Department of Food and Nutritional Sciences

Effect of Processing on the Composition, Structure and Digestibility of Cow's Milk Protein

Thesis submitted for the requirement for the degree of Doctor of Philosophy in Food & Nutritional Science

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Milk provides essential nutrients and serves as an energy source in our diet. The quality of protein in milk is closely linked to its nutritive value, which reflects its ability to support key metabolic functions essential for optimal health. Important aspects of dietary protein quality include amino acid composition, protein digestibility, and amino acid bioavailability.

Before consumption, commercial milk undergoes various processing methods for safety and improving shelf-life, which can significantly alter its nutritional protein value. Processing techniques may impact the nutritional quality of milk proteins, either positively or negatively. Given the necessity of processing, it is essential to manage these methods carefully to preserve the protein functionality and nutritional value of milk, especially as it is a core component of many healthy and functional foods and beverages. Microfiltration of fresh milk is a relatively new commercial process designed to extend its shelf life in combination with pasteurisation.

The aim of this study is to expand our understanding of the effects of microfiltration on milk proteins. This work could facilitate research and development of food products containing milk protein as a crucial nutritional component, as well as improve processing methods to optimize milk protein attributes. This thesis specifically addresses changes in protein structure, digestibility, and peptide profiles in commercially available filtered milk in the UK market, in comparison to pasteurized milk.

By 2024, the production of filtered semi-skimmed milk had increased by about 25 %, representing 83 % of the brands available in the UK market, according to the data collected in this study. Meanwhile, filtered whole milk saw a 58 % increase, now comprising approximately 66 % of brands that sell fresh milk, as observed in this research.

The combination of particle size measurements, thiol content analysis, and Confocal Laser Scanning Microscopy (CLSM) provided a comprehensive understanding of how microfiltration influences milk's structural properties in comparison to pasteurisation. A significant increase in Z-average particle size (average ~+12 %) and reduction in thiol content (average ~-24 %) indicate that filtration promotes the formation of larger aggregates, potentially through thiol-disulfide exchange interactions. CLSM imaging further revealed enhanced protein-fat interactions in filtered

milk, suggesting a strengthened association between milk proteins and fat globule membrane proteins.

Protein digestibility and peptides released after *in vitro* digestion were analysed by examining the static *in vitro* gastrointestinal digested milk samples using high-resolution quadrupole time of flight instruments (TOF LC/MS). Although no significant differences were observed in the *in vitro* gastrointestinal digestion between filtered and pasteurised milk, notable changes in peptide distribution were identified. These variations suggest that the filtration process may influence the types or quantities of peptides released. First, β-casomorphin 7 (BCM7), is an opioid peptide released during the digestion of β-casein, which has been associated with various health concerns, was measured in filtered and pasteurised samples following the *in vitro* digestion. Interestingly, the presence of fat appears to limit BCM7 release and has a greater impact on BCM7 release than microfiltration alone, suggesting that fat content may play a more prominent role in moderating bioactive peptide release. Microfiltration appears to influence BCM7 release more significantly when fat is present. In semi-skimmed filtered milk, quantification of BCM7 revealed no significant difference in levels compared to pasteurised milk, though correlations between fat content, processing methods, and protein digestion percentages highlighted distinct impacts of microfiltration. Comparing the percentage increase in BCM7 release between filtered and pasteurized milk of the same fat content (indicating the effect of processing) and between semi-skimmed and whole milk under the same process (indicating the effect of fat content) provides clearer insight into how these factors individually affect BCM7 production.

This finding warrants further investigation to understand the mechanisms behind these differences in protein structure and peptide distribution and their potential implications for milk's bioactivity and nutritional properties.

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Abbreviations

Casein proteins: **alpha S1 casein:** αS1-casein, **alpha S2 casein**: αS2-casein, **beta casein:** β-casein or βcas and **kappa casein: ϰ-**casein or **ϰcas**.

Whey proteins: **αLac:** αLactalbumin, **βLg:** β-lactoglobulin and **BSA:** bovine serum albumin.

BCM7: β-casomorphin 7.

MFGM: milk fat globule membrane

IgE: Immunoglobulin E

CLSM: Confocal Laser Scanning Microscopy

SGF: simulated gastric fluid

SIF: simulated intestinal fluid

OH: Old Hill Farm

Single Letter Amino Acid Codes

A = Alanine **C** = Cysteine **D** = Aspartic acid **E** = Glutamic acid **F** = Phenylalanine

G = Glycine **H** = Histidine **I** = Isoleucine **K** = Lysine **L** = Leucine

M = Methionine **N** = Asparagine **P** = Proline **Q** = Glutamine **R** = Arginine

S = Serine **T** = Threonine **V** = Valine **W** = Tryptophan **Y** = Tyrosine

Chapter 1. Introduction

Milk is an important part of the human diet, primarily due to its high nutritional content, particularly proteins and fats. While raw milk consumption carries a risk of bacterial infection, processing is essential to improve the microbiological safety of milk without significantly altering its nutritional value or sensory properties (Fox et al., 2015). On the other hand, food processing can lead to structural and chemical changes and the formation of bioactive peptides (Fox et al., 2015; Loveday, 2023), which can significantly influence milk protein quality, gastrointestinal digestion, and potential allergenicity (Roy et al., 2020; Van Lieshout et al., 2020). Several studies already described the effect of thermal processes on milk proteins (Aguilera, 2019; Borad et al., 2017; Bu et al., 2013). However, more recently filtered milk, which undergoes pasteurisation and microfiltration, offering a longer shelf life compared to pasteurised milk has become available in UK supermarkets. Microfiltration is a process in which milk is passed through a membrane with a specific pore size to remove spores, bacteria, and somatic cells from skim milk. This treatment consequently extends the shelf life of dairy products by reducing the microbial load. In 2020, the sales of filtered milk witnessed a significant boost, attributed to its extended shelf life and reduced milk wastage caused by spoilage or expiration, when compared to pasteurised milk (Mintel, 2021). The shear force applied during microfiltration causes membrane fouling, as well as intermolecular interactions and structural rearrangements. Most research on milk microfiltration to date has primarily focused on its effects on extending shelf life and separating protein fractions. However, there is still limited information available on the impact of microfiltration on milk protein structure, digestibility, and allergenicity.

1.1. Research hypothesis and objectives

This research aims to develop a more detailed understanding of commercially processed fresh cow's milk, examining aspects such as protein composition, structure, and digestion. Numerous studies have extensively investigated the properties of heat-treated (particularly pasteurised) cow's milk however limited studies are available on filtered milk. Our research hypothesis is that the shear forces and membrane fouling occurring during milk microfiltration may induce structural changes in filtered milk that differ from those in regular pasteurized milk. Thus, the objectives of this research are:

- Analyse market trends of filtered milk in the UK (2022 - 2024): investigate the growth of filtered milk in the UK market. Assess changes in supermarket offerings for filtered versus pasteurised milk (Chapter 3).

- Evaluate the impact of microfiltration on milk protein structure: examine the structural properties of filtered milk proteins. Compare these structural effects to those of pasteurised milk Chapter 4).

- Assess BCM7 release in filtered milk: compare the levels of BCM7 released in filtered and pasteurised milk, evaluating how factors like process, β-casein composition, fat content, and digestion conditions impact its release. Develop a comprehensive understanding of how milk composition and digestion parameters interact to influence BCM7 release (Chapter 5Chapter 6).

- Analyse peptide profiles after *in vitro* digestion in filtered and pasteurised milk: characterise and compare the peptide profiles resulting from *in vitro* digestion of semi-skimmed filtered and pasteurised milk Chapter 7).

1.2. Significance of the research

There is a growing need for scientific research on how milk processing affects protein structure and digestibility, as these factors may influence the bioactivity of certain peptides, including those with potentially negative health effects. Addressing these gaps is essential for guiding the development of dairy products with optimised health benefits. In particular, there is limited knowledge about the impact of microfiltration on milk protein structure, digestion, and bioactivity, highlighting a clear area for further investigation.

This study attempts to shed light on the potential impacts of microfiltration on milk protein structure and peptide release after digestion. It also provides foundational information on how fat content influences the release of BCM7. These findings are valuable for milk manufacturers and dairy researchers, as they may help refine microfiltration conditions to improve product quality and enhance bioavailability.

1.3. Thesis outline

The current research thesis has been written in the format of a series of papers and it consists of 7 main chapters. This thesis focuses solely on cow's milk, as such throughout the thesis, the term milk is referring only to cow's milk. Following this introduction, the **second chapter** contains a narrative literature view about cow's milk protein composition, digestion, release of BCM7 and the effect of processing on milk protein structure. The **third chapter** of the thesis assessed changes in supermarket offerings for filtered versus pasteurised milk between 2022 and 2024.

C**hapter four** focuses on the structural changes in filtered milk protein compared to pasteurised milk. This chapter has been presented (oral presentation) at the Nutrition Society London: Winter Conference 2022/23 – Architecture of food: processing, structure and health, 24 - 25 January 2023.

Shuayb, R., Clegg, M. and Oruna-Concha, M. (2023) 'Effect of microfiltration on milk protein microstructure', Proceedings of the Nutrition Society, 82 (OCE1), p. E3. doi:10.1017/S0029665123000113.

In **chapter five**, the content of β-casomorphin 7 (BCM7) peptide after *in vitro* digestion of commercially available filtered milk was studied compared to pasteurised milk. This chapter has been presented at the 4th International Electronic Conference on Foods, 15–30 October 2023; Available online: https://foods2023.sciforum.net/ and has been published in the Biology and Life Sciences Forum journal:

Buatig R, Clegg M, Michael N, Oruna-Concha M-J. Quantification of β-Casomorphin 7 in Commercially Available Filtered and Pasteurized Cow's Milk. Biology and Life Sciences Forum. 2023; 26(1):125. https://doi.org/10.3390/Foods2023-15157.

Chapter Six examined factors that may impact the release of BCM7, including β-casein composition, fat content, and protein digestion. **Chapter Seven** explores the peptide profiles of *in vitro*-digested filtered milk compared to pasteurized milk, highlighting differences in bioactive peptide release due to processing methods. Finally, **Chapter eight** presents an overall summary and conclusions of the research and directions for future work.

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Chapter 2. Literature Review

2.1. Milk Composition.

Cow's milk is a rich source of nutrients in the human diet such as proteins, fat, carbohydrates, vitamins and minerals (**Table 2.1**) and it is the most consumed milk representing about 81 % of total milk production (FAO, 2017). The concentration of these nutrients varies according to breed, milk type, processing, sessions etc. Analysing these compounds is important to understand the nutritional value, quality and impact of milk and its products on human health, in addition to understanding the effect of processing, storage conditions and feed on milk composition (Foroutan et al., 2019). Milk protein is a source of high-quality protein and indispensable amino acids the body requires for tissue growth and maintenance. They play a crucial role in milk structure.

Table 2.1: Approximate composition of cow's milk*.

Component	Range content in milk (% w/w)
Water	85.3 – 88.7
Solid non-fat	7.9 – 10.0
Lactose	3.8 -5.3
Fat	2.5 – 5.5
Protein	2.3 – 4.4
Casein	1.7 – 3.5
Minerals	0.57 – 0.83

* (Fox, 2003).

2.1.1 Protein.

The primary structure of a protein is its amino acid sequence which is linked by peptide bonds; those polypeptide chains fold into the secondary structure by hydrogen bonds. The secondary structure is then folded into a three-dimensional arrangement stabilised by covalent and non-covalent interactions, some proteins form quaternary. The protein content in cow's milk is about 30 - 35 g / L. The major protein fractions of milk protein are casein and whey. Casein micelles represent about 80 % of total milk protein and whey proteins represent~20 % of total protein (**Table 2.2**) (Bonizzi et al., 2009; Farrell et al., 2004; Fox, 2003).

Table 2.2: Major proteins of bovine milk and some of their properties*.

Protein	Concentration (g / L milk)	Genetic variants	Molecular weight (kDa)	Amino acid (n)	Thiol (s-s)
αS1	12–15	B	23,615	199	-
		C	23,542		
αS2	3–4	A	25,226	207	0 (1)
β-casein	9–11	A1	24,023	209	-
		A2	23,983		
		B	24,092		
κ-casein	2–4	A	19,037	169	0 (1)
		B	19,006		
βLg	2–4	A	18,363	162	1 (2)
		B	18,277		
αLac	0.6–1.7	B	14,178	123	0 (4)
BSA	0.4	A	66,399		1 (17)

* (Bonizzi et al., 2009; Farrell et al., 2004; Fox, 2003).

Casein Proteins

Casein is the main protein in milk (1.7 - 3.5 % of milk w/w), which originates from the family of phosphoproteins. The main fractions of casein are: alpha S1-casein (αS1), alpha S2-casein (αS2), beta-casein (β-casein) and kappa casein (κ-casein), and these fractions have several variants that differ in the amino acid sequence, structure, and phosphorylation and glycosylation (Table **2.2**). Casein particles are concentrated in the colloidal fraction of milk, in the form of hydrated and mineralised spherical, so-called casein micelles (Fox, 2003; Mercier & Vilotte, 1993). These micelles contain about 92 % protein and 8 % inorganic components. The caseins sizes range from 50 to about 600 nm in diameter (on average 150 nm), and these particles are present in the form of essentially spherical particles with excellent surfactant properties in emulsions and foams, gelling properties, and thermal resistance to denaturation (Anema et al., 2005; Fox, 2003). The casein micelles contain αS1-, αS2-, β-, and κ-casein in proportions of about 40, 10, 35, and 15 %, respectively (Fox, 2003). Casein molecules are phosphorylated due to their capacity to bind calcium phosphate, and 50 % of κ-casein is glycosylated, which makes the C-terminal part of the casein hydrophilic. The presence of prolyl residues confers open and flexible conformations, and

this flexibility confers the casein's molecules excellent surface-active and stabilising properties (De Kruif et al., 2012). Micellar organisation consist of three proposed models (1) a sub-micellar model where the micelles correspond to submicelle assemblages linked together by nanoclusters of calcium phosphate (2) a model with an open structure (3) an open model like sponge (Rehan et al., 2019). Although there are different micellar suggested models, in all of these models the glycosylated forms of ϰ-casein are located at the surface of casein micelles, conferring them a negative charge and stability (Hristov et al., 2016).

The four casein fractions lack stable secondary structures, however, in contrast, the whey proteins are highly structured. For this reason, the caseins are very flexible molecules unable to form stable structures. This is due to their high content of the structure-breaking amino acid proline. β-casein is rich in proline, consisting of 35 of the 209 residues. The open, flexible structure of the caseins provides them with the ability to proteolysis, which facilitates their natural function as a source of amino acids (De Kruif et al., 2012; Rehan et al., 2019).

The caseins aggregate at pH 4.6 at ~4°C, while the whey is a secondary product obtained as a result of the coagulation of the casein. Coagulation of milk is achieved using acids such as hydrochloric or lactic acid. There are many other methods to separate casein from whey such as ultracentrifugation, ultrafiltration, microfiltration, gel filtration, precipitation by ethanol, rennet coagulation and salting-out methods (De Kruif et al., 2012; Fox, 2003; Rehan et al., 2019).

Overall, casein features include (Fox, 2003; Huppertz, 2012; Miranda et al., 2020; Rehan et al., 2019): (i) heat stable (casein starts to become gradually insoluble if heated at above 120 ^{o}C and becomes insoluble at 140 ^{o}C for 15 -20 min), (ii) sensitive to pH which makes it precipitate at pH 4.6. (iii) caseins are hydrophobic with high charge in order to be kept in solution And (iv) the formation and stability of casein micelles are modified glycosylation (ϰ-casein) and phosphorylation (αS-, β- and ϰ-casein), then the casein exhibits a high degree of heterogeneity.

These features impact the primary structure of the peptide chain, and the activity of peptides produced after digestion of caseins by proteases in the digestive tract. Therefore, the accurate identification of all the protein fractions is important to determine the wide range of bioactive peptides released during digestion (Agudelo et al., 2004; De Kruif et al., 2012; De Noni, 2008; Egger & Ménard, 2017).

The core of the micelle is further cross-linked by colloidal calcium phosphate nanoclusters and electrostatic interactions with phosphoserine residues of αS- and β-casein, while ϰ-casein is thought to be the formation of a polyelectrolyte brush on the surface of the micelle. Whereas ϰ-casein's role is allowing colloidal stabilisation due to electrostatic repulsion between micelles, and determining the size of the micelle by preventing further casein aggregation, so responsible of variability in casein micelle size (Broyard & Gaucheron, 2015; Lambers et al., 2021).

Beta-casein (β-casein)

β-casein proteins make up approximately 30 % of the total protein of cows' milk (Fox, 2003). β-casein is released from the casein micelle thus increasing its solubility under low-temperature conditions due to the weakening of hydrophobic attraction, which is called cold denaturation (Markoska et al., 2021). β-casein fraction can be subjected to chemical changes such as phosphorylation, glycosylation and proteolytic action yielding gamma-casein (γ -casein). There are at least 12 variants of β-casein and the most common variants are A1, A2, and B. These variants significantly differ in their mineral content, particularly Ca, P, Mg, Zn, salts, and fat. β-casein has 209 amino acids, and the position of these amino acids differs between β-casein variants (Daniloski et al., 2022; De Kruif et al., 2012; Fox, 2003; Huppertz, 2012). Among milk, there are variations in their casein composition, for example, milk produced by modern European-type cattle contains a mixture of A1 and A2, whereas cow species such as purebred Asian and African cattle produce milk with β-casein containing A2 β-casein only and free of A1 type (Agudelo et al., 2004; Brooke-Taylor et al., 2017; Mercier & Vilotte, 1993). Furthermore, the ratio of A1:A2 is approximately 1:1 in some herds in Western countries, while Guernsey and Fleckvieh breeds are generally considered to have a particularly high A2 (Pal et al., 2015). Undoubtedly, A1 and A2 levels in milk will differ from breed to breed. There are different types of milk depending on its amino acid sequence, bovine milk containing Pro^{67} is called A2/A2 β-casein milk, compared to A1/A2 and A1/A1 β-casein milk, which are known to carry His^{67} as part of their β-casein structure. Over the last number of years, β-casein genetic variants have received much attention, mainly due to the potential health benefits of A2/A2 β-casein milk compared to the other β-casein variants. Although as of yet, there is no consensus that A1/A1 β-casein milk has a detrimental impact on human health, A1/A1 β-casein milk has been potentially implicated with juvenile diabetes mellitus type-1, ischemic heart disease, and digestive discomfort (Giribaldi et al., 2022; Kamiński et al., 2007; Pal et al., 2015; Quintieri et al., 2024; Sun et al., 2024). The mechanism behind the associated digestive

discomfort of A1/A1 β-casein milk has been associated with the formation of opioid peptides such as β-casomorphin 7 (BCM7) during the digestion process. This peptide is more likely to be produced from A1/A1 β-casein than A2/A2 β-casein due to the ease of cleavage of His at position 67 compared to Pro. The A2 β-casein has a proline at position 67 so BCM7 is much less and probably minimal amounts released (Kamiński et al., 2007; Lambers et al., 2021; Nielsen et al., 2023; Ul Haq, 2020; Ul Haq et al., 2014) (more details in section 2.4).

Alpha S1 casein (αS1)

This fraction is a phosphoprotein and contains 199 amino acid residues, although no cysteine residues are present in its molecular structure. It has a molecular weight of 22.9 kDa and is present in milk at a concentration of 19.5 g / L (Farrell et al., 2004). This protein plays a crucial role in the stabilization of casein micelles due to its high phosphorylation, which facilitates calcium binding (Fox, 2003; McSweeney & Fox, 2015). The presence and expression level of αS1-casein can vary significantly between breeds and is also associated with milk allergenicity, as it is one of the more immunogenic casein fractions (Carira et al., 2012). Its digestion can lead to the release of bioactive peptides with various physiological effects (Nielsen et al., 2023).

Alpha S2 casein (αS2)

This fraction contains 207 amino acid residues including 2 cysteine and 11 phosphorylated serine residues in its molecular structure and has a molecular weight of 25,226 kDa and is present in milk at a concentration of 3 g/L (Farrell et al., 2004). The high number of phosphate groups contributes significantly to the calcium-binding capacity of casein micelles, playing a key role in their stability and structure (Fox, 2003; McSweeney & Fox, 2015). Although present in lower concentrations than αS1-casein, αS2-casein is important in the overall nutritional quality and functionality of milk proteins. Upon digestion, αS2-casein can also release bioactive peptides, which may have antihypertensive and antimicrobial properties (Nielsen et al., 2023). Like αS1-casein, its expression and composition vary with breed, stage of lactation, and individual genetics, potentially influencing allergenicity and technological functionality (Bu et al., 2013).

Kappa-casein (ϰ-casein)

κcas is calcium-insensitive because it is less phosphorylated than the other fractions, and because it is located on the surface of the casein micelle it protects the calcium-sensitive fractions (β- and α-cas) from precipitation by calcium ions (Farrell et al., 2004). ϰ-casein is located on the

surface of micelles and is soluble in water, and these features make the whole micelle highly soluble. The micelle aggregation/ solubility is dependent on the integrity of the hydrophilic C-terminal of ϰ-casein, if these sites break, the micelles lose their solubility and interaction between calcium phosphate and the hydrophobic site will occur (De Kruif et al., 2012; Huppertz, 2012).

Whey proteins

Whey is the soluble fraction after precipitation of casein at pH 4.6 and 20 °C. The branched-chain amino acids particularly leucine, isoleucine and valine are present at high levels, which is important for tissue growth and repair. In addition, it contains a significant amount of sulphur amino acids (cysteine and methionine) which are essential for enhancing immune function upon intracellular conversion to glutathione, a potent antioxidant. Thence scientific and commercial interest is focused on whey protein properties (Tovar Jiménez et al., 2012). The major whey fractions are beta-lactoglobulin (βLg), alpha-lactalbumin (αLac), bovine serum albumin (BSA) and immunoglobulin (Ig). The minor fractions are lactoferrin, blood transferrin, lanolin and proteose-peptone (Cayot & Lorient, 2017; Fox, 2003).

Beta-lactoglobulin (βLg)

Native βLg is a globular protein that occurs as a 36 KDa dimer is composed of 162 amino acid residues and is not present in human milk. Its average concentration in cow milk is 3-4 mg / ml (about 50 % of total protein in whey) (Cayot & Lorient, 2017; Fox, 2003). There are two variants of βLg: βLg A and βLg B. The difference between variants A and B lies in their amino acid sequence: variant A has aspartic acid and valine, while variant B has glycine and alanine at positions 64 and 118 in the polypeptide chain. This change in amino acid sequence changes the isoelectric point of βLg A at pH 5.1 and B at pH 5.3. βLg monomer has two disulphide bonds between cysteine residues (Cys^{66}-Cys^{160} and Cys^{106}-cys^{119}) and one thiol group (Cys^{121}) that is buried within the native structure, and becomes exposed and active after protein denaturation and can then undergo sulfhydryl-disulphide interactions with itself or other proteins (Cayot & Lorient, 2017; Fox, 2003; Le Maux et al., 2014). A significant feature of βLg is that is heat sensitive and starts being denatured at 74 °C with decreased solubility, particularly in the presence of calcium. βLg structure has a free thiol group and amphiphilic character, and those features have an impact on βLg behaviour during heat treatments (Bu et al., 2013; Le Maux et al., 2014). βLg dimer dissociates with heating and therefore this property should be taken into consideration for the

hydrolysis of whey products because it changes the protein solubility and hence enzyme hydrolysis. βLg can be denatured by alkali, heat, cold, pressure, ions or organic compounds to yield coagulate. Moreover, this protein is an allergen for many people particularly infants, mainly because it is not present in human milk and thus resistant to gut hydrolysis (Bu et al., 2013; Cayot & Lorient, 2017; Simmons et al., 2007; Tovar Jiménez et al., 2012).

Alpha-lactalbumin (αLac)

Alpha-lactalbumin (αLac) is an albumin, very soluble in water, representing between 1 – 1.5 g / L of bovine milk, about 20 % of whey protein and 3.5 % of total milk protein. It has a molecular weight of about 14 kDa and is formed by a chain of 123 amino acids with high amounts of tryptophan, which causes favourable effects on serotonin release and promotes the psychological health of patients under stress. αLac starts being denatured at ~ 62 °C, but 90 % of this change is reversible. An irreversible change occurs when heating at 70 or 80°C and at neutral pH (Cayot & Lorient, 2017; Fox, 2003; Stănciuc & Rapeanu, 2010).

Bovine serum albumin (BSA)

This fraction represents between 0.1 – 0.4 g / L. It has a molecular weight of about 66 kDa with 582 amino acids. Although BSA has several functions, it has little effect on the physicochemical properties of milk because it is present at very low levels. BSA starts to denature at 64 °C (Fox, 2003).

Other minor proteins

Immunoglobulins, lactoferrin and proteose–peptones are minor proteins in bovine whey, about 0.8, 0.1 and 0.6 g/L of milk, respectively. Ig are antibodies produced in response to viruses, bacteria and animal antigens; LF transports iron from serum to tissues and PP is a product of the enzymatic degradation of casein (Fox, 2003).

2.1.2 Fat.

Milk fat is a complex mixture of mono-, di- and triglycerides, free fatty acids phospholipids, fat-soluble vitamins and other minor components that vary depending on some factors such as the diet of the cow, processes, season of year, stage of lactation and breed. The fat content in cow milk ranged between 3 to 5 % in the form of oil-in-water emulsions called fat globules. There are several health benefits of milk fat, it is a source of essential fatty acids, and fat-soluble vitamins and

contributes to satiety (German & Dillard, 2006). Milk fat plays a significant role in the flavour, texture, and mouthfeel of dairy products, and it also contributes to the stability and structure of various processed foods (Argov et al., 2008; Mercier & Vilotte, 1993). Milk fats are insoluble globules with diameters ranging between 1 – 10 μm. The membrane of the fat globules is composed of proteins, fat and glycolipids, this membrane plays a role in the physical, functional and health properties such as stabilising the globules in an emulsion in the aqueous phase of milk and delivering the bioactive nutrients (Argov et al., 2008; German & Dillard, 2006). Milk fat globule membrane is a heterogeneities particle consisting of polar lipids (phosphatidylcholine, glycoproteins, sphingolipids, glycerophospholipids, cholesterol, proteins and enzymes) with the interior phase consisting of hydrophobic lipids, that provide nutrition and medicinal benefits such as a source of unsaturated fatty acids (Argov et al., 2008; Gallier et al., 2012; Lopez et al., 2011). The protein content in the fat globule membrane is about 0.04 % of total milk protein, however, the changes in proteins during processing may influence some properties of dairy products such as creaming (Keenan et al., 1983; Ye et al., 2004). During milk processing, the fatty acid tails of the membrane interact with the hydrophobic regions of proteins, which disperses fat globules and enhances the stability of emulsions and gels. In addition, the protein-fat interaction impacts the enzyme catalytic activity and nutrient transport depending on how this interaction affects the degree of coalescence or the binding place (Berton et al., 2012; Ding et al., 2023). Homogenisation is a process that is used to reduce fat globule size and ensure they are uniformly distributed throughout the milk. This process prevents cream separation, thereby improving texture, stability, and mouthfeel, and facilitates the adsorption of skim milk proteins onto the newly exposed fat globule surfaces. While, the heat treatment denatures the milk fat globule membrane proteins, promoting their interaction with whey proteins through thiol-disulfide interchange reactions. These interactions during these processes occur through thiol-disulfide interchange reactions, (Bu et al., 2013; Singh, 2019). These thiol-disulfide interchange reactions in milk protein-fat globule membrane proteins become easily available at lower temperatures, below the denaturation of whey proteins (Singh, 2019; Ye et al., 2004). These changes can alter the functional and structural properties of milk, consequently, bioavailability and digestibility (German & Dillard, 2006; Liang et al., 2017; Singh, 2019; Ye et al., 2004).

2.2. Milk type.

Thousands of years ago, mutations in cow breeds led to the emergence of the A1 variant of β-casein, particularly in European herds. Consequently, conventional milk from these herds, which primarily contains the A1 variant, is widely available commercially and often called "conventional or new milk." In contrast, Jersey and Guernsey breeds produce predominantly A1-free milk, containing the A2 variant. The milk types refer to variations in the compositional profile of milk according to the genetic variations of cow breeds. The most common A1 variant breeds are Holstein, Friesian and British Shorthorn, while Jersey and Guernsey breeds are common as A1 free or A2 milk (Bell et al., 2006; Bodnár et al., 2018; Cieślińska et al., 2022). The most studied area is β-casein composition, in which variation in the β-casein may impact some properties such as the peptide profile, milk coagulation, and the quality of milk products (Carroll et al., 2006). In addition, Jersey milk contains higher fat content with different fatty acid profiles in comparison to other conventional breeds (Drackley et al., 2001). These variations in milk composition impact the proteins and fat composition, which consequently affect the physic-chemical, rheological and nutritional properties of milk and its products (Carroll et al., 2006; Drackley et al., 2001). One of the most studied areas in the differences between conventional and A2 or Jersey milk is β-casomorphin 7 released.

2.3. Milk processing.

Milk processing is any treatment applied to milk to ensure its safety for human consumption. The process of heating milk for health purposes has been carried out since the beginning of the 19th century and was applied to decrease milk-borne illness and death rates in infants in that period (Currier & Widness, 2018). Raw milk consumption may incur bacterial infections, while thermal treatment remains the most common and effective method used to increase the microbiological safety of milk without substantially changing the nutritional value or the sensory properties of milk (Aguilera, 2019; Claeys et al., 2013).

Milk processing can however change the milk protein structure in several ways, depending on the conditions under which it has been processed. The main protein changes occurring during milk processing are aggregation and denaturation of the protein and its amino acid chemical modifications. These modifications in protein composition and structure may affect digestion and the overall physiological impact the consumption of these proteins has on human health. The

digestibility and bioavailability of proteins are the most physiological consequences of the effect processing that have been studied. However, the protein modifications may also cause changes along the gastrointestinal tract such as related to microbiota, epithelial physiology and immune responses (Aguilera, 2019; Bu et al., 2013; Van Lieshout et al., 2020). It is widely known that the processing of milk can result in modification of the protein structure, resulting in altered interactions between proteins and other nutrients (Aguilera, 2019; Ding et al., 2022). Milk processing generates a large number of dairy products that have different effects on the physiological functions of the human body (Augustin & Udabage, 2007; Han et al., 2020; **V**an Lieshout et al., 2020).

2.3.1 Effect of milk processing on milk protein structure.

The main aims of food processing are to extend the shelf life of foods and add value to diets by providing safety, convenience, variety, and nutrition. Several processes have been applied to obtain these purposes, with associated changes in the physical, chemical, biochemical, microbiological, organoleptic and nutritional properties of foods. Commonly applied processes in fresh milk production include pasteurisation and homogenisation, with microfiltration recently introduced to further enhance product taste, safety and shelf stability (Aguilera, 2019; Augustin & Udabage, 2007; Bhat et al., 2021; Borad et al., 2017).

Pasteurisation.

Heat treatment is an important step in milk manufacture; as such this step is aimed at killing microorganisms, specifically pathogens, inhibiting enzymes which could produce reversible or irreversible changes; and maintaining the desired quality (Bu et al., 2013; Van Lieshout et al., 2020; Verhoeckx et al., 2015). This thermal process is a mild heat treatment which involves heating to a sufficient temperature (below 100 °C) and time to inactivate and destroy the contaminating pathogenic microorganisms (Qi et al., 2015). Pasteurization conditions should be one of the following: (i) Holder method (62.8 – 65.6 °C for 30 min), (ii) High-temperature short time (HTST) (71.7 °C for 15 s) (Özer & Yaman, 2014). Compared with many other foods, milk is heat stable to some thermal processing and this allows to the application of many types of thermal processing on milk (McSweeney & Fox, 2013). However, some reactions occur in milk composition during thermal processing, such as chemical reactions (Maillard reaction, oxidation, etc.), biochemical (inactivation of enzymes, etc.) and physical (coalescence, aggregation, flocculation, etc.)

(Aguilera, 2019; Bogahawaththa et al., 2021; Borad et al., 2017; Bu et al., 2013; Fox, 2009; Mauron, 1990; Verhoeckx et al., 2015). Milk pasteurisation under 80 °C is more recommended than ultra-high temperature (UHT) (135 °C for 2 s) treatment because it has less significant effects on amino acid bioavailability and, consequently, nutritional value (Efigênia et al., 1997). Milk protein denaturation, irreversible or reversible, is dependent on the processing conditions and protein fraction.

Heating milk at temperatures ranging from 70 to 100°C denatures the whey protein, while casein is less affected (Bu et al., 2013; Efigênia et al., 1997; Qian et al., 2017; Verhoeckx et al., 2015). During the heating process, conformation changes start by exposing the thiol group of βLg which then will associate with other active thiol groups that will be generated. Furthermore, βLg will polymerise with ϰ-casein and αs2-casein, which can lead to irreversible denaturation and aggregations (Bu et al., 2013; Qian et al., 2017).

At pasteurisation temperatures, βLg and αLac will form aggregates with each other (Hurt et al., 2015) or/and with casein (Zamora et al., 2012). Qian et al. (2017) evaluated the whey denaturation by using Native-PAGE and showed that whey denaturation started from heating milk for a few minutes at 65ºC while almost all of the whey protein was denatured at 85ºC. Denatured whey protein would aggregate with casein during heating milk due to the poor thermal stability of whey protein. Whey-casein complex precipitation could be obtained by high-speed centrifugation. (Singh & Creamer, 1991). The degree of whey protein-casein aggregation could be successfully determined by the polyacrylamide gel electrophoresis method, by comparing the contents of whey protein in centrifugal supernatant of unheated and heat-treated milk (Qian et al., 2017).

Pasteurisation, however, has minimal effect on the secondary structure of milk proteins when compared with other severe heat treatments (UHT, sterilization, etc.). Although there is a positive correlation between the heat treatment conditions (temperature and time) and the denaturation of whey protein and the formation of whey-casein aggregates. Whereas the compositions and structure changes will increment with expanding temperature and treatment time (Bogahawaththa et al., 2021; Efigênia et al., 1997; Mauron, 1990; Qian et al., 2017). As a result, heat treatment of milk could cause an increase and/or decrease in the percentage of total protein of casein and whey, respectively, depending on the heat treatment conditions, because whey protein is denatured and becomes associated with the casein micelle; particularly, βLg forms disulfide bonds with ϰ-casein

(Hurt et al., 2010). For example, compared with raw milk, Hurt et al. (2010) found that heating milk at 72 °C for 16 s does not cause a significant change in the total protein of casein, however, Ma et al. (2000) reported that the percentage of total protein in casein increased by about 3% when heating the milk at a higher temperature and longer time (74 °C for 34 s). Sequentially, the whey-casein complex causes an increase in casein micelle size (Hougaard et al., 2009). Heat treatments will tailor the functional properties of whey proteins depending on process conditions (Galani et al., 1999).

Singh (2019) reviewed the impact of heat treatments on milk fat globule membrane protein denaturation. The denaturation temperature of these proteins is lower than the denaturation temperature of whey proteins. As a result, the stability of fat globules and their ability to interact with other proteins increase when heating begins. At 60 ºC, the proteins on the fat globules denature and interact with whey proteins through thiol-disulfide interchange reactions. During cooling following the heat treatment, some of the fat globule proteins migrate into the aqueous phase of milk.

From all above, it can be concluded that heat treatment (depending on the conditions used) alters the structure of whey proteins by unfolding the globular structure thus increasing their sensitivity to enzymatic digestion. On the other hand, the caseins are more stable due to their loose and flexible structure.

Homogenisation.

