein	A0A6J0WBI5	*Odocoileus virginianus texanus*	AHSG	4.7	6.90E-03
Alpha-S1-casein	A0A6J0VVP2	*Cervus elaphus hippelaphus* *Muntiacus muntjak* *Odocoileus virginianus texanus*	CSN1S1	4.3	4.26E-05
Alpha-S2-casein	A0A212D6E7	*Odocoileus virginianus texanus* *Cervus elaphus hippelaphus*	CSN1S2	4.8	6.08E-04
Butyrophilin subfamily 1 member A1	A0A212D5D9	*Cervus elaphus hippelaphus*	BTN1A1	4.8	9.82E-05
H15 domain-containing protein	A0A5N3W0N5	*Muntiacus reevesi* *Muntiacus muntjak*	H1-5	2.6	2.45E-04
Haptoglobin	B6D985	*Cervus elaphus*	HP	1.3	3.57E-02
Histone H1.3 isoform X1	A0A6J0WWZ2	*Odocoileus virginianus texanus*	H1-3	2.6	2.45E-04
Histone H1.3 isoform X2	A0A6J0WX52	*Odocoileus virginianus texanus*	H1-4	2.6	2.45E-04
Kappa-casein	Q95149	*Cervus elaphus* *Cervus nippon*	CSN3	6.4	4.60E-04
Lactoferrin	A0A212C9V5	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus* *Muntiacus muntjak*	LTF	2.2	2.65E-02
Osteopontin	A0A212CLU0	*Cervus elaphus hippelaphus* *Cervus nippon*	SPP1	3.6	7.14E-04
Ubiquitinyl hydrolase 1	A0A5N3WQE0	*Muntiacus muntjak*	TNFAIP3	3.2	2.55E-02
Xanthine dehydrogenase/oxidase	A0A212CXS3	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	XDH	4.7	2.99E-07
Downregulated					
Alpha-lactalbumin	A0A212DFY0	*Cervus elaphus hippelaphus* *Cervus elaphus xanthopygus*	LALBA	-5.0	1.97E-04

Apolipoprotein AI	A0A212DI82	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	APOA1	-6.4	3.46E-07
Ig-like domain-containing protein	A0A212CM59	*Cervus elaphus hippelaphus* *Muntiacus reevesi*		-4.8	4.83E-03
Immunoglobulin J	A0A212D5R7	*Cervus elaphus hippelaphus*	JCHAIN	-3.1	3.12E-03
Joining chain of multimeric IgA and IgM	A0A5N3V8L0	*Muntiacus muntjak* *Muntiacus reevesi*	JCHAIN	-3.1	3.12E-03
Lactadherin	A0A212CT53	*Cervus elaphus hippelaphus*	MFGE8	-6.5	1.09E-04
Secretoglobin family 1A member 1	A0A5N3XVI2	*Muntiacus muntjak* *Muntiacus reevesi*	SCGB1A1	-2.9	1.12E-02
Secretoglobin family 1D member-like	A0A6J0ZFK	*Odocoileus virginianus texanus*	SCGB1D	-2.9	1.84E-02
Serpin A3-3 isoform X1	A0A6J0XZZ8	*Odocoileus virginianus texanus*	SERPINA3-3	-4.3	4.80E-03
Serpin A3-3 isoform X2	A0A6J0XZL1	*Odocoileus virginianus texanus*	SERPINA3-4	-4.3	4.80E-03
Serpin domain-containing protein	A0A5N3XDE4	*Muntiacus reevesi*		-8.2	5.88E-06
Serum albumin	A0A212D5P0	*Cervus elaphus hippelaphus* *Cervus nippon*	ALB	-9.1	2.39E-03
Vitamin D-binding protein	A0A5N3V3L3	*Muntiacus muntjak* *Muntiacus reevesi* *Odocoileus virginianus texanus*	GC	-9.2	2.16E-03

Appendix Table 3. Significantly different red deer milk proteins from the whey fraction over lactation based on Log fold change values between week 3 and 16.

Protein name	Uniprot ID	Species	Gene	LogFC	P-value
Upregulated					
Actin	A0A6J0VYA4	*Odocoileus virginianus texanus* *Muntiacus reevesi*	ACTB	2.3	6.30E-03
Alpha-2-HS-glycoprotein	A0A6J0WBI5	*Odocoileus virginianus texanus*	AHSG	1.4	3.13E-02
Fatty acid-binding protein	A0A6J0WPP2	*Odocoileus virginianus texanus*	FABP3	0.3	9.45E-03
Glycosylation-dependent cell adhesion molecule 1	A0A212DFU4	*Cervus elaphus hippelaphus*	GLYCAM1	0.6	1.37E-02
Kappa-casein	Q95149	*Cervus elaphus* *Cervus nippon*	CSN3	0.9	3.77E-02
Lactoferrin	A0A212C9V5	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus* *Muntiacus muntjak*	LTF	1.6	8.05E-04
Osteopontin	A0A212CLU0	*Cervus elaphus hippelaphus* *Cervus nippon*	SPP1	2.3	1.21E-03
Peptidoglycan-recognition protein	A0A212DE23	*Cervus elaphus hippelaphus*	PGRP	0.5	1.38E-02
Polymeric immunoglobulin receptor	A0A212CRH2	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	PIGR	0.4	3.01E-02
Ubiquitinyl hydrolase 1	A0A5N3WQE0	*Muntiacus muntjak*	TNFAIP3	1.3	2.12E-02
Downregulated					
Alpha-lactalbumin	A0A212DFY0	*Cervus elaphus hippelaphus* *Cervus elaphus xanthopygus*	LALBA	-1.4	2.08E-02
Apolipoprotein AI	A0A212DI82	*Cervus elaphus hippelaphus* *Odocoileus virginianus texanus*	APOA1	-1.4	5.03E-03
Ig-like domain-containing protein	A0A212CM59	*Cervus elaphus hippelaphus* *Muntiacus reevesi*		-0.8	9.70E-03
Immunoglobulin J	A0A212D5R7	*Cervus elaphus hippelaphus*	JCHAIN	-1.6	5.72E-03
Joining chain of multimeric IgA and IgM	A0A5N3V8L0	*Muntiacus muntjak* *Muntiacus reevesi*	JCHAIN	-1.6	5.72E-03

Secretoglobin family 1A member 1	A0A5N3XVI2	*Muntiacus muntjak* *Muntiacus reevesi*	SCGB1A1	-0.7	1.65E-02
Serpin A3-3 isoform X1	A0A6J0XZZ8	*Odocoileus virginianus texanus*	SERPINA3-3	-1.0	3.02E-02
Serpin A3-3 isoform X2	A0A6J0XZL1	*Odocoileus virginianus texanus*	SERPINA3-4	-1.0	3.02E-02
Serpin domain-containing protein	A0A5N3XDE4	*Muntiacus reevesi*		-1.1	1.32E-03
Serum albumin	A0A212D5P0	*Cervus elaphus hippelaphus* *Cervus nippon*	ALB	-0.3	4.03E-02
Vitamin D-binding protein	A0A5N3V3L3	*Muntiacus muntjak* *Muntiacus reevesi* *Odocoileus virginianus texanus*	GC	-0.5	1.96E-02