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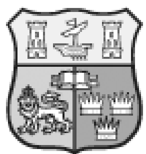

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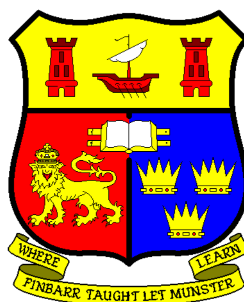

Generation and characterisation of biologically active milk-derived protein and peptide fractions

Thesis presented by

Brian Andrew McGrath, B.Sc. (NUI)

for the degree of

Doctor of Philosophy

in

Food Science and Technology

March, 2014

Table of Contents

Declaration

Acknowledgements

Abstract

		Page
Chapter 1	A review of the composition and physico-chemical properties of bovine colostrum	1
Chapter 2	Composition and physico-chemical properties of bovine colostrum during the first 3 days *post partum*	83
Chapter 3	Characterisation of bovine colostrum and potential anti-inflammatory and insulinotrophic properties of fractions thereof	177
Chapter 4	Characterisation of the protein component of milk from cows with lipopolysaccharide-induced mastitis and its potential immunomodulatory properties	256
Chapter 5	Heat-induced hydrolysis of sodium caseinate and potential production of biologically active peptides	296
Chapter 6	Proteomic characterisation of sodium caseinate hydrolysed by chymosin and its potential effect on satiety	349
Chapter 7	General discussion	413

Declaration

I hereby declare that, unless otherwise stated, the work submitted is entirely my own and has not been submitted to any other university or higher education institute, or for any other academic award in this university.

______________________ Date: ____________________

Brian Andrew McGrath

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Abstract

In recent years, extensive research has been carried out on the health benefits of milk proteins and peptides. Biologically active peptides are defined as specific protein fragments which have a positive impact on the physiological functions of the body; such peptides are produced naturally *in vivo,* but can also be generated by physical and/or chemical processes, enzymatic hydrolysis and/or microbial fermentation. The aims of this thesis were to investigate not only the traditional methods used for the generation of bioactive peptides, but also novel processes such as heat treatment, and the role of indigenous milk proteases, e.g., in mastitic milk, in the production of such peptides. In addition, colostrum was characterised as a source of bioactive proteins and peptides. Firstly, a comprehensive study was carried out on the composition and physical properties of colostrum throughout the early-lactation period. Marked differences in the physico-chemical properties of colostrum compared with milk were observed. Various fractions of colostrum were also tested for their effect on the secretion of pro- and anti-inflammatory cytokines from a macrophage cell line and bone marrow dendritic cells, as well as insulin secretion from a pancreatic beta cell line. A significant reduction in the secretion of the pro-inflammatory cytokines, TNF-α, IL-6, IL-1β and IL-12, a significant increase in the secretion of the anti-inflammatory cytokine, IL-10, as well as a significant increase in insulin secretion were observed for various colostrum fractions. Another study examined the early proteomic changes in the milk of 8 cows in response to infusion with the endotoxin lipopolysaccharide (LPS) at quarter level in a model mastitic system; marked differences in the protein and peptide profile of milk from LPS challenged cows were observed, and a pH 4.6-soluble fraction of this milk was found to cause a substantial induction in the secretion of IL-10 from a murine macrophage cell line. Heat-induced hydrolysis of sodium caseinate was investigated from the dual viewpoints of protein breakdown and peptide formation, and, a peptide fraction produced in this manner was found to cause a significant increase in the secretion of the anti-inflammatory cytokine, IL-10, from a murine macrophage cell line. The effects of sodium caseinate hydrolysed by chymosin on the gut-derived satiety hormone glucagon-like peptide-1 (GLP-1) were investigated;

the resulting casein-derived peptides displayed good *in vitro* and *in vivo* secretion of GLP-1. Overall, the studies described in this thesis expand on current knowledge and provide good evidence for the use of novel methods for the isolation, generation and characterisation of bioactive proteins and/or peptides.

Chapter 1

A review of the composition and physico-chemical properties of bovine colostrum

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1.1 Introduction

Milk is a fluid secreted by the female of all (approximately 4500) mammalian species (Darewicz *et al.,* 2011). It is a complete source of carbohydrate, protein, fat and minerals, the primary function of which is to meet the complete nutritional requirements of the neonate, while also serving several physiological functions (Fox, 2009a). In addition to its principal components, milk contains several hundred minor constituents, e.g., vitamins, hormones, enzymes, metal ions and flavour compounds (Fox and McSweeney, 1998). The majority of the non-nutritional functions of milk are carried out by proteins and peptides, including immunoglobulins, enzymes and enzyme inhibitors, binding or carrier proteins, growth factors and antibacterial agents (Fox and McSweeney, 1998).

Milk is an aqueous solution consisting of lactose, organic and inorganic salts and several compounds at trace levels (milk serum). In the milk serum, colloidal particles of three size ranges are dispersed: whey proteins dissolved at the molecular level, the caseins dispersed as large (50-500 nm) colloidal aggregates (micelles), and lipids emulsified as large (1-20 µm) globules (Fox, 2009a).

The composition of milk is highly variable between species, reflecting the different nutritional and physiological requirements of the young, which is the result of a long and slow adaptive evolutionary process that started 150 million years ago (Zeder, 2008). In addition to interspecies differences, the composition of milk of any particular species varies due to a number of factors, including individuality of the animal, breed, health (mastitis and other diseases), nutritional status, stage of lactation, age and interval between milkings (Walstra *et al.,* 2006). The milk of the principal dairying species, i.e., cow, goat, sheep and buffalo, and the human are among those that are well characterised (Jensen, 1995).