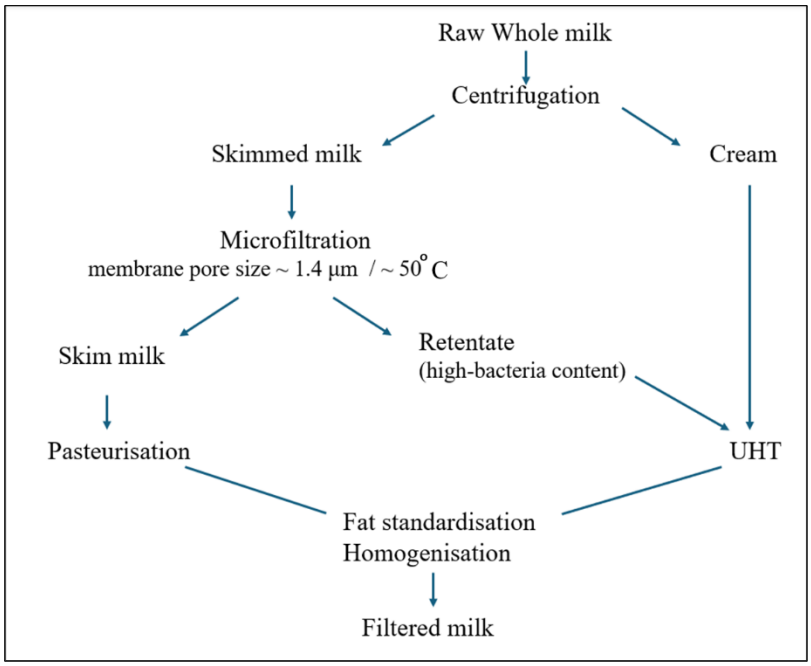
Homogenisation is a non-thermal process that is used to reduce the milk fat globule size by pumping milk at high pressure (15 – 40 MPa) through a small valve. The process breaks fat particles (average diameter approximately 3.5 μm) into much smaller globules and alters their structure. In addition, this process rearranges casein, serum protein, and milk fat globule membrane, and changes protein-protein interaction (Qi et al., 2015). The combination of homogenisation and heat treatment caused increases in the whole milk viscosity that could be related to the unfolding of βLg and the subsequent association with the casein micelles, while a positive correlation was found between increasing the viscosity and the levels of βLg denaturation or the level of aggregation of βLg and casein micelles (Hougaard et al., 2009). Heat treatment of milk can lead to an integration of βLg and αLac into the milk fat globule membrane while

homogenisation can cause the adsorption of casein micelles to the surface of the fat globules (Hougaard et al., 2009; Zamora et al., 2012).

Microfiltration.

Microfiltration (MF) is a non-thermal treatment that can be used to extend the shelf life of dairy products (Elwell & Barbano, 2006; Saboya & Maubois, 2000). Microfiltration is a separation process that uses a membrane with different pore sizes depending on the components that need to be separated (Elwell & Barbano, 2006; Saboya & Maubois, 2000). This treatment can physically remove spores, bacteria, fat globules, and somatic cells and enrich the casein micellar in the cheese-making from skim milk with little effect on milk components such as protein, lactose and ash. However, MF alone cannot completely remove the pathogenic bacteria, thus MF is usually combined with heat treatment (Cheryan, 1998; Crowley, 2016; Saboya & Maubois, 2000; Zhang et al., 2021). Although heat treatments can produce free-pathogenic bacteria milk, these treatments have an effect on the component and nutritional value of milk (Bogahawaththa et al., 2021; Borad et al., 2017; Efigênia et al., 1997). Many studies aim to produce milk with a long refrigerated shelf life using minimum heat treatment while retaining the nutritional value and flavour quality of fresh milk (Elwell & Barbano, 2006).

During MF (as it can be seen in **Figure 2.1**), raw milk is centrifuged to separate fat or cream (retentate), leaving the permeate (skimmed milk) which is free of bacteria. The next stage is then to filter the skimmed milk through a microfiltration membrane to produce skimmed milk that is free of bacteria. Then the retentate with high bacterial content is mixed with the cream and heat-treated to eliminate the bacteria which is then mixed with skimmed milk to produce filtered milk



359

360 **Figure 2.1:** The process of milk microfiltration (Elwell & Barbano, 2006; Saboya & Maubois, 2000).
361

362 Microfiltration at low temperature (< 50 ºC) causes the dissociation of β-casein from the micelles
363 thus migrating into the serum phase due to reduce hydrophobic interactions as well as an increased
364 solubility of calcium phosphate, which leads to an enhanced release of micellar-bound β-casein
365 (Schiffer et al., 2020; Schiffer et al., 2021). Thus, microfiltration presents a significant opportunity
366 to improve the functional and physical properties of casein micelles such as heat stability and
367 coagulation.

368 No changes in size or the composition of the water phase of casein micelles have been detected in
369 skimmed filtered milk at 50 ºC (E. Hurt et al., 2015). However, the small fat globules separated by
370 microfiltration contain higher moisture than large fat globules, and this increase in available water
371 enhances the enzymatic activities, consequently increasing the proteolysis of these small fat
372 globules. During microfiltration, these small fat globules are entrapped in the casein matrix
373 increasing the surface area of the milk fat globule membrane and altering casein strands. These
374 changes have been reported to affect the physic-chemical and sensory properties of Camembert
375 cheese as stated by Michalski et al. (2003). Most microfiltration membranes have protein binding
376 characteristics which consist of an isotropic network of polymer fibres resulting in a highly
377 interconnected pore structure (Cheryan, 1998; Elwell & Barbano, 2006). In addition, the filtration

process uses physical factors that can also have significant effects on the performance of the membrane device. Consequently, during filtration, two distinct phenomena could be described; protein adsorption and protein deposition. Protein adsorption describes the interaction between proteins and the membrane polymer that occurs in the absence of any convective flow through the membrane, and protein deposition, refers to any additional protein that becomes associated with the membrane during filtration (Yang, 2011).

The data obtained Steinhauer et al. (2015) study undoubtedly indicates that the rate and degree of flux decrease —where flux is defined as the volumetric flow rate of permeate per unit membrane area (commonly expressed in $L/m^2 \cdot h$) (Elwell & Barbano, 2006)—during protein microfiltration is directly linked to the structural characteristics of the protein molecule. Especially, the presence of a free thiol group induces an initial boost in the rate of flux decline due to the chemical attachment of the native protein to the growing deposit via intermolecular thiol-disulfide interchange reactions. Blocking the free thiol in the solution eliminates the chemical accumulation of native protein in the growing deposit. Those proteins without free thiol were only able to degrade growing deposits (Steinhauer et al., 2015). However, under microfiltration conditions that have been used by Kelly and Zydney (1997) on some protein solutions, there are two different suggestions of the changes that could occur to the solution during microfiltration: pore blockage associated with aggregate deposition and chemical attachment of native solution to the growing sediment via the formation of an intermolecular disulfide linkage.

Microfiltration can influence the extent of mineral exchange between diffusible and colloidal phases, potentially affecting the behavior of micelles during acidification or rennet-induced processes. Additionally, microfiltration tends to trap colloidal aggregates in the membrane pores, which may result in changes to the composition or ratios of milk proteins(Sachdeva & Buchheim, 1997). Thus, microfiltration presents a significant opportunity to improve the functional and physical properties of casein micelles such as heat stability and coagulation (Krstić et al., 2002; Saboya & Maubois, 2000), and these changes in milk proteins properties may affect milk protein digestibility and its potential allergenicity.

Commercial filtered milk usually undergoes two different treatments (**Figure 2.1**), pasteurisation, and microfiltration to ensure milk is safe to be consumed. While thermally processed milk has

been intensely investigated, some gaps can be identified in the literature in relation to filtered milk and the protein structure and the impact on protein digestibility peptide profile.

2.4. Digestion of milk protein.

Milk digestion begins at the low pH environment of the stomach, where the enzyme pepsin starts protein digestion by breaking down milk proteins into smaller peptides. These smaller fragments of peptides and fats then move into the small intestine for further digestion. In the small intestine, the digestion of milk proteins, fats and their fragments by proteases and lipases will continue, while bile is also secreted into the small intestine to help with fat digestion. Milk proteins become a smaller chain of amino acids and free amino acids, and milk fats become diglycerides, monoglycerides and free fatty acids. The hydrolysis of lactose is done by secreted lactase by the brush border cells to produce its constituent monosaccharides, glucose and galactose. All these nutrients (such as monosaccharides, peptides and amino acids, etc.) are absorbed from the small intestine into the liver, which is responsible to regulate the distribution of these nutrients to the rest of the body. Gut muscle activity (gut motility) is serves to mix luminal contents with the digestive enzymes and move these contents through the tract. The rate of gut motility is measured as gastrointestinal transit time (Dupont & Tomé, 2020; Petrat-Melin, 2014). The delivery of protein to the small intestine is critically affected by the changes in the physical and structural of the coagulate fraction of the gastric contents (Roy et al., 2022).

The digestibility of milk proteins is an important factor in protein nutritional value and their bioavailability. The gastrointestinal transit time of cow's milk and its proteins are subject to individual differences. Prior to their digestion and absorption, some bioactive peptides may cause gastrointestinal symptoms (Claeys et al., 2013; Dupont & Tomé, 2020).

Milk protein aggregation, as mentioned above, starts as dimerisation between the appropriate orientation of unfolded protein to each other via thiol interchange and also involves hydrophobic interactions. This aggregation could interfere the milk enzymatic coagulation due to a layer of molecules becomes adsorbed to the surface of casein micelles (McMahon et al., 1993). Under digestion conditions, whey protein stays soluble and rapidly passes from the stomach to the intestine without being hydrolysed by pepsin and increasing the plasma amino acid, in contrast,

436 the casein is coagulated under these conditions which causes a slower gastric emptying rate than
437 whey. Therefore, whey proteins have a faster gastric emptying rate than casein. The digestion and
438 gastric emptying rates of casein, as casein micelles in milk mixture, were slower than in the
439 digestion of pure casein or caseinate, due to altering the casein by both acid and enzyme
440 coagulation (Wang et al., 2018).

441 2.4.1 Effect of milk processing on milk protein digestibility.

442 It has been shown that skimmed milk is digested faster than whole milk, which showed some
443 persistence of the peptides throughout digestion, due to the adsorption of protein with fat (Tunick
444 et al., 2016). Mauron (1990) reported that milk processing can influence stomach emptying time,
445 showing that UHT milk had a stomach emptying time faster than raw and pasteurised milk. Caseins
446 are extensively and rapidly degraded under the gastric phase conditions, about 75 % of casein is
447 hydrolysed during the first 30 min after meal intake. Under both *in-vivo* and *in vitro* conditions,
448 the casein released medium-sized peptides (750 – 1050 kDa), about 60 and 25 % of these peptides
449 are released from β-casein and αS1-casein, respectively. These peptides contain two or more
450 proline residues that resist gastric and pancreatic digestive enzymes (Dupont & Tomé, 2020).

451 In contrast, the globular structure of whey proteins is not affected by gastric enzymes and survived
452 under gastric conditions for 60 min. However, the conformational changes in β-lactoglobulin, such
453 as binding with fat globules, increase their sensitivity to enzymatic hydrolysis. β-casein represents
454 about 50 % of these peptides were low and no peptides from β-lactoglobulin and α-lactalbumin,
455 respectively, suggest that these proteins were highly resistant to infant gastric digestion (Dupont
456 & Tomé, 2020).

457 The combination of homogenisation with heat treatment increases the susceptibility of proteins to
458 hydrolysis by pepsin, due to the unfolding of the proteins at the fat globule membrane. During
459 gastric digestion, heat-treated and homogenised whole milk formed a coagulum with fragmented
460 and crumbled structures with more pores. These pores allowed a better diffusion of the digestive
461 enzymes during simulated digestion, leading to an increase in the rate of proteolysis and the
462 bioavailability of amino acids, as a result (Ye et al., 2017).

463 2.4.2 Released peptides after digestibility.

Milk has numerous positive nutritional properties and contains bioactive components, most of which have beneficial effects on human health. However, in certain contexts, some components may have potential negative effects (Bidasolo et al., 2012; Dupont & Tomé, 2020; Truswell, 2005). Bioactive peptides are generally 3 – 20 amino acid residues in length, derived during protein hydrolysis by proteolytic enzymes or in the gastrointestinal tract (Rutherfurd-Markwick & Moughan, 2005), these peptides may have two or more different bioactivities such as opioid peptides, immunostimulant peptides (Dupont & Tomé, 2020). Protein hydrolysis produces a complex mixture of peptides and free amino acids. Some peptides, and free amino acids, are more readily absorbed in the gastrointestinal tract when compared to native proteins, while proteins and peptides exhibit specific biological activities in addition to their established nutritional value (EFSA, 2009; Meisel, 1998). Recent research by Nielsen et al. (2023) identified 202 bioactive peptides, including those with dipeptidyl peptidase (DPP)-IV inhibitory, anti-inflammatory, antimicrobial, angiotensin-converting enzyme (ACE)-inhibitory, opioid, and antioxidant properties.

2.4.3 Beta-Casomorphin7 (BCM7).

β-casomorphins (BCMs) are exogenous opioid peptides isolated from an enzymatic digest of β-casein. There are many BCMs released during the digestion of β-casein (from BCM3 to 11 peptides) (**Table 2.3**). The number depicts the count of amino acids in the peptide and all of the BCMs contain the same first three amino acids (i.e., -Tyr-Pro-Phe-) in the sequence. Among them, the most active peptides contain 7 amino acids, beta-casomophin 7 (BCM7) (**Figure 2.2**) (De Noni & Cattaneo, 2010; Nguyen et al., 2017). β-casomorphin 7 (BCM7) represents fragments 60–66 of bovine β-casein, with amino acid sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile (**Figure 2.2**). Many studies mentioned that the release of BCM7 during hydrolysis of β-casein seems to be dependent on the presence of histidine in some variants of this protein, such as A1 and B β-casein (De Noni & Cattaneo, 2010; Nguyen et al., 2017; Truswell, 2005; Ul Haq et al., 2014). Since the first identification of BCM7 in 1979 by Brantl and Teschemacher (1979), milk has been classified as A1 or A2 (or A1 free) based on the β-casein variant it contains. This division is due to the discovery that A1 β-casein releases BCM7 during digestion, while A2 β-casein releases significantly less or none, depending on the conditions. The bioactive opioid peptide BCM7 is released by gastrointestinal digestion from the milk protein containing the A1 β-caseins under digestive

enzymes but not released by the A2 β-caseins as reported in earlier studies (De Noni, 2008; Jinsmaa & Yoshikawa, 1999). However, more recent studies indicate that while A2 milk also releases BCM7, it does so at levels approximately 2 - 4 times lower than A1 milk (Cattaneo et al., 2023; Lambers et al., 2021; Nguyen et al., 2021).

Table 2.3: Sequence of bovine β-Casomorphins (BCMs)*.

BCMs	Sequences	Corresponding β-casein location
BCM3	Tyr-Pro-Phe	60–62
BCM4	Tyr-Pro-Phe-Pro	60–63
BCM5	Tyr-Pro-Phe-Pro-Gly	60–64
BCM 6	Tyr-Pro-Phe-Pro-Gly-Pro	60–65
BCM 7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	60–66
BCM 8	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro	60–67
BCM 9	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn	60–68
BCM 10	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser	60–69
BCM 11	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu	60–70

* (Brooke-Taylor et al., 2017; Nguyen et al., 2017).

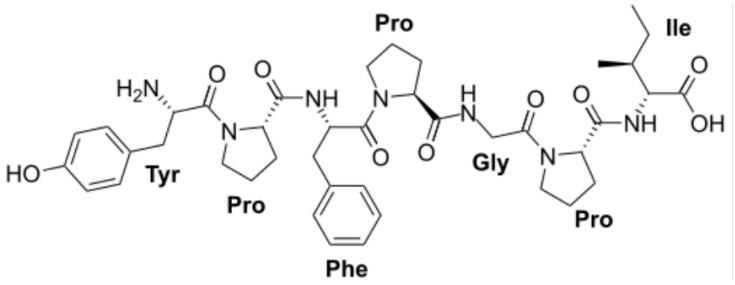


Figure 2.2: Chemical formula and chemical structure of the BCM7 (Roushani et al., 2020).

2.4.4 BCM7 and health.

Numerous studies suggest that BCMs, in particular, BCM7 may be implicated in many illnesses, and a risk factor for the development of type 1 diabetes, autism in children, sudden infant death and heart diseases (EFSA, 2009; Nguyen et al., 2017). BCM7 peptide binds to the receptors located in the central nervous system, gastrointestinal tract, and some immune cells. Moreover, the BCM7 can be absorbed in the gut, circulate in the blood, and inflame other tissues. BCM7 exhibits more resistance to enzymatic hydrolysis, higher opioid potency than the natural peptide, and many immunological activities such as allergy and skin reaction (Cieślińska et al., 2022; EFSA, 2009;

Nguyen et al., 2017; Ul Haq et al., 2014). Many studies mentioned that A2 milk is easier to digest than A1 milk and related that to the presence of that specific digest-resistance BCM7 (Brooke-Taylor et al., 2017; Truswell, 2005; Ul Haq et al., 2014). Jianqin et al. (2016) and Ho et al. (2014) have mentioned that milk containing both A1 and A2 β-casein causes an increase in systemic inflammation and gastrointestinal disorders similar to those of lactose intolerance, may be related to the presence of A1 β-casein rather than lactose. While consumption of milk that only contained the A2 β-casein type did not adversely affect these variables. Gastrointestinal symptoms such as longer gastrointestinal transit times, softer stools, and diarrhoea, are associated with the consumption of milk containing both β-casein variants more than the consumption of milk containing only the A2 β-casein type (Ho et al., 2014; Jianqin et al., 2016).

2.4.5 Effect of processing on formation and degradation of BCM7.

The BCM7 is the most important peptide with an important opioid property and is currently being studied extensively for its physiological significance (Lambers et al., 2021; Nguyen et al., 2017). Many studies have shown that BCM7 releases much less and probably minimal amounts from A2 in milk (Ho et al., 2014; Lambers et al., 2021). Moreover, these outcomes were affected by the differences between single cows of multiple breeds, seasons and model products, which have been tested using different digestion procedures and analytical methods to detect BCM peptide formation (Lambers et al., 2021). The BCM7 is released from milk, yoghurt, cheese, and milk products. Although the release of BCM7 in cheese and yoghurt is modest, certain bacteria present in yoghurt may hydrolyse BCM7 (Cattaneo et al., 2023; De Noni et al., 2015; Nguyen et al., 2017; Pal et al., 2015). In human infant jejunal, each 30 g of casein released about 4 mg of BCM7 after 2 h of digestion, may with further release thereafter and BCM7 has been identified in the blood and urine of human infants (Pal et al., 2015). Lambers et al. (2021) reported that, in intestinal digestion, the pasteurisation (85 ºC/30 s) and UHT (140 ºC/15 s) of milk decreased the formation of BCM7, however, Nguyen et al. (2021)reported that opposite thought which this processing increased the formation of BCM7 in A1 milk. Lambers et al. (2021) found that pasteurisation reduces the release of BCM7 due to protein denaturation that impacts the protein hydrolysis. Overall, the digestion conditions impact the protein hydrolysis and the resulting peptides (Cattaneo et al., 2023; Lambers et al., 2021), and milk processing alters the release of BCM7 by altering the

protein structure which consequently impacts protein digestion and its products (Bhat et al., 2021; Kopf-Bolanz et al., 2014; Lambers et al., 2021).

2.5. Allergenicity of cow's milk proteins.

Milk is the main food for infants and children as a main source of high-quality protein, however, cow milk is one of the foods reported to cause allergic reactions (milk, eggs, fish, crustaceans, peanuts, nut trees, wheat and soybeans) (Monaci et al., 2006; Pekar et al., 2018; Sharma et al., 2001). Milk allergy is a negative immune reaction triggered by the ingestion of milk or its derivatives. It has been reported that milk allergy affects approximately 1 to 3 % of children aged 1 to 5 years around the world (Caira et al., 2012; Chatchatee et al., 2001), and it affects more than 20 % of the UK population (Wong et al., 2022). There are two types of immunological reactions depending on the period of resulting allergy symptoms after ingestion of the allergic food. First, is the immunoglobulin E (IgE) reaction which appears immediately after protein ingestion because it triggers the immune system. The other type takes up between 1 h to a couple of days to develop involving the immune system after ingestion of an allergic source, which is called non-IgE mediated immunological reactions. The capability to induce the production of IgE is called 'allergenicity'. In individuals with a milk allergy, their immune system identifies certain milk proteins regions (epitopes) as harmful invaders. The immune system reacts to the presence of epitopes by generating specific IgE, which upon binding to the epitopes, triggers the degranulation of mast cells and basophils, releasing histamine, resulting in allergic symptoms such as skin reaction, digestive and respiratory problems and even severe reactions such as anaphylaxis (Monaci et al., 2006; Sathe & Sharma, 2009; Villa et al., 2018). Epitopes that have allergenic elicit could be short sequential segments of amino acids or may be unfolding of the structure due to the conformational changes. According to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) official list of allergens, milk allergen proteins are classified with the following designation: Bos d 5 (βLg), Bos d 4 (αlac), Bos d 6 (BSA), Bos d 7 (Ig), Bos d 9 (αS1), Bos d 10 (αS2), Bos d 11 (β-casein), Bos d 12 (ϰ-casein) (Venter et al., 2024). The allergenicity of the βLg is attributed to no βLg in the human milk protein, IgE response against βLg starts since birth. However, the IgE response against casein may starts from 1 year old (Lajnaf et al., 2022). The major problem of milk allergy is the patient's present immune reaction to two or more cow milk allergens, thus none of the main milk protein allergens can be regarded as the only

one responsible for the allergenicity (Lajnaf et al., 2022). To demonstrate what makes proteins allergenic, most of allergy research focuses on understanding the allergenicity and adjuvanticity of allergens of these proteins. The allergens have to pass via the epithelial barriers, in the respiratory and gastrointestinal tract or through the skin, to develop IgE-mediated responses (Caira et al., 2012; Deifl & Bohle, 2011; Fan et al., 2023; Goodman et al., 2007; Graversen et al., 2020; Venter et al., 2024). There are two categories of epitopes: conformational and linear. During milk processing and/or digestion, conformational epitopes are formed by discontinuous amino acid sequences brought together through thiol-disulfide exchange interactions, relying on a specific three-dimensional structure to form the antigenic site and maintain their function. While linear epitopes are short continuous amino acid sequences (7 to 20 amino acids) (Monaci et al., 2006; Panchaud et al., 2012; Sharma et al., 2001).

2.5.1 The effect of different processes on the allergenicity of cow's milk proteins.

Food processing and added ingredients induce differences in the allergenic properties of proteins, these properties could be increased, decreased or not affected depending on the conditions of the process (Bu et al., 2013; Verhoeckx et al., 2015; Xu et al., 2016). Previous studies mentioned that heat treatments could decrease or increase the potential of milk protein allergenicity depending on the time and temperature of heating. For example, heating milk above 90 ºC may lead to a decrease in the allergenicity, however, heating the milk below this temperature increases the allergenicity of βLg (Bu et al., 2013; Xu et al., 2016). Heating milk at temperatures > 60 °C will cause the destabilisation and unfolding of protein structure and lead to the exposition of disulfide bonds and the free thiol as active epitopes (Taheri-Kafrani et al., 2009; Xu et al., 2016). On the other hand, food processing can be related to the decrease in the allergenic properties of proteins that are attributed to the mask of sequential epitopes by disulfide bond during the aggregation or the damage of conformational epitopes (Bu et al., 2013; Xu et al., 2016).

Feng and Collins (1999) reported the effect of homogenisation and pasteurisation on milk protein allergenicity from different points of view. During the homogenisation process, an association of milk proteins with fat globule membranes will occur. Fat particles are known as vehicles for vaccine delivery, and these particles are proven to deliver the antigen within the gastrointestinal tract effectively and can transfer their contents into blood circulation. Furthermore, protein-fat interactions have been explained as a relevant key to an understanding of immunological

responsiveness to dietary antigens introduced to the gastrointestinal membrane. The fat is delivered to the liver from the gastrointestinal tract via the lymphatic system. Fat taken up from the gastrointestinal tract is present in lipophilic protein in the immune system (Feng & Collins, 1999).

The allergenicity assessment provided an evaluation of the possibility that exposure components might lead to increased allergies due to similarities of sequence/structure of the new component with known allergenic proteins from other sources (Goodman et al., 2007). The bioinformatics analysis was conducted to compare the sequences of the proteins to those of known allergens to evaluate the potential for allergic reactions (Goodman et al., 2007; Lajnaf et al., 2022). The evaluation of the sequential and structural similarities between the known allergens sequences and the query sequences by sequence searches have been performed to evaluate the possibility the unknown sequences may share one or more common epitopes with an allergen. Thus, the possibility of the query sequence provoking an allergic response in people with existing allergies might be increased (Goodman et al., 2007). The major common assumed features and physicochemical parameters, such as hydrophobicity, protein stability, structural features, molecular surface motifs, glycosylation, dimerization/oligomerization and enzymatic activity have been suggested (Bu et al., 2013; Deifl & Bohle, 2011; Lajnaf et al., 2022). Furthermore, allergenicity may depend on the ability of allergens to bind to fat (Deifl & Bohle, 2011). Investigating the ligand-binding properties of allergens and identifying interaction sites through structural studies are essential for understanding the relationship between allergenicity and biological function. Food allergenicity is intrinsically connected to the physicochemical properties of the food, its structural stability during milk processing, and the impact of these processes on protein hydrolysis (Bavaro et al., 2019; Bu et al., 2013; Burnett et al., 2002; Caira et al., 2012; Chatchatee et al., 2001; Dall'Antonia et al., 2014; Deifl & Bohle, 2011). Some authors have hypothesized that the interaction between proteins and other components of the food matrix can change protein structure and hide IgE binding sites (Bavaro et al., 2019; Schulten et al., 2011). From all the above, milk protein composition, structure and released peptides are the most features were used to study the influence of milk processing and its potential allergenicity. Heat treatments under different conditions were extensively studied. Although filtered milk is widely commercially available, the effect of microfiltration on features that influence the potential allergenicity of milk proteins is still needs investigation. Studying these shared properties could spot the light on cross-reactivity between different food allergens. Focusing attention on these shared properties and

analysing recent works and findings in cross-reactivity of the biochemical and allergenic properties of food allergens is a good chance to understand the allergenicity of the protein.

2.6. Conclusion.

Milk protein is an important source of essential nutrients, including high-quality protein, calcium, and vitamins, making it a vital component of a balanced diet. It plays a crucial role in muscle development, bone health, and overall growth, especially in children. However, for some individuals, milk proteins, such as casein and whey, can trigger allergic reactions. Milk allergy is one of the most common food allergies in children, and it can cause a range of symptoms, from mild, such as digestive issues, to severe, including anaphylaxis. Milk digestion can significantly influence the severity of allergic reactions. During digestion, enzymes break down milk proteins into smaller peptides. In individuals with a milk allergy, the immune system mistakenly identifies these peptides as harmful, triggering an allergic response. Additionally, milk processing can unfold protein structures, exposing previously hidden reactive groups and altering protein digestion and the peptides released. These exposed peptides and reactive groups can further exacerbate the immune system's recognition of these components as harmful, leading to an allergic reaction. The effects of thermal processes such as pasteurisation, UHT, and homogenisation have been widely investigated. However, microfiltration is a more recent process being used in milk manufacturing. The impact of microfiltration on milk protein structure, digestion, and potential allergenicity remains relatively underexplored.

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Chapter 3. The Rise of Filtered Milk: Analysis of Market Trends in the UK (2022-2024)

3.1. Abstract

Milk is a staple food product in the UK, widely consumed due to its nutritional value and versatility. Over the years, the dairy industry has evolved to meet changing consumer demands, introducing developments like filtered milk. This chapter aims to evaluate the availability of fresh cow's milk in UK markets, focusing on overall production in the country, the main types of milk available, and the recent emergence of filtered milk, which is marketed as having a longer shelf life and a fresher taste. The data about available liquid milk was collected from the main 10 supermarkets and online groceries in Reading, UK during 2021 - 2024. Analysis of the collected data shows that the number of available filtered milk options has increased by 14% over the past two years. This increase in filtered milk availability could reflect the customers' willingness to buy filtered milk due to its longer shelf-life, leading to a demand for more in-depth research to study the effect of microfiltration on milk's nutritional and functional composition.

3.2. Introduction.

Cow's milk is an important ingredient in the human diet across all ages, providing essential nutrients such as protein, minerals and vitamins. It is important for bone maintenance and development, supporting muscle function and overall human health (Brisson & Singh, 2013; Foroutan et al., 2019; Fox, 2003). In addition, its versatility in cooking and beverage forms also makes it a staple in food manufacturing. The selection of liquid cow milk choices in supermarkets has expanded considerably in recent years. According to Mintel (2023), about 89 % of consumers use liquid cow's milk as a part of their diet, despite rising milk prices and competition from alternative milk products. The traditional methods for processing milk include pasteurisation and ultra-high temperature (UHT) treatment, alongside microfiltration, which is a more modern process (Brisson & Singh, 2013; Lewis & Deeth, 2009; Villamiel, 2009). While pasteurised and UHT milk have been extensively studied, filtered milk is a newer type of cow's milk that has recently been introduced to the UK market The first filtered milk in the UK markets was Cravendale, a brand launched by Arla Foods in 2001. This milk was marketed as offering a longer shelf life milk compared to traditional pasteurised milk while maintaining a fresh taste

(Cravendale, 2024; Elwell & Barbano, 2006). Microfiltration is the process that passes milk through a membrane with a pore size from 0.1 to 1.5 μm (Elwell & Barbano, 2006). Filtered milk undergoes two processes, pasteurisation and microfiltration, to extend its shelf-life up to 21 days by removing the micro-organisms and osmotic cells (Elwell & Barbano, 2006; Hoffmann et al., 2006). Around twenty years ago, there was only one brand that sold filtered milk (Arla Cravendale) in the main UK markets. According to Mintel (2018), this filtered milk experienced a 2.7 % decrease in volume sales, due to its higher price compared to pasteurised milk. However, the volume sales of filtered milk increased during 2021-22 and 2022-23 by 5 % and 10 % respectively, driven by consumer preference for its longer shelf life (Mintel, 2023). The main reason for using microfiltration in milk processing is to decrease food waste by increasing the shelf-life of milk (Mintel, 2021). Most studies focused on the effect of microfiltration on the shelf-life as a main reason to process filtered milk (Dinkçi & Sirbu, 2024; García & Rodríguez, 2014; Hoffmann et al., 2006; Wang et al., 2019). This report examined the availability of filtered milk in the major UK retail market from April 2022 to April 2024, as a first step to evaluate the filtered milk availability.

3.3. Method.

Data of available processed conventional fresh cow's milk was collected in April 2022 and April 2024 via the main supermarkets' websites in the UK (collectively covering ~ 94% of the grocery market share between 2022 2024 (Kantar, 2024). This data collection included the major companies/ brands of processed fresh cow's milk in the UK with their respective market shares, including Tesco (28.0 %), Sainsbury's (15.8 %), ASDA (12.6 %), Morrisons (8.6 %), Aldi (Cowbelle) (9.8 %), Lidl (Dairy Manor) (8.1 %), Co-operative (Co-op) (5.9 %), Waitrose (4.6 %), Ocado (1.8%), Marks and Spencer food (M&S), Muller and Cravendale (Kantar, 2024). Fresh conventional cow's milk was categorised into 2 groups, based on the process applied (pasteurised and filtered). Each category was split based on fat content into whole, semi-skimmed and skimmed milk. The nutritional, prices and processing information were collected via the local supermarkets and their websites. The comparison of nutritional information per 100 mL (energy (kcal), fat, saturated fat, sugar, protein, salt (g), calcium (mg) and expiration date (days)), bottle volume (0.25, 0.5, 1, 2 and 3 L) and price (pence/L) were conducted between filtered and pasteurised milk with different fat content in April 2022 and April 2024.

Two-way ANOVA was used to analyse the main effect of processing method (filtered and pasteurised) and time period (April 2022 and April 2024). T-tests were conducted to identify specific differences between filtered and pasteurised milk within each time period and across time periods. A significance level of $p < 0.05$ was used to determine statistical significance with 95 % confidence. XLSTAT (version 2022.2.1) was used for statistical analyses.

3.4. Results and Discussion

From the collected database about the available conventional fresh cow's milk in the main UK markets, almost all the companies/brands sold fresh milk as a whole, semi-skimmed and skimmed in different volumes and subject to different processes. **Table 3.1** shows the number of brands selling fresh filtered and pasteurised milk (whole, semi-skimmed and skimmed) in 2022 and 2024. More brands were offering whole filtered milk in 2024 than in 2022. Pasteurised whole and semi-skimmed milk were the most common milk among all studied brands, followed by semi-skimmed filtered (**Table 3.1**). Supporting this, Mintel (2021) data reveals that pasteurised semi-skimmed milk is the most popular choice accounting for 63 % of milk consumption. The popularity of semi-skimmed milk could be due to its appeal to consumers who want to control their fat intake. It offers a balanced nutritional profile with lower fat content than whole milk while retaining good taste and nutritional benefits (Delley & Brunner, 2020). In 2022, semi-skimmed filtered milk accounted for about 58 % of total brands that sell fresh conventional pasteurised milk, based on data collected in this study. At that time, there was only one brand (Cravendale) offering filtered milk with different fat content (whole, semi-skimmed and skimmed). In recent years, in the UK market, the percentage of brands selling filtered milk with different fat content has increased. As such, the production of whole filtered milk has increased by about 80 % among the brands that sell filtered milk and by 66 % among all brands regardless of the type of milk from 2022 to 2024 (**Table 3.1**). More brands (M&S and Morrisons) launched whole and semi skimmed filtered milk by the 2024. While Tesco, Sainsbury's, Waitrose and Aldi (Cowbelle) lunched whole and skimmed filtered milk beside the semi-skimmed.

According to Mintel (2018) report, sales of filtered milk (shown as Cravendale brand the top filtered milk brand) declined more rapidly than those of pasteurised cow's milk. This decline can be attributed to its higher price and the fact that most customers were satisfied with the shelf life of standard (pasteurised) milk. However, the 2020 COVID-19 pandemic significantly impacted

the dairy market by shifting the retail value and volume of sales of long-life milk, particularly, filtered milk (Cravendale milk) by about 30 and 24 %, respectively (Mintel, 2021). In addition, Delley and Brunner (2020) survey showed that consumers place great attention on milk taste, lower fat content, longer shelf life and packaging size, all while seeking the lowest price. This trend might also reflect a growing interest in long-life processed milk, potentially leading dairy companies to invest more in filtered milk.

Table 3.1: Available commercially processed conventional fresh milk in the main UK markets in 2022 and 2024. The coloured dots represent the fat content of each milk: blue = whole milk, green = semi-skimmed milk, and red = skimmed milk.

	2022		2024	
Brand	Filtered	Pasteurised	Filtered	Pasteurised
ASDA		🔵🟢🔴	🔵🟢	🔵🟢🔴
CO-OP	🟢	🔵🟢🔴	🟢	🔵🟢🔴
Aldi	🟢	🔵🟢🔴	🟢	🔵🟢🔴
Cravendale	🔵🟢🔴		🔵🟢🔴	
Lidl	🟢	🔵🟢🔴	🔵🟢	🔵🟢🔴
M&S		🔵🟢🔴	🔵🟢	🔵🟢🔴
Morrisons		🔵🟢🔴	🔵🟢	🔵🟢🔴
Müller		🔵🟢🔴		🔵🟢🔴
Ocado British		🔵🟢🔴		🔵🟢🔴
Sainsbury's	🟢	🔵🟢🔴	🔵🟢	🔵🟢🔴
Tesco	🟢	🔵🟢🔴	🔵🟢🔴	🔵🟢🔴
Waitrose	🟢	🔵🟢🔴	🔵🟢🔴	🔵🟢🔴

* These data were collected from the main supermarkets in Reading -UK and their websites.

Table 3.2, Table 3.3 and Table 3.4 show the cow's milk prices (pence/L) and volume of bottles available in the main UK supermarkets between April 2022 and April 2024. A total of 115 and 123 conventional fresh cow milk bottles, with varying fat content and volumes, were identified during 2022 and 2024, respectively. In 2024, the available bottles of fresh milk consisted of 34 whole pasteurised, 3 whole filtered, 36 semi-skimmed pasteurised, 10 semi-skimmed filtered, 30 skimmed pasteurised and 3 skimmed filtered milk bottles. The data indicates an increase in the availability of filtered milk, particularly whole filtered milk. Previously, Cravendale was the primary brand offering whole filtered milk. However, by 2024, six additional brands (Tesco,

Waitrose, M&S, Lidl, Sainsbury's and Morrisons) have entered the market, expanding the variety of options available to consumers. Notably, the availability of skimmed pasteurised milk declined in 2024 compared to 2022, especially in 0.5 L volumes. However, two additional brands (Tesco and Waitrose) began selling skimmed filtered milk alongside the Cravendale brand, expanding the market in this category. Consistent demand for semi-skimmed milk remains, but the shift towards filtered milk is clear, with two additional brands now offering filtered semi-skimmed milk.

As shown in **Table 3.2**, **Table 3.3** and **Table 3.4**, there are significant differences in the mean prices of filtered and pasteurised milk across different bottle volumes, both within each year and between 2022 and 2024 ($p < 0.05$). All pairwise comparisons between the two years for each milk category and volume show significant differences, indicating notable changes over time. There were statistically significant differences ($p < 0.05$) between the price of filtered and pasteurised milk prices. The pooled analysis showed that the average prices of whole, semi-skimmed and skimmed filtered milk were higher than those of pasteurised milk by 37, 34 and 41 %, respectively ($p < 0.05$). This price increase could be attributed to the operational and cleaning costs associated with the microfiltration process (Brans et al., 2004; Papadatos et al., 2003). Fresh conventional cow's milk is available in four different sizes (0.5, ~ 1, ~ 2 and ~ 3 L), with smaller bottles (0.5 and 1 L) being more expensive per litre than the larger bottles (2 and 3 L). The most commonly available bottle volume was 2 L of semi-skimmed milk, for both filtered and pasteurised, followed by whole milk. Skimmed pasteurised milk was most frequently available in 1 L bottle. Cravendale was the only brand offering small bottles of semi-skimmed filtered milk (0.25 and 0.5 L) and a large volume of 3 L. By April 2024, the prices of pasteurised and filtered milk had increased. The price of 2 L bottle of semi-skimmed pasteurised milk rose by 25 %, while the price of filtered semi-skimmed milk increased by 38 %, and whole pasteurised and filtered milk prices were increased by 18 and 12 %, respectively.

Table 3.2: The price (pence/L) of different bottle volumes of filtered and pasteurised whole milk available in the UK markets during April 2022 and April 2024.

2022														
Litre		Asda	Co-op	Aldi	Waitrose	Lidl	M&S	Morrison	Tesco	Sainsbury's	Ocado	Muller	Cravendale	Average ± SD
0.25	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	104.0	96.8	88.0	96.8	84.7	96.8	96.8	88.0	97.0	-	-	-	94.3 ± 6.1
1	F	-	-	-	-	-	-	-	-	-	-	-	110.0	110.0 ± 0.0
	P	70.4	78.0	70.4	79.2	70.2	74.8	70.4	71.0	70.0	70.4	-	-	72.4 ± 3.5
2	F	-	-		-	-	-	-	-		-	-	115.0	115.0 ± 0.0
	P	52.4	77.0	48.0	50.6	48.0	50.6	48.0	48.0	48.0	50.6	62.5	-	53.0 ± 8.9
3	F	-	-	-	-	-	-	-	-	-	-	-	108.0	108.0 ± 0.0
	P	46.9	-	-	-	-	-	46.9	47.0	47.0	-	-	-	46.9 ± 0.05
2024														
0.25	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	150.0	167.0	150.0	168.0	150.0	150.0	180.0	150.0	150.0	-	158.0	-	157.3 ± 10.7
1	F	-	-	-		-	-	-	-	-	-	-	135.0	135.0 ± 0.0
	P	106.0	118.0	106.0	111.0	106.0	106.0	106.0	106.0	106.0	106.0	-	-	107.7 ± 3.9
2	F	95.0	95.0	85.0	95.0	85.0	95.0	95.0	95.0	95.0	-	-	120.0	95.5 ± 9.5
	P	63.8	72.2	64.0	68.2	64.0	63.8	63.8	64.0	64.0	63.8	99.5	-	68.2 ± 10.6
3	F	-	-	-	-	-	-	-	-	-	-	-	120.0	120.0 ± 0.0
	P	63.0	-	-	-	-	-	63.0	-	63.0	-	-	-	63.0 ± 0.0

***** All pairwise comparisons between years within each milk process and volume are significantly different ($p < 0.05$). F = filtered milk. P = pasteurised milk. SD = standard deviation.

Table 3.3: The price (pence/L) of different bottle volumes of filtered and pasteurised semi-skimmed milk available in the UK markets during April 2022 and April 2024.

2022														
Litre		Asda	Co-op	Aldi	Waitrose	Lidl	M&S	Morrison	Tesco	Sainsbury's	Ocado	Muller	Cravendale	Average ± SD
0.25	F	-	-	-	-	-	-	-	-	-	-	-	200.0	200.0 ± 0.0
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	104.0	96.8	88.0	96.8	84.7	96.8	96.8	88.0	97.0	-	-	-	94.3 ± 6.1
1	F	-	-	-	-		-	-	-	-	-	-	110.0	110.0 ± 0.0
	P	70.8	78.9	70.4	79.2	70.2	74.8	70.4	71.0	70.0	70.4	-	-	72.6 ± 3.6
2	F	-	75.0	59.9	70.0	59.5	-	-	68.0	68.0	-	-	95.0	70.7 ± 12.2
	P	52.4	66.1	48.0	50.6	48.0	50.6	48.0	48.0	48.0	48.4	62.5	-	51.8 ± 6.3
3	F	-	-	-	-	-	-	-	-	-	-	-	120.0	120.0 ± 0.0
	P	46.9	-	46.9	-	46.9	-	46.9	47.0	47.0	-	-	-	46.9 ± 0.05
2024														
0.25	F	-	-	-	-	-	-	-	-	-	-	-	240.0	240.0 ± 0.0
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	150.0	167.0	150.0	168.0	150.0	150.0	180.0	150.0	150.0	-	-	-	-
1	F	-	-	-	-	-	-	-	-	-	-	-	135.0	135.0 ± 0.0
	P	106.0	118.0	106.0	111.0	106.0	106.0	114.0	106.0	106.0	106.0	-	-	108.5 ± 4.3
2	F	95.0	95.0	85.0	95.0	85.0	95.0	95.0	95.0	95.0	-	-	120.0	95.5 ± 9.5
	P	63.8	72.7	64.0	68.2	64.0	63.8	63.8	64.0	64.0	63.8	99.5	-	68.3 ± 10.7
3	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	63.0	-	63.0	-	63.0	-	63.0	-	63.0	-	-	-	63.0 ± 0.0

* All pairwise comparisons between years within each milk process and volume are significantly different ($p < 0.05$). F = filtered milk. P = pasteurised milk. SD = standard deviation.

Table 3.4: The price (pence/L) of different bottle volumes of filtered and pasteurised skimmed milk available in the UK markets during April 2022 and April 2024.

2022														
Litre		Asda	Co-op	Aldi	Waitrose	Lidl	M&S	Morrison	Tesco	Sainsbury's	Ocado	Muller	Cravendale	Mean ± SD
0.25	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
0.50	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	104.0	96.8	88.0	96.8	84.7	96.8	96.8	88.0	97.0	-	-	-	94.3 ± 6.1
1	F	-	-	-	-	-	-	-	-	-	-	-	115.0	115.0 ± 0.0
	P	70.8	78.9	70.4	79.2	70.2	74.8	70.4	70.0	70.0	70.4	-	-	72.5 ± 3.7
2	F	-	-	-	-	-	-	-	-	-	-	-	95.0	95.0 ± 0.0
	P	52.4	66.1	48.0	50.6	48.0	50.6	48.0	48.0	48.0	48.0	62.5	-	52.2 ± 6.6
3	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
2024														
0.25	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	150.0	-	-	168.0	-	150.0	180.0	158.0	150.0	-	-	-	159.3 ± 12.3
1	F	-	-	-	-	-	-	-	-	-	-	-	155.0	155.0 ± 0.0
	P	106.0	118.0	106.0	111.0	106.0	106.0	106.0	106.0	106.0	106.0	-	-	107.7 ± 3.9
2	F	-	-	-	95.0	-	-	-	95.0	-	-	-	125.0	105.0 ± 17.3
	P	63.9	72.2	64.0	68.2	64.0	63.8	63.8	64.0	64.0	63.8	99.5	-	68.2 ± 10.6
3	F	-	-	-	-	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-	-	-	-	-

* All pairwise comparisons between years within each milk process and volume are significantly different ($p < 0.05$). F = filtered milk. P = pasteurised milk. SD = standard deviation.

Table 3.5 presents cow's milk samples' nutritional components and shelf-life date, categorised by processing method (filtered vs. pasteurised) and fat content (whole, semi-skimmed, skimmed). There were no changes in the food label information between 2022 and 2024. As expected, removing fat from milk significantly impacts its composition (Brisson & Singh, 2013; Luisa, 1995). Whole cow's milk had higher energy values and fat content compared to both semi-skimmed and skimmed cow's milk. In contrast, the protein, sugar and calcium content of skimmed milk was higher than in semi-skimmed and whole milk, due to the higher fat content in whole milk which means that there is less room for protein and other nutrients within the same volume, resulting in a slightly lower protein content compared to skimmed milk (Brisson & Singh, 2013; Luisa, 1995). This report compares filtered and pasteurised milk to assess the differences in nutritional content and shelf life, based on information provided by the milk brands websites and packaging. There was a significant difference ($p < 0.05$) in some of the milk components protein, sugar and energy. The pooled analysis showed that filtered and pasteurised differs in protein, sugar and consequently energy. During microfiltration, some protein loss is expected due to fouling, aggregation, or adsorption onto fat globules, which prevents the protein from passing through the membrane pores. As a result, filtered milk generally contains less protein than pasteurized milk (Elwell & Barbano, 2006; Kelly & Zydney, 1997; Lay et al., 2021). Interestingly, the protein loss in semi-skimmed filtered milk is higher than the reduction observed in whole and skimmed milk. Whole and skimmed filtered milk contained more energy than whole and skimmed pasteurised milk, however, semi-skimmed filtered milk contained lower energy compared to semi-skimmed pasteurised milk. This could be related to interactions within the food matrix during processing (Ding et al., 2022). While whole and skimmed milk exhibited similar trends in composition, semi-skimmed milk demonstrated a different pattern. This deviation suggests that there may be unique factors influencing the composition of semi-skimmed milk during processing. To better understand these differences, further investigation is required to uncover the underlying reasons for the observed variations, particularly focusing on the role of fat content and its interaction with the milk matrix during processing.

In addition to nutritional differences, filtered milk has a longer shelf life (up to 21 days unopened and 7 days after opening) compared to pasteurised milk (about 7 to 10 days unopened and 3 days after opening) (Table 3.5). The processing method significantly affects the shelf life of milk. The microfiltration process removes more microorganisms and somatic cells that contribute to

spoilage, allowing filtered milk to stay fresh for a longer period (Elwell & Barbano, 2006). While pasteurisation effectively reduces harmful bacteria, it does not eliminate as many spoilage organisms as microfiltration, resulting in a shorter shelf life (Elwell & Barbano, 2006; García & Rodríguez, 2014; Hoffmann et al., 2006). This extended shelf life of filtered milk makes it a preferred choice for consumers who prioritise convenience and reducing food waste. As mentioned in the Delley and Brunner (2020) survey and Mintel (2021) report, shelf life is one of the key factors influencing consumer choices.

Table 3.5: The average of nutritional components and shelf-life date of milk samples, categorised by processing method (filtered vs. pasteurised) and fat content (whole, semi-skimmed, skimmed). No differences in the food label information between 2022 and 2024.

	Whole		Semi-skimmed		Skimmed	
Variable /100 g	Filtered (n=8)	Pasteurised (n = 14)	Filtered (n = 10)	Pasteurised (n = 14)	Filtered (n = 3)	Pasteurised (n = 11)
Energy (kcal)	66.1 ±1.11a	65.47 ±0.55b	47.0 ±1.45a	48.4 ±2.02b	37.0 ±0.00a	35.9 ±0.98b
Fat (g)	3.64 ±0.10a	3.60 ±0.04a	1.7 ±0.05a	1.7 ±0.21a	0.30 ±0.00a	0.31 ±0.18a
Saturated fat (g)	2.36 ±0.05a	2.40 ±0.04a	1.05±0.05a	1.04 ±0.11a	0.10 ±0.00d	0.1 ±0.00d
Sugar (g)	4.75 ±0.01c	4.71 ±0.05b	4.78 ±0.08a	4.70 ±0.07b	4.99 ±0.10a	4.90 ±0.04b
Protein (g)	3.34 ±0.04a	3.45 ±0.08b	3.40 ±0.24a	3.60 ±0.00b	3.50 ±0.010a	3.6 ±0.000b
Salt (g)	0.10 ±0.01a	0.10 ±0.01b	0.10 ±0.01a	0.10 ±0.00b	0.11 ±0.00a	0.11 ±0.010a
Calcium (mg)	124.0 ±0.0a	123.2 ± 1.59a	122.4 ±2.1a	124.3 ±1.02b	129.0 ±0.00a	129.1 ±1.120a
Expire date (days)	up to 21	> 7	up to 21	> 7	up to 21	> 7
Use within (days)	7	3	7	3	7	3

* Values (mean ± standard deviation) with different letters within the same milk category indicate significant differences between filtered and pasteurised ($p < 0.05$).

3.5. Conclusion

This database collection examined the availability of fresh conventional cow's milk in the UK market in 2022 and 2024. There is a variety of milk categories, including whole, semi-skimmed and skimmed, available in different volumes and processes. While pasteurised semi-skimmed milk

remains the predominant type, filtered milk has seen a notable increase in market share during these two years. This report suggests that filtered milk is becoming one of the main fresh milk options alongside pasteurised milk. The effect of microfiltration on milk protein structure and peptide released after *in vitro* digestion will be discussed in the following chapters. Further in-depth research is needed to investigate the effect of microfiltration on the biological and physicochemical properties of milk components.

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Chapter 4. Effect of microfiltration on cow's milk protein microstructure

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4.1. Abstract.

Milk proteins are essential components due to their nutritional value, functional properties in food systems, and potential health implications, including support for bone strength and responses related to allergens. These properties are significantly influenced by the protein structure. In this study, key parameters measured to assess protein structure include free thiol groups, which indicate protein folding and stability, and particle size, which reflects the interactions between proteins and fat in milk. Microfiltration is a process often used in conjunction with pasteurisation to produce filtered milk. This study aimed to evaluate the effect of microfiltration on the protein structure of cow's milk compared to pasteurisation. To achieve this aim, commercially available semi-skimmed filtered-pasteurized cow's milk samples were collected from major food retailers in the UK. Semi-skimmed pasteurised (non-filtered) milk samples from the same brands were used for comparison. The Z-average as measured by DLS of filtered milk (168 to 198 nm) samples was significantly ($p < 0.05$) larger than pasteurised milk (159 to 185 nm) across all the commercial milk brands. Furthermore, differences were observed in the content of the free thiol group, with filtered milk having significantly ($p < 0.05$) lower free thiol concentration compared to pasteurised milk for all commercial milk brands analysed (1.04 to 1.29 mM and 0.79 to 0.95 mM, respectively). The Confocal Laser Scanning Microscopy (CLSM) indicated heterogeneities in the distribution of fat and proteins associated with milk processing. All filtered samples showed that there was additional interaction between the fat globules and proteins. The results bring new interesting insights on the potential impact of microfiltration on protein structure. Further investigations are needed to determine the benefits of these changes on protein bioavailability and human health.

4.2. Introduction.

Milk proteins are an essential component of the human diet, for both nutritional and functional purposes. The structure of milk proteins can be modified by processing conditions, leading to altered interactions between proteins and other nutrients in the milk; consequently, this affects the properties and functionality of the milk proteins (Bhat et al., 2021; Krishna et al., 2021; Nunes & Tavares, 2019; van Lieshout et al., 2020). The composition of processed milk, particularly as a milk matrix, and the impact of its properties on physiological and physicochemical functions are significant focuses of research (Capuano & Janssen, 2021; Ding et al., 2022; Iqbal et al., 2024; Liu et al., 2022; Lopez et al., 2011; Ye et al., 2017). Milk microfiltration is an emerging process that offers similar or better nutritional value, microbial removal and shelf stability compared to thermal processes (Elwell & Barbano, 2006). Commercial microfiltered milk usually undergoes two different treatments, pasteurization, and microfiltration to ensure milk is safe to be consumed (Cheryan, 1998, Elwell and Barbano, 2006, Solanki and Rizvi, 2001, Maubois, 1991). The combination of pasteurisation and microfiltration extends the shelf-life of pasteurised milk by removing the somatic cells and microorganisms and stopping the native proteinase enzymes (Dinkçi & Sirbu, 2024; Wang et al., 2019). Commercially filtered milk undergoes microfiltration, pasteurisation, and homogenisation. The process begins with skimming the raw milk. The skimmed milk then passes through a microfiltration membrane, while the cream undergoes ultra-high temperature (UHT) treatment. After microfiltration, the filtered skimmed milk is homogenised with the cream and subsequently pasteurised to ensure that the filtered milk is ready for consumption (Elwell & Barbano, 2006).

Previous research indicates that shear force during microfiltration can destabilise the native structure of proteins (Kelly & Zydney, 1997), thus it is necessary to examine the impact of this force on the milk matrix during commercial processing. The thiol and disulfide groups are important active groups that undergo several reactions and interactions during milk processing, impacting the biochemical and biological properties of milk (Owusu-Apenten, 2005). The highest amount of thiol groups is present in whey proteins, however, during milk processing the disulfide groups present in the casein fraction are involved in thiol-disulphide exchange reactions with the milk fat globule membrane proteins (Ding et al., 2022; Owusu-Apenten, 2005; Ye et al., 2004). One of the key activities of thiol groups is their role in modifying allergenicity (Bu et al., 2013;

Miciński et al., 2013; Rahaman et al., 2015). Allergic reactions occur when an allergen, typically a protein or peptide, is recognised by the immune system as harmful, leading to its binding with immunoglobulin E (IgE) antibodies. Changes in protein structure, such as unfolding or aggregation, can either expose or mask (or bury) these thiol groups, directly influencing the allergenic potential of the protein (Bu et al., 2013; Miciński et al., 2013; Rahaman et al., 2015; Wilson et al., 2008; Wu et al., 2018; Zhou et al., 2024). In addition to protein interactions, the Activity of fat globules during milk processing is crucial. Pasteurization causes the denaturation of both milk proteins and milk fat globule membrane (MFGM) proteins (Argov et al., 2008; Nunes & Tavares, 2019). In contrast, microfiltration can lead to the migration or retention of small fat globules within skim milk, thereby altering its overall composition (Anderson & Brooker, 1975; Jhanwar & Ward, 2014). These processes highlight how processing methods significantly influence protein and fat interactions within the milk matrix, contributing to changes in the structure, bioavailability, and functionality of milk.

Most of the studies about filtered milk have been focused on the self-life and the microbiological load, indicating that filtered milk has a longer shelf-life and low microbiological load in comparison with pasteurised milk (Bellassi et al., 2020; García & Rodríguez, 2014; Hoffmann et al., 2006). We hypothesise that milk microfiltration may impact protein structure and therefore this study aimed to evaluate the effect of microfiltration on protein structure, as understanding these structural changes is crucial for assessing protein properties such as bioavailability and functionality.

4.3. Materials and Methods.

4.3.1 Materials

All chemicals were of analytical grade. Nile Red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, was purchased from MedChemExpress (Milwaukee, WI, USA)) and used to stain fat globules (1 mg/mL in acetone). Fast Green was purchased from Sigma-Aldrich (UK) to stain protein (0.1 mg/mL in water). Ellman's reagent DTNB (5,5-dithio-bis-2-nitrobenzoic acid) and L-Cysteine, 98 % were purchased from Thermo Scientific (UK).

4.3.2 Sample collection.

Eight filtered, pasteurised cow's milk samples from different brands were bought from the main retailers in Reading (UK). Seven pasteurised cow milk samples from the same brands were also bought for comparison. The Cravendale (Cr) brand was only available in filtered milk format. All milk samples were semi-skimmed and homogenised. The sample codes, label information, brand, and process details are shown in **Table** 4.1. Three different batches (between March and August 2022) of each milk sample were used to conduct the analyses. All milk samples were transported to the laboratory in a cool box within 60 min of purchase. All samples were mixed, aliquoted and stored at -20 ^{o}C until analysis.

Table 4.1: Commercially available filtered (F) and pasteurised (P) milk used in this study, including macronutrient content as indicated on label information.

Sample code	Brand	Process	Label information (g/100 mL)		
			Fat	Protein	Sugar
A	ASDA	F	1.8	3.6	4.8
		P	1.8	3.6	4.8
Co	CO-OP	F	1.8	3.6	4.8
		P	1.8	3.4	5.0
DM	Dairy Manor / Aldi	F	1.8	3.6	4.8
		P	1.7	3.5	4.7
CB	Cow Belle / Lidl	F	1.8	3.6	4.8
		P	1.8	3.6	4.8
T	Tesco	F	1.8	3.3	4.9
		P	1.8	3.6	4.8
S	Sainsbury's	F	1.6	3.1	4.9
		P	1.8	3.6	4.8
W	Waitrose	F	1.6	3.3	4.9
		P	1.8	3.6	4.8
Cr	Cravendale	F	1.7	3.6	4.8

4.3.3 pH determination.

The pH was determined by standardizing the pH meter (Thermo Scientific, Orion Star A111 Benchtop pH Meter, UK) with buffer solutions pH 4 and 9 and the pH of the milk was determined at ~ 10 °C.

4.3.4 Proximate analysis by Lactoscope

Semi-skimmed milk samples were analysed for total protein, fat, solids contents and lactose by Lactoscope (Delta Instruments Type C4-2.3, Sofia, Bulgaria). The device was calibrated for semi-skimmed milk analysis and samples (200 mL) were heated in a water bath until they reached a temperature of 40 ºC before being analysed by the Lactoscope. Measurements were performed in triplicate.

4.3.5 Particle size measurement by Dynamic Light Scattering.

Particle size analysis was measured using a Malvern Instrument (Zetasizer software Version 7.13 Orsay, France) according to Mootse et al. (2014). Milk samples were diluted with water (1:200 (s/w)) and filtered using a 0.45 µm pore size disposable syringe filter (Millex MCE, Merrek, Germany) syringe filter and kept overnight at 4 ºC before analysis. Water was considered the solvent with a refractive index of 1.330, measured at 25 ºC. For each sample, triplicate measurements were performed and ten readings from individual samples were collected.

4.3.6 Free thiol group content by Ellman's reagent.

The reactive thiol groups were determined on diluted samples (1:100, milk: distilled water) with Ellman's reagent (Guingamp et al., 1993; ThermoScientific, 2011). A solution of thiol content was expressed as cysteine; for this purpose, a standard curve was constructed with a standard cysteine solution at pH 8.2 (0.25 to 1.5 mM, r = 0.99). A 50 µL of Ellman's Reagent solution (4 mg DTNB/1 mL sodium phosphate, 0.1 M, pH 8.0), 2.5 mL of sodium phosphate, 0.1 M and 250 µL of each standard or samples mixed in a test tube and incubated at room temperature for 15 min. Absorbance measurements at 412 nm were performed on Perkin Elmer (Lambada XLS and XLS+, 8382 V3.0.1, Norwalk, USA). Sodium phosphate buffer was used instead of sample, as a reagent blank. Each determination was made in triplicate.

4.3.7 Fat (cream) Separation

Due to the well-established fact that homogenisation reduces the size of the fat globules and causes milk proteins to become associated with milk fat (Berton et al., 2012; Michalski et al., 2002; Ye et al., 2017), this step aimed to determine if there was any adsorption between the fat globule membrane protein and milk proteins in these commercially processed milk samples. Therefore, all

milk samples were skimmed under centrifuge conditions of 2500 *x g* for 30 min at 4 °C (Thermo Scientific Medifuge Centrifuge, Germany). This step was performed specifically to identify any differences in the adsorption or intermolecular interactions between fat and proteins in filtered and pasteurised milk samples. The separated cream from the milk samples was analysed by Confocal Laser Scanning Microscopy (CLSM).

4.3.8 Protein microstructure evaluation by Confocal Laser Scanning Microscopy.

The microstructure of semi-skimmed filtered and pasteurised milk samples was studied using CLSM analysis (Nikon A1R, USA) with a 60x oil immersion objective lens, as previously described by Gallier et al. (2010) and Gallier et al. (2012). Milk samples were diluted with distilled water (1:50), then 200 µL of diluted milk was stained with Fast Green (5 µL) and Nile Red (10 µL). For casein structure analysis, 200 mL of milk was acidified with HCL (0.5 M) to pH 4.6 to separate the casein from milk serum, then centrifuged at 2500x g for 20 min at 4 ºC (Thermo Scientific Medifuge Centrifuge, Germany). The crude casein was washed three times with distilled water, then redissolved in water at pH 6.8 and diluted for staining. The cream separated from the milk samples was diluted, and the same stain steps were followed. The fat globule, stained with Nile Red, was excited at 488 nm, whereas milk proteins, stained with Fast Green, were excited at 633 nm. The stained sample (20 µL) was transferred to the cavity slide (75x26 mm) (Eisco Microscope, BI0086B, UK), covered with a glass coverslip (0.17 mm thick) and secured with nail polish (Rimmel London, UK). All images were acquired at room temperature. Three brands were randomly selected, ASDA (A), CO-OP (Co) and Tesco (T), to study the protein structure of filtered and pasteurized milk, casein and cream. The images were processed by Nikon NIS-Elements Imaging Software version 5.42.02 (Czech Republic). Image analysis was performed using ImageJ Fiji-64 software (USA) to count the number of fat globules and measure the area of the fat globules. Three images per sample were analysed, the software counted particles, and the average particle surface area was expressed in μm^2.

4.3.9 Statistical analysis

Results were expressed as the mean of triplicate determinations ± standard deviation. XLSTAT (version 2022.2.1) was used for all statistical analyses. The significance of differences between filtered and pasteurized milk samples within the same brand was assessed using an independent samples t-test, with a p-value of less than 0.05 considered statistically significant. To evaluate the

overall effect of milk processing methods (filtered vs. pasteurised) across all brands, a one-way ANOVA was employed. This analysis allowed for the assessment of differences in the selected variables due to processing methods, irrespective of the brand. Comparisons between samples under the same treatment (such as filtered-filtered or pasteurised-pasteurised) were not discussed in this study. Additionally, Pearson's correlation coefficient was calculated to assess the strength and direction of linear relationships between key variables, including Z-average, particle size distribution, and free thiol group content.

4.4. Results and Discussion

4.4.1 Proximate analysis and pH

The pH of filtered and pasteurised milk samples ranged from 6.73 to 6.80. No significant differences ($p > 0.05$) were observed between the samples independent of their treatment (**Table 4.2)**. These values are in agreement with previously published data (Tsioulpas et al. 2007, On-Nom et al. 2010), where the pH of cow milk varies between 6.55 and 6.8 and is dependent on many factors such as feeding, stage of lactation, processes, measurement conditions, etc.

Within the same brand, no significant differences were found in the protein values in filtered milk compared to the pasteurised counterparts **Table 4.2**. However, the overall analysis of the filtered milk samples showed a significantly ($p < 0.05$) lower protein content (3.42 ± 0.11 g/100 mL) than pasteurised samples (3.61 ± 0.15 g/100 mL). The decrease in protein content due to microfiltration could be attributed to the removal of some of the aggregated protein through membrane fouling in the retentate phase (Elwell & Barbano, 2006; Hoffmann et al., 2006; Kelly & Zydney, 1997; Lay et al., 2021).

The lactose and total solids are shown in **Table 4.2**. Lactose is a major carbohydrate of milk ranging between 4 – 5 %, it is a disaccharide sugar consisting of glucose and galactose (Fox, 2003). The lactose content of filtered and pasteurised milk samples ranged from 4.7 to 4.9 % with no significant differences ($p > 0.05$) between them. As expected, the total solids content for all filtered milk samples was 10.52 ± 0.07 g/100 mL, which was significantly lower than in pasteurised milk samples (10.73 ± 0.05 g/100 mL) ($p < 0.05$). This reduction is due to the protein fouling during the microfiltration (Kelly & Zydney, 1997), as shown in the protein content **Table 4.2**.

Table 4.2: Proximate analysis (protein, lactose and solids) and pH in filtered and pasteurised milk. Mean ± standard deviation.

Milk brand	Process	pH	(g/100 mL milk)		
			Protein	Lactose	Solids
A	F	6.77 ± 0.04	3.44 ± 0.18	4.86 ± 0.25	10.50 ± 0.25
	P	6.78 ± 0.05	3.53 ± 0.27	4.84 ± 0.36	10.64 ± 0.36
Co	F	6.73 ± 0.04	3.41 ± 0.25	4.91 ± 0.47	10.70 ± 0.47
	P	6.76 ± 0.03	3.55 ± 0.36	4.88 ± 0.36	10.77 ± 0.53
DM	F	6.75 ± 0.06	3.45 ± 0.34	4.94 ± 0.29	10.50 ± 0.22
	P	6.76 ± 0.04	3.61 ± 0.27	4.87 ± 0.47	10.76 ± 0.39
CB	F	6.72 ± 0.03	3.33* ± 0.33	4.89 ±0.54	10.42* ± 0.31
	P	6.73 ± 0.04	3.73 ± 0.54	4.87 ± 0.38	10.77 ± 0.59
T	F	6.77 ± 0.05	3.43 ± 0.38	4.92 ± 0.26	10.59 ± 0.29
	P	6.76 ± 0.06	3.47 ± 0.27	4.89 ± 0.34	10.66 ± 0.37
S	F	6.75 ± 0.03	3.31 ± 0.28	4.90 ± 0.47	10.59 ± 0.34
	P	6.78 ± 0.05	3.53 ± 0.45	4.98 ± 0.55	10.79 ± 0.47
W	F	6.76 ± 0.04	3.43 ± 0.36	4.88 ± 0.38	10.71 ± 0.21
	P	6.77 ± 0.06	3.57 ± 0.28	4.91 ± 0.25	10.84 ± 0.41
Cr	F	6.79 ± 0.05	3.41 ± 0.34	4.90 ± 0.48	10.58 ± 0.33

* donate that there is a significant difference ($p < 0.05$) between the protein content of filtered and pasteurised milk within the same brand.

Within the same brands, filtered samples showed lower total solids than pasteurised milk samples ($p > 0.05$). However, only one brand (CB) showed significantly lower total solids in filtered milk than in pasteurised milk. Additionally, this sample had lower protein content compared to the pasteurised CB.

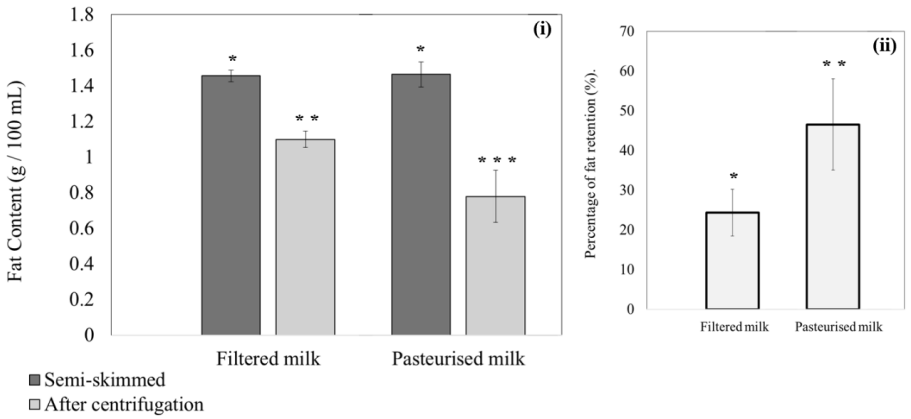
The Lactoscope measures the fat as well as protein, lactose and total solids. Overall, there were no significant differences *($p > 0.05$)* in the fat content between filtered and pasteurised milk. This was also observed for each of the milk brands (**Table 4.3**). Since some food processing, such as pasteurisation, ultra-high temperature and homogenisation alters protein structure and enhances protein-fat interaction, they consequently impact the physicochemical and biological properties of proteins (Han et al., 2020; Kopf-Bolanz et al., 2014; Loveday, 2023; Ye et al., 2017). We hypothesise that the shear force during the microfiltration (Kelly & Zydney, 1997) could affect protein structure and protein-fat interaction. To infer the interaction between the fat globule membrane and proteins in these commercially processed milk samples, all milk samples underwent centrifugation to remove fat (cream) as a first step to identify differences in the fat retention rate to predict the protein-fat interaction before conducting further experiments.

Homogenisation and pasteurisation significantly alter the structure and composition of milk proteins and fat globules. During homogenisation, the size of fat droplets is reduced, and the interfacial membrane surrounding the fat globules is modified. Furthermore, pasteurisation further alters the milk proteins, impacting their conformation and interactions with fat, thereby enhancing protein-fat interactions. The combination of these processes affects the structural composition of milk, making it difficult to remove all fat after these processes. Additionally, these changes can influence the digestion of milk fat, as droplets coated with proteins (as a result of homogenisation and pasteurisation) exhibit different digestive characteristics compared to those surrounded by native fat globule membranes (Fox et al., 2015; Kopf-Bolanz et al., 2014). Although complete skimming of these commercially homogenised milk samples was not expected, all filtered milk samples showed different results compared to pasteurised milk samples **(Figure 4.1)**. Combined analysis of the fat content in all filtered milk, after centrifugation and removing the cream layer, revealed higher fat content compared to pasteurised milk ($p < 0.05$) (**Figure 4.1** (i)), while the fat retention rate was higher in the pasteurised milk ($p < 0.05$) (**Figure 4.1** (ii)). Additionally, the fat (cream) separated from filtered milk samples exhibited a softer and more fragile texture than the fat from pasteurised milk (**Figure 4.1** (iii)). This led to the assumption that microfiltration could have an additional effect on protein-fat interactions. Thus, this interaction may affect fat texture and may impact protein properties.

Table 4.3: Total fat content in filtered (F) and pasteurised (P) milk samples before (semi-skimmed) and after centrifugation and percentage of fat retention.

Milk brand	Process	Fat content (g/100 mL)		Percentage of fat retention (%) [(1)]
		Semi-skimmed*	After centrifugation** [(1)]	
A	F	1.51 ± 0.08	1.22 ± 0.05	19.21 ± 2.1
	P	1.52 ± 0.07	1.07 ± 0.06	29.60 ± 3.2
Co	F	1.54 ± 0.05	1.03 ± 0.07	32.57 ± 3.0
	P	1.48 ± 0.06	0.69 ± 0.06	52.91 ± 4.4
DM	F	1.45 ± 0.04	1.12 ± 0.09	23.28 ± 2.2
	P	1.41 ± 0.07	1.03 ± 0.07	26.59 ± 2.9
CB	F	1.45 ± 0.03	1.03 ±0.04	28.57 ± 3.1
	P	1.44 ± 0.04	0.72 ± 0.08	49.84 ± 4.5
T	F	1.45 ± 0.08	1.19 ± 0.06	17.59 ± 1.9
	P	1.42 ± 0.07	0.87 ± 0.04	38.66 ± 3.3
S	F	1.45 ± 0.08	1.06 ± 0.07	27.02 ± 2.8
	P	1.48 ± 0.05	0.66 ± 0.05	55.23 ± 3.9
W	F	1.49 ± 0.06	1.14 ± 0.08	23.15 ± 2.0
	P	1.45 ± 0.08	0.77 ± 0.05	46.81 ± 4.3
Cr	F	1.38 ± 0.04	1.16 ± 0.08	15.58 ± 2.1

(*) in the column header indicates that there is no significant difference ($p > 0.05$) between the fat content of filtered and pasteurised milk within the same brand. (**) Semi-skimmed milk samples were centrifuged at 3500 x g for 30 min at 4 °C. The fat layer was then removed, and the remaining fat content was measured. ([1]) All pairwise comparisons between the fat content after centrifugation and the percentage of fat retention in the filtered (F) and pasteurised (P) milk within each milk brand are significantly different ($p < 0.05$). The values are mean ± standard deviation.