Colostrum is the milk secreted following parturition. The duration for which milk is classified as colostrum varies considerably amongst authors, i.e., immediately after parturition (Levieux and Ollier, 1999; Nakamura *et al.,* 2003; Godhia and Patel, 2013), two days (Playford *et al.,* 2000; Playford, 2001), three to four days (Foley and Otterby, 1978; Gopal and Gill, 2000; Davis *et al.,* 2007; Zhang *et al.,* 2011) or five to

seven days *post partum* (Marnila and Korhonen, 2002; Georgiev, 2008; Zarcula *et al.,* 2010; Abd El-Fattah *et al.,* 2012). As with mature milk, the composition of colostrum is highly variable between different species, as shown in Table 1.1. For the purposes of this review, colostrum, unless otherwise defined, will refer to the early *post partum* milkings from dairy cows.

Table 1.1 Composition of colostrum from various species (per 100 mL) (data taken from Emmett and Rogers, 1997; El-Agamy, 2006; Park, 2006; Silk *et al.,* 2006; Kehoe *et al.* 2007; Csapo *et al.,* 2009; Pecka *et al.,* 2012)

Nutrient	Bovine	Human	Camel	Yak	Mare	Sow
Total solids (g)	27.6	11.8	25.1	33	19.3	20.5
Protein (g)	14.9	2	17.8	16.1	15.2	10.6
Fat (g)	6.7	2.6	0.45	14	1.7	5.8
Lactose (g)	2.5	6.6	4.3	1.9	2.5	3.4
Ash (g)	0.05	na	2.6	1	0.59	0.7
Calcium (mg)	472	28	156	na	74.8	na
Phosphorus (mg)	445	14	255	na	74.2	na
Sodium (mg)	106	47	na	na	32	na
Potassium (mg)	285	70	na	na	92.8	na
Iron (Fe)	0.53	0.07	na	na	0.1	na
Magnesium (mg)	73.3	3	45	na	14	na
Zinc (mg)	3.8	0.6	na	na	0.3	na

na = information not available

The composition and physical properties of colostrum are highly variable, due to a number of factors, including individuality, breed, parity, prepartum ration, length of dry period of cows, and time *post partum* (Parrish *et al.,* 1947, 1948, 1949; Moody *et al.,* 1951). In general, colostrum contains less lactose and more fat, protein, peptides, non-protein nitrogen, ash, vitamins and minerals, hormones, growth factors, cytokines and nucleotides than mature milk and, except for lactose, the levels of these compounds rapidly decrease during the first 3 days of lactation (Campana and Baumrucker, 1995; Blum and Hammon, 2000a,b; Uruakpa *et al.,* 2002). Colostrum is best characterised by its very high concentration of immunoglobulin G (IgG), in particular from the IgG_1 subclass (Butler, 1981). This is of

particular importance to the neonate, whose gut, immediately following parturition, allows the passage of large immunoglobulins, thereby conferring passive immunity (Marnila and Korhonen, 2002; Tsioulpas *et al.,* 2007; Stelwagen *et al.,* 2009). It is essential that the newborn calf receives an adequate supply of colostrum as both the concentration of immunoglobulins and permeability of the gut decrease rapidly over the first 48 h following parturition (Bush and Staley, 1980; Moore *et al.,* 2005). Additionally, colostrum intake influences metabolism, endocrine systems and the nutritional state of neonatal calves (Guilloteau *et al.,* 1997; Blum and Hammon, 2000a,b) and stimulates the development and function of the gastrointestinal tract (Hadorn *et al.,* 1997; Guilloteau *et al.,* 1997; Buhler *et al.,* 1998; Blum and Hammon, 2000a,b).

Colostrum accounts for approximately 0.5% of a cow's annual milk output (Scammell, 2001). Most healthy dairy cows produce colostrum far in excess of the calf's nutritional requirements (Muller *et al.,* 1975; Rindsig, 1976; Oyeniyi and Hunter, 1978). In the past, milk collected during the colostrum period was considered unmarketable (Foley and Otterby, 1978) and was excluded from bulk milk collection (Marnila and Korhonen, 2002). The high protein content of colostrum leads to multiple problems in industrial processes, e.g., poor heat stability and subsequent low clotting temperature interferes with pasteurisation (Marnila and Korhonen, 2002; McMartin *et al.,* 2006). Also, the high content of antimicrobial components in colostrum may affect the fermentation process (Marnila and Korhonen, 2002). Despite this, colostrum has attracted considerable interest as a functional food ingredient (Korhonen, 1998). Colostrum preparations are currently available in the US and Europe as health food supplements. These products contain large amounts of potent growth factors and have been shown to influence cell growth and migration *in vitro* and prevent gastric and small intestinal injury induced by the non-steroidal anti-inflammatory drug (NSAID) indomethacin *in vivo* (Playford *et al.,* 1993, 1999; Chinery and Playford, 1995). Colostrum has also been used for treatment of other ulcerative conditions of the bowel (Simmen *et al.,* 1990; Playford *et al.,* 1999). The high concentrations of growth factors in colostrum, namely transforming growth factors alpha and beta (TGF-α and β), have been linked

with muscle and cartilage repair and promotion of wound healing (Tollefsen *et al.,* 1989; Wilson, 1997). Several authors have shown that TGF-β has a suppressive effect on the cytotoxic activity of monocytes (Ho and Lawton, 1978; Kohl *et al.,* 1980). Several studies have correlated the effectiveness of a colostrum supplement (Intact^{TM}) (Whyte, 1994) with improved athletic performance (Buckley *et al.,* 1998, 1999, 2000, 2002; Smeets, *et al.,* 2001; Kreider *et al.,* 2001a,b). In general, these trials have shown improved rates of recovery from exhaustive exercise, improved muscle power and performance, and increased body mass. Hofman *et al.* (2002) and Brinkworth *et al.* (2002) reported an improvement in athletic performance in elite hockey players and elite female rowers, respectively, with colostrum supplementation. Several authors have reported the benefit of colostrum for the treatment of chronic diarrhoea among persons with immune deficiency syndromes. In general, supplementation resulted in improved stool volume and decreased frequency of diarrhoea (Stephan *et al.,* 1990; Rump *et al.,* 1992; Kelly, 2003).

Boosting the natural concentrations of immune components in colostrum through vaccination of cows with appropriate antigens offers great potential for their use as prophylactic or therapeutic products in humans (Stelwagen *et al.,* 2009). Several commercial immune milk products are available on the market which target a variety of conditions (Pakkanen and Aalto, 1997), e.g., Gastrogard (Northfield Laboratories, Oakden, Australia) helps prevent diarrhoea caused by rotavirus in young children (Davidson *et al.,* 1989, 1994), Pro-Immune 99 (GalaGen Inc., Minnesota, USA) helps prevent scours caused by *E. coli* in young calves, Lactimmunoglobulin Biotest (Biotest Pharm GmbH, Frankfurt, Germany) is used in the treatment of severe diarrhea in AIDS patients (Stephan *et al.,* 1990), Bioenervi (Viable Bioproducts Ltd., Turku, Finland) provides growth and antimicrobial factors during strenuous physical activity (Mero, 1995). Hyperimmune colostrum has shown some degree of efficacy in either prophylaxis or treatment against *Cryptosporidia* (Tzipori *et al.,* 1987; Ungar *et al.,* 1990), *Helicobacter pylori* (Tarpila *et al.,* 1995; Oona *et al.,* 1997), rotavirus (Mach and Pahud, 1971; Mitra *et al.,* 1995), *Clostridium difficile* (Van Dissel *et al.,* 2005; Numan *et al*., 2007; Young *et al*.,

2007), *Escherichia coli* (Tawfeek et al., 2003) and *Shigella* (Tacket *et al.,* 1992; Ashraf *et al*., 2001).

Foley and Otterby (1978) reviewed the literature on the composition of colostrum, but, to the best of the authors' knowledge, no comprehensive review on the composition of colostrum has been published since. Changes in the composition and physical properties of milk throughout lactation have been studied extensively (White and Davies, 1958a,b,c; Cerbulis and Farrell, 1976; Donnelly and Horne, 1986; Horne *et al.,* 1986; Rodriguez *et al.,* 2001), however, little is known about the composition and stability of colostrum. The objectives of this review are to discuss comprehensively the composition and physico-chemical properties of colostrum.

1.2 Chemical constituents

1.2.1 *Carbohydrate*

1.2.1.1 *Lactose*

Lactose, β-D-galactopyranosyl-(1-4)-D-glucose, is the principal carbohydrate in milk. It is a reducing disaccharide, composed of galactose and glucose linked through a β 1-4 glycosidic linkage (Walstra *et al.,* 2006). Lactose synthesis, which occurs in mammary epithelial cells, is catalysed by the enzyme lactose synthase, which consists of the membrane-bound enzyme β1,4-galactotransferase and the regulatory protein α-lactalbumin (Meser and Tadasu, 2002). The lactose content of bovine milk ranges from 4.4 to 5.2%, averaging at 4.8% anhydrous lactose (Ganzle *et al.,* 2008). Several factors influence the lactose content of milk, i.e., breed of cow, individuality factors, udder infection and, in particular, the stage of lactation (Fox and McSweeney, 1998). The concentration of lactose in milk is higher at the beginning of lactation but then decreases to about 70% of its maximum towards the end of lactation (Fox, 2009b). The concentration of lactose is reduced in colostrum and behaves inversely to other constituents such as solids, protein and ash (Parrish *et al.,* 1950; Kehoe *et al.,* 2007). A low level of lactose in early *post partum* milkings, followed by a steady increase thereafter, until normal levels are

reached, has been reported by several authors (Parrish *et al.,* 1948, 1950; Kilmes *et al.,* 1986; Madsen *et al.,* 2004; Georgiev, 2005; Kehoe *et al.,* 2007; Tsioulpas *et al.,* 2007; Georgiev, 2008; Abd El-Fatah *et al.,* 2012; Morill *et al.,* 2012). Lactose concentrations as low as 1.2% have been reported in colostrum (Kehoe *et al.,* 2007; Morrill *et al.,* 2012). In general, lactose concentration reaches normal concentrations within 7 days *post partum*; however, Tsioulpas *et al.* (2007) reported that the lactose content of milk did not reach normal levels until 60 days *post partum*; however, no samples were taken between 30 and 60 days *post partum*.