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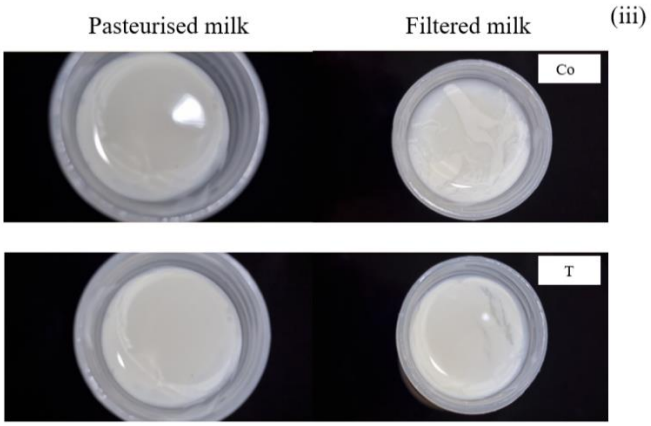


232

233 **Figure 4.1**: (i) Total fat content in all filtered and pasteurised milk before and after centrifugation and removing the
234 fat layer, (ii) Total percentage of fat retention after milk centrifugation (%) of filtered and pasteurised milk. (iii)
235 Cream separated from filtered and pasteurised milk, the tested samples are Co= CO-OP and T = Tesco. The vertical
236 lines in the graph indicate the standard error. All differences marked with *, ** and *** are statistically significant at
237 $p < 0.05$.
238

239 4.4.2 Particle Size Analysis

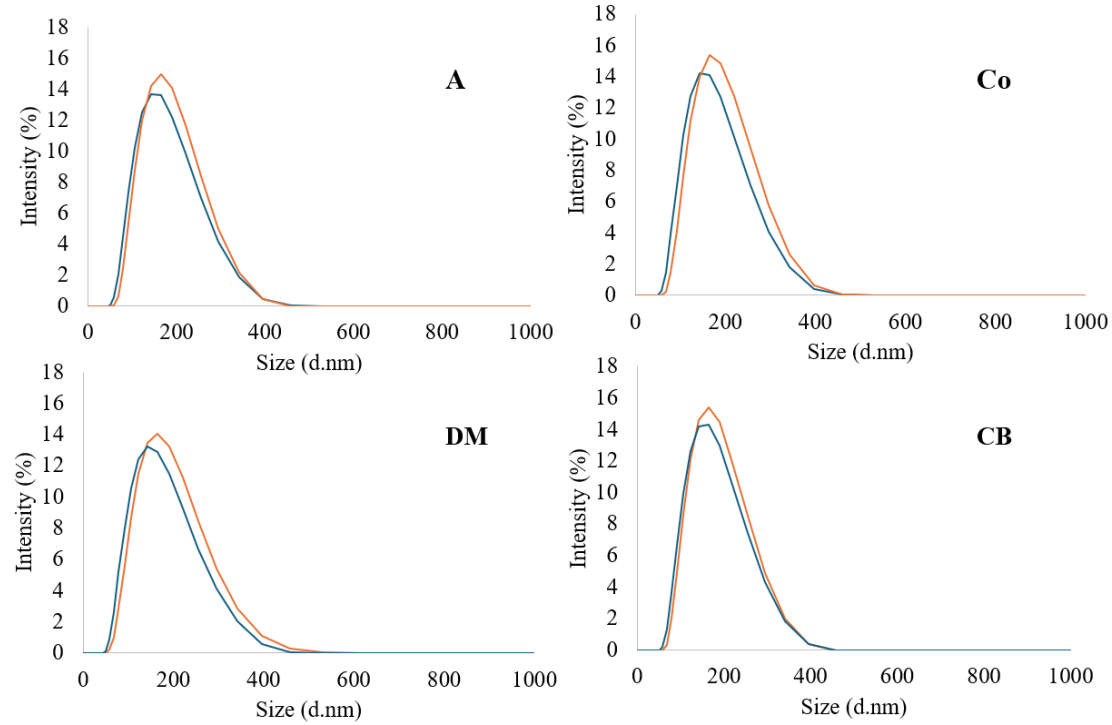
240 The particle size of milk influences its microstructure and defines many properties of dairy
241 products such as colloidal stability, texture etc (Augustin & Udabage, 2007). The average
242 diameters (Z-average) and particle size distribution of casein micelles and fat in semi-skimmed
243 homogenised filtered and pasteurised milk with sizes smaller than 0.45 μm were investigated by
244 Dynamic Light Scattering. The Z-average of particles in filtered and pasteurised milk samples
245 ranged between 186 to 198 and 159 to 185 nm, respectively (**Table 4.4**). Overall, filtered milk
246 samples showed significantly ($p < 0.05$) larger particle size diameters when compared to
247 pasteurised samples.

A single peak was observed in the size distribution in all samples, the differences in the particle size distribution in filtered and pasteurised milk samples were examined as shown in **Figure 4.2** and **Table 4.4**. All samples exhibited a single peak with the majority of particle sizes distributed between 100 to 400 nm, with no significant differences ($p > 0.05$) between filtered and pasteurised milk samples. Overall, the size distribution in all samples was similar although the size intensity or the particle concentration of filtered milk was slightly higher than that of pasteurised milk, without significant differences ($p > 0.05$). This may indicate a slight increase in the number of most common particle sizes, due to the shear force during the microfiltration, which enhances the milk matrix intermolecular interactions and subsequent different aggregation or adsorption rates between milk components (Kelly & Zydney, 1997; Lay et al., 2021). The identification of particle types was not attempted in this study. However, casein micelles typically have diameters ranging between 30 and 300 nm (Anema et al., 2005; Olson et al., 2004), leading to the assumption that larger particles are likely small fat globules or aggregated particles. Thus, the larger particles are slightly more abundant in all filtered milk compared to pasteurised milk, regardless of the source.

Table 4.4: Mean values of Z-average, primary particle size (size) and the free thiol content in filtered and pasteurised milk

Milk brand	Process	Z-average (nm)	Free thiol (μM)
A	F	196 ± 10.2	0.91 ± 0.10
	P	185 ± 9.9	1.31 ± 0.15
Co	F	188 ± 11.5	1.01 ± 0.11
	P	168 ± 8.7	1.29 ± 0.09
DM	F	194 ± 9.2	0.81 ± 0.07
	P	167 ± 11.2	1.15 ± 0.12
CB	F	197 ± 12.3	0.88 ± 0.10
	P	169 ± 9.1	1.20 ± 0.18
T	F	198 ± 14.1	1.00 ± 1.11
	P	159 ± 11.5	1.22 ± 0.98
S	F	189 ± 9.5	0.95 ± 0.08
	P	165 ± 15.2	1.24 ± 0.20
W	F	195 ± 13.9	0.93 ± 0.10
	P	172 ± 10.1	1.15 ± 0.11
Cr	F	192 ± 11.9	0.79 ± 0.16

All pairwise comparisons marked between filtered (F) and pasteurised (P) milk within each brand are significantly different ($p < 0.05$). The values are mean ± standard deviation.



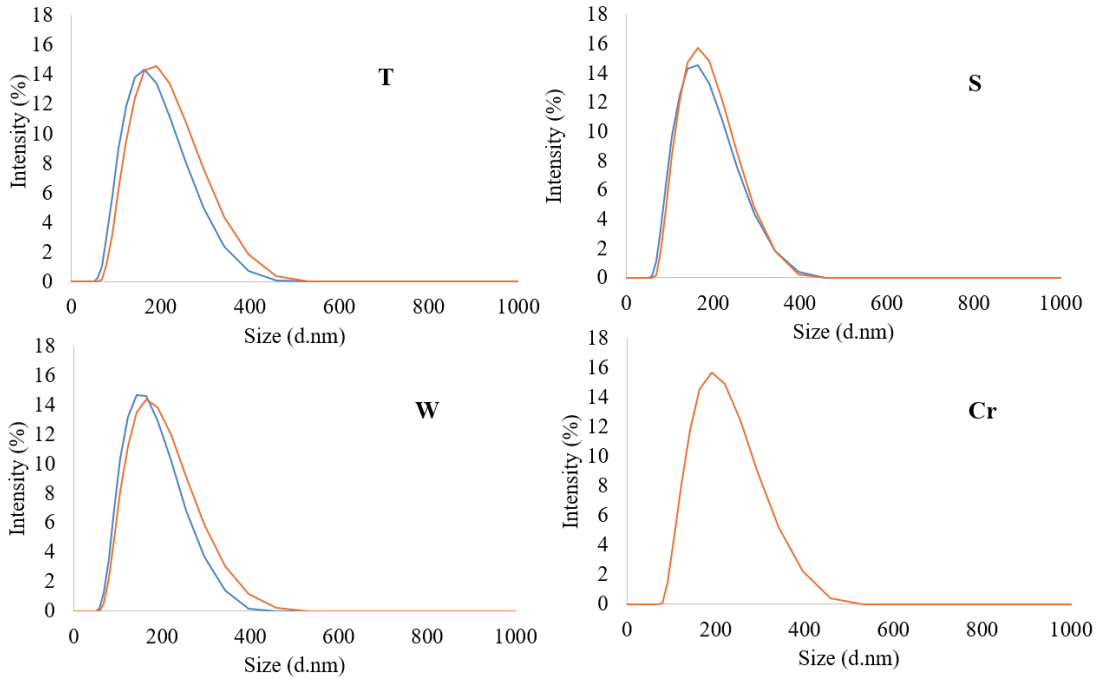


Figure 4.2: Distribution of particle sizes in DLS micrographs of *all milk samples,* filtered milk = orange line and pasteurised = blue line. Milk sample details are shown in Materials and Method.

4.4.3 Free thiol groups.

The concentration of exposed free thiol groups, which are chemical groups that can play a role in protein structure and stability, in milk samples is shown in **Table 4.4**. There were significant differences ($p < 0.05$) in the content of the free thiol group between pasteurised and filtered cow's milk (**Table 4.4**). Filtered milk consistently exhibited lower free thiol concentration compared to pasteurised milk across all brands. The lower content of free thiol groups in the filtered samples could be attributed to protein-fat interaction during the filtered milk process. Microfiltration, which separates the milk from the cream, could unfold protein structures in the skimmed milk produced due to shear forces (Kelly & Zydney, 1997; Verruck et al., 2019). This unfolding might expose more thiol groups on the proteins. During the heating of the cream, the protein on the surface of the fat globules is denatured, and its structure unfolds, exposing the thiol group. These unfolded proteins (on fat globules surface and proteins) with exposed thiols could potentially interact, leading to disulfide bond formation resulting in a decrease in free thiol groups available.. Additionally, other factors during microfiltration, such as protein adsorption onto the filtration membrane, could also contribute to the lower free thiol content in filtered milk. Supporting this concept, Ye et al. (2004) established that milk proteins can interact with milk fat membrane proteins even at temperatures lower than the protein denaturation temperature. This suggests that protein-fat interactions could occur during processing steps like microfiltration in filtered milk production. This aligns with the findings shown in **Figure 4.1** (i and ii), where the remaining fat in filtered milk after the centrifugation was higher than in pasteurised milk samples. These results show that there is a probability of intermolecular interaction (thiol-disulfide exchange reactions/ intra- and interprotein thiol-disulfide interchange reactions) between proteins and fat, thus we can correlate the decrease of the free thiol groups in filtered milk with the increase of the Z-average and decrease of the fat separation. Previous studies have shown that homogenisation and pasteurisation enhance the casein, whey and fat interaction (Berton et al., 2012; Ding et al., 2022; Ye et al., 2004), therefore, it is plausible that filtration may similarly enhance these interactions.. Correlation analysis between the particle size and free thiol group content reveals significant insight into the impact of microfiltration on milk particle characteristics compared to pasteurisation. A strong and moderate negative correlation was observed between the Z-average ($r = -0.76$, $p < 0.05$) and particle size distribution ($r = -0.63$, $p < 0.05$) with the free thiol group content, respectively. These correlations highlight the interplay between microfiltration and

particle properties. While microfiltration increases the particle size, it decreases the free thiol group content, potentially due to the shear force enhancing the intermolecular interactions. Previous research has shown that milk processing, such as heat treatments and homogenisation, can induce interactions between milk proteins (whey, casein and fat globule membrane proteins), mediated by thiol-disulphide exchange reactions. In this study, all samples (commercially filtered and pasteurised milk) underwent pasteurisation and homogenisation; however, filtered milk samples were additionally subjected to microfiltration to extend shelf-life. The results showed that filtered milk has a lower content of free thiols and a larger particle diameter compared to pasteurised milk. Since filtered milk has a lower content of free thiols, it suggests these groups might be involved in protein-fat linkages via thiol-disulphide exchange reactions. Additionally, the larger particle size in filtered milk could be another consequence of this interaction. Furthermore, **Figure 4.1** (iii) demonstrates that after centrifugation, the percentage of fat separated from filtered milk was less than the fat separated from pasteurised samples. Mechanical processes cause a migration of the membrane fat globule proteins from the surface of the membrane to the aqueous face resulting in an interaction between these proteins as mentioned by Anderson and Brooker (1975). This observation supports the hypothesis that, during the microfiltration, more proteins interact with fat globule membrane proteins via thiol-disulfide bonds and cause an increase in the particle diameter size. This interaction links the fat with protein in the aqueous phase and prevents fat separation. As protein-fat interaction occurs in some milk processing techniques, this effect may also occur during microfiltration. Further research is needed to fully elucidate the specific mechanisms behind the observed protein-fat interactions during microfiltration.

4.4.4 Protein microstructure.

The CLSM is a widely used tool to visualise changes in protein and fat as a result of food processing or digestion (Gallier et al., 2010; Gallier et al., 2012). The distribution of protein aggregates and fat globules in filtered and pasteurised milk samples, as well as in the casein and cream separated from these milk samples was observed by CLSM. Fast Green fluorescent dye was used to label the protein, and the Nile Red fluorescent probe was used to label the fat. The distribution of protein aggregates and fat globules in the milk samples is shown in **Figure 4.3** The images visualise the interaction between the proteins and fat globules in filtered and pasteurised semi-skimmed milk samples. These samples underwent heating and homogenisation leading to

protein denaturation and aggregation (Ye et al., 2017). Filtered milk samples (**Figure 4.3**), which underwent heating, homogenisation, and microfiltration, showed more interaction between protein (stained green) and fat (stained red), resulting in more yellow-orange colouration compared to pasteurised milk samples due to the interaction areas stained with Fast Green and Nile Red stains **Figure 4.3.** The dark areas correspond to the aqueous phase. The structure of the casein separated from filtered and pasteurised milk samples is shown in **Figure 4.4**. Casein from filtered milk showed more fat globule content (more red-stained particles) than the casein separated from pasteurised milk, furthermore, the casein separated from filtered milk appeared fluffier and had a cloud-like appearance with less clear, green-stained particles. These clear green areas or particles are indicative of no protein-fat interaction, as only Fast Green stain was observed in these areas. On the other hand, the cream separated from filtered milk samples showed more yellow-orange areas, indicating protein-fat interactions as visualised by the combined Fast Green (protein stain) and Nile Red (fat stain) in the same aggregates or particles (**Figure 4.5)**. By contrast, the cream separated from pasteurised milk showed mainly green particles, stained by Fast Green, suggesting the presence of proteins with less association with fat, as indicated by the reduced yellow-orange staining compared to the filtered milk. The overall number and total surface area of fat globules within the casein matrix, separated from the filtered and pasteurised milk samples, are presented in **Table 4.5**, representing the combined analysis of the three samples (filtered and pasteurised milk from A, T and Co). Casein separated from filtered milk had a significantly ($p < 0.05$) higher total fat area than casein separated from pasteurised samples. CLSM images revealed that more protein aggregates or more interactions between protein and fat were present in filtered milk, casein and cream separated from filtered milk compared to pasteurised samples. This observation agrees with the size measurements and free thiol results, where filtered milk samples showed larger particle size and less free thiol groups compared with pasteurised milk samples which led to the conclusion that protein and fat interact together via exchange reactions, intra- and interprotein thiol-disulfide interchange reactions. Microfiltration could be a promising process that can lead to structural changes in proteins and result in properties that cannot be achieved through pasteurisation. Such structural changes or thiol-related reactions can influence protein structure, potentially affecting nutrient bioavailability and digestibility, as well as allergenicity (Bu et al., 2013; Liu et al., 2022; Monaci et al., 2006; Zhou et al., 2024), while also impacting functional properties (Augustin & Udabage, 2007; Nunes & Tavares, 2019).

Table 4.5: Average fat count and total fat area in casein separated from filtered and pasteurised milk obtained by CLSM and analysed by ImageJ software.

Casein separated from	Fat count	SD	average total area (μm^2)	SD
Filtered milk	579.60*	133.62	302.02*	123.66
Pasteurised milk	325.44	183.13	161.39	102.40

SD = standard deviation. (*) indicates a significant difference ($p < 0.05$) within the column.

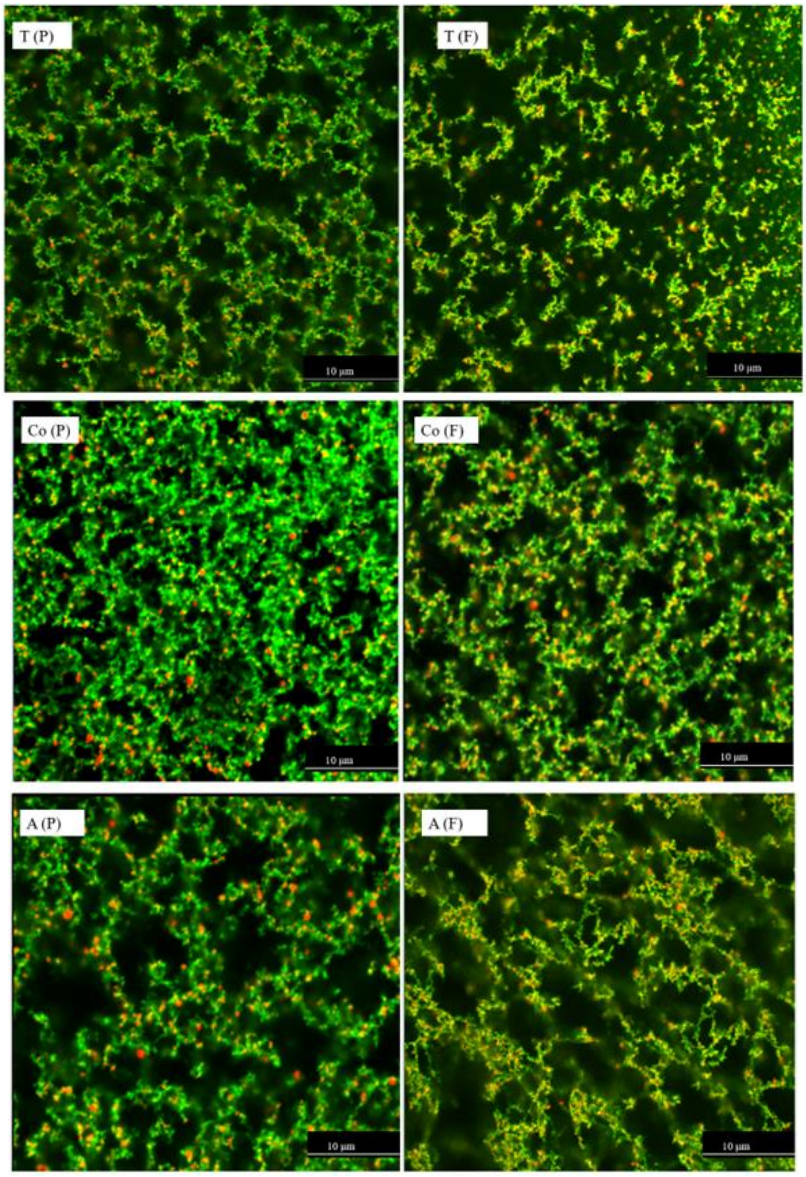


Figure 4.3: CLSM micrographs of pasteurised (P) and filtered (F) milk from three different brands (T, Co and A) stained with Nile Red-stained fat globules appearing red and Fast Green-stained proteins appearing green. The dark areas correspond to the serum. Images were captured with a 60x oil objective lens. The top image has a 2x magnified area to highlight detail. Samples were diluted 50 times with distilled water. The scale bars are 10 μm in length.

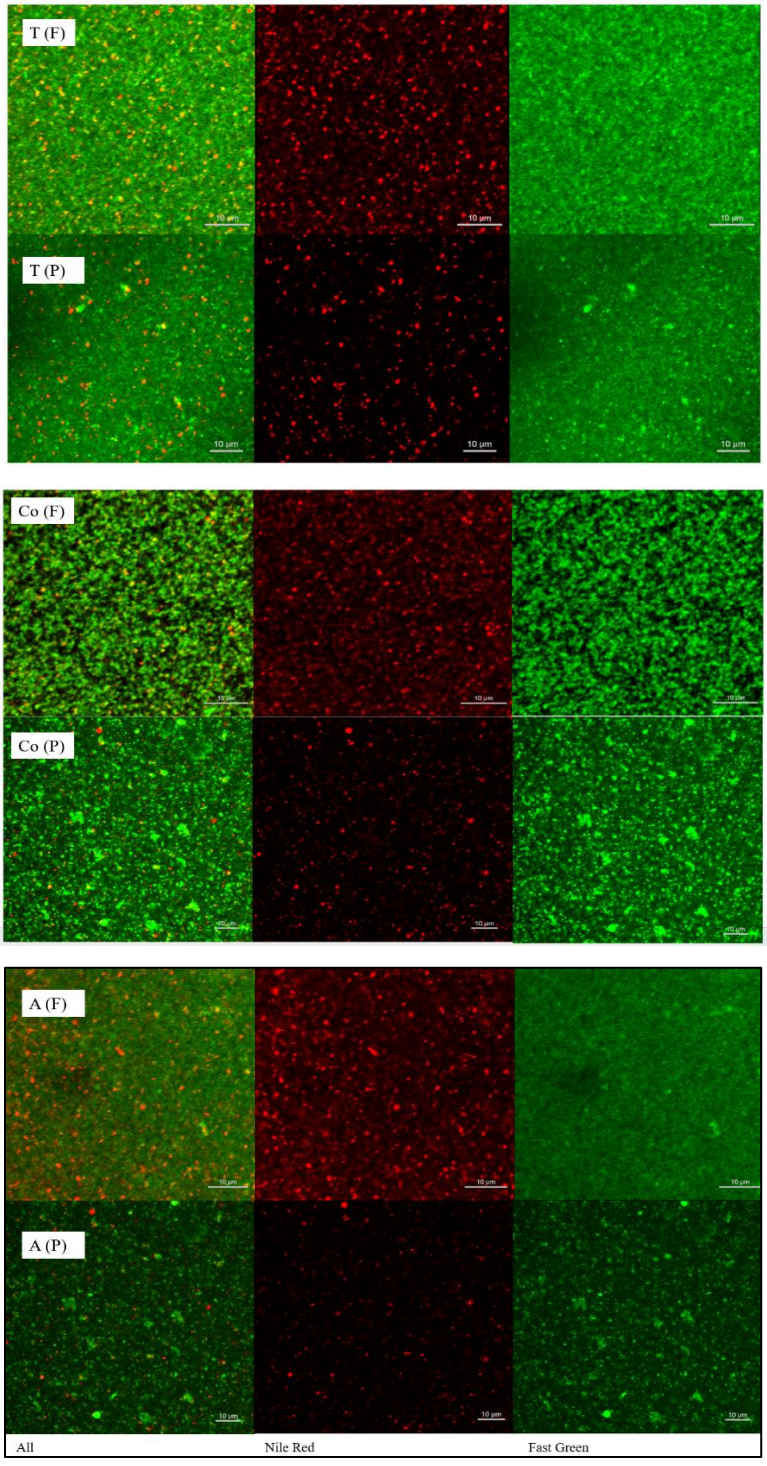


Figure 4.4: CLSM micrographs of casein separated from pasteurised (P) and filtered (F) from three different brands (T, Co and A) stained with Nile Red-stained fat globules appearing red and Fast Green-stained proteins appearing green. The dark areas correspond to the serum. Images were captured with a 60x oil objective lens. The scale bars are 10 µm in length.

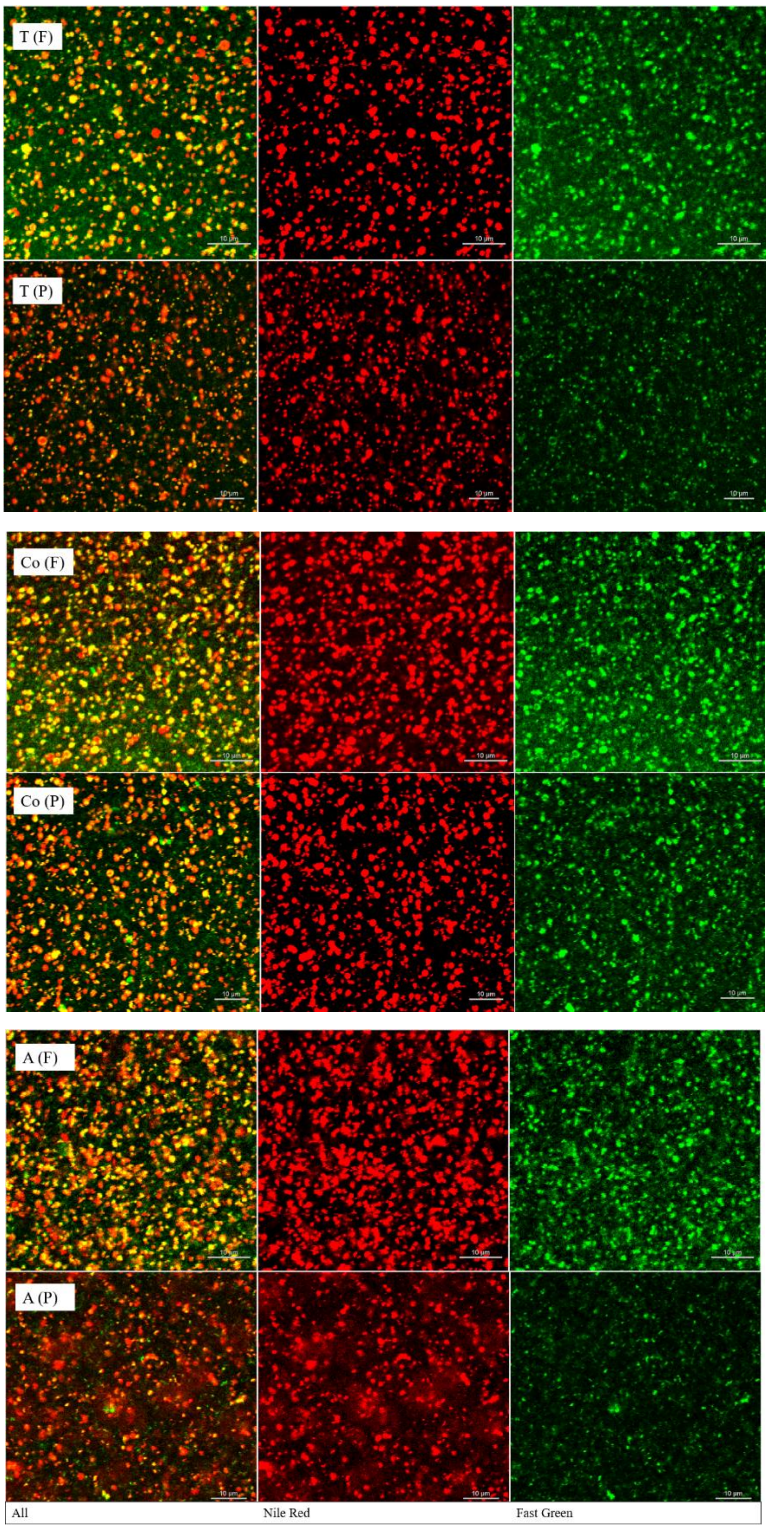


Figure 4.5: CLSM micrographs of cream separated from pasteurised (P) and filtered (F) from three different brands (T, Co and A) stained with Nile Red-stained fat globules appearing red and Fast Green-stained proteins appearing green. The dark areas correspond to the serum. Images were captured with a 60x oil objective lens. The scale bars are 10 µm in length.

4.5. Conclusions.

In summary, this study demonstrates that the free thiol content in filtered milk was lower than in pasteurised milk, while the particle size in filtered milk was larger. CLSM analysis revealed additional protein-fat interactions in filtered milk, as well as in the casein and cream fractions. This suggests that these enhanced interactions between milk proteins and fat globule membrane proteins may occur through thiol-disulfide interchange reactions. The findings indicate that microfiltration induces more protein-fat interactions compared to pasteurisation. Such structural differences in filtered milk proteins could influence protein bioavailability and functionality. These findings provide valuable insights into the impact of microfiltration on dairy processing, particularly regarding changes in protein structure and composition. The observed modifications in the interactions among casein, whey proteins, and fat may alter the exposure of allergenic epitopes, potentially influencing the allergenicity of milk. In addition, such structural changes could affect digestibility and the release of bioactive peptides, thereby contributing to both nutritional benefits and possible health risks. Future research should investigate the mechanistic links between microfiltration-induced protein modifications and allergenicity, as well as explore strategies to optimize processing parameters to enhance the health-promoting properties of dairy products.

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Chapter 5. Quantification of β-casomorphin 7 in commercially available filtered and pasteurized cow's milk.

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5.1. Abstract.

β-casomorphin 7 (BCM7) is a bioactive peptide that is released during the digestion of β-casein (in particular, A1 variant) present in cow's milk. BCM7 has been linked to several health concerns such as gastrointestinal disorders. Milk processing alters the composition of milk, which in turn may affect its digestion thus impacting the amount of BCM7 that is released. This study aimed to understand the impact of microfiltration on BCM7 release after *in vitro* digestion (mimicking in vivo digestion) of semi-skimmed filtered milk compared to pasteurized milk and pasteurized Jersey milk (which does not contain A1 β-casein the main source of BCM7). LC/MS was used to quantify BCM7. Results indicated that the β-casein variants present in milk rather than the milk treatments themselves are the key factors for the release of BCM7. Similar BCM7 levels were found in filtered and pasteurized milk samples, whereas Jersey milk released just half the amount.

5.2. Introduction.

β-casein (~ 30 % of total casein) has 12 variants (A1, A2, A3, B, C, D, E, F, G, H1, H2 and I) that differ in the amino acid sequence. The most common in cattle breeds of European origin are a mix of these variants, while, Guernsey or Jersey cows produce milk that has the A2 as a major variant (Ul Haq et al., 2014).

β-casomorphin 7 (BCM7) is an opioid peptide, that has effects like morphine, that has been isolated from an enzymatic digest of β-casein (in particular A1) and may be implicated in many illnesses such as type 1 diabetes, autism in children, sudden infant death and induce pseudo-allergic reactions (Bell et al., 2006; Cieślińska et al., 2022; Ul Haq et al., 2014). It has also been shown to

slow gastrointestinal motility (Andıran et al., 2003). Minimal amounts of BCM7 are however released from milk containing A2 β-casein as a main protein (Asledottir et al., 2018; Cieślińska et al., 2012; De Noni & Cattaneo, 2010). The difference between A1 and A2 β-casein lies in the specific amino acid at a particular position (the 67th position) in the protein sequence. A1 β-casein contains the amino acid histidine at this position in its protein sequence whereas A2 β-casein has the amino acid proline (Asledottir et al., 2018; Cieślińska et al., 2012). Pasteurisation (85 ºC/30 s) and UHT (140 ºC/15 s) of milk inhibit the formation of BCM7 during intestinal digestion which could be due to protein denaturation altering the protein digestion (Cattaneo et al., 2023; Cattaneo et al., 2020; De Noni & Cattaneo, 2010; Lambers et al., 2021), or due to the formation of radicals during Maillard reaction that could attack the protein backbone subsequently modifying some of the peptides that are formed (Meltretter et al., 2008). Traditionally milk has been subjected to heat treatments that differ in time and temperature, with pasteurisation and UHT being the most commonly used (Cattaneo et al., 2023; Cieślińska et al., 2007; Cieślińska et al., 2012; Lambers et al., 2021). However, more recently a new filtered milk, which undergoes pasteurization and microfiltration, offering a longer shelf life compared to pasteurized milk has become available in the UK supermarkets. In 2020, the sales of filtered milk witnessed a significant boost, attributed to its extended shelf life that reduced milk wastage caused by spoilage or expiration, when compared to pasteurized milk (Mintel, 2021). Although research has investigated BCM peptides resulting from the digestion of heat-treated milk (Cattaneo et al., 2023; Cattaneo et al., 2020; Lambers et al., 2021), there are gaps in the literature concerning filtered milk and the generation of BCM7 during digestion. Hence, this study aimed to assess the proportions of the main β-casein variant proteins and characterize the release of BCM7 during *in vitro* digestion of commercially filtered milk. Pasteurized milk from the same brand of filtered milk and Jersey milk (A2 milk) were used for comparison of process effect and A1 variant content, respectively.

5.3. Materials and Methods.

5.3.1 Materials

All chemicals were of analytical grade. HPLC water from Fisher Scientific (UK) was used throughout the study. Urea (99 %), dithiothreitol (≥ 98 %), trifluoroacetic acid (TFA, ≥ 99 %) and purified bovine β-casein proteins (≥ 98 %) from bovine were obtained from Sigma-Aldrich (UK). LC-MS grade water, formic acid and acetonitrile ≥ 99.9 % were sourced from Fisher Scientific

(UK). Pepsin from porcine gastric mucosa (P7000; 800 to 2,500 U/mg of protein), porcine bile extract (no. B8631); and pancreatin (P1750; 4 × USP) were purchased from Sigma-Aldrich (UK).

5.3.2 Samples

Commercially conventional available semi-skimmed filtered, pasteurised cow's milk (F) samples from seven different brands were bought from the main retailers in Reading (UK). Seven pasteurised cow milk (P) samples from the same brands were also bought for comparison. A Jersey whole milk sample (A1 free milk) was used as the negative control. Three different batches for each of the milk samples were used to carry out the analysis. All milk samples were homogenised. The sample codes, label information, brand, and process details are shown in **Table 5.1**. Three different replicates of each milk sample were used to conduct the analyses. All milk samples were transported to the laboratory in a cool box within 60 min of purchase. All samples were stored at –20 ºC until analysis.

Table 5.1: Conventional filtered (F) and pasteurised (P) milk and Jersey (A1 free) milk were used in this study.

Sample code	Brand	Process	Label information (g / 100 mL)		
			Fat	Protein	Sugar
A	ASDA	F	1.8	3.6	4.8
		P	1.8	3.6	4.8
Co	CO-OP	F	1.8	3.6	4.8
		P	1.8	3.4	5.0
DM	Dairy Manor / Aldi	F	1.8	3.6	4.8
		P	1.7	3.5	4.7
CB	Cow Belle / Lidl	F	1.8	3.6	4.8
		P	1.8	3.6	4.8
T	Tesco	F	1.8	3.3	4.9
		P	1.8	3.6	4.8
S	Sainsbury's	F	1.6	3.1	4.9
		P	1.8	3.6	4.8
W	Waitrose	F	1.6	3.3	4.9
		P	1.8	3.6	4.8
Jersey	Aldi	P	4.9	3.8	4.8

Milk samples purchased between January and March 2022.

5.3.3 β-casein characterisation by TOF LC/MS.

Identification and relative quantitation of β-casein variants were done according to a previously published method by Givens et al. (2013). Milk samples (1mL) were mixed with 1 mL of 8 M urea buffer containing 20 mM dithiothreitol and were kept at room temperature for 1 h. The residual fat was removed by centrifugation (Thermo Scientific Medifuge Centrifuge, Germany) for 5 min at 1200 *x g* and 4 ºC. Then 0.5 mL of the aqueous layer was diluted with 2 mL HPLC grade water and filtered by using a 0.45 µm pore size disposable syringe filter before analysis. Separation of the milk proteins was achieved using an Agilent 1100 HPLC interfaced with a Bruker Microtof QII high-resolution quadrupole time of flight instrument (Bruker Instruments, Coventry, UK). Separations were performed on a C18 reversed-phase analytical column (150 mm x 2.1 mm internal diameter) with 30 nm pore size and 5 µm particle size. The column was thermostatically controlled at 45 ºC. Mobile phase A consisted of a solution of 0.01% TFA in HPLC grade water and mobile phase B, 0.01 % TFA in LC-MS grade acetonitrile. The flow rate was set at 0.2 mL/min, the injection volume was 0.2 µL, and the total run time was 50 min. Identification was possible as a standard of β-caseins was available and analyzed alongside the milk samples. Identification was carried out by means of the MS spectra and UV chromatograms of the β-casein region, together with extracted ion chromatograms of the A1, A2, and B variants at 1144.95, 1143.05, and 1148.16 m/z, respectively (Bonfatti et al., 2008; Givens et al., 2013). The mass range was calibrated at a range of 500-3500 *m/z.* Bruker software (Data analysis version 4.0, Bruker Daltonik GmbH, Bremen) was used to identify the protein variants and relative quantitation. An external calibration curve of the β-casein standard was used for the quantification of the protein (R2 = 0.98).

5.3.4 Simulated *In vitro* digestion of milk samples.

The *in vitro* gastric and intestinal digestion model used in this study was previously described by Brodkorb et al. (2019) and Gallier et al. (2012). Twenty millilitres of milk was mixed with 10 mL of simulated gastric fluid (SGF) containing 2 g of NaCl/L and 7 mL of HCl/L at pH 1.2. The mixture was acidified with 6 M HCl to pH 1.5 and was incubated in a shaking water bath (Grant OLS 200, Grant Instruments, Cambridge, UK) at 37°C for 10 min at 95 rpm/min. Pepsin (3.2 mg/mL of SGF) was added, and the temperature and shaking were maintained for 2 h.

For the intestinal stage, the simulated intestinal fluid (SIF) was prepared with 6.8 g of K_2HPO_4/L and 190 mL 0.2 M NaOH/L and maintained at pH 7.5. The milk-SGF mixture was mixed with SIF

(1:1) to a total volume of 30 mL, adjusting the pH to 7, and adding bile extract (5 mg/mL) and Pancreatin (1.6 mg/mL). The mixture was incubated at 37 °C in a shaking water bath (Grant OLS 200, Grant Instruments, Cambridge, UK) at 95 rpm/min for 3 h. To inactivate the enzymes, samples were immediately transferred to a water bath at 95 °C for 5 min (Wen et al., 2015). All digestions were performed in duplicate and an enzyme-reagent control, matched to digestion conditions, was conducted with each set of digested samples.

5.3.5 Identification of BCM7 through Liquid Chromatography–Mass Spectrometry Analysis (LC-MS).

An Agilent 1100 HPLC interfaced with a Bruker Microtof QII high-resolution quadrupole time of flight instrument (Bruker Instruments, Coventry, UK) was used for the identification and quantification of BCM7 released after the *in vitro* digestion of milk samples. Elution solvents A and B were (A) 0.1% formic acid in water and (B) 0.1 % formic acid in acetonitrile with a gradient elution using LC/MS-grade elution solvents (Fisher Chem-ical™, Loughborough, UK) on an ACE® C-18 column (300 Å 5 µM 150 mm × 2.1 mm). The quantification of BCM7 in the samples and in the deuterated BCM7 standards (1 – 100 µg/mL, R2 = 0.9871) was accomplished by comparing the peak areas in the extracted ion chromatogram at 790.4 m/z.

5.3.6 Statistical Analysis.

Statistical analyses were performed using XLSTAT version 2022.2.1 (Addinsoft, New York, NY, USA) to assess the differences in BCM7 release between processed milk samples. A one-way ANOVA was conducted to evaluate the effects of milk type (conventional vs. Jersey) and processing method (filtered vs. pasteurized). Tukey's tests were then performed to compare filtered and pasteurized milk within the same milk type, as well as to compare conventional and Jersey milk. A significance level of $p < 0.05$ was set to determine statistical significance. No comparisons were made between individual brands.

5.4. Results and Discussion.

5.4.1 β-casein levels

The concentration of β-casein (mg/mL) and relative content of β-casein variants (as % of total β-casein) present in the conventional milk (filtered and pasteurised) and Jersey (A1 free) milk

samples is shown in **Table 5.2**. Although there are variations in β-casein content within the same processing method, these differences are most likely attributed to the milk sourced from different brands. Since this study focuses on the effect of processing, comparisons were made between filtered and pasteurised milk within the same brand. β-casein concentrations in filtered milk ranged from 10.5 to 11.3 mg/mL, while pasteurised milk contained β-casein levels ranging from 10 to 11.6 mg/mL. No significant differences ($p > 0.05$) in β-casein levels were found between filtered and pasteurised milk within the same brand. Jersey milk (A1-free) was found to contain significantly higher β-casein levels (13.80 mg/mL, $p < 0.05$). These data are consistent with previous studies reporting β-casein levels in conventional milk ranging from 9 to 13.4 mg/mL (Bonizzi et al., 2009; Farrell et al., 2004; Hallén et al., 2008), while higher levels have been reported in Jersey milk, ranging from 13.5 to 17.3 mg/mL (Auldist et al., 2004).

Table 5.2: β-Casein concentration (mg/mL) and its relative content (% of total protein) in filtered (F) ($n = 21$), pasteurised (P) ($n = 21$) and Jersey (A1 free) milk ($n = 3$).

Milk sample	β-Casein (mean ± SD)			
	Concentration mg/mL		% of total casein	
	F	P	F	P
A	10.51 ± 1.27	10.01 ± 1.20	43.55 ± 1.85	42.85 ± 2.8
Co	11.08 ± 1.15	10.82 ± 1.04	42.90 ± 2.87	41.70 ± 2.19
DM	10.71 ± 2.26	10.95 ± 1.38	42.09 ± 2.4	42.18 ± 1.90
CB	10.78 ± 1.73	11.30 ± 1.05	40.10 ± 2.97	41.62 ± 1.93
S	11.12 ± 1.80	10.75 ± 1.57	43.17 ± 3.00	42.25 ± 2.6
T	11.28 ± 1.11	11.68 ± 1.53	39.41 ± 2.64	39.23 ± 1.86
W	10.88 ± 1.61	11.08 ± 2.14	40.33 ± 2.10	41.65 ± 1.79
Jersey		13.8* ± 1.31		

All pairwise comparisons marked between filtered (F) and pasteurised (P) milk within each brand are not significantly different ($p > 0.05$). (*) indicates a significant difference between Jersey vs conventional ($p < 0.05$). The values are mean ± standard deviation.

The spectra were deconvoluted to provide the molecular mass of the proteins in the purified samples (**Figure 5.1)**, which enabled the identification of the main β-casein variants by confirming the genotype with masses of 24023, 23968 and 24092 Da for variants A1, A2 and B, respectively (Fuerer et al., 2020). All conventional milk samples had a higher content of the A2 variant compared to the A1 (**Table 5.3**). The relative content of the main β-casein variants: A1, A2, and B, ranged from 34 – 40, 56 – 63, and 2 – 3 % of total β-casein, respectively, in conventional milk. As expected, the main β-casein variant in conventional milk available in the UK market was A2, followed by A1 in agreement with (Givens et al., 2013). In alignment with previous findings,

conventional milk has been reported to exhibit A1, A2, and B variant proportions ranging from 51 – 71, 28 – 40, and 2 – 10 % of the total β-casein, respectively (Foroutan et al., 2019; Fuerer et al., 2020; Givens et al., 2013). On the other hand, A2 was the main variant in Jersey milk (~ 75 % of total β-casein), with A1 not detected (**Table 5.3**), with B β-casein as the second most prominent variant in Jersey milk, accounting for approximately 25 % of total β-casein. The results were consistent with previous data, which demonstrated a characteristic distribution of A2 and B variants in casein separated from Jersey milk. Specifically, the proportions were found to range from 48 – 60 and 28 – 40 % of the total β-casein, respectively (Foroutan et al., 2019; Fuerer et al., 2020). Understanding the distribution of β-casein variants in milk samples from different processes is important in order to monitor process-induced changes in Pro^{67} and His^{67} balance in milk and dairy products, avoiding a possible excessive increase in His^{67} in the processed milk due to milk protein denaturation or/and conformation.

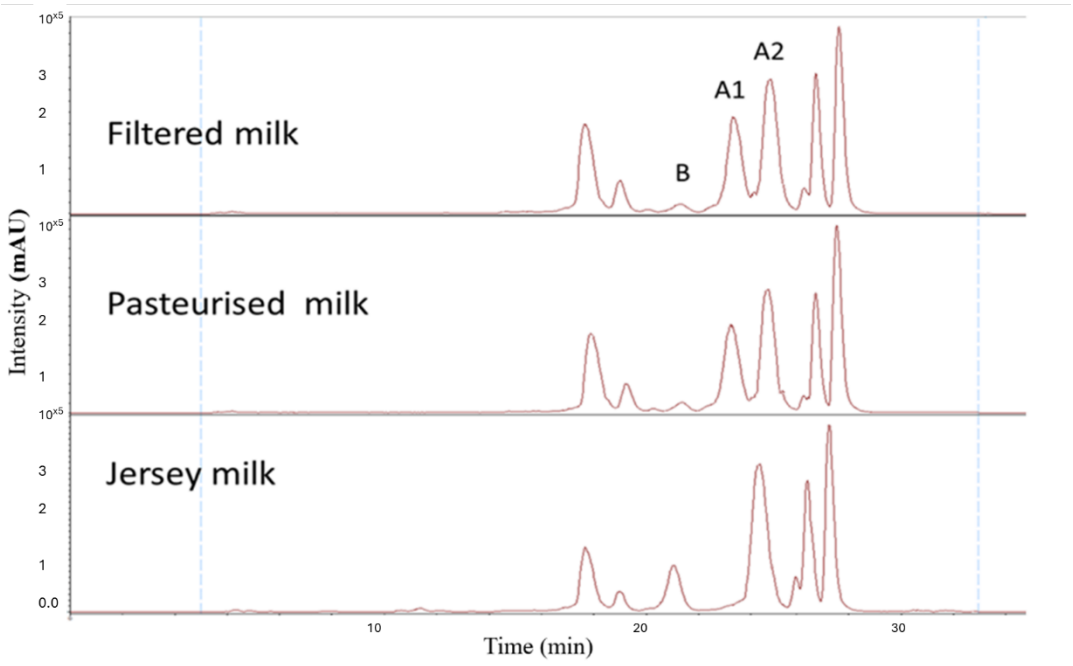


Figure 5.1: Separation of bovine β-casein proteins (A1, A2 and B) from filtered, pasteurised and Jersey milk by high resolution LC/MS at 214 nm.

Table 5.3: Relative content of β-casein variants (A1, A2 and B) (% of total β-casein) in filtered (F) (n = 21), pasteurised (P) (n = 21) and Jersey (A1 free) milk samples (n = 3).

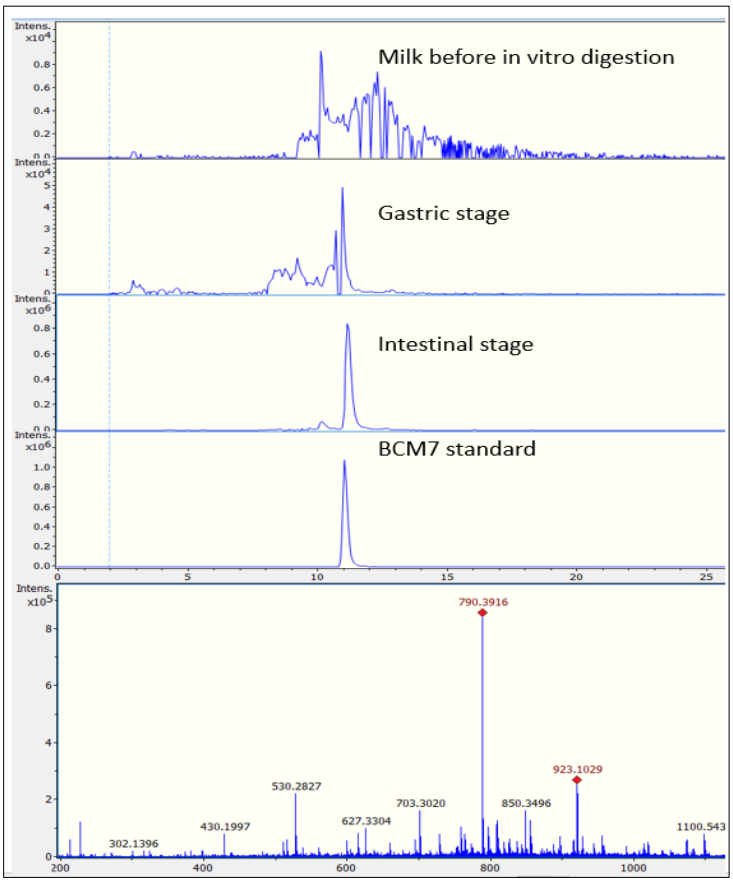
Milk brand	A1		A2		B	
	F	P	F	P	F	P
A	39.13 ± 4.20	40.08 ± 3.85	58.50 ± 3.92	57.46 ± 2.95	2.37 ± 0.90	2.46 ± 0.70
Co	40.12 ± 2.90	40.63 ± 3.61	57.48 ± 2.99	56.81 ± 3.02	2.40 ± 1.21	2.56 ± 0.73
DM	38.27 ± 4.11	37.69 ± 2.98	59.65 ± 4.20	60.07 ± 3.19	2.08 ± 0.21	2.24 ± 0.82
S	34.22 ± 3.87	33.84 ± 2.88	63.09 ± 2.01	63.42 ± 2.34	2.69 ± 0.86	2.74 ± 0.98
CB	34.19 ± 4.01	34.64 ± 3.66	62.88 ± 2.58	62.47 ± 1.90	2.93 ± 0.67	2.86 ± 0.72
T	37.34 ± 3.52	37.69 ± 192	60.15 ± 4.30	59.84 ± 2.42	2.51 ± 0.72	2.47 ± 0.90
W	34.49 ± 3.10	34.60 ± 4.61	63.06 ± 4.21	63.11 ± 2.44	2.45 ± 1.40	2.29 ± 1.11
Jersey	-	0.0	-	75.91* ± 4.32	-	24.9* ± 2.74

All pairwise comparisons marked between filtered (F) and pasteurised (P) milk within each brand are not significantly different ($p > 0.05$). (*) indicates a significant difference between Jersey vs conventional ($p < 0.05$). The values are mean ± standard deviation.

5.4.2 BCM7 peptide release by digestion of milk samples

Milk samples (filtered, pasteurised and Jersey) were also subjected to *in vitro* gastrointestinal digestion and BCM7 was quantified by TOF LC/MS. In the present work, no BCM7 was detected in either conventional or Jersey milk before and after digestion with Pepsin alone, irrespective of the milk type or processing methods employed (**Figure 5.2**). However, BCM7 was detected in all milk samples after the intestinal stage (**Table 5.4** and **Figure 5.2**). The BCM7 released after the intestinal stage ranged from 4.55 to 7.05 mg/g β-casein in filtered milk and from 4.99 to 6.89 mg/g β-casein in pasteurised milk samples, respectively. No significant differences were found in the BCM7 content between the filtered and pasteurised milk samples from the same brand ($p > 0.05$). The amount of BCM7 released from conventional milk samples was approximately double that released by Jersey milk (2.5 – 4.5 mg/g β-casein). While the BCM7 levels observed in this study were higher than those reported by Asledottir et al. (2018) (3.8 mg BCM7/g β-casein), the differences in BCM7 amounts are likely attributable to variations in sample preparation and digestion methods. Despite these differences, in agreement with Asledottir et al. (2018), milk without the A1 variant consistently released significantly lower amounts of BCM7, further

198 confirming the influence of the A1 variant on BCM7 production. While most studies correlate the
199 BCM7 released after intestinal digestion with the presence of A1 (Brooke-Taylor et al., 2017;
200 Duarte-Vázquez et al., 2017; Jianqin et al., 2016), the absence of A1 in Jersey milk was not
201 sufficient to eliminate the presence of BCM7. These results indicate that the A1 variant is not
202 solely responsible for the release of BCM7 after milk digestion, with the B variant potentially
203 acting as a contributing factor.



204

205 **Figure 5.2**: Extracted chromatographic peak of BCM7 (at mass 790.4 Da) in milk samples before and after
206 gastrointestinal digestion (picture shown Tesco pasteurised milk).
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Table 5.4: BCM7 concentration (mg/g β-casein) in filtered (F) (n =21), pasteurised (P) (n = 21) and Jersey (A1 free) (n = 3) milk samples after the *in vitro* intestinal digestion stage.

Milk brand	BCM7 (mg/g β-casein)	
	F	P
A	6.53 ± 0.20	6.44 ± 0.80
Co	4.68 ± 0.41	4.99 ± 0.66
DM	6.25 ± 0.61	6.61 ± 0.53
S	7.02 ± 0.83	6.80 ± 0.78
CB	6.14 ± 0.57	6.61 ± 0.71
T	7.05 ± 0.52	7.29 ± 0.92
W	4.55 ± 0.60	4.5 ± 0.53
Jersey*	-	3.5 ± 0.64

All pairwise comparisons marked between filtered (F) and pasteurised (P) milk within each brand are not significantly different ($p > 0.05$). (*) indicates a significant difference between Jersey vs conventional ($p < 0.05$). The values are mean ± standard deviation.

Understanding the distribution of β-casein variants in different milk types from different processes is important in order to monitor the changes in the released peptides in milk and dairy products. Many of studies have shown that several factors can influence the release of bioactive peptides, such as process-induced structural changes (Kopf-Bolanz et al., 2014; Lambers et al., 2021; Nguyen et al., 2015), milk composition (Ding et al., 2022; Iqbal et al., 2024; Liu et al., 2022), and digestion conditions (Asledottir et al., 2018; Cattaneo et al., 2023). In this part of the study, we compared semi-skimmed conventional milk to whole Jersey milk since we couldn't find any commercially available semi-skimmed Jersey milk. On the other hand, most previous studies have used skimmed milk (either conventional or Jersey), highlighting a gap in the literature regarding the effects of other factors such as different fat content (in both the conventional and Jersey milk), β-casein variants and processing methods to impact the release of BCM7. In the next chapter (Chapter **6**), we will discuss the effects of β-casein variants, fat content, and processing techniques (like microfiltration and pasteurisation) on the release of BCM7. More investigation is needed to study the effect of other β-casein variants and milk matrix on the release of BCM7.

5.5. Conclusion.

BCM7 is released during the intestinal digestion stage. Through simulated *in vitro* digestion of milk and utilization of a BCM7 standard for peptide quantification, it was clear that BCM7

formation can arise from both conventional (with A1) and Jersey (A1 free) milk. Among the filtered and pasteurised milk samples, there was no significant difference in the content of BCM7. This suggests that microfiltration has no significant effect on the proportions of β-casein variants. However, Jersey milk exhibited a significantly lower BCM7 content. This study suggests that the relatively lower concentration of BCM7 in Jersey milk compared to conventional samples may be attributed to differences in β-casein composition. More investigation is needed to understand the effect of β-casein composition and milk matrix on the BCM7 released after milk digestion.

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Chapter 6. Determination of the effect of β-casein composition, fat content and protein digestion on the release of BCM7.

6.1. Abstract.

This study aimed to investigate the release of the β-casomorphin-7 (BCM7), the opioid peptide derived from β-casein, from cow milk that differs in β-casein composition (A1, A2 and B), and fat content (whole, semi-skimmed and skimmed) and underwent different processes (filtered and pasteurised milk). An *in vitro* gastrointestinal digestion model was used to digest the conventional and Jersey milk (contains only A2 β-casein) samples. Liquid chromatography time-of-flight mass spectrometry (TOF LC-MS) was used to measure BCM7 after milk digestion. Results revealed that BCM7 was released after the intestinal stage digestion in all milk types, the average BCM7 amount ranged from 0.08 to 2.94 μg/g protein. Although numerous studies on BCM7 link the release of this peptide to A1 β-casein, some of the milk samples (specifically Jersey milk) that were free from A1 β-casein still exhibited comparable amounts of BCM7. Moreover, an important relationship was observed between BCM7 concentration, fat content, and the percentage of protein digestion, suggesting that these factors, along with processing methods, may interact to influence the release of BCM7.

6.2. Introduction.

β-casein represents about 30 % of total milk protein, containing 13 different variants of β-casein (A1, A2, A3, A4, B, C, D, E, F, H1, H2, I, G) (Farrell et al., 2004). The composition of β-casein is affected by cow breed as a main factor. For example, the A1 and A2 content of Jersey milk is 1-12 and 48 – 97 % respectively, in contrast, the A1 and A2 of Friesian milk are 51 - 71 and 28 – 40 % of total β-casein, respectively (Kamiński et al., 2007; McLean et al., 1984; Vincent et al., 2016). During the 1990's, the link between milk type consumption and some proinflammatory responses in some individuals began to be unveiled due to the release of the β-casomorphin 7 (BCM7), the opioid-active peptide that is released during the intestinal digestion stage of β-casein (in particular from the A1 variant) (Elliott et al., 1999). However, recent research has found that A1-free milk releases a lower amount of BCM7 than milk containing A1 (Sun et al., 2024). Since then, the A1 and A2 variants of β-casein have been widely researched including factors that could affect the release of BCM7 such as the processing methods (Lambers et al., 2021; Nguyen et al., 2015) and

digestion conditions (Cattaneo et al., 2023). Within the food matrix, macronutrients interact with one another both before and during milk processing and digestion. The release of peptides is influenced by the conditions of processing and digestion which could impact their bioavailability and physiological effects. Understanding the interaction of these factors is essential for optimizing the properties of food products, as well as for managing their derivatives which may induce health benefits or negative health consequences (Capuano & Janssen, 2021; Ding et al., 2022; Thorning et al., 2017). The digestion of protein, influenced by its content, processing, and structure, is interconnected with molecular interactions with fat within the stomach indicating that fat and protein are easier to hydrolyse during the subsequent digestion process (Capuano & Janssen, 2021; Ding et al., 2022). Consequently, studying the influence of a single nutrient alteration on health can lead to significant differences between the nutritional expectations of a particular food and the actual outcomes. A high-fat content in the food matrix may affect the solubility, hydrophobicity, and secondary structures of the protein (Ding et al., 2022). For example, when the protein and fat are dispersed, and the protein is adsorbed at the oil-water interface evenly, while the fat droplets are small and more scattered, a high specific surface area could help improve the hydrolysis of digestive enzymes on the protein (Ding et al., 2022).

As BCM7 is a digestion by-product, it is crucial to assess the variability in protein digestion percentages within each type of milk, which can be attributed to various factors, such as variations in β-casein composition, fat content and processing methods. Additionally, it is important to consider the inherent variations in the release of BCM7 among the analysed milk samples. This study aims to investigate the factors that may affect the release of BCM7. While previous studies have primarily focused on the A1 and A2 β-casein variants, this study hypothesizes that the milk matrix (particularly fat content), the percentage of protein digested, and processing methods (such as pasteurisation and microfiltration) could significantly impact the release of BCM7. By examining these additional variables, this research seeks to provide a more comprehensive understanding of the mechanisms influencing BCM7 release across different milk types.

6.3. Material and Methods.

6.3.1 Chemicals.

All chemicals were of analytical grade. HPLC water from Fisher Scientific (UK) was used throughout the study. Urea (99 %), dithiothreitol (≥ 98 %), trifluoroacetic acid (TFA, ≥ 99 %) and

purified bovine β-casein proteins (≥ 98 %) were obtained from Sigma-Aldrich (UK). LC-MS grade water, formic acid and acetonitrile ≥ 99.9 % were sourced from Fisher Scientific (UK). Pepsin from porcine gastric mucosa (no. P7000; 800 to 2,500 U/mg of protein), porcine bile extract (no. B8631); and porcine pancreatin (no. P1750; 4 × USP) were purchased from Sigma-Aldrich (UK).

6.3.2 Samples.

All sample information and descriptions are illustrated in **Table 6.1.** Various brands of commercial conventional milk samples were collected from local supermarkets in Reading (Berkshire, UK), including ASDA, Cravendale, and Co-op. These samples consisted of 2 whole filtered (n = 6), 3 semi-skimmed filtered (n = 9), 1 skimmed filtered (n = 3), 2 whole pasteurised (n = 6), 2 semi-skimmed pasteurised (n = 6), and 2 skimmed pasteurised milk (n = 6). Commercial Jersey whole milk samples (n = 9), which are expected to have a different β-casein composition compared to conventional milk, were collected from three different brands at local supermarkets in Reading (Marks and Spencer (M&S), Aldi (Jw) and Graham (G) milk from ASDA. Furthermore, raw whole, semi-skimmed and skimmed Jersey milk samples were collected from Old Hill Farm (OH) (Woodton, UK) (this brand was selected because the milk was labelled as A2 milk) and underwent pasteurisation treatment in the Food Processing Plant at the University of Reading (75 ºC for 15 s using the Armfiel HTST/UHT system (FT74XTS) pasteurisation unit). All milk samples were collected between the Summer and Autumn of 2023. Three different bottles for each of the milk samples were collected and used to carry out the analysis. Two different batches of OH milk were collected, with each batch consisting of four litres of each whole, semi-skimmed and skimmed OH Jersey milk. This milk was delivered to the University of Reading on the day of milking. The collected samples were rapidly transported to the laboratory in ice boxes. Two litres of each OH sample (whole (n = 2), semi-skimmed (n = 2) and skimmed (n = 2)) underwent the pasteurisation treatment in the Food Processing Plant at the University of Reading as previously indicated, this is presented as a whole, semi-skimmed and skimmed pasteurised OH samples. The remaining 2 litres are presented as whole, semi-skimmed and skimmed raw OH milk samples. All samples were aliquoted and stored at -20 ºC until analysis.

Table 6.1: List of cow milk samples collected and analysed. The information about process, fat content, fat % and protein % were sourced from the food labels.

Sample code	Milk Type	Process	Fat content	Fat %	protein %	Brand
AFW	Conventional	Filtered-pasteurised-homogenised	Whole	3.7	3.5	ASDA
AFS	Conventional	Filtered-pasteurised-homogenised	Semi-skimmed	1.8	3.6	ASDA
APW	Conventional	Pasteurised-homogenised	Whole	3.7	3.5	ASDA
APS	Conventional	Pasteurised-homogenised	Semi-skimmed	1.8	3.6	ASDA
APK	Conventional	Pasteurised-homogenised	Skimmed	<0.5	3.6	ASDA
CoPW	Conventional	Pasteurised-homogenised	Whole	3.7	3.5	CO-OP
CoPS	Conventional	Pasteurised-homogenised	Semi-skimmed	1.8	3.6	CO-OP
CoFS	Conventional	Filtered-pasteurised-homogenised	Semi-skimmed	1.8	3.6	CO-OP
CoPK	Conventional	Pasteurised-homogenised	Skimmed	0.5	3.6	CO-OP
CrW	Conventional	Filtered-pasteurised-homogenised	Whole	3.6	3.4	Cravendale
CrS	Conventional	Filtered-pasteurised-homogenised	Semi-skimmed	1.7	3.6	Cravendale
CrK	Conventional	Filtered-pasteurised-homogenised	Skimmed	0.3	3.6	Cravendale
Jw	Jersey	Pasteurised-homogenised	Whole	4.9	3.8	Aldi
MS	Jersey	Pasteurised-homogenised	Whole	5.7	3.6	M&S
G	Jersey	Pasteurised-homogenised	Whole	5.0	3.7	Graham
RW	Jersey	Raw	Whole	5.3	4.6	Old Hill Farm
RS	Jersey	Raw	Semi-skimmed	1.3	4.7	Old Hill Farm
RK	Jersey	Raw	Skimmed	0.1	4.8	Old Hill Farm
PW	Jersey	Pasteurised	Whole	5.3	4.6	Old Hill Farm
PS	Jersey	Pasteurised	Semi-skimmed	1.3	4.7	Old Hill Farm
PK	Jersey	Pasteurised	Skimmed	0.1	4.8	Old Hill Farm

6.3.3 Analysis of β-casein by high-resolution HPLC–MS.

Identification and quantitation of β-casein variants were done according to Givens et al. (2013). Milk samples (1 mL) were mixed with 1 mL of 8 M urea buffer containing 20 mM dithiothreitol and were kept at room temperature for 1 h. The residual fat was removed by centrifugation for 5 min at 1200 x g and 4 ºC (Thermo Scientific Medifuge Centrifuge, Germany). Then 0.5 mL of the aqueous layer was diluted with 2 mL HPLC grade water and filtered by using a 0.45 µm pore size disposable syringe filter before analysis. Separation of the milk proteins was achieved using an Agilent 1100 HPLC interfaced with a Bruker Microtof QII high-resolution quadrupole time of flight instrument (Bruker Instruments, Coventry, UK). Separations were performed on a C18

reversed-phase analytical column (150 mm x 2.1 mm internal diameter) with 30 nm pore size and 2.7 µm particle size (ACE HPLC Columns, UK). The column was thermostatically controlled at 45 ºC. Mobile phase A consisted of a solution of 0.01 % TFA in HPLC grade water and mobile phase B, 0.01 % TFA in LC-MS grade acetonitrile. The flow rate was set at 0.2 mL/min, the injection volume was 0.2 µL, and the total run time was 50 min. The main β-casein variants A1, A2 and B were detected by using both UV at 214 and high-resolution mass spectrometry in series with the UV detector, and the mass range was calibrated at a range 500-3500 *m/z.* A standard curve for β-casein was created by preparing standard solutions at concentrations of 0.1-1 mg/mL (R^2 = 0.98). Bruker software (Data analysis version 4.0, Bruker Daltoni GmbH, Bremen) were used to identify the protein variants and relative quantitation.

6.3.4 *In vitro* digestion of milk.

The *in vitro* gastric and intestinal digestion model used in this study was previously described by Brodkorb et al. (2019) and Gallier et al. (2012). A 20 mL of milk was mixed with 10 mL of simulated gastric fluid (SGF) containing 2 g of NaCl/L and 7 mL of HCl/L at pH 1.2. The mixture was acidified with 6 M HCl to pH 1.5 and was incubated in a shaking water bath (Grant OLS 200, Grant Instruments, Cambridge, UK) at 37 °C for 10 min at 95 rpm/min. Pepsin (3.2 mg/mL of SGF) was added, and the temperature and shaking were maintained for 2 h.

For the intestinal stage, the intestinal fluid (SIF) was prepared with 6.8 g of K_2HPO_4 /L and 190 mL 0.2 M NaOH/L and maintained at pH 7.5. The milk-SGF mixture was mixed with SIF (1:1) to a total volume of 30 mL, adjusting the pH to 7, and adding bile extract (5 mg/mL) and Pancreatin (1.6 mg/mL). The mixture was incubated at 37 °C in a shaking water bath (Grant OLS 200, Grant Instruments, Cambridge, UK) at 95 rpm/min for 3 h. To inactivate the enzymes, samples were immediately transferred to a water bath at 95 °C for 5 min (Wen et al., 2015). All digestions were performed in triplicate and an enzyme-reagent control, matched to digestion conditions, was conducted with each set of digested samples.

For the quantification of the percentage of protein digestion of the samples before and after *in vitro* digestion, the Lowry assay was conducted (Waterborg, 2009). A series of dilutions of known concentrations of bovine serum albumin (BSA) was prepared and assayed alongside the unknowns to determine the concentration within the working range (0.10 – 2 mg). A blank was included in the analysis, and its absorbance was subtracted from the absorbance values of the samples. The

percentage of protein digestion before and after *in vitro* digestion was calculated by the following Equation:

$$Percentage\ of\ protein\ digestion = \frac{protein\ b - \ protein\ a}{protein\ b}\ x\ 100$$

Where:

Protein b = protein concentration before *in vitro* digestion

Protein a = protein concentration after *in vitro* digestion.

6.3.5 Determination of β-casomorphin 7 (BCM7) in milk digests by LC/MS-MS.

An Agilent 1100 HPLC interfaced with a Bruker Microtof QII high-resolution quadrupole time of flight instrument (Bruker Instruments, Coventry, UK) was used for the identification and quantification of BCM7 released after the *in vitro* digestion of milk samples. Elution solvents A and B were (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile with a gradient elution using LC/MS-grade elution solvents (Fisher Chemical™, Loughborough, UK) on an ACE® C-18 column (300 Å 5 µM 150 mm × 2.1 mm). The quantification of BCM7 in the samples and in the deuterated BCM7 standards (1 – 10 µg/mL, R^2 = 0.9871) was accomplished by comparing the peak areas in the extracted ion chromatogram at 790.4 m/z.

6.3.6 Statistical Analysis.

The statistical analysis was conducted using IBM SPSS Statistics version 29.0.2.0 (20), and results are presented as means ± standard deviations. A two-way analysis of variance (ANCOVA) was used to examine the effects of fat content (whole, semi-skimmed, skimmed) and process type (filtered vs. pasteurised) on BCM7 release. The independent variables were fat content and process, both treated as nominal variables, while BCM7 concentration served as the continuous dependent variable. Additionally, two continuous covariates, B β-casein and percentage of digestion, were included in the model to account for their potential influence on BCM7 release. The main effects for both fat content and process type, as well as their interaction, were assessed. In this study, statistical analyses were performed without direct comparisons between different brands of milk. Instead, combined analyses were conducted to assess the overall effects of various processing methods and fat content on BCM7 release, regardless of the brand. The relationship

between BCM7 levels and fat content and percentage of digestion was assessed using Spearman's rank correlation coefficient. A significance threshold of $p < 0.05$ was set for all tests.

6.4. Results and discussion.

The factors influencing the release of BCM7 during the digestion of cow milk, whether in its native state or as a result of technological processes, have long been a subject of inquiry without definitive answers. Our objective was to generate data that could contribute to human health. To investigate the study aims, all milk samples underwent *in vitro* digestion, and BCM7 peptide levels were quantified using LC-MS. The majority of studies investigating BCM7 release after digestion have utilised raw, skimmed milk or extracted β-casein (Cieślińska et al., 2007; De Noni, 2008; Lambers et al., 2021), rather than commercially processed milk which contains all its natural components. To the best of our knowledge, limited research has been conducted on commercial milk, and yet practically all consumed milk is processed. This study employed milk samples from various sources (ASDA, COOP, Cravendale, M&S, Aldi, Grahams and Old Hill farm). Despite the diversity in sample types and sources, results consistently indicate that both β-casein variants and fat content significantly influence the release of BCM7. In the forthcoming discussion, emphasis will be placed on elucidating how this consistency in trends across various sample sources and types, sheds light on the impact of these factors (β-casein variants, fat content, the percentage of protein digestion and some processes such as pasteurisation and microfiltration) on the release of BCM7, thereby which shows their overall influence. The main results are presented in **Table 6.2**, and each result will be discussed in detail in the following sections.

Table 6.2: The relative content of β-casein variants, percentage of protein digestion (% Dig), and levels of BCM7 (μg/g protein) released from the milk samples analysed in this study.