Lactose is responsible for about 50% of the osmotic pressure of milk (Fox, 2009b); its production causes the movement of water from the cytoplasm of mammary epithelial cells into the secretory vesicles and subsequently into milk (Kuhn *et al.,* 1980). This influx of water into milk through osmotic effects regulates the volume of milk produced and the concentration of casein in milk (Jenness and Holt, 1987). A low level of lactose results in the production of milk that is extremely viscous and contains little water due to the absence of the osmoregulator lactose (Bleck *et al.,* 2009). In the study of Madsen *et al.* (2004), the density of the first *post partum* milking was found to be 1.048 g/mL, which is significantly higher than the average density of milk (1.029 g/mL), as reported by Walstra *et al.* (2006). This value decreased over the first six milkings until a normal level was reached, while in parallel the lactose concentration increased from 2.6% to 4.4% (Madsen *et al.,* 2004). Similarly, Strekozov *et al.* (2008) reported the density of first *post partum* milkings from 62 cows ranged from 1.054 g/mL to 1.072 g/mL. Czister (2003) reported that low level of lactose in colostrum helps to ensure a high viscosity, which may play a role in prevention of diarrhoea in the calf. In order to maintain osmotic pressure, milk with a low level of lactose has an elevated level of inorganic salts (Holt and Jenness, 1984).

1.2.1.2 *Oligosaccharides*

In addition to lactose, milk contains trace amounts of other sugars, including glucose, fructose, glucosamine, galactosamine, neuraminic acid and oligosaccharides (Fox and McSweeney, 1998). Oligosaccharides are defined as carbohydrates that contain 3 to 10 monosaccharides covalently linked through glycosidic bonds (Mehra and Kelly, 2006). Oligosaccharides are divided into two broad classes, i.e., neutral and acidic. Neutral oligosaccharides, or galacto-oligosaccharides (GOS), contain no charged carbohydrate residues, whereas, acidic oligosaccharides contain one or more negatively charged residues of *N*-acetylneuraminic acid (sialic acid) (Gopal and Gill, 2000). The concentration of oligosaccharides in colostrum is approximately 0.7 to 1.2 g/L (Veh *et al.,* 1981; Nakamura *et al.,* 2003), the majority of which are acidic, whereas mature milk contains only trace amounts (Gopal and Gill, 2000). To date, 40 oligosaccharides have been identified in bovine colostrum (Tao *et al.,* 2008, 2009; Barile *et al.,* 2010). The total number of oligosaccharides in colostrum varies between individual cows due to unique genetic variability (Ninonuevo *et al.,* 2006). In one study, the total number of oligosaccharides in colostrum from individual cows ranged between 14 and 32 (Barile *et al.,* 2010). 3'Sialylactose (3'SL), 6'sialylactose (6'SL), 6'sialylactosamine (6'SLN), and disialyllactose (DSL) are the predominant oligosaccharides in colostrum, with 3'SL accounting for 70% of the total oligosaccharide content (Martin-Sosa *et al.,* 2003; Nakamura *et al.,* 2003; McJarrow and van Amelsfort-Schoonbeek, 2004; Tao *et al.,* 2009; Urashima *et al.,* 2009). Nakamura *et al.* (2003) reported that levels of 3'SL, 6'SL and 6'SLN in colostrum were highest immediately following parturition and decreased rapidly by 48 h *post partum*, whereas neutral oligosaccharides increased. McJarrow and van Amelsfort-Schoonbeek (2004) found the concentrations of 3'SL, 6'SL, 6'SLN and DSL to be 681, 243, 239 and 201 mg/L, respectively, in Holstein colostrum and 867, 136, 220 and 283 mg/L, respectively, in Jersey colostrum immediately after parturition.

1.2.2 *Protein*

Bovine milk contains about 3.5% protein but the level varies considerably during lactation (Fox and McSweeney, 1998). Proteins in bovine milk can be divided into two groups based on their solubility at pH 4.6. Caseins are insoluble at pH 4.6 and represent approximately 80% of milk protein, whereas whey proteins are soluble at pH 4.6 and account for the remaining 20% of milk protein. Caseins are synthesised exclusively in the mammary gland; some whey proteins (e.g., β-lactoglobulin, α-lactalbumin and lactoferrin) are also synthesised in the mammary gland while others (e.g., serum albumin and immunoglobulins) are derived from the blood. Both the caseins and whey proteins are heterogeneous groups of proteins. There are four caseins, i.e., α_{S1}-, α_{S2}-, β- and κ-casein, which are present in the relative proportions 37:10:35:12, respectively. The whey proteins are a much more heterogeneous group, consisting primarily of β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin and the proteose-peptone fractions (Farrell *et al.,* 2004); however, bovine whey contains more than 200 different proteins, the majority of which are present at trace amounts (Golinelli *et al.,* 2011).

Few reports exist in the literature on changes in the properties of the caseins in milk from the colostrum period to early lactation. The concentration of casein is higher in colostrum than in milk (Crowther and Raistrick, 1916; Cerbulis and Farrell, 1975). Parrish *et al.* (1948) reported that the concentration of casein decreased from 6.4% to 2.8% over the course of the first 16 *post partum* milkings. Madsen *et al.* (2004) reported a similar trend, but, the concentration of casein in the first *post partum* milking was 9.24%, i.e., significantly higher than the value reported by Parrish *et al.* (1948). Sobscuz-Szul *et al.* (2013), who investigated changes in the relative proportions of the individual caseins in colostrum, found that early *post partum* milkings contained reduced proportions of α_s-casein, which increased with time *post partum*, and elevated proportions of κ-casein, which decreased with time *post partum*, while the proportion of β-casein remained constant throughout. A negative correlation between κ-casein and α_s-casein was also observed by Wielgosz-Groth, (2004). Glycomacropeptide (GMP) is a hydrophilic glycopeptide (f106-169) which is released from the C-terminal region of κ-casein by the action of

chymosin (Korhonen, 2012) and comprises 15-20% of cheese whey proteins (Saito *et al.*, 2001). Furlanetti and Prata (2003) reported that the level of free-GMP in milk is highest during the colostral period and decreases rapidly following parturition.