Samples Code	Process	β-casein variants (% of total β-casein)			% Dig	BCM7 (μg/g protein)
		A1	A2	B		
ASDA						
AFW	F	$37.9^{a} \pm 3.4$	$58.8^{a} \pm 4.0$	$3.2^{a} \pm 0.5$	$88.8^{a} \pm 4.2$	$1.80^{a} \pm 0.55$
AFS	F	$39.7^{a} \pm 3.1$	$56.3^{ac} \pm 3.1$	$3.8^{b} \pm 0.3$	$83.2^{b} \pm 6.6$	$2.93^{b} \pm 0.83$
APW	P	$41.0^{b} \pm 4.9$	$55.4^{b} \pm 4.9$	$3.5^{b} \pm 0.7$	$87.4^{a} \pm 5.7$	$1.20^{c} \pm 0.29$
APS	P	$42.6^{c} \pm 3.9$	$54.8^{bc} \pm 4.2$	$2.5^{c} \pm 0.6$	$82.1^{b} \pm 6.9$	$2.21^{b} \pm 0.98$
APK	P	$42.7^{c} \pm 3.7$	$54.9^{d} \pm 3.2$	$2.4^{c} \pm 0.4$	$85.2^{c} \pm 5.9$	$2.33^{d} \pm 0.35$
COOP						
CoPW	P	$42.9^{a} \pm 3.8$	$54.7^{a} \pm 4.4$	$2.1^{a} \pm 0.6$	$87.2^{a} \pm 5.8$	$1.19^{a} \pm 0.27$
CoPS	P	$42.9^{a} \pm 3.5$	$55.0^{ac} \pm 5.0$	$1.9^{b} \pm 0.4$	$86.8^{a} \pm 4.5$	$2.29^{b} \pm 0.19$
CoFS	F	$39.5^{a} \pm 4.0$	$58.6^{bc} \pm 4.0$	$1.7^{b} \pm 0.4$	$87.0^{a} \pm 4.0$	$2.53^{b} \pm 0.45$
CoPK	P	$39.6^{a} \pm 2.6$	$58.9^{b} \pm 2.6$	$1.3^{c} \pm 0.3$	$84.5^{c} \pm 6.7$	$2.10^{c} \pm 0.30$
Cravendale						
CRW	F	$38.1^{a} \pm 4.3$	$56.5^{a} \pm 5.0$	$5.2^{a} \pm 1.1$	$87.5^{a} \pm 4.7$	$1.24^{a} \pm 0.25$
CRS	F	$40.2^{b} \pm 4.5$	$54.9^{b} \pm 4.4$	$4.7^{a} \pm 0.6$	$87.3^{a} \pm 3.9$	$1.61^{b} \pm 0.11$
CRK	F	$39.4^{b} \pm 2.2$	$55.1^{b} \pm 3.0$	$5.3^{a} \pm 0.4$	$82.2^{b} \pm 6.2$	$2.94^{c} \pm 0.35$
*						
Jw	P	-	$80.6^{a} \pm 4.2$	$19.4^{a} \pm 2.4$	$90.8^{a} \pm 4.3$	$0.67^{a} \pm 0.25$
MS	P	$5.7^{a} \pm 1.8$	$81.5^{b} \pm 3.9$	$12.5^{b} \pm 3.1$	$88.6^{a} \pm 5.2$	$0.86^{a} \pm 0.40$
G	P	$12.1^{b} \pm 3.3$	$72.3^{c} \pm 3.5$	$15.6^{c} \pm 4.4$	$89.9^{a} \pm 4.7$	$0.69^{a} \pm 0.31$
Old Hill						
RW	R	-	$83.4^{a} \pm 4.6$	$16.6^{a} \pm 4.0$	$89.1^{a} \pm 5.6$	$0.90^{a} \pm 0.12$
RS	R	-	$83.3^{a} \pm 3.4$	$16.7^{a} \pm 2.3$	$89.6^{a} \pm 4.9$	$1.10^{b} \pm 0.06$
RK	R	-	$83.4^{a} \pm 2.9$	$16.6^{a} \pm 2.0$	$90.5^{a} \pm 6.0$	$1.10^{b} \pm 0.03$
PW	P	-	$84.0^{a} \pm 3.9$	$16.0^{a} \pm 3.7$	$89.6^{a} \pm 5.5$	$0.80^{a} \pm 0.10$
PS	P	-	$83.9^{a} \pm 2.5$	$16.1^{a} \pm 2.9$	$89.7^{a} \pm 6.6$	$1.04^{b} \pm 0.03$
PK	P	-	$83.4^{a} \pm 2.1$	$16.6^{a} \pm 2.4$	$90.0^{a} \pm 4.4$	$1.00^{b} \pm 0.04$

***** Commercial whole Jersey milk (Jw from Aldi, MS from Marks & Spencer and G from Graham brand). In the sample code, W = whole, S = semi-skimmed and K = skimmed. F = filtered milk. P = pasteurised milk. R = raw milk. Mean values ± standard deviation. Different letters within the same milk brand/group column indicate a significant difference ($p < 0.05$).

6.4.1 β-casein variants and releasing the BCM7.

Figure 6.1 shows the relative content of β-casein variants across all conventional and Jersey milk. A2 β-casein was the predominant variant in all samples. The content and proportions of A1, A2, and B variants of β-casein can vary depending on the type of milk (e.g., conventional, Jersey/A2-labeled). The pooled analysis showed a significant difference ($p < 0.05$) in the proportion of β-casein variants between the conventional and Jersey milk.

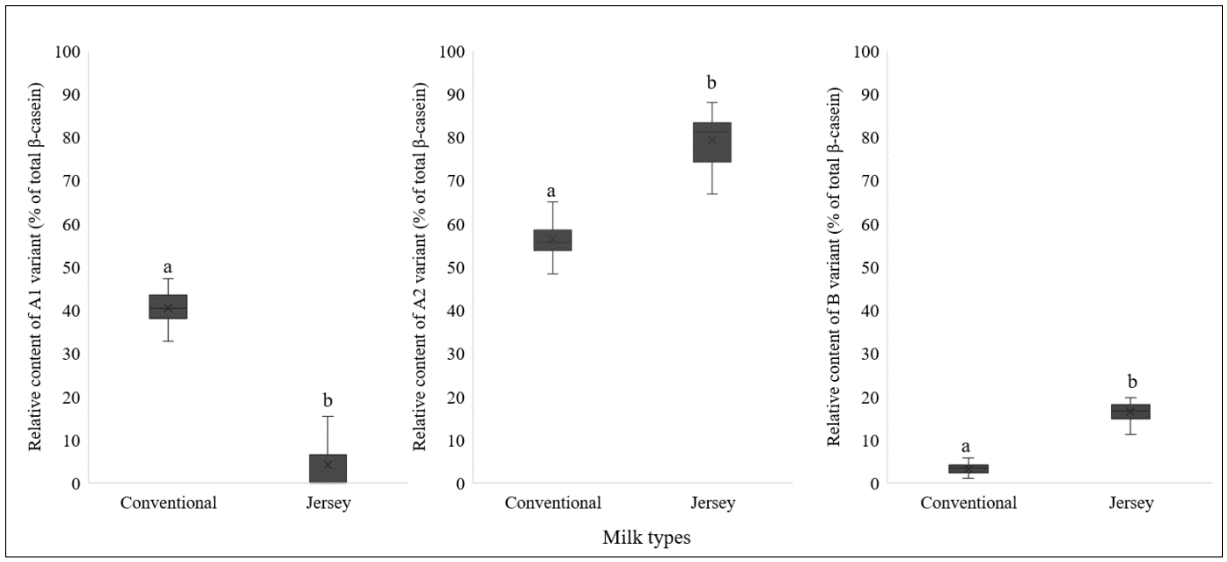


Figure 6.1: The proportions of A1, A2, and B variants of β-casein (% of total β-casein) in all conventional (n = 36) and all Jersey whole milk (n = 15). Different letters within the same variant denote a significant difference ($p < 0.05$). Milk sample details are shown in Materials and Methods.

The pooled analysis of all conventional milk showed that A2 β-casein was the most abundant variant, accounting for over half of the total β-casein content, with an average of 56.56 ± 4.6 %. A1 β-casein was present at a significant level (an average of 40 ± 4.7 % of total β-casein) but at a lower concentration compared to A2. B β-casein was the least abundant variant in the conventional milk, making up only an average of ~ 3 ± 1.3 % of the total β-casein. **Table 6.2** shows the proportions of β-casein variants in conventional and Jersey milk samples. There is a notable difference in the proportion of β-casein variants among various milk brands, which can be attributed to variations in milk sources or breed. Interestingly, Cravendale filtered milk samples (Crw, Crs, and CrK) contained the highest content of the B variant among conventional milk brands ($p < 0.05$). Cravendale is the first UK milk brand known for specializing in filtered milk (Cravendale, 2024). The elevated levels of B β-casein in Cravendale filtered milk compared to other brands may be linked to the genetic makeup or breed of the cows supplying the milk to this brand. Additionally, the microfiltration process could be a potential factor that may influence this concentration by affecting the retention of certain proteins. Microfiltration operates at low temperatures (below 50 °C) (Elwell & Barbano, 2006), which can induce structural changes in β-casein through a phenomenon known as cold denaturation. This process reduces hydrophobic interactions and leads to the dissociation of β-casein from the casein micelle, potentially impacting casein retention during microfiltration. Further investigations are needed to establish the potential

effects of microfiltration conditions on milk protein composition, which would enhance our understanding of protein behaviour during food processing.

In commercial Jersey milk samples (G and MS) containing the A1 variant, the most abundant variant was A2, followed by B and then A1, accounting for an average of 75.7 ± 8.4 %, 14.6 ± 2.8 %, and 8.9 ± 5.7 % of total β-casein, respectively. In the A1-free Jersey milk samples (Jw and OH), the most abundant variant was A2, followed by B, accounting for an average of 80 ± 4.9 % and 20 ± 2.8 % of total β-casein, respectively. However, the studied Jersey milk samples show that not all Jersey milk are free of the A1 variant, some Jersey milk has a low content of A1 **(Table 6.2)**. In the Jersey samples containing A1, the A1:A2 ratio was observed to be 1:9, and a similar ratio (1:7.5) was observed by Venn et al. (2006). The conventional milk samples contain a higher proportion of the A1 variant, with the average ratio of A1:A2 being 2:3, consistent with the findings of Jianqin et al. (2016). Although the Jersey samples with A1 contain a significantly ($p < 0.05$) lower content of A1 in comparison with conventional milk samples, all Jersey milk samples contain the B variant more than conventional milk.

6.4.2 *In vitro* digestibility analysis.

Protein digestibility is affected by various factors, such as milk composition (fat content, protein structure, particle size, etc..) (Berton et al., 2012; Garcia et al., 2014; Roy et al., 2020), or by the effect of processing on milk protein structure and composition (Loveday, 2023; van Lieshout et al., 2020). It is widely acknowledged that the rate of protein digestibility significantly influences the profile of peptides released during digestion (Agudelo et al., 2004; Loveday, 2023).

Despite the variations between samples from both milk types, the percentage of protein digestion ranged from 84 to 92 % (**Table 6.2**), which is in agreement with a previous study by Van Hekken et al. (2017). The pooled analysis of all whole milk samples showed no significant difference ($p > 0.05$) in the percentage of protein digestion between conventional and Jersey whole milk. However, the results have shown a trend towards slightly higher levels of protein digestion in Jersey milk samples (**Figure 6.2**)**.** The findings of Gai et al. (2021); Giribaldi et al. (2022); Rahaman et al. (2015) suggest that A2 milk has advantages over conventional A1-containing milk in terms of digestion and gut health. These studies reported that consuming A2 milk reduced gut discomfort compared to A1 milk, which may be attributed to the higher proline content in the A2 variant. High proline content significantly affects the hydrophobicity of the protein, resulting in

structural differences such as longer rennet coagulation times and looser curds compared to A1 milk. Additionally, Ramakrishnan et al. (2023) found that the stomach emptied conventional A1 milk faster than A2 milk, which may lead to negative abdominal symptoms in some individuals.

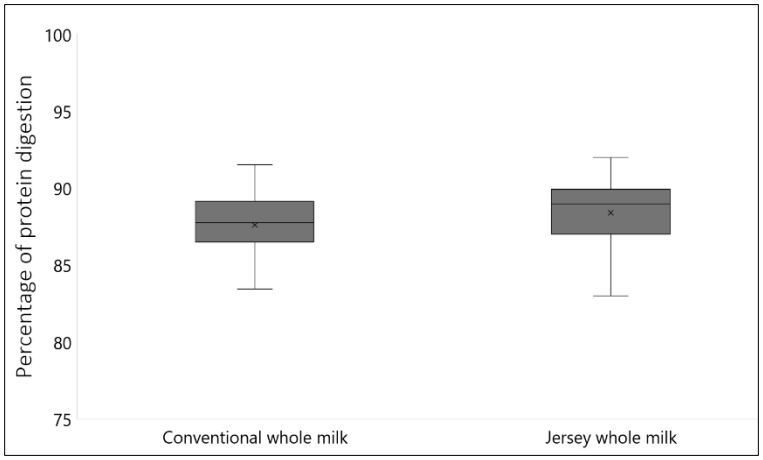


Figure 6.2: Percentage (%) of protein digestion of conventional and Jersey whole milk samples.

Earlier studies on milk gastric digestion have highlighted that a higher fat content leads to the formation of a more fragile curd due to fat-protein interactions, rather than protein-protein aggregation. This fragility, in turn, provides better access to digestive enzymes, promoting more efficient digestion and nutrient absorption (Capuano & Janssen, 2021; Ding et al., 2022; Garcia et al., 2014; Loveday, 2023; Roy et al., 2020). There exists a positive correlation between the softness of the curd and the rate of protein digestibility and its absorption (Roy et al., 2020; Ye et al., 2016, 2017). This phenomenon was observed in both conventional pasteurised and filtered milk, as well as Jersey milk samples in the current study. Whole conventional milk consistently exhibited the highest percentage of protein digestion, followed by semi-skimmed and skimmed milk. In addition, there is a noticeable difference in the percentage of protein digestion between conventional pasteurised and filtered milk samples. The combined analysis between filtered milk samples demonstrated higher levels of protein digestion compared to pasteurised milk, although is not significant ($p > 0.05$) (**Figure 6.3**)**.** Within the same brand, the significant differences ($p < 0.05$) in the percentage of protein digestion were more related to the fat content than the process (**Table 6.2**). However, the data also highlights an interesting point, while semi-skimmed milk showed lower protein digestion percentages compared to whole milk, the difference was less pronounced in filtered milk samples (**Table 6.2**). This could be attributed to the microfiltration

process conditions, which may alter the milk protein structure and potentially impact protein digestibility (Kelly & Zydney, 1997; Shuayb et al., 2023). This suggests that the microfiltration process might have a more significant impact on the digestibility of semi-skimmed milk compared to whole milk. Among the commercial Jersey milk samples, no statistically significant differences ($p > 0.05$) in the percentages of protein digestion were observed between Jw, G and MS samples **(Table 6.2)**.

The data in **Table 6.2** suggests the percentage of protein digestion was similar among the different types of OH milk samples. Raw skimmed milk (RK) exhibited the highest percentage of protein digestion at 90.5 ± 6.0 %, while raw whole milk (RW) showed the lowest at 89.1 ± 5.6 %. However, it's important to note that the differences between the percentages of protein digestion for each type of milk are relatively small and not significant ($p > 0.05$).

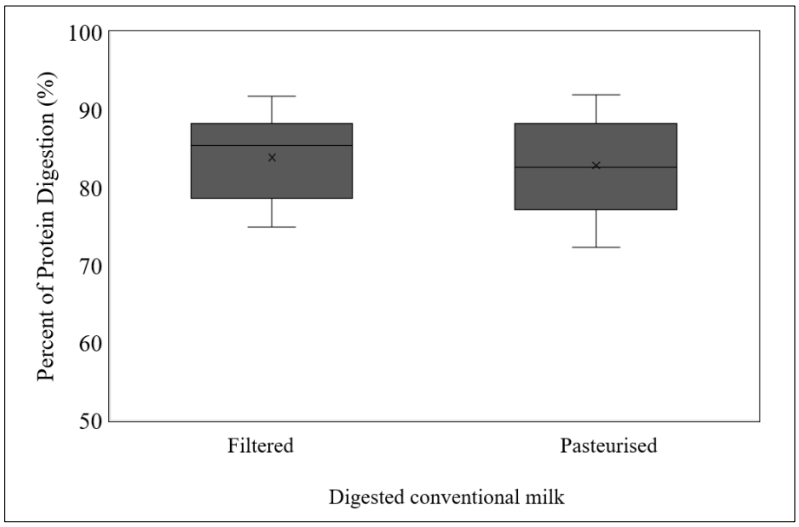


Figure 6.3: Percentage (%) of protein digestion of conventional Filtered and pasteurised milk samples.

6.4.3 Level of BCM7 released from milk samples after *in vitro* digestion.

All milk type samples (conventional and Jersey) underwent *in vitro* digestion, and BCM7 peptide levels were quantified using LC-MS. **Figure 6.4** illustrates that, following *in vitro* digestion, conventional milk samples released approximately twice the amount of BCM7 compared to Jersey

milk samples. These results are in agreement with previous studies that mentioned that the main factor that affects the release of BCM7 is the presence of the A1 β-casein variant (Cieślińska et al., 2007; Cieślińska et al., 2012; Nguyen et al., 2015). Milk with lower relative abundance of A1 β-casein also exhibited lower BCM7 release (Duarte-Vázquez et al., 2017). The A1 variant was more common in the conventional milk samples than Jersey milk **(Figure 6.1** and **Table 6.2)**.

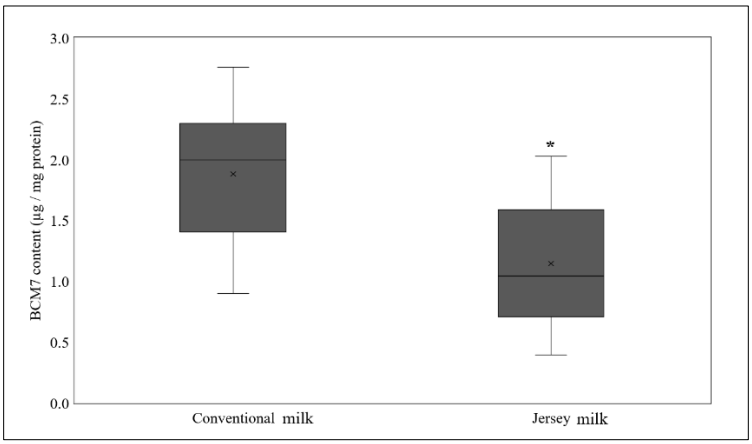


Figure 6.4: The level of BCM7 detected in conventional and Jersey milk after *in vitro* digestion. (*) indicates a significant difference ($p < 0.05$). Milk sample details are shown in Materials and Methods.

However, all Jersey milk samples released BCM7, even though the A1 variant was not detected in some samples (Jw and OH) (**Table 6.2**). Thus, the results indicate that the release of BCM7 from Jersey (A1 free) milk does not necessarily depend solely on the presence of the A1 β-casein variant. Moreover, there was no significant difference ($p > 0.05$) in the amount of BCM7 released between A1-free Jersey milk and Jersey milk containing the A1 variant. This suggests that other casein variants, such as the B variant, may also play a role in influencing BCM7 release, rather than A1 being the sole factor.

A1-free Jersey milk sample contained a higher percentage of the B variant compared to Jersey milk with the A1 variant (**Table 6.2**). Most BCM7 research has focused on the A1 variant as the source, but our results indicate that Jersey milk samples with a higher B variant, including those with A1, released comparable amount of BCM7. The findings of our study reveal that G Jersey milk (with A1 and B comprising 12.1 % and 15.6 % of total β-casein, respectively) released less BCM7 than MS Jersey milk (with A1 and B at 5.7 % and 12.5 % of total β-casein, respectively) **(Table 6.2)** ($p > 0.05$). On the other hand, A1-free Jersey milk (Jw and OH), containing a higher

B variant than G and MS samples, released a comparable amount of BCM7 within Jersey samples specifically. This suggests that the B variant or other minor variants could be one of the factors influencing BCM7 release, not solely the A1 variant. The latest review article by Sun et al. (2024) primarily focused on the A1 and A2 β-casein variants as being responsible for the release of BCM7 and its potential role in gut disorders, without addressing other β-casein variants. Observing the BCM7 levels in each Jersey sample, we find it challenging to correlate the release of BCM7 with the β-casein variant solely. Having a significantly higher amount of A2 compared to A1 in samples doesn't necessarily mean they will release less BCM7. We hypothesize that other factors may influence the release of this peptide such as milk composition, processes and digestion.

In the current study, the release of BCM7 following *in vitro* digestion was observed in Jersey milk with different fat contents, as well as in conventional milk with varying fat content and processing methods (filtered and pasteurised). Additionally, correlations between fat content, processing methods, and the percentage of protein digestion were calculated. In the conventional milk samples, regardless of the source and processes, significantly lower BCM7 was released from whole milk compared to semi-skimmed and skimmed milk from the same brands ($p < 0.05$). The combined analysis of conventional milk processed by microfiltration and pasteurisation, categorised by fat content (whole, semi-skimmed, and skimmed), shows that both processing method and fat content may influence the release of BCM7 in milk **(Figure 6.5)**. Filtered milk consistently exhibits slightly higher levels of BCM7 compared to pasteurised milk across all fat content categories **(Table 6.2)**. In both processing methods, whole milk releases the lowest amount of BCM7, while semi-skimmed and skimmed milk show higher levels ($p > 0.05$). Although a significant difference ($p < 0.05$) was observed in one sample, whole milk from the ASDA brand (AFW and APW), no significant differences were found in the release of BCM7 in other samples, including semi-skimmed milk from ASDA and COOP (AFS vs. APS, CoPF vs. CoFS) (**Table 6.2**). However, significant differences ($p > 0.05$) were noted among whole, semi-skimmed, and skimmed Cravendale filtered milk (**Table 6.2**). In addition, this trend was observed among all whole and low-fat content OH milk (A1-free) samples (**Table 6.2**). The raw and pasteurised OH Jersey milk samples (whole, semi-skimmed, and skimmed) released varying amounts of BCM7 depending on their fat content (**Table 6.2**). Both raw and pasteurised OH Jersey whole milk released significantly lower amounts of BCM7 compared to raw and pasteurised semi-skimmed and skimmed milk from the same source ($p < 0.05$). Although the difference was not statistically

significant, raw OH milk showed slightly higher BCM7 content compared to pasteurised milk, which aligns with Lambers et al. (2021), who reported that pasteurisation could reduce BCM7 levels. The results showed there are differences in the release of BCM7 among all whole and low-fat content samples regardless of milk type or β-casein variant content (**Table 6.2**). This suggests that reduced-fat milk may promote greater BCM7 release, possibly due to alterations in protein structure or interactions during digestion (Bao et al., 2023; Ding et al., 2022; Iqbal et al., 2024). The difference between semi-skimmed and skimmed milk is minimal, indicating that fat reduction may have a more significant effect when transitioning from whole milk to lower-fat milk. A substantial inverse relationship was observed between fat content and the release of BCM7 ($r = -0.70$, $r^2 = 0.49$) (**Figure 6.6**). Additionally, a statistically significant inverse relationship was found between the percentage of protein digestion and BCM7 release ($r = -0.55$, $r^2 = 0.31$, $p < 0.05$) (**Figure 6.6**).

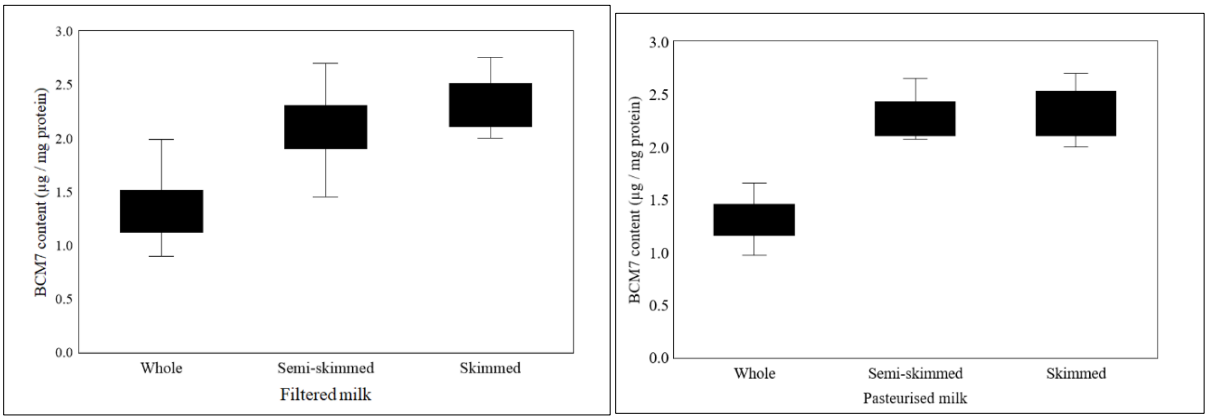
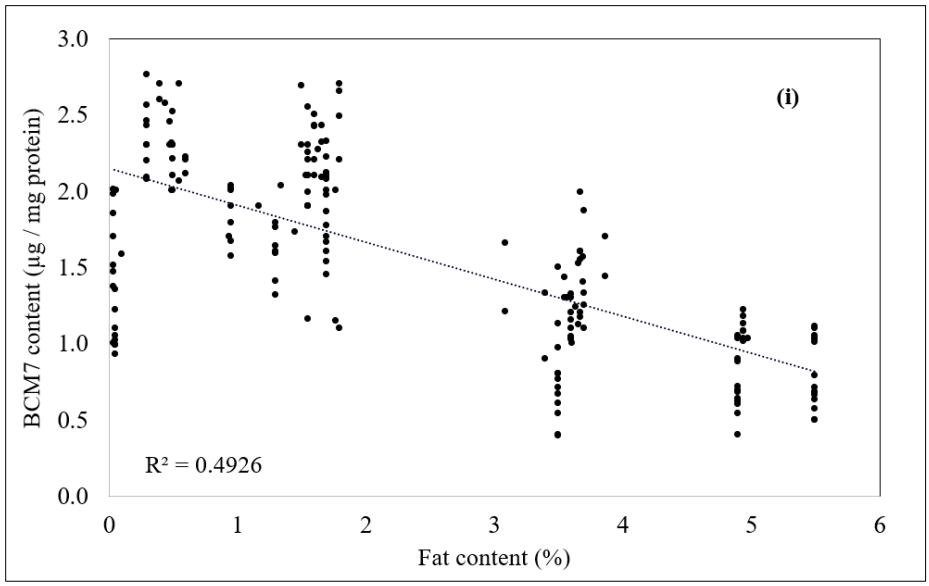


Figure 6.5: Combined analysis of BCM7 release from conventional milk after *in vitro* digestion, categorised by fat content and process ($p > 0.05$) for filtered and pasteurised milk.



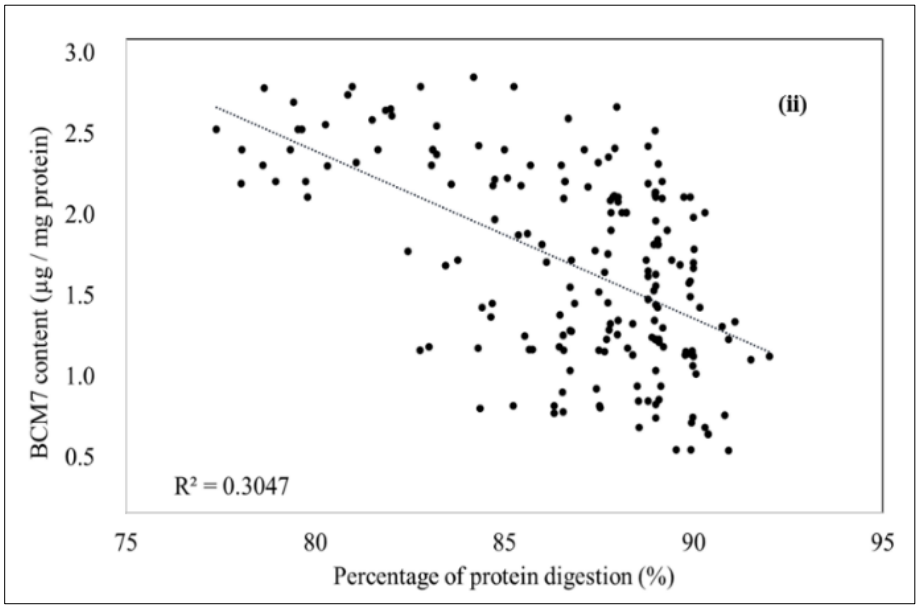


Figure 6.6: The correlation between the release of BCM7 after *in vitro* digestion of all conventional and Jersey milk samples with (i) the fat content (%) and (ii) the percentage of protein digestion (%).

While De Noni et al. (2015) and Nguyen et al. (2015) indicated that protein breakdown influences peptide release, our results demonstrate that milk composition also plays a significant role. Consistent with previous studies (Argov et al., 2008; Bao et al., 2023; Ding et al., 2022; Garcia et al., 2014), suggested that fat content plays a key role in milk structure and digestion. Milk processing alters the microstructure and composition of fat globules and milk proteins, impacting their physicochemical properties, digestion, and peptide release (Agudelo et al., 2004; Egger &

Ménard, 2017; Lambers et al., 2021; van Lieshout et al., 2020). Our findings suggest that fat content and microfiltration conditions could play a role in the release of BCM7. Therefore, this study emphasises the need for further research into the effects of microfiltration conditions and the milk matrix on peptide release. The dual nature of these findings highlights the need for further in vivo investigations to determine whether the higher BCM7 content in filtered milk poses a health risk or could offer benefits in terms of digestibility and allergenicity. Such insights are crucial for informing dietary recommendations, particularly for vulnerable groups such as infants and individuals with milk sensitivities.

Furthermore, **Figure 6.7** demonstrates the impacts of processing methods and fat content on BCM7 release. By comparing the percentage increase in BCM7 between filtered and pasteurised milk with the same fat content (as the effect of process), and between semi-skimmed and whole milk under the same process (as the effect of fat content), this figure helps to highlight how these two factors influence BCM7 production, providing clearer insight into their relative effects. In the first comparison between filtered and pasteurised milk with the same fat content, 33 % more BCM7 was released from whole filtered milk (AFW) compared to whole pasteurised milk (APW) from the same brand (**Table 6.2**). In the case of semi-skimmed filtered milk 25 % and 9 % more BCM7 was released from AFS and CoFS compared to their pasteurised counterparts (APS and CoPS), respectively. This suggests that microfiltration increased BCM7 release, having a more pronounced effect in whole milk than in semi-skimmed milk. Interestingly, the percentage increase was notably different, implying that the microfiltration process conditions may influence the release of BCM7 or other peptides.

In the second comparison, between semi-skimmed and whole milk processed similarly, AFS and CrS semi-skimmed filtered milk released 39 % and 25 % more BCM7 than whole filtered milk from the same brand (AFW and CrW), respectively. Similarly, semi-skimmed pasteurised milk APS and CoPS showed a higher increase in BCM7 release by 46 % and 48 % compared to whole pasteurised milk (APW and CoPW), respectively. This indicates that both fat content and processing type (microfiltration vs. pasteurisation) may affect BCM7 release, with microfiltration increasing the release of BCM7 and playing a particularly notable role in whole milk. However, the presence of fat seems to limit the release of BCM7 and showed a greater impact on BCM7 release than microfiltration.

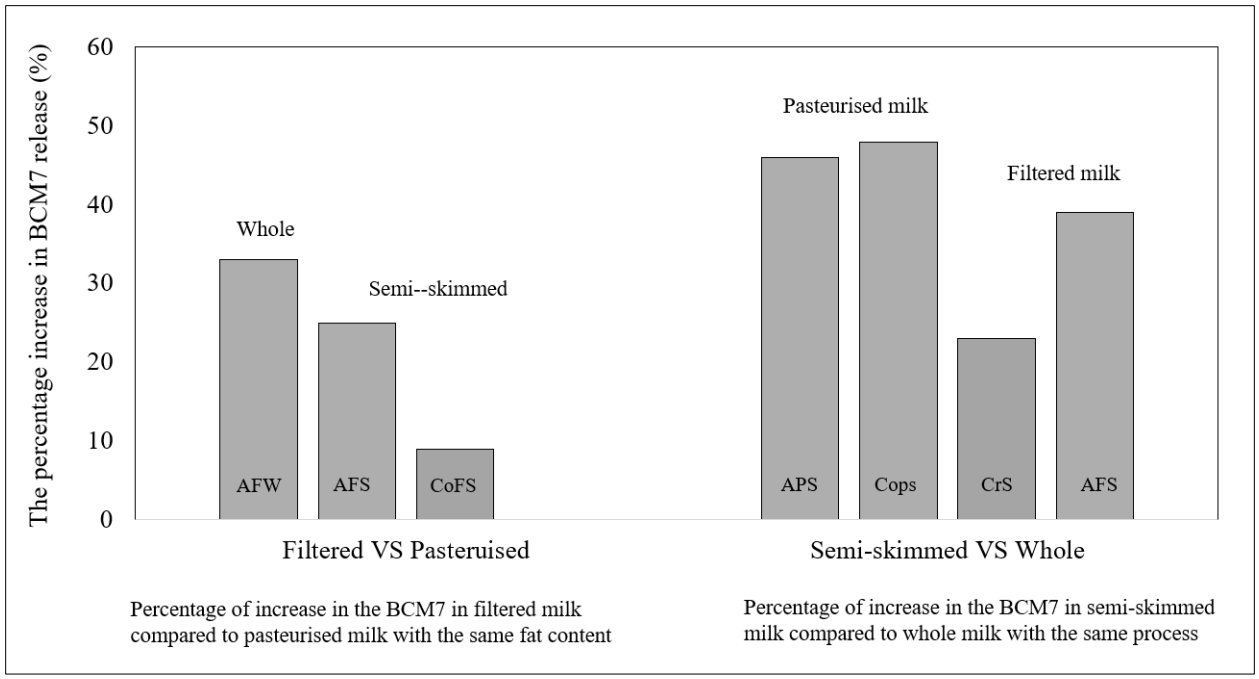


Figure 6.7: The percentage increase in BCM7 release after the *in vitro* digestion of filtered and pasteurised conventional milk with different fat content**.**

6.5. Conclusion.

Our study aligns with previous research regarding the significant differences in BCM7 release between conventional and Jersey milk samples following *in vitro* digestion, with conventional milk releasing approximately twice the amount of BCM7 compared to Jersey milk. Notably, Jersey milk, even when A1-free, still released BCM7, suggesting that these factors contribute to this mechanism. While earlier studies have primarily focused on the A1 β-casein variant as the main factor influencing BCM7 release, our findings indicate that other factors, such as the presence of the B β-casein variant, milk composition, processing and digestion, also play a critical role. Additionally, we observed a strong inverse relationship between fat content, the percentage of protein digestion, and BCM7 release. These results indicate that the interaction between milk fat and proteins, along with processing methods, significantly impacts BCM7 release. Therefore, further research is essential to explore how microfiltration conditions and the milk matrix affect the release of BCM7 and other bioactive peptides. This study contributes to a broader understanding of the milk matrix, processing, and digestibility, while also challenging the exclusive focus on A1 β-casein in prior research.

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Chapter 7. Peptide Profile Analysis of Commercial Semi-Skimmed Filtered and Pasteurised Milk After *In vitro* Digestion.

7.1. Abstract.

This study aimed to analyse the peptide profile released after *in vitro* digestion of commercially available filtered and pasteurised milk. The existing literature exhibited a deficiency in detailed knowledge about the effect of microfiltration on milk protein digestion and peptide profiles. Static *in vitro* gastrointestinal digestion protocol was used to digest selected commercially available semi-skimmed filtered cow's milk from different brands alongside semi-skimmed pasteurised milk as a control. The resulting peptide profile of digested milk samples was characterised by Quadrupole Time-of-Flight Mass Spectrometry Q-TOF LC/MS. After gastrointestinal digestion, the number of peptides released from filtered milk exceeded, by an average of 5 %, that of peptides released from pasteurised milk. The major milk proteins that were sources of peptides included αS1-casein (αS1) and β-casein (β-casein) followed by αS2-casein (αS2), β-lactoglobulin (βLg), kappa-casein (ϰcas) and α-lactalbumin (αLac). Some differences were noticed in the peptide size and number distribution between filtered and pasteurised milk samples, although there were no significant differences in the percentage digestion of filtered and pasteurised milk samples from the same brand. More investigation is needed to explore the effect of microfiltration under different conditions on milk protein digestion and the released peptide.

7.2. Introduction.

Milk protein is not only a good source of essential amino acids that are important to human health but also a multitude of bioactive peptides that could have different uses (such as in drug manufacture) and exhibiting multiple health-promoting properties, including antimicrobial, antioxidant, and anti-inflammatory effects (Quintieri et al., 2024). Most of the peptides are formed during enzymatic hydrolysis where proteins are cleaved into small peptides or during milk processing (Egger & Ménard, 2017; Kopf-Bolanz et al., 2014). Some peptides have multiple bioactivities, such as βLg-derived peptide (TPEVDDEALEK), which was used to determine the amount of whey that was added to raw milk in the cheese processing as a marker of adulteration (Stastna, 2024), or as a tool to detect and identify food allergens (Villa et al., 2018). Regarding potential health benefits, several milk-derived peptides have been reported to have bioactive

properties or are mentioned as promising alternatives to drugs with no side effects. For example, some milk protein-derived peptides inhibit the angiotensin-converting enzyme (ACE-inhibitory) or increase the endothelial nitric oxide synthase lowering blood pressure (Egger & Ménard, 2017; Nielsen et al., 2023). Several peptides have been shown to enhance insulin signalling and lower blood glucose concentration by dipeptidyl peptidase IV inhibitory activity (DPP-IV) (Egger & Ménard, 2017; Quintieri et al., 2024). Furthermore, some peptides have been reported as to promote immunomodulatory, anti-inflammatory and osteoanabolic activity (Loveday, 2023; Nielsen et al., 2023; Zhou et al., 2024). However, some milk peptides have also been reported to have undesirable effects. For example: peptides with opioid activity, such as β-casomorphin-7 (BCM7), have been associated with health-related issues (e.g., gastrointestinal symptoms or heart disease [EFSA, 2009]); certain peptides can cause gastrointestinal discomfort, respiratory problems, or skin irritation in individuals with milk allergies; and some peptides may interact with medications or promote inflammatory effects (Monaci et al., 2006; Zhou et al., 2024). Peptides required for the unique identification of proteins and those shown to possess potential health-promoting properties typically range from 7 to 25 amino acids in length (800 to 2500 Da) (Panchaud et al., 2012; Xu et al., 2024).

Milk processing can influence the release of peptides during milk digestion thus impacting their bioavailability and physiological effects. The changes in the physicochemical properties of the protein alter milk digestibility which may consequently alter the peptides that are released (Kopf-Bolanz et al., 2014; van Lieshout et al., 2020). Understanding how structural changes during milk processing impact the length and amino acid composition of released peptides and subsequently affect their bioactivity after digestion, is one of the most studied topics in dairy science (Cui et al., 2023; Jiang et al., 2024; Nielsen et al., 2023; Pi et al., 2023; Quintieri et al., 2024; Stastna, 2024; Yang et al., 2024). The structures of whey and casein are different, while whey is a globular folded structure, the casein has a flexible and open structure (Cayot & Lorient, 2017; Dall'Antonia et al., 2014; De Kruif et al., 2012). The process-induced changes in protein significantly differ according to processing conditions. For example, heating unfolds the whey structure and does not impact the casein structure, on the other hand, the casein coagulates at an acidic pH unlike whey, which does not coagulate. These changes in protein structure impact the resulting digestion products (Bu et al., 2013; Lorieau et al., 2018; Tunick et al., 2016). However, recent studies show that the changes in the protein structure during the processes are not solely

about the process conditions and protein structures, the food matrix plays a key role as well (Capuano & Janssen, 2021; Ding et al., 2022; Loveday, 2023).

Recently, filtered milk has become more widely available in some UK markets, according to the database analysis conducted in Chapter **3**. This type of milk undergoes microfiltration in addition to pasteurisation and homogenisation, unlike traditional pasteurised milk, which undergoes only pasteurisation and homogenisation. However, detailed information on the released peptides in digested filtered milk with complex matrices is limited. The study aimed to compare the peptide profile of *in vitro* digested filtered and pasteurised milk to gain insight into the potential impact of microfiltration on the presence and formation of the peptides after protein digestion. A preliminary evaluation of some peptide bioactivities was also conducted. For this purpose, commercially available semi-skimmed filtered milk was subjected to *in vitro* gastrointestinal digestion, and pasteurised milk samples from the same brand were used for comparison. For structural assignments of the detected signals, ion spectra were generated by Q-TOF LC/MS, and analysed with Bioconfirm MassHunter software.

7.3. Material and Methods.

7.3.1 Chemicals.

Chemicals of analytical grade were used throughout the study. HPLC water, LC-MS grade water; formic acid and acetonitrile ≥ 99.9 % were sourced from Fisher Scientific (UK). Pepsin from porcine gastric mucosa (no. P7000; 800 to 2,500 U/mg of protein), porcine bile extract (no. B8631); and porcine pancreatin (no. P1750; 4 × USP) were purchased from Sigma-Aldrich Corp (UK).

7.3.2 Samples.

Seven semi-skimmed filtered cow milk samples from different brands (A, Co, DM, CB, T, S, and W) (n=14) were bought from the local supermarkets in Reading (UK). Seven pasteurised semi-skimmed cow milk samples (n=14) from the same brands were also purchased for comparison. **Table 7.1** shows samples information according to their information label. Two different batches of each milk sample were used to conduct the analyses. All milk samples were delivered to the laboratory under cool conditions in less than 60 min of purchase.

Table 7.1: Commercially available filtered (F) and pasteurised (P) milk used in this study, including macronutrient content as indicated on label information.

Sample code	Brand	Process	Label information (g/100 mL)		
			Fat	Protein	Sugar
A	ASDA	F	1.8	3.6	4.8
		P	1.8	3.6	4.8
Co	CO-OP	F	1.8	3.6	4.8
		P	1.8	3.4	5.0
DM	Dairy Manor / Aldi	F	1.8	3.6	4.8
		P	1.7	3.5	4.7
CB	Cow Belle / Lidl	F	1.8	3.6	4.8
		P	1.8	3.6	4.8
T	Tesco	F	1.8	3.3	4.9
		P	1.8	3.6	4.8
S	Sainsbury's	F	1.6	3.1	4.9
		P	1.8	3.6	4.8
W	Waitrose	F	1.6	3.3	4.9
		P	1.8	3.6	4.8

7.3.3 *In vitro* digestion of milk

The *in vitro* gastric and intestinal digestion model used in this study was previously described by Brodkorb et al. (2019) and Gallier et al. (2012). A 20 mL milk sample was mixed with 10 mL of simulated gastric fluid (SGF), which contained 2 g of NaCl/L and 7 mL of HCl/L at pH 1.2. The mixture was acidified with 6 M HCl to pH 1.5 and was incubated in a shaking water bath (Grant OLS 200, Grant Instruments, Cambridge, UK) at 37 °C for 10 min. Pepsin (3.2 mg/mL of SGF) was then added, and the temperature 37 °C and shaking 95 rpm were maintained for 2 h.

For the intestinal stage, the intestinal fluid (SIF) was prepared with 6.8 g of K_2HPO_4/L and 190 mL 0.2 M NaOH/L and maintained at pH 7.5. The milk-SGF mixture was mixed with SIF (1:1) to a total volume of 30 mL, adjusting the pH to 7, and adding bile extract (5 mg/mL) and Pancreatin (1.6 mg/mL). The mixture was incubated at 37 °C in a shaking water bath (Grant OLS 200, Grant Instruments, Cambridge, UK) (95 rpm) for 3 h. To inactivate the enzymes, samples were immediately placed in a water bath (Grant JB 300W, Cambridge, UK) at 95 °C for 5 min (Wen et al., 2015). All digestions were performed in triplicate and an enzyme-reagent control, matched to digestion conditions, was conducted with each set of digested samples.

For the quantification of the percentage of protein digestion of the samples before and after *in vitro* digestion, the Lowry assay was conducted (Waterborg, 2009). A series of dilutions of known concentrations of bovine serum albumin (BSA) were prepared and assayed alongside the unknowns to determine the concentration within the working range (0.10 – 2 mg). A blank was included in the analysis, and its absorbance was subtracted from the absorbance values of the samples. The percentage of protein digestion before and after *in vitro* digestion was calculated by the following equation 1:

$$Percentage\ of\ protein\ digestion = \frac{protein\ b - \ protein\ a}{protein\ b}\ x\ 100$$

Where:

Protein b = protein concentration before *in vitro* digestion.

Protein a = protein concentration after *in vitro* digestion.

7.3.4 Peptide profile analysis

The LC-MS peptide analysis was performed on a Q-TOF (Agilent, MassHunter workstation, 1290 Infinity Advance Bio, 6545XT). A reversed-phase (C18, 150 mm x 2.1 mm internal diameter) with 120 Angstrom pore size and 2.7 µm particle size) (ACE HPLC Columns, UK) analytical column was used for peptide separation and the flow rate was kept at 0.2 mL/min. The LC gradient started with 90 % mobile phase A (water/0.1% Formic acid), 10 % B (Acetonitrile/0.1 % Formic acid) at 0 and 2 min and an increase to 30 % B for 5 min, and a 51 min linear gradient to 40 % B, followed by 50 % B for 5 min. Mobile phase B increased from 10 % to 90 % over 25 minutes, followed by column wash at 90 % B for 15 minutes. Full scan mass spectra were acquired at a rate of 3 spectra/s in sensitivity mode from 100 to 3000 m/z. MS/MS scan spectra were acquired at a rate of 2 spectra/s over the same mass range using data-dependent acquisition (DDA; acquisition). The raw data from Q-TOF measurements were processed with the software MassHunter (BioConfirm 2.7). The following parameters were selected to create the project: nonspecific (enzyme), reduced disulphides, and variable modifications (oxidation of M, Phosphorylation of S, T, Y). A database containing the following sequences were used: α-S1-casein (P02662), α-S2-casein (P02663), β-casein (P02666), ϰ-casein (P02668), β-lactoglobulin (P02754) and α-lactalbumin (P00711). Data was searched with MS and MS/MS mass tolerances set at 10 ppm and 50 ppm, respectively.

7.3.5 Identification of bioactive peptides.

Peptide sequences obtained from Q-TOF LC/MS analysis of *in vitro* digested milk samples were screened for bioactive peptide identification. The peptide sequences were searched using the Milk Bioactive Peptide Database MBPDB (Nielsen et al., 2023). This database contains all the potentially bioactive peptide sequences and has recently been commonly used by many researchers. The search outcomes were further refined by comparison with the literature.

7.3.6 Statistical analysis

All of the assays were performed in duplicate. The results shown are the mean values ± standard deviation. Results were analysed by paired *t-test* using XLSTAT software version 2022.1.2 (Addinsoft, New York, NY, USA) with a significance level of $p \leq 0.05$. No direct comparisons were made between different brands; instead, analyses focused on assessing the overall effects of processing methods (filtered vs. pasteurised) across all brands.

7.4. Results and Discussion

The goal of the present study was to identify and quantify the peptides released after *in vitro* digestion of commercially available filtered and pasteurised semi-skimmed milk and to assess the impact of milk processing treatments, particularly microfiltration, on peptide release. A comparison between brands (filtered vs. filtered and pasteurised vs. pasteurised) was not conducted due to expected variations from different milk sources and processing conditions, but a comparison was made between filtered and pasteurised milk within the same brand.

7.4.1 Percentage of protein digestion after gastric stage

The percentage of protein digestion (% Dig) at the gastric stage was assessed by measuring the protein content in milk before and after incubation with pepsin at 37 °C for 120 min at pH = 1.5 (**Table 7.2)**. At this stage, filtered and pasteurised milk displayed a relatively low % Dig, with values ranging between 15 – 39 % and 21 – 43.9 % for filtered and pasteurised milk, respectively. These results align with the findings of Huang et al. (2022), who reported that some intact proteins remained undigested after the gastric digestion stage of fresh milk. When comparing the % Dig between filtered and pasteurised samples from the same brand, three filtered milk samples (A, S and W) among the seven brands showed lower % Dig, although differences were only significant

for S milk ($p < 0.05$). On the other hand, DM, CB and T brands showed the opposite trend, with significant differences between treatments observed in the T and CB samples ($p < 0.05$). Brand A had the highest % Dig, while filtered and pasteurised from Co brand had similar % Dig. In both cases, no significant differences were observed between treatments ($p > 0.05$). Overall, the % Dig after the *in vitro* gastric digestion of filtered and pasteurised samples was 29 ± 7.4 %. However, brand-to-brand variability in % Dig suggests that factors beyond basic milk composition, such as differences in protein structure or food matrix (data shown in Chapter Chapter 4) (Ding et al., 2022; Iqbal et al., 2024), as well as the differences in process conditions (Bhat et al., 2021) might influence digestion.

Table 7.2: Percentage of protein digestion of filtered (F) and pasteurised (P) milk samples after the *in vitro* gastric stage.

	% Dig after gastric stage	
Milk brand	F	P
A	39.1 ± 5.1	43.9 ± 6.5
Co	26.2 ± 3.2	26.5 ± 2.0
DM	32.5 ± 6.1	27.3 ± 5.9
CB	31.2* ± 5.2	23.1 ± 5.6
T	32.9* ± 5.5	21.1 ± 4.7
S	15.1* ± 4.3	29.5 ± 6.3
W	29.4 ± 3.3	32.1 ± 4.5

* Significant differences ($p < 0.05$) were observed between filtered and pasteurised samples within the same brand**.**

7.4.2 Percentage of protein digestion after intestinal stage

Determining protein digestion and derived products is essential to understanding the biological effect, nutritional and health implications of protein (Capuano & Janssen, 2021; Egger & Ménard, 2017; Kopf-Bolanz et al., 2014). Investigating the relation between the protein peptide profiles as a result of the digestion process could provide valuable insights into protein structure functionality, allergenicity and bioactivities (Cui et al., 2023; Graversen et al., 2021; Huang et al., 2022).

Table 7.3 reports the % Dig of protein after 2 h of gastric digestion and 3 h of intestinal digestion. The % Dig in both filtered and pasteurised samples was within the range reported by Van Hekken et al. (2017) (80 – 95 %). No significant differences ($p > 0.05$) were observed between filtered and

pasteurised samples within the same brand. After the intestinal stage, a higher degree of hydrolysis of proteins was observed compared to the gastric phase among tested samples.

Table 7.3: Percentage of protein digestion of filtered (F) and pasteurised (P) milk samples after the *in vitro* intestinal stage.

	% Dig after intestinal stage	
Milk brand	F	P
A	85.2 ± 6.3	86.8 ± 5.5
Co	84.5 ± 5.0	85.0 ± 6.1
DM	83.3 ± 4.3	84.6 ± 5.7
CB	84.1 ± 5.2	82.6 ± 4.1
T	86.3 ± 4.5	87.2 ± 3.1
S	84.2 ± 5.6	85.7 ± 4.4
W	83.4 ± 3.7	82.6 ± 4.2

A comparison of % Dig between filtered and pasteurised milk after the intestinal stage showed no significant differences. However, the gastric stage exhibited notable variation in % Dig across samples from different brands. This suggests that milk processing conditions influence protein structure and digestion at this stage more than during intestinal digestion. This protein digestion variation may affect the resulting peptide profiles, leading to differences in size and length. These variations could, in turn, influence the bioactivity and potential allergenicity of the resulting peptides (Huang et al., 2022; Roy et al., 2020; Sharma et al., 2001).

7.4.3 Peptides after *in vitro* gastric stage.

The length (number of amino acids) of peptides is measured because it provides insights into protein digestion and bioavailability, which can impact nutrition, health, and immune responses (Cui et al., 2023; Huang et al., 2022; Miciński et al., 2013; Monaci et al., 2006). According to Fan et al. (2023); Xu et al. (2024) and Villa et al. (2018) reviews, the bioactive peptides have peptide lengths ranging from 6 to 27 amino acids. Overall, as can be seen in **Figure 7.1 (i),** the number of peptides identified during the gastric stage was higher in the pasteurised samples compared to the filtered samples with values ranging between 163 – 227 and 176 – 228, respectively. However, the differences between filtered and pasteurised samples within the same brand were not statistically significant ($p > 0.05$). Notably, only two digested filtered milk samples (DM and T) released

significantly fewer peptides compared to their pasteurised counterparts ($p < 0.05$), while most other samples showed similar results ($p > 0.05$) (**Figure 7.1 (ii)**).

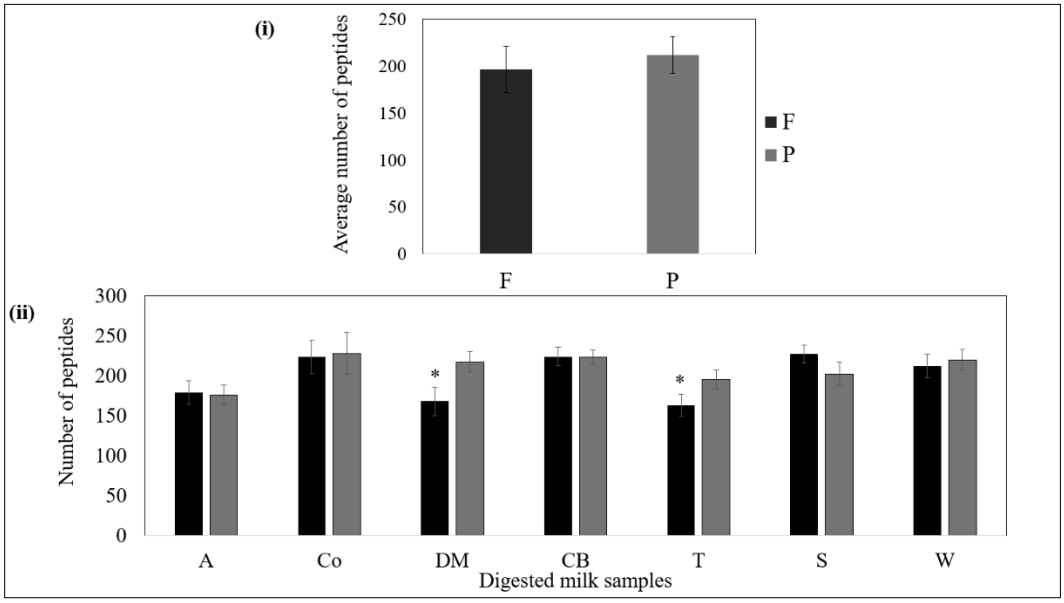


Figure 7.1: (i) The average number of peptides released from all filtered and pasteurised milk after the gastric stage. (ii) The total number of peptides released from each filtered and pasteurised milk brand. (*) indicates a significant difference ($p < 0.05$) between F and P samples from the same brand.

Figure 7.2 visualises the distribution of peptide lengths (number of amino acids) across all studied samples following *in vitro* gastric digestion. In the heatmap, the green colour indicates low abundance, the yellow colour indicates medium abundance and red indicates high abundance. The white area in a heatmap indicates that no peptide was identified.

The data evaluation of all identified peptides (after 2 h of *in vitro* gastric digestion) revealed that the most abundant peptides have a length size of 16 amino acids or lower, represented by the maximum areas of the curve and dark orange in the heat map in **Figure 7.2.** At the gastric stage, there is a wide variation of peptide length starting from 2 amino acids to 62 amino acids, however, peptides with length > 27 amino acids were the least abundant. To better understand the peptide profiles of filtered and pasteurised milk following *in vitro* gastric digestion, peptide lengths were categorised into four groups (2 – 8, 9 – 14, 15 – 24 and > 24 amino acids). As shown in **Figure 7.3,** peptides from the gastric stage accounted for approximately one-third of the total amino acids in both filtered and pasteurised samples. In filtered milk, peptides with lengths of 2 – 8, 9 – 14, and 15 – 24 amino acids comprised about 32, 28, and 29 % of the total peptides, respectively, while

in pasteurised milk, these lengths represented about 30, 27 and 30 % of the total peptides, respectively. Peptides longer than 24 amino acids constituted about 9 % and 11 % of total peptides in filtered and pasteurised samples, respectively. Comparing processing treatments (microfiltration vs. pasteurisation) revealed variations in peptide length distribution, although no consistent trend was observed ($p > 0.05$). However, filtered milk exhibited greater variability in peptide length distribution, potentially suggesting that microfiltration conditions varied across brands. This variation may have led to structural differences in the proteins, impacting peptide release during *in vitro* gastric digestion.

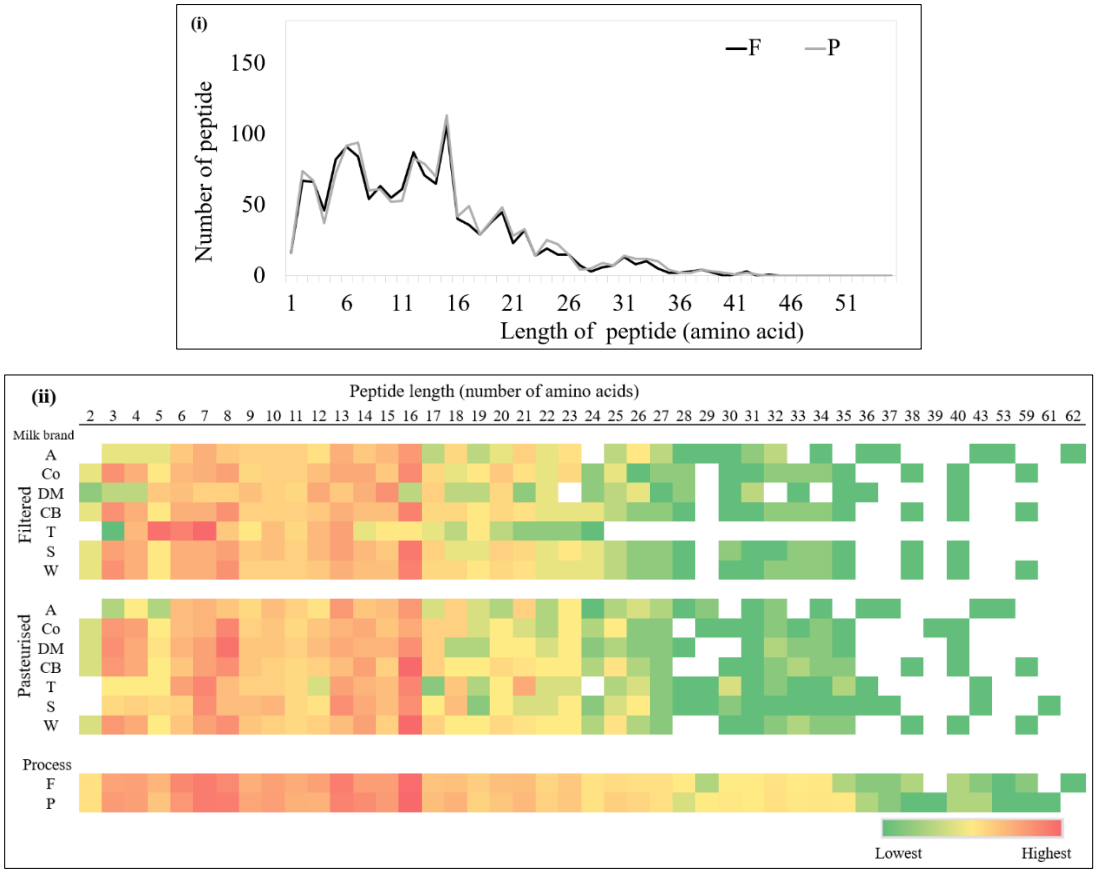


Figure 7.2: Peptide length distribution. (i) The average number of peptides dependent on the peptide length (number of amino acids), identified peptides in all filtered (F) and pasteurised (P) after *in vitro* gastric digestion. (ii) Heatmaps of the peptide length distribution in each *in vitro* gastric digested filtered and pasteurised milk brand (A, Co, DM, CB, T, S, and W). The colours range from green to red, indicating low and high occurrence of specific peptides, respectively. Unidentified peptide sequences are shown as white stretches.

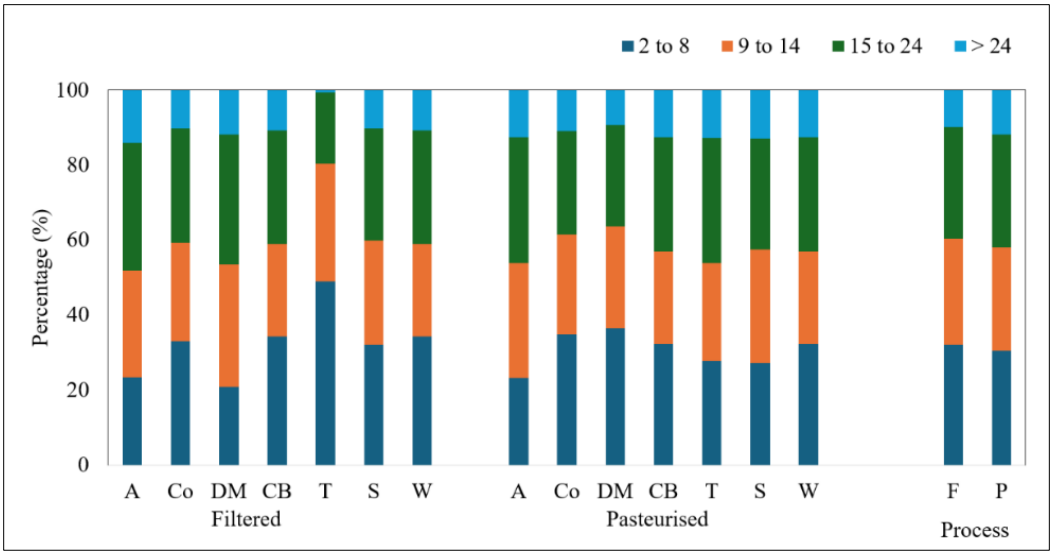


Figure 7.3: Length distribution of the identified peptides from *in vitro* gastric digestion of filtered and pasteurised milk brands (A, Co, DM, CB, T, S and W) and overall process (F = filtered and P = pasteurised).

In terms of peptide size, the distribution of peptides released after *in vitro* gastric digestion ranged from 317 to 7140 Da. The percentage of the peptide size distribution was grouped according to their mass as shown in **Figure 7.4**. The highest percentage of peptides had a size ranging between > 600 Da to < 2400 Da. Larger peptides (> 3000 Da) were detected but in small amounts. Both filtered and pasteurised milk showed similar percentages of peptide size distribution ($p > 0.05$), with both releasing a higher proportion of peptides with molecular weights less than 2400 Da. This is consistent with the findings of Cui et al. (2023) and Huang et al. (2022), which found that in the enzymatic hydrolysis of milk proteins for 2 - 3 h, different combinations of peptides with a mass of less than 3000 Da were found. However, pasteurised samples showed a more prevalence of larger peptides (> 2400 Da) compared to filtered milk ($p > 0.05$). Most of the biologically active peptides have a molecular weight between 700 to 1600 Da (Panchaud et al., 2012). This suggests that the processing method may influence the peptide size distribution, potentially affecting bioactivity.

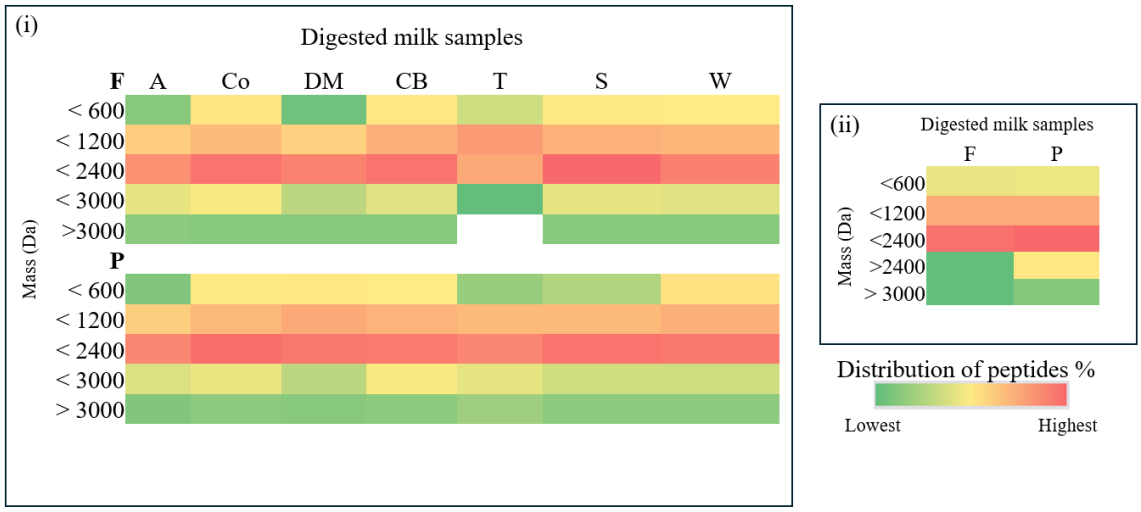


Figure 7.4: Heatmap of the percentage of peptide size distribution in: (i) filtered (F) and pasteurised (P) milk samples (A, Co, DM, CB, T, S and W) after the *in vitro* gastric stage. (ii) overall filtered samples (F) and all pasteurised samples (P). The colours range from green to red, indicating low and high occurrence of specific peptides, respectively. Unidentified peptide sequences are shown as white stretches.

The diversity of peptide distribution released from each milk protein among the filtered and pasteurised samples presented in **Figure 7.5**. The average distribution of peptides across different milk proteins in all milk samples was observed in the following order: β-casein (~28 ± 2.8 %), followed by αS1 (~ 24 ± 5.4 %), αS2 (~ 17 ± 2.6 %), ϰcas (~15 ± 1.2 %), βLg (~ 9 ± 3.1 %), and αLac (~ 6 ± 1.8 %). However, the heat map in **Figure 7.5** highlights that casein proteins were the predominant source of peptides across all brands for both filtered and pasteurised milk after the *in vitro* gastric digestion. This aligns with previous studies, which suggest that the flexible and open structure of casein makes it more susceptible to enzymatic hydrolysis compared to the more compact, globular structure of whey proteins (Egger & Ménard, 2017; Kopf-Bolanz et al., 2014; Punia et al., 2020) or could be attributed to a high abundance of casein in milk proteome (Baum et al., 2013). The high prevalence of peptides from β-casein and αS1, as indicated by the red and orange colours in the heat map, reinforces the idea that caseins are more readily hydrolysed during gastric digestion. In contrast, whey proteins (βLg and αLac) released fewer peptides, as shown by the green and yellow colours in the heatmap. This observation is in line with previous research indicating that βLg is resistant to gastric digestion due to its stable, globular structure (Bhat et al., 2021; Jiang et al., 2024; Loveday, 2023). The relatively lower peptide release from whey proteins, in both filtered and pasteurised milk, highlights the resistance of these proteins to gastric digestion. The combined analysis showed that certain proteins such as ϰcas, β-casein, and βLg released

slightly fewer peptides in filtered milk compared to pasteurised samples ($p > 0.05$) **Figure 7.5** (i). Interestingly, filtered milk from different brands showed greater diversity in peptide distribution than pasteurized samples, with some significant differences observed ($p < 0.05$). However, no clear trend was identified **Figure 7.5** (ii). Such variability in peptide release could be influenced by differences in processing conditions and the milk source, as suggested by (Loveday, 2023). This finding suggests that the microfiltration process may impact the protein digestion products, potentially influencing the nutritional value and functional properties of the milk. These observations highlight the potential impact of processing conditions on gastric digestion and the bioavailability of milk proteins or peptides, which may ultimately influence the functional and nutritional properties of the milk.

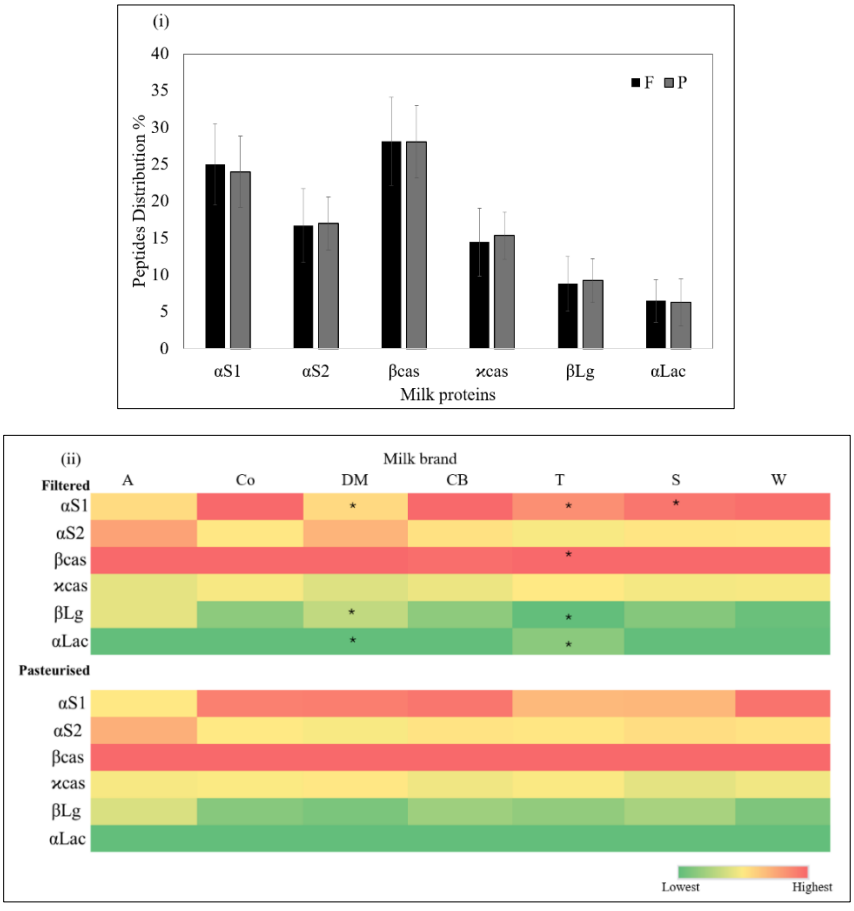


Figure 7.5: (i) The distribution of peptides from the parent proteins αS1, αS2, β and ϰ-casein, βLg and αLac from all *in vitro* gastric digested filtered and pasteurised semi-skimmed milk. (ii) Heatmap of peptides from the parent proteins from each milk brand (A, Co, DM, CB, T, S and W). The colours range from green to red, indicating low and high occurrence of specific amino acids, respectively. (*) indicates a significant difference between filtered and pasteurised samples within the same brand ($p < 0.05$).

The pooled analysis of the peptide size (length) distribution from the milk proteins (αS1, αS2, β-casein and ϰcas, βLg and αLac*)* in filtered and pasteurised *in vitro* gastric digested milk is illustrated in **Figure 7.6.** Variations in the peptide size distribution were observed between filtered and pasteurised samples. Protein fractions from filtered and pasteurised milk samples mostly release peptides within the 7 – 21 amino acid range, with a particularly high abundance of peptides between 12 – 16 amino acids. This pattern is consistent across both processes, suggesting similar digestion profiles, as there were no statistically significant differences in peptide length distribution ($p > 0.05$) between filtered and pasteurised samples. Casein proteins, particularly α- and β-caseine released a broader range of peptide lengths (7 – 26 amino acids), indicating their higher susceptibility to enzymatic digestion (Agudelo et al., 2004). In contrast, ϰ-casein and whey proteins (βLg and αLac) released fewer range of peptide with > 36 amino acids. Notably, significant differences ($p < 0.05$) were observed between filtered and pasteurised samples in the peptide distribution, in particular whey protein fractions **Figure 7.6**. These differences may be attributed to the effects of microfiltration, which could alter the protein structure and thereby impact enzymatic breakdown by pepsin. As explained by Ye et al. (2016), during the gastric stage, the peptic cleavage sites in whey protein are hydrophobic amino acids, which are buried within the hydrophobic core. Processing methods or conditions that induce conformational changes in whey fractions can increase the exposure of these sites, thereby enhancing the susceptibility of protein to pepsin action. Further investigation is needed to determine the potential differential effects of these processes on peptide size distribution. Such insights could provide valuable information regarding the biological activities of peptides.

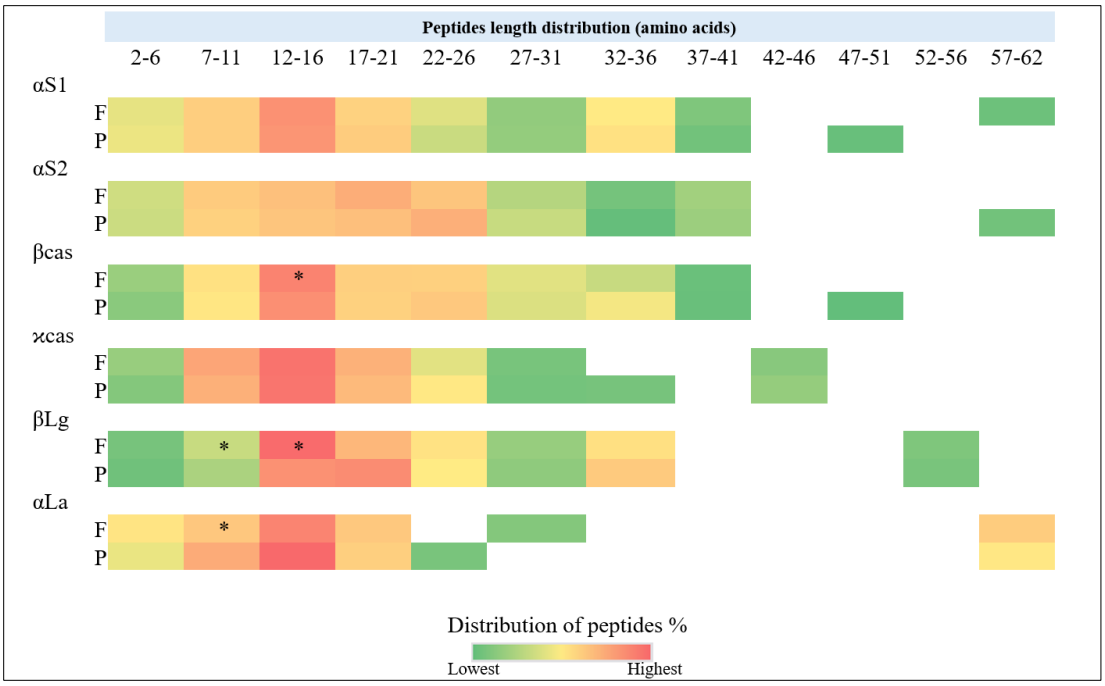


Figure 7.6: Heatmap of peptide length distribution from the parent proteins αS1, αS2, β and κ-casein, βLg and αLac from all filtered (F) and pasteurised (P) milk samples after *in vitro* gastric digestion. The colours range from green to red, indicating low and high occurrence of specific peptides, respectively. Unidentified peptide sequences are shown as white stretches. (*) indicates a significant difference between filtered and pasteurised samples within the same brand ($p < 0.05$).

7.4.4 Peptides after *in vitro* intestinal stage

Figure 7.7 (**i**) shows that, overall, filtered samples released slightly more peptides than pasteurised samples. In **Figure 7.7** (**ii**) showed the peptide numbers released from filtered and pasteurised samples within the same brand, although filtered samples generally tended to release more peptides than pasteurised samples ($p = 0.14$).

This finding contrasts with the gastric stage (**Figure 7.1**), where differences in peptide count release were observed between filtered and pasteurised samples in some brands, with certain filtered samples releasing fewer peptides than pasteurised samples. This suggests that the processing method (microfiltration vs. pasteurisation) has a more pronounced effect during gastric digestion but becomes less impactful in the subsequent intestinal stage.

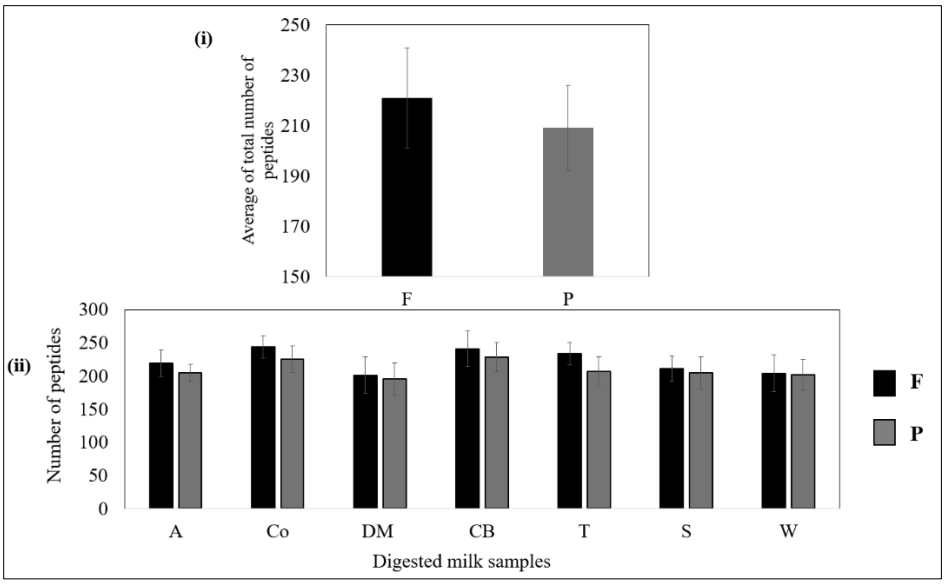


Figure 7.7: (i) The average number of peptides released from all filtered and pasteurised milk after the intestinal stage. (ii) The total number of peptides released from each filtered and pasteurised milk brand. No significant difference ($p > 0.05$) between F and P samples within the same brand.

The peptide length distribution of peptides that were released after the *in vitro* intestinal digestion can be seen in **Figure 7.8.** The length of the peptides identified ranged between 2 to 24 amino acids. The most abundant peptides contained 4, 8, 10, 11 and 12 amino acids. Digested-filtered milk released larger peptides containing 14, 18 and 19 amino acids compared to digested-pasteurised milk ($p = 0.21$). In comparison with the gastric stage, the length of peptides released from the intestinal stage was shorter than the gastric stage which showed a wider and longer range of peptide length.

The peptide length distribution in filtered and pasteurized milk after the intestinal stage reveals differences in peptide profiles among the milk brands (**Figure 7.9**). On average, peptides with lengths of 2 – 8 amino acids made up the majority, accounting for approximately 75 ± 6.1 % of total peptides in filtered milk and 76 ± 3.6 % of total peptides in pasteurized milk ($p > 0.05$). Slightly more variability was observed in the filtered samples, with percentages ranging from 73 - 78 % of total peptides, compared to a narrower range of 75 to 78 % of total peptides in pasteurised samples.

For peptides longer than 14 amino acids, filtered milk showed a distribution range of 0.4 to 2 % of total peptides, while pasteurised milk ranged from 0.4 to 1.4 % of the total peptides, representing

only a small fraction of the peptide profile in both milk types. Whereas the percentage of peptide length distribution in the length 9–14 amino acids represent the second largest group, comprising about 22 ± 2.4 % of the total peptides in both milk types. The variability in filtered milk, suggests some differences in proteolysis between brands during digestion may be due to variation in microfiltration conditions that impact structure changes.

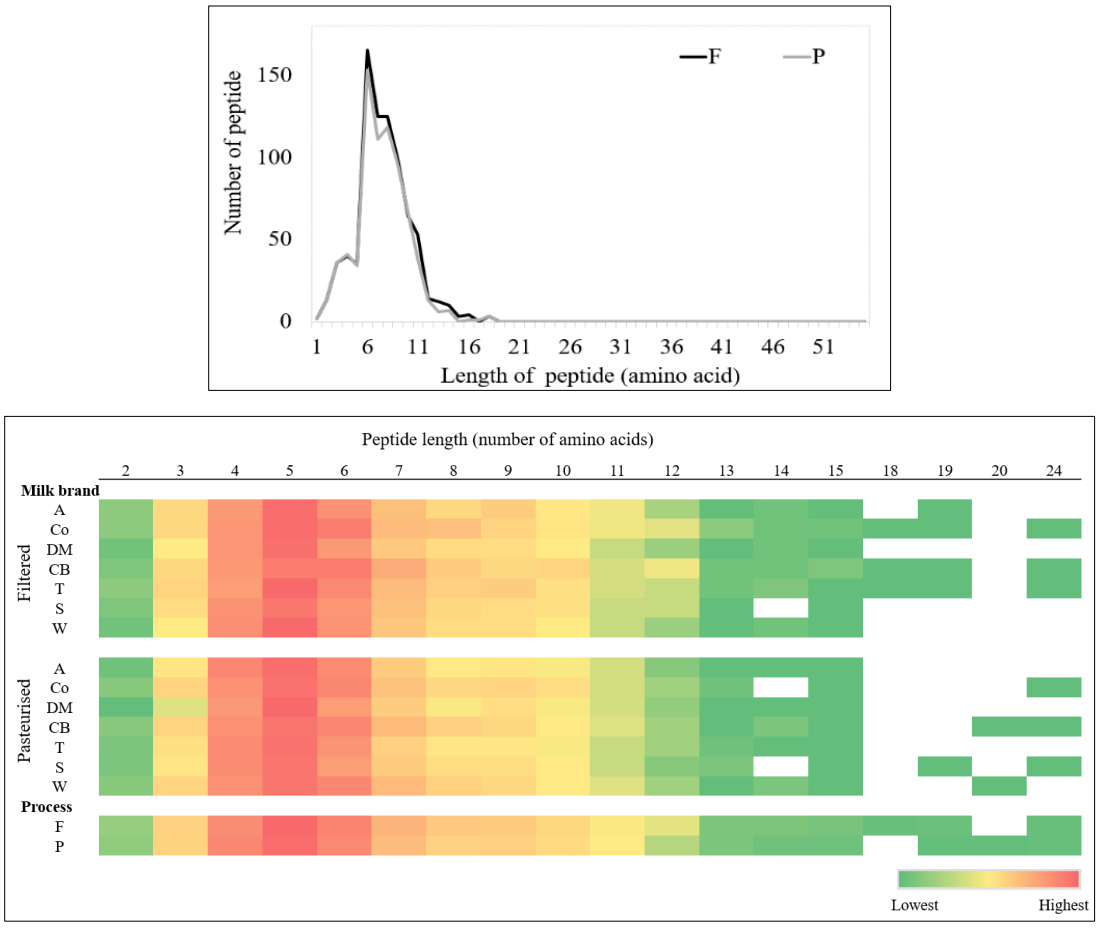


Figure 7.8: Peptide length distribution after the *in vitro* intestinal stage. The number of peptides dependent on the peptide length (number of amino acids), identified peptides in all filtered (F) and pasteurised (P) after *in vitro* intestinal digestion (top). Heatmaps of the peptide length distribution in each *in vitro* intestinal digested filtered and pasteurised milk brand (A, Co, DM, CB, T, S, and W). The colours range from green to red, indicating low and high occurrence of specific peptides, respectively. Unidentified peptide sequences are shown as white stretches.

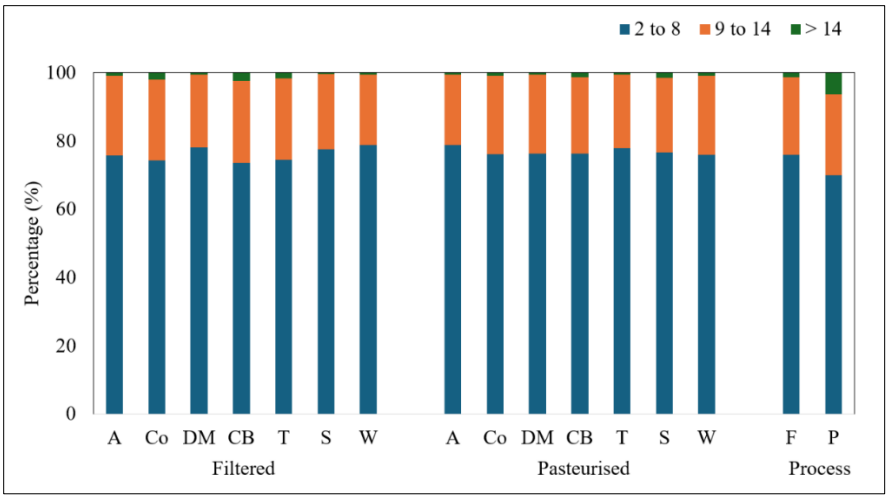


Figure 7.9: Length distribution of the identified peptides from *in vitro* intestinal digestion of filtered and pasteurised milk brands (A, Co, DM, CB, T, S and W) and overall process (F = filtered and P = pasteurised).

A heatmap of the size of the peptides released after *in vitro* intestinal digestion can be seen in **Figure 7.10**. Overall, both digested filtered and pasteurised milk showed that the highest proportion of peptides were those with a mass less than 1200 Da (between 600 Da – 1200 Da) followed by smaller peptides (less than 600 Da).

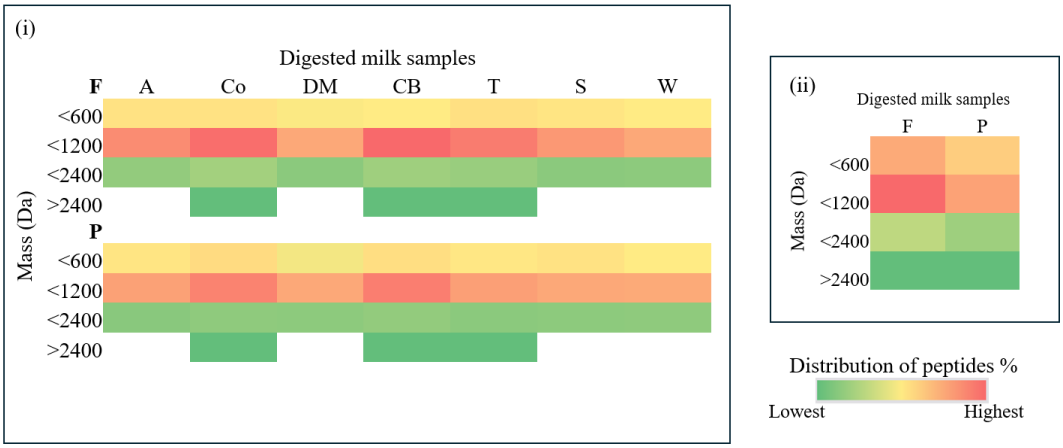
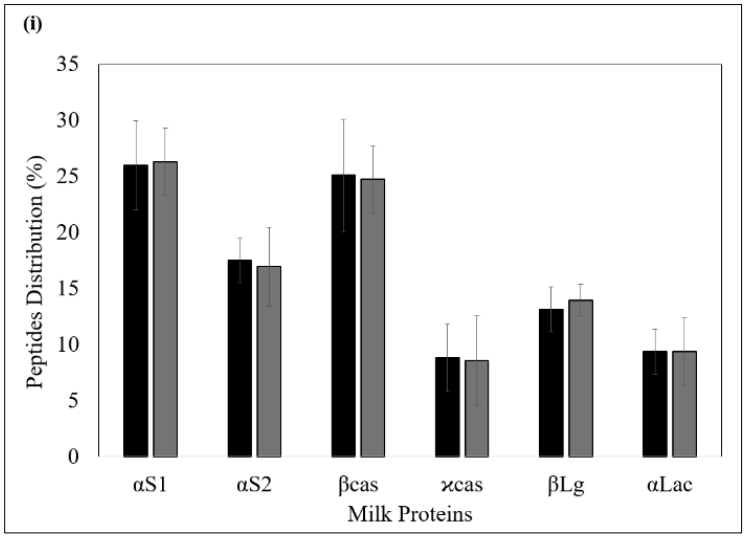


Figure 7.10: (i) Heatmap of the percentage of peptide size distribution in *in vitro* intestinal digested filtered (F) and pasteurised (P) milk samples (A, Co, DM, CB, T, S and W). (ii) Heatmap of peptide size distribution in all filtered and pasteurised samples. The colour ranges from green to red, indicating low and high occurrence of specific peptides, respectively. Unidentified peptide sequences are shown as white stretches.

Overall, the peptides released after *in vitro* intestinal digestion of filtered and pasteurised milk were derived from αS1, followed by β-casein, αS2, and βLg, accounting about 26 ± 5.3 %, 25 ±

395 3.9 %, 17 ± 3.5 %, and 13 ± 2.8 % of the total peptides, respectively **Figure 7.11 (i)**. No significant
396 difference was found the peptides released after *in vitro* intestinal digestion of filtered and
397 pasteurised milk within the same brand (**Figure 7.11**).



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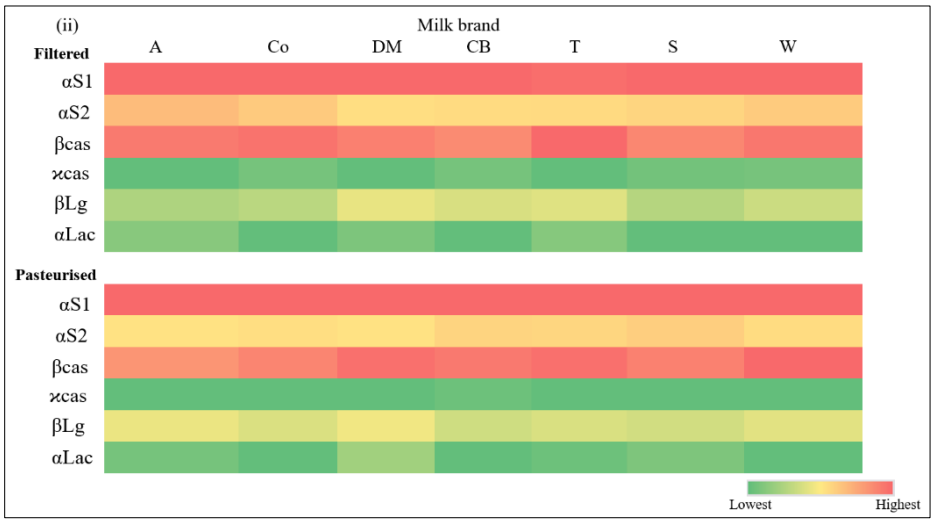

399

400 **Figure 7.11:** (i) The distribution of peptides from the parent proteins αS1, αS2, β and ϰ-casein, βLg and αLac from
401 all *in vitro* intestinal digested filtered and pasteurised semi-skimmed milk. (ii) Heatmap of peptides from the parent
402 proteins from each milk brand (A, Co, DM, CB, T, S and W). The colours range from green to red, indicating low
403 and high occurrence of specific amino acids, respectively.
404

405 **Figure 7.12** presents a heatmap showing the distribution of peptide lengths (as a percentage of
406 total peptides) derived from different milk proteins after *in vitro* intestinal digestion of filtered and
407 pasteurised milk. From the heatmap, it is evident that certain peptide lengths are more frequently
408 generated from specific proteins. Small peptides (< 7 amino acids) are abundantly released from

αS1 and αLac, followed by β-casein and κ-casein, while shorter peptides (2–6 amino acids) are less common in βLg. Both αLac and κ-casein in filtered samples released fewer small peptides compared to pasteurised samples. Medium peptides (7–11 amino acids) are released at a higher percentage from αS2, β-casein, and κ-casein in both filtered and pasteurised samples. In addition, medium peptides released from βLg in pasteurised samples in higher percentage than filtered samples. In contrast, αS1 and αLac in both filtered and pasteurised samples a showed the lowest percentage of medium peptides. This visualisation provides insights into how different proteins release peptides of varying lengths and highlights some difference between filtered and pasteurised samples.

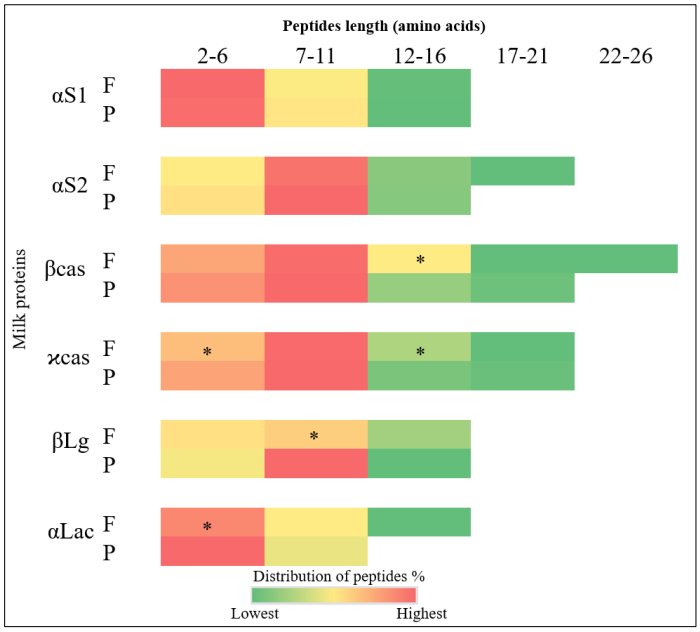

Figure 7.12: Heatmap of peptide length distribution from the parent proteins αS1, αS2, β and ϰ-casein, βLg and αLac from all *in vitro* intestinal digested filtered (F) and pasteurised (P) milk samples. The colours range from green to red, indicating low and high occurrence of specific amino acids, respectively. Unidentified protein sequences are shown as white stretches. (*) indicates a significant difference ($p < 0.05$) between F and P samples from the same brand.

7.4.5 Bioactive peptides identification

The peptide profiles of digested milk were characterised by detecting the most abundant peptides, which were then compared with a milk bioactive peptide database (MBPDB) (Nielsen et al., 2023). The pooled analysis for the peptides released from all filtered and pasteurised milk samples revealed about 579 and 510 identified peptides, respectively. The main parent proteins of the

identified bioactive peptides were β-casein followed by αS1, βLg, αLac and αS2. The peptide distribution among the filtered and pasteurised samples varied and some differences in peptide profiles were noticed. Approximately 7.3 % and 6.0 % of the total peptides released from filtered and pasteurised milk, were potential bioactive peptides, representing a possibility of 12 different categories of bioactivity (**Table 7.4**). The most abundant bioactive peptides observed were ACE-inhibitory, antioxidant, antimicrobial, DPP-IV Inhibitory and opioid peptides. Considering the relative abundance, the ACE-inhibitory peptides were the largest group of bioactive peptides identified in filtered samples; ACE-inhibitory peptides represented 65 % of all the bioactive peptides in the pasteurised samples. Interestingly, the relative abundance of DPP-IV Inhibitory peptides was higher in filtered milk than in pasteurised milk. Whereas the antioxidant and antimicrobial peptides represented similar abundance in both treatments. β-casein and βLg are the main sources of peptides that affect satiety or have DPP-IV inhabitation bioactivity (Kondrashina et al., 2020), and those proteins and derived peptides may be affected by microfiltration which needs further investigation in this area.

The peptide sequences released after *in vitro* intestinal digestion of filtered and pasteurised milk were compared with reported epitopes reviewed by Xu et al. (2024) and Fan et al. (2023). Many of these bioactive peptides have the capacity to bind IgE, acting as linear epitopes that correspond to established allergenic sequences (**Table 7.4**). While numerous peptides are known to trigger allergic symptoms, only a few of these peptides were detected in our study. This may be attributed to the digestion method used, as most studies on allergenic peptides rely on trypsin (Fan et al., 2023; Villa et al., 2018; Xu et al., 2024). In contrast, our study employed pepsin and pancreatin. The peptide distribution suggests that filtered milk may have a distinct peptide profile. Additional studies are necessary to understand how microfiltration influences the release of bioactive and allergenic peptides, particularly regarding their IgE-binding properties.

460 **Table 7.4:** The most abundant bioactive peptides from all *in vitro* intestinal digested filtered (F) and pasteurised (P)
461 milk samples. (√) means present and blank means not present.

Peptide	Milk sample		Bioactivity of the peptide				
	F	P	ACE-inhibitory	antimicrobial	DPP-IV Inhibitory	antioxidant	IgE-binding
AMKPW	√	√	Δ				*
AYFYPE	√		Δ	●		◊	
DVENLHLPLPL	√	√					*
EMPFPK	√	√	Δ			◊	
EQLTK	√	√				◊	
FFVAP	√		Δ				*
FVAPFPEVFG	√	√	Δ				
FYPEL	√	√	Δ	●			
GLDIQK	√	√	Δ				*
GVSLPEW	√	√	Δ				*
HLPLP	√		Δ				
IPAV		√					
IVP	√	√	Δ				
LHLPLP	√	√	Δ				
LIVTQTMK	√						*
LNVPGEIVE	√		Δ				*
LPQ	√				□		
LVYPFPGP	√		Δ				*
LVYPFPGPI	√	√	Δ				*
MPFPKYPVEP	√		Δ				
NVPGEIVESL	√	√		●			
PIVLNP	√	√					
PMHIR	√		Δ				
PVVVPPFLQPE	√	√				◊	*
RELEEL	√	√		●			*
SDIPNPIGSENSEK	√	√				◊	*
TEDELQDKIHPF	√	√				◊	
TPEVDDEALEK	√				□	◊	*
TTMPLW	√	√	Δ			◊	
VLDTDY	√		Δ		□		*
VLPVPQ	√	√					
VPSERYL	√	√	Δ				*
VSLPEW	√	√	Δ				
VYPFPGPI	√	√					*
VYPFPGPIPN	√	√	Δ	●			*
YFYPEL	√	√	Δ	●			
YPFPGPIP	√	√	Δ	●			
YPVEPF	√	√		●	□	◊	
YQEPVLGPVRGPFPI	√					◊	

7.5. Conclusion.

Comprehensive profiles of peptides released from *in vitro* digested filtered and pasteurised milk from seven different brands were established by Q-TOF LC/MS. Some milk proteins released peptides which differed in the peptide distribution between filtered and pasteurised samples. The search for bioactive peptides, such as DPP-IV, showed that filtered milk released about 25% more peptides with DPP-IV inhibitory activity than pasteurised samples. The variation observed in filtered samples may reflect differences in microfiltration conditions, which could, in turn, impact milk protein digestion and the resulting peptide profile. Further investigations are now required to clarify the effect of microfiltration on protein digestion and peptide bioactivities, which is mostly unknown. These primary results show that microfiltration may impact the resulting peptide profiles following digestion. An in-depth analysis will be needed to show if these changes may have a significant effect on the biological properties of proteins. For this purpose, however, more controlled processed filtered milk and more detailed studies on the effect of microfiltration on milk protein are necessary.

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597

Chapter 8. Overall discussion, conclusion and future research.

8.1. Overall discussion and conclusion

Milk is a nutrient-rich whole food matrix containing various essential nutrients, which can be affected by milk processing conditions. Therefore, understanding the relationship between processing conditions and milk quality is crucial for the dairy industry. Milk processing is essential for ensuring milk safety, creating a variety of dairy products, and extending shelf life. However, the purpose of food processing extends beyond ensuring safety, shelf life, and taste; there has been growing interest in understanding how various processing methods affect the physicochemical properties and structure of milk proteins, as these factors directly influence protein digestibility, bioavailability, and potential allergenicity. Several studies (Bhat et al., 2021; Bu et al., 2013; Cui et al., 2023; Graversen et al., 2020; Graversen et al., 2021; van Lieshout et al., 2020; Wu et al., 2018; Zhou et al., 2024) have primarily focused on how processing techniques can modify cow's milk protein epitopes, potentially altering their allergenicity. The allergenicity of proteins (epitopes) is often assessed by their capacity to bind to IgE. When milk proteins enter the bloodstream, they can specifically bind to IgE on the surface of mast cells. This binding triggers mast cells to release histamine and other inflammatory mediators, which lead to the clinical symptoms associated with cow's milk allergy. These studies defined the epitopes as specific regions on an allergenic protein that the immune system recognizes, particularly through IgE antibodies. These epitopes can be classified into two types: conformational and linear. Conformational epitopes consist of discontinuous amino acid sequences that rely on the protein's tertiary or quaternary structure and are stabilised by disulfide bonds under the acidic gastric environment (Pekar et al., 2018). On the other hand, linear epitopes are continuous amino acid sequences forming part of the primary protein structure and are generally resistant to digestive enzymes. Changes in conformational epitopes can occur through intramolecular thiol or disulfide exchange reactions, which can affect the protein hydrolysis, resulting in alteration of their IgE-binding capability. On the other hand, enzymatic hydrolysis can modify the ability of linear epitopes to elicit an immune response.

Certain processing conditions that disrupt the IgE-binding action of proteins may reduce their allergenicity. It is not just the processing method itself but the specific conditions under which it

is applied that determine the effect on allergenicity. Factors like temperature, heating duration, and the presence of certain compounds can influence allergenic properties. For example, heating milk above 65 °C for 30 min significantly reduces the allergenicity of βLg, while heating above 90 °C can increase it (Xu et al., 2016). Additionally, compounds such as lactose (Pi et al., 2023) or polyphenol (Yang et al., 2024) may facilitate covalent interactions with the amino or thiol side chains of allergens. Such interactions can mask or destroy dominant linear epitopes, leading to a reduction in IgE-binding capacity.

Thus, processing conditions that cause protein denaturation or/and disulfide- or thiol-mediated interactions which can impact allergenicity by either disrupting IgE-binding epitopes or exposing additional epitopes from within the protein structure or generation of insoluble protein aggregates (Rahaman et al., 2015; Simmons et al., 2007; Wal, 2001). This can lead to either an increase or decrease in allergenic potential, depending on the specific conditions applied. However, heating milk for extended periods at high temperatures can result in undesirable sensory properties (Fox et al., 2015). Thus, combining heat treatment with other food processing technologies, particularly non-thermal methods, may offer an opportunity to develop low-allergenicity cow's milk products. For instance, Rahaman et al. (2015) found that heat- and shear-induced aggregation may further disrupt conformational epitopes via intermolecular disulfide-mediated aggregation, consequently decreasing the antigenic response.

While thermal processing is the most commonly used method for shelf-life extension, microfiltration has recently been adopted commercially as an extra step to achieve this. The objective of this thesis was to identify the differences between commercially available filtered and pasteurised milk in the UK markets, specifically focusing on protein structure and peptide release following protein digestion, with an emphasis on discussing potential allergenicity.

As filtered milk becomes increasingly available in UK supermarkets, analysing these commercially available products provides a realistic basis for studying the milk that consumers are now encountering. This approach offers a practical means of assessing the characteristics of milk as it is actually consumed. The initial step of this study involved a thorough investigation to collect data on the availability of filtered cow's milk in the UK market. Filtered milk has shown a notable increase in market share, while pasteurised remains the predominant type. Between 2022 and 2024, the availability of whole, semi-skimmed, and skimmed filtered milk had increased by 74, 25 and

17%, respectively, across UK markets (Chapter **3**). These findings indicate that filtered milk is gaining ground towards becoming a leading consumer choice alongside pasteurised milk. Despite its growing popularity, limited research exists on the effects of microfiltration on milk protein structure and the release of bioactive peptides, such as antihypertensive, antioxidant, or immunomodulatory peptides, that could have significant nutritional implications. Therefore, this study conducted an in-depth investigation into the impact of microfiltration on milk structure. Filtered milk samples displayed additional interactions between fat globules and proteins, with a significantly larger Z-average ($p < 0.05$) than pasteurised milk across all commercial brands. Furthermore, filtered milk showed a significantly lower free thiol content ($p < 0.05$) compared to pasteurised milk across all brands analysed. Despite all samples undergoing pasteurisation and homogenisation, applying centrifugation after these processes yielded different outcomes for filtered milk compared to pasteurised samples. Filtered milk retained more fat than pasteurised samples, providing an initial insight into the potential for protein-fat interactions. This preliminary finding allowed for further investigation into protein-fat interactions without immediately resorting to complex and costly experiments. CLSM visualisation of filtered milk further revealed distinct fat and protein distribution influenced by milk processing, suggesting that microfiltration may impact intermolecular structure by enhancing protein-fat interactions through thiol-disulfide interchange reaction between milk protein and fat globule membrane protein.

To compare digestion and peptide release between filtered and pasteurised milk, BCM7 levels and overall peptide profiles were analysed (Chapter 5Chapter 6). Numerous studies indicate that BCM7 is primarily released from A1 β-casein, with lower amounts released from low or free A1 variant (i.e Jersey milk) (Brooke-Taylor et al., 2017; Lambers et al., 2021; Nguyen et al., 2015). This peptide's role in health and allergenicity is an area of interest, particularly given its potential effects on the gastrointestinal and immune systems (Cattaneo et al., 2023; Cattaneo et al., 2020; Cieślińska et al., 2007; De Noni & Cattaneo, 2010; EFSA, 2009; Nguyen et al., 2015; Ul Haq, 2020). To investigate the effect of microfiltration on milk digestibility and bioactive peptide release, specifically the opioid peptide BCM7, an *in vitro* static gastrointestinal digestion of conventional (filtered and pasteurised) semi-skimmed milk was applied. The resulting BCM7 and peptide profiles were then analysed using LC/MS. Jersey (A1-free) whole milk was included for comparison, as all commercially available Jersey milk is whole milk. Previous studies (Cieślińska et al., 2007; Lambers et al., 2021; Nguyen et al., 2021) have indicated that Jersey milk releases

little to no BCM7, making it a useful reference in evaluating BCM7 release across different milk types.

Results showed that BCM7 was released primarily during the intestinal digestion stage, with comparable amounts detected in both filtered and pasteurised milk after digestion ($p > 0.05$), indicating no significant difference between the two processing methods in terms of BCM7 release. The digested Jersey milk released approximately half the amount of BCM7 compared to conventional milk samples ($p < 0.05$). The comparison between semi-skimmed conventional milk and whole Jersey milk, which differ in β-casein variants and fat content, provided insights into additional factors that may influence BCM7 release. This led the research to further investigate the roles of β-casein variants, fat content, and protein digestion in potentially affecting BCM7 release, shifting the focus to explore how these variables might interact and contribute to differences in BCM7 production. For this purpose, more commercially available conventional (filtered and pasteurised) and Jersey milk samples with different fat content were collected. Interestingly, whole milk released less BCM7 than the semi-skimmed samples from the same brand. The results showed there are differences in the release of BCM7 among all whole and low-fat content samples regardless of β-casein variants content. This suggests that reduced-fat milk may promote greater BCM7 release, possibly due to alterations in protein structure or/and the presence of fat that results in altered digestion processes (Bao et al., 2023; Ding et al., 2022; Iqbal et al., 2024). The difference between semi-skimmed and skimmed milk was minimal, indicating that fat reduction may have a more significant effect on releasing BCM7 when transitioning from whole milk to lower-fat milk. A strong, statistically significant inverse relationship was observed with both fat content and processing type (microfiltration vs. pasteurisation) appearing to influence BCM7 release, with microfiltration notably increasing BCM7 release, especially in whole milk. However, the presence of fat seems to have a limiting effect on BCM7 release, exerting a greater impact than the processing method itself.

The analysis of overall peptide profiles revealed that, although the percentage of protein digestion after the intestinal stage showed no significant difference between filtered and pasteurised milk samples, filtered milk exhibited a slight increase in the number of peptides released post-digestion compared to pasteurised milk. Additionally, variability in peptide length distribution was observed among filtered samples, potentially indicating differences in microfiltration conditions across

brands. This variation in processing may have contributed to structural differences in milk proteins, which, in turn, impacted their digestion and peptide release.

Allergenicity is influenced not only by the degree of protein hydrolysis but also by the structure or sequence of the resulting digestion products. Moreover, changes in the food matrix can affect protein digestibility, and consequently, allergenicity (Bøgh et al., 2024; Cui et al., 2023; Dall'Antonia et al., 2014; Huang et al., 2022; Liu et al., 2022). Pekar et al. (2018) demonstrated that a protein-rich matrix containing fats and carbohydrates can prolong the stability of certain milk allergens. For example, when this matrix was introduced, βLg initially degraded and became undetectable after 10 min in the intestinal phase, but IgE antibody detection extended up to 120 min. Additionally, Rahaman et al. (2015) found that shear force can disrupt IgE binding capacity by altering thiol-disulfide interchange reactions. These findings suggest that protein-fat interactions, such as those observed in filtered milk, may impact the allergenic potential of milk proteins by influencing their structural stability during digestion. Thus, protein-fat interactions in filtered milk may have significant implications for milk allergenicity.

To conclude, microfiltration has shown significant potential in promoting protein-fat interactions, which can alter protein structure. Observed variations among commercially filtered samples suggest that differences in microfiltration conditions (such as temperature, shear force, etc.) may lead to distinct effects on protein structure and peptide release. This study provided valuable insights into how microfiltration influences milk protein structure and peptide profiles, underscoring the potential for using these findings to develop dairy products. Although microfiltration alters milk protein structure, the treatment alone may not be sufficient to reduce milk allergenicity. Further research is needed to directly measure allergenic potential.

8.2. Contribution to Knowledge

While there is abundant literature on the effects of heat and homogenisation on cow's milk physical, chemical, and nutritional properties, this study is the first comprehensive investigation into the impact of microfiltration on such cow's milk properties. The contributions of this research to scientific knowledge can be summarised as follows:

- The findings offer practical insights for food scientists and the dairy processing industry, laying a foundation for future research aimed at leveraging the structural changes induced by microfiltration.
- A reliable, rapid method for peptide separation and quantification was developed using the Q-TOF LC/MS technique.
- Conducting experiments directly on milk and digested milk samples, without additional extraction or purification, yielded comparative results that provided valuable insights while also reducing costs and environmental impact by minimizing the use of chemicals and specialized equipment typically required for sample purification.

This study opens new pathways for exploring protein-fat interactions and their implications for the dairy industry, especially in terms of product formulation and potential hypoallergenic applications.

8.3. Future research

Throughout this research, several interesting observations were made regarding the effects of microfiltration on milk properties. However, not all aspects could be fully explored due to the limited time frame, study scope, and the absence of a microfiltration unit. The following areas that worth further exploration to fill current knowledge gaps and offer deeper insights into cow milk processing characteristics:

- Investigating the effect of microfiltration under controlled conditions, considering the influence of variables like temperature, shear force, and milk matrix, would be valuable for understanding its detailed impact.
- Studying the effect of microfiltration on IgE binding capacity, while factoring in these processing conditions and variations in milk types (e.g., Jersey milk), would help assess potential allergenic responses.
- To establish the full commercial potential of filtered milk properties, further research on how microfiltration impacts functional and sensory qualities, compared to traditional heat treatments, is essential.

- Conducting consumer research studies would be invaluable to assess consumer acceptability and preferences, providing insights into the perceptions and market potential for filtered milk.

These areas of inquiry would offer valuable contributions to both scientific knowledge and the practical application of microfiltration in the dairy industry.

8.4. References

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