Table 1.2 Concentration of major whey proteins in mature milk and colostrum (data taken from Marnila and Korhonen, 2002; Pihlanto and Korhonen, 2003)

Protein	Concentration (mg/mL)	
	Colostrum	Milk
Immunoglobulins (IgG, IgM, IgA)	20-200	0.5-1.0
β-lactoglobulin	8	3.3
α-lactalbumin	3	1.2
Glycomacropeptide	2.5	1.2
Lactoferrin	1.5	0.1
Lactoperoxidase	0.02	0.03
Lysozyme	0.0004	0.0004
Growth factors	50 μg - 40 mg/L	<1 μg - 2 mg/L

The immunoglobulins, of which there are three major classes in milk, i.e., IgG, IgM and IgA, account for about 1% of the total milk protein or about 6% of the total whey protein (Farrell *et al.,* 2004). Factors which affect the Ig concentrations in milk include breed, age of the cow, lactation number and volume of the first *post partum* milking (Oyeniyi and Hunter, 1978; Muller and Ellinger, 1981; Pritchett *et al.,* 1991; Mechor *et al.,* 1992; Vacher and Blum, 1993; McFadden *et al.,* 1997). Colostrum has a distinct protein composition that differs substantially from that of milk and contains elevated levels of IgG, IgA and IgM (Smolenski *et al.,* 2007). Immunoglobulins make up 70-80% of the total protein in colostrum (Larson, 1992), which is of particular importance to the neonate, as transfer of passive immunity to the calf occurs through colostrum and not via the placenta (Zhang *et al.,* 2011). Changes in the level and relative proportions of the Igs in colostrum compared with milk have been studied by several authors (Quigley *et al.,* 1994; Levieux and Ollier,

1999; Korhonen *et al.,* 2000; Elfstrand *et al.,* 2002; Zhao *et al.,* 2010). The concentration of Igs in the first *post partum* milking can vary considerably, from 30 to 200 mg/mL (Hancock, 1985; Larson, 1992; Korhonen *et al.,* 1995; Gapper *et al.,* 2007). IgG_1 comprises over 75% (46.4 mg/mL) of the Igs in colostral whey, followed by IgM (6.8 mg/mL), IgA (5.4 mg/mL) and IgG_2 (2.9 mg/mL) (Butler, 1974). The concentration of Igs in milk declines rapidly following parturition to around 0.7-1.0 mg/mL (Korhonen *et al.,* 2000). In blood serum, both IgG subclasses are present at roughly equal concentrations (Korhonen *et al.,* 2000). The preferential occurrence of IgG_1 in milk and colostrum suggests a subclass-specific transport mechanism (Butler *et al.,* 1972; Morgan *et al.,* 1981), however, the precise mechanism remains unclear. One possible explanation is that a significant proportion of IgG_2 taken up by mammary epithelial cells from blood serum during colostrum formation does not pass on to the alveolar lumen (Hurley and Thiel, 2011).

The concentrations of β-lg and α-la are higher in colostrum than in mature milk (see Marnila and Korhonen, 2002; Georgiev, 2008). Marnila and Korhonen (2002) reported that the initial concentration of β-lg in colostrum ranges from 7.9 to 30 g/L, the average being 14 g/L in the first milking and falling sharply thereafter to 8 g/L in the second to fourth milkings. The decrease is more gradual until the sixteenth milking, when the average is 5 g/L. These results are in agreement with those reported by Levieux and Ollier (1999) and Sobczuk-Szul *et al.* (2013). Marnila and Korhonen (2002) reported that the level of α-la in colostrum decreases more gradually from 2 g/L in the first milking to 1.4 g/L in the sixteenth milking, which was as reported by Levieux and Ollier (1999). However, the results mentioned above differ substantially from those reported by Sobczuk-Szul *et al.* (2013), who observed that the initial concentration of α-la in colostrum from Jersey and Polish Holstein-Friesian cows was 13.82 and 7.91 g/L, respectively. The concentration ratio of β-lg:α-la is four times greater in colostrum than in mature milk, suggesting that β-lg may play a specific role during the early *post partum* period (Perez *et al.,* 1990).

Serum albumin represents about 1.5% of the total protein in mature milk and about 8% of total whey protein (Farrell *et al.,* 2004). The concentration of serum albumin in colostrum is higher than in milk (Zhang *et al.,* 2011). Perez *et al.* (1989) reported

that the first milking *post partum* contains 2.63 mg/mL albumin. This value decreases substantially within 24 h and reaches normal levels by the second week *post partum* (0.2 mg/mL). A similar trend was observed by Levieux and Ollier (1999), but the initial concentration of serum albumin was slightly lower (1.2 mg/mL) than that reported by Perez *et al.* (1989). The increased concentration of serum albumin in colostrum is a result of increased leakage from the circulating blood (Zhang *et al.,* 2011), which may play a role in the transport of small molecules, i.e., free fatty acids released from adipocytes (Evans, 2002).

Lactoferrin is a cationic iron-binding glycoprotein that is of mammary origin (Farrell *et al.,* 2004) which, in milk, plays a key role in the defense of the mammary gland (Cheng *et al.,* 2008). The concentration of lactoferrin in milk is significantly associated with somatic cell count (SCC), levels of serum albumin, stage of lactation, and milk production (Harmon *et al.,* 1975), and ranges from 0.02 to 0.75 mg/mL (Hahn *et al.,* 1998; Fox and Kelly, 2003; Turner *et al.,* 2003; Chen and Mao, 2004). Several authors have reported an increased concentration of lactoferrin in colostrum (Pakkanen and Aalto, 1997; Hahn *et al.,* 1998; Zhang *et al.,* 2002*,* 2011; Sobczuk-Szul *et al.,* 2013). Reiter (1978) reported that the concentration of lactoferrin in colostrum is 30-fold higher than that in milk, while Indyk and Filonzi (2005) observed a 100-fold higher concentration of lactoferrin in colostrum (5 mg/mL) than in milk (0.05 mg/mL). Typically, the concentration of lactoferrin in colostrum ranges from 1.5 to 5 mg/mL (Korhonen, 1977; Tsuji *et al.,* 1990; Hahn *et al.,* 1998).

There are few reports in the literature on the occurrence of minor proteins in colostrum. This is because detection and identification of minor proteins is difficult due to the high concentration of the principal proteins, which reduce detection sensitivity of mass spectrometry. Yamada *et al.* (2002) used immunoabsorption to remove major proteins (β-casein and IgG) from colostrum and milk prior to 2D-PAGE, which enabled the detection of several otherwise undetectable minor milk proteins. In the same study, a total of 29 minor proteins were identified in colostrum and milk, of which several were only observed in colostrum, i.e., fibrinogen β-chain, chitinase 3-like 1, α-antitrypsin, complement C3 α-chain,

gelsolin and apolipoprotein H. Smolenski *et al.* (2007), using a combination of liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) and two-dimensional gel electrophoresis (2-DE) followed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS), and D'Amato *et al.* (2009), using combinatorial peptide ligand libraries, identified 53 and 149 minor proteins in whey from colostrum or milk, respectively, some of which were identified only in colostrum. Using ion-exchange chromatography, Le *et al.* (2011) identified a total of 293 whey proteins in colostrum and milk, of which 36 were identified exclusively in colostrum. Nissen *et al.* (2012) identified 403 minor proteins in colostrum using 2D-LC-MS/MS, which, to the best of the authors' knowledge, is the most comprehensive characterisation of whey proteins in colostrum to date. It is interesting to note that, in a follow-on study, Nissen *et al.* (2013), also using 2D-LC-MS/MS, identified a total of 742 minor proteins in milk, however; 366 of these proteins were identified only in one replicate and are therefore regarded as tentative results.

To date, over 120 proteins have been identified in the milk fat globule membrane (MFGM) (Reinhardt and Lippolis, 2006; Affolter *et al.,* 2010). The major MFGM proteins are butyrophilin (BTN), adipophilin (ADPH), periodic acid Schiff 6/7 (PAS 6/7, also known as lactadherin) and xanthine dehydrogenase/oxidase (XDH/XO) (Fong *et al.,* 2007). Minor proteins include polymeric immunoglobulin receptor protein, apolipoprotein E (APOE), apolipoprotein A1 (APOA1), 71 kDa heat-shock cognate protein, clusterin, lactoperoxidase, immunoglobulin heavy chain, peptidylprolyl isomerase A, actin, fatty acid-binding protein (FABP), cluster of differentiation 36 (CD36) and mucin (MUC) (Dewettinck *et al.,* 2008). Reinhardt and Lipollis (2008) studied changes in the MFGM proteome during the transition from colostrum to milk. MUC, BTN, FABP, XDH were upregulated 2.6- to 7.7-fold in MFGM from milk compared with colostrum MFGM. APOA1, APOE and clusterin were downregulated 2.6- to 3.6-fold in MFGM milk compared with colostrum MFGM while no changes in the concentrations of polymeric immunoglobulin receptor, peptidylprolyl isomerase A, 71 kDa heat-shock cognate protein, CD 36, actin and lactadherin were noted.

1.2.3 *Growth Factors*

Growth factors may be defined as proteins or polypeptides which bind to receptors on cell surfaces, resulting in cellular proliferation and/or differentiation (Gauthier *et al.,* 2006). The main growth factors present in milk and colostrum are epidermal growth factor (EGF) (Yagi *et al.,* 1986; Iacopetta *et al.,* 1992), betacellulin (BTC) (Dunbarr *et al.,* 1999; Bastian *et al.,* 2001), insulin-like growth factor I (IGF-I) (Malven *et al.,* 1987; Collier *et al.,* 1991), IGF-II (Vega *et al.,* 1991; Schams, 1994), transforming growth factor-β1 (TGF-β1) (Jin *et al.,* 1991; Ginjala and Pakkanen, 1998), TGF-β2 (Cox and Burk, 1991; Jin *et al.,* 1991), fibroblast growth factor 1 and 2 (FGF1 and FGF2) (Kirihara and Ohishi, 1995; Rogers *et al.,* 1995; Hironaka *et al.,* 1997), and platelet-derived growth factor (PGDF) (Belford *et al.,* 1997). The concentration of growth factors in colostrum and milk are highly variable according to the stage of lactation and also the method used for quantification; however, in general, the concentration of growth factors in colostrum is highest during the initial hours *post partum* and declines significantly in a time-dependant manner (Collier *et al.,* 1991; Kishikawa *et al.,* 1996; Ginjala *et al.,* 1998; Bastian *et al.,* 2001). The most abundant growth factors in colostrum are IGF-1 and IGF-II (Marnila and Korhonen, 2002). Pakkanen and Aalto (1997) reported that the concentration of IGF-I and IGF-II in colostrum ranges from 50-2000 µg/L and 200-600 µg/L, respectively, whereas milk contains < 10 µg/L of each. Yagi *et al.* (1986) found the concentration of EGF in colostrum and milk to be 324 µg/L and 155 µg/L, respectively; however, the study of Iacopetta *et al.* (1992) reported concentrations of EGF in colostrum and milk to be 4-8 µg/L and < 2 µg/L, respectively. The concentration of TGF-β1 in colostrum ranges from 12 to 43 µg/L, compared with 0.8 to 3.5 µg/L in milk (Ginjala and Pakkanen, 1998). Similarly, the concentration of TGF-β2 is higher in colostrum than in milk, i.e., 150-1150 µg/L and 13-71 µg/L, respectively (Pakkanen, 1998).

1.2.4 *Enzymes*

Approximately 70 indigenous enzymes have been reported in milk (Fox *et al.,* 2003), most of which have no obvious physiological role in the biosynthesis or secretion of milk. There are four principal sources of enzymes in milk, i.e., blood plasma, secretory cell cytoplasm, milk fat globule membrane (MFGM) and somatic cells (Fox and Kelly, 2006 a,b). In general, the enzyme content of colostrum is higher than in milk (Shahani *et al.,* 1973).

1.2.4.1 *Antioxidants*

Lactoperoxidase (LPO) is a member of the peroxidase family whose primary function, in the presence of hydrogen peroxide, is to catalyse the oxidation of thiocyanates, leading to the generation of intermediate compounds with a wide range of antimicrobial activities (Fox and Kelly, 2006a). Following xanthine oxidoreductase (XOR), LPO is the most abundant enzyme in milk (Kussendrager and van Hooijdonk, 2000). It accounts for about 0.5% of the whey proteins and is present at approximately 30 mg/L in milk (de Wit and van Hooydonk, 1996). Hahn *et al.* (1998) reported that the concentration of LPO in milk ranges from 11 to 45 mg/L, while colostrum contains 13 to 30 mg/L. Korhonen (1977) observed that the concentration of LPO in colostrum is low initially but increases rapidly to reach a maximum after 3-5 days *post partum*, followed by a slow decrease until a plateau is reached after about 2 weeks.

Catalase is a haemoprotein which catalyses the decomposition of hydrogen peroxide; its activity is higher in colostrum than in milk, and decreases during lactation (Farkye, 2002).

Superoxide dismutase (SOD) scavenges superoxide radicals and is present in milk at levels similar, albeit lower, than XOR (Fox and Kelly, 2006b). The concentration of SOD in milk ranges from 0.15 to 2.5 mg/L (Farkye, 2002), but is not affected by the stage of lactation (Przybyslaka *et al.,* 2007).

1.2.4.2 *Proteinases*

Plasmin, a serine protease derived from the proenzyme, plasminogen (Bastian and Brown, 1996), is the principal indigenous proteinase in milk (Fox and Kelly, 2006 a,b). Dupont *et al.* (1998) reported that the concentration of plasmin in colostrum is about 10 times higher than in milk, i.e., 0.49 µg/mL and 0.04 µg/mL, respectively. Madsen *et al.* (2004) observed a 2-fold increase in plasmin activity in colostrum compared with milk. Similarly, Pyorala and Kaartinen (1988) found a decrease in plasmin activity during the transition from colostrum to milk.

Cathepsin D is a lysosomal proteinase and, as with plasmin, it is part of a complex system including inactive precursors (Hurley *et al.,* 2000). Larsen *et al.* (2006) reported that cathepsin D activity is significantly lower in colostrum than in milk.

Other proteinases which have been identified in bovine milk include cathepsins B, L and G, and elastase (Kelly and McSweeney, 2003), but, to best of the authors' knowledge, these enzymes have not been studied in colostrum.

1.2.4.3 *Lipases and esterases*

Lipoprotein lipase (LPL) is the principal indigenous lipase in milk (Olivecrona *et al.,* 1992). Mammary LPL activity increases markedly prior to parturition and remains high throughout lactation (Shirley *et al.,* 1973; Liesman *et al.,* 1988). Saito and Kim (1995) observed that LPL activity in colostrum is low initially but increases rapidly during the first few days of lactation and, thereafter, remains constant for the remainder of lactation. Andersen (1982) reported that LPL activity in the serum phase of colostrum fell from 45% to 34% during the first 24 h following parturition, but, these figures are not representative of total LPL activity in milk as, in milk, more than 90% of LPL is associated with the casein micelles (Fox and McSweeney, 1998).

In addition to the lipase system, milk contains several other carboxyl ester hydrolases, collectively referred to as esterases (Deeth and Fitz-Gerald, 2006).

Compared with LPL activity, the total esterase activity in milk is quite low, about ten percent (Downey, 1974). Forster *et al.* (1959) and Marquardt and Forster (1965) reported that the concentration of arylesterase in colostrum is higher than in milk. Similarly, the level of carboxylesterase activity is higher in colostrum than in milk (Fitz-Gerald *et al.,* 1981).

1.2.4.4 *Others*

Milk contains several phosphatases, the principal ones being alkaline phosphatase and acid phosphatase (Fox and Kelly, 2006b). The concentration of alkaline phosphatase is very high in colostrum and decreases to a minimum within 1-2 weeks of parturition before reaching a constant level after approximately 25 weeks (Schlimme and Thiemann, 1992; Shakeel ur-Rehman and Farkye, 2002; Fox and Kelly, 2006b). The activity of acid phosphatase in milk is much lower (about 2%) than that of alkaline phosphatase and ranges from 2.6 x 10^{-4} to 2.6 x 10^{-3} IU/mL (Shakeel ur-Rehman and Farkye, 2002). The concentration of acid phosphatase is low in colostrum initially, increases to reach a maximum over 5-6 days *post partum,* and then decreases and remains low until the end of lactation (Shakeel ur-Rehman and Farkye, 2002).

Ribonucleases catalyses the cleavage of RNA into smaller components. Ribonuclease A, B, C, D and II-1 have all been detected in milk (Bingham and Kalan, 1967; Bingham and Zittle, 1964). Meyer *et al.* (1987) reported that colostrum contains 10 to 15 times more ribonuclease II-1 than milk and three times more total ribonuclease activity. Roman *et al.* (1990) reported that the concentration of ribonuclease in colostrum is highest in the third milking *post partum* (63 mg/L), followed by an abrupt decrease in concentration until normal levels were reached 1 month after parturition (14 mg/L).

Lysozyme is a lysosomal enzyme which catalyses the cleavage of β1,4-linkages between muramic acid and *N*-acetylglucosamine in bacterial cell walls (Fox and McSweeney, 1998). Generally, lysozyme activity is higher in colostrum than in milk

(Goudswaard *et al.,* 1978; Farkye, 2002). The concentrations of lysozyme in colostrum and milk are about 0.14-0.7 and 0.07-0.6 mg/L, respectively (Korhonen, 1977).

γ-Glutamyl transferase (γ-GT) is an enzyme that is localised on the outer surface of the alveolar cell membrane which catalyses the transport of free amino acids across the membrane into the cell (Baumrucker and Pocius, 1978). Hadorn *et al.* (1997) and Ontsouka *et al.* (2003) reported that the activity of γ-GT is much higher in colostrum than in milk. Vacher and Blum (1993) reported 2.5-3 fold higher γ-GT activity in colostrum than in milk.

1.2.5 *Enzyme inhibitors*

Colostrum and milk contain a number of enzyme inhibitors, the concentration of which are highest initially and decrease rapidly with time *post partum* (Georgiev, 2008). It is thought that these inhibitors play an important role in the mechanism of absorption of immune components by the calf, i.e., protecting Igs from proteolytic cleavage and allowing absorption of the intact molecule (Carlson *et al.,* 1980).

Several authors have reported the presence of trypsin inhibitors in colostrum (Laskowski and Laskowski, 1951; Cehova *et al.,* 1971; Pineiro *et al.,* 1978), the concentration of which is nearly 100 times greater than in milk (Sandholm and Honkanen-Buzalski, 1979; Honkanen-Buzalski, 1981). Quigley *et al.* (1995) found that colostrum contains approximately 560 mg trypsin inhibitor/L and reported a positive correlation with IgG, which is in agreement with the study of Pineiro *et al.* (1978).

Cysteine protease inhibitors form a cystatin superfamily consisting of three subfamilies, i.e., family I (the stefins), family II (the cystatins) and family III (the kininogens) (Saitoh and Isemura, 1993). Hirado *et al.* (1984, 1985) reported the presence of two types of cysteine protease inhibitors in colostrum, belonging to the kininogen and cystatin subfamilies, which have been isolated and well characterised. Kirihara *et al.* (1995) purified a third type of cysteine protease

inhibitor from colostrum; however, information relating to its taxonomy was not available.

α2-Macroglobulin is a large plasma glycoprotein (718 kDa) with broad-spectrum proteinase inhibitory activity (Barret *et al.,* 1973). The concentration of α2-macroglobulin in colostrum is higher than that in milk (Honkanen-Buzalski and Sandholm, 1981). Perez *et al.* (1989) reported that the first milking *post partum* contained the highest concentration of α2-macroglobulin (386.7 µg/mL), which decreases very rapidly until a plateau is reached during the second week *post partum* (4.5 µg/mL).

Christensen *et al.* (1995) reported the presence of seven plasma-derived protease inhibitors in colostrum, i.e., α2-macroglobulin, α2-antiplasmin, antithrombin III, C1-inhibitor, inter-α-trypsin inhibitor, bovine plasma elastase inhibitor and bovine plasma trypsin inhibitor. The concentration of these inhibitors was highest in colostrum initially and decreased dramatically during the first 3 days *post partum*, at which point stable levels were reached, e.g., the concentration of bovine plasma trypsin inhibitor and bovine plasma elastase inhibitor is approximately 200 mg/L in colostrum compared with 5-10 mg/L in milk (Christensen *et al.,* 1995).

1.2.6 *Nucleotides and nucleosides*

Nucleotides and nucleosides belong to the non-protein-nitrogen (NPN) fraction of milk and are present in the sub-milligram range per litre (Schlimme *et al.,* 2000). These compounds have important roles in biochemical synthesis, i.e., nucleic acid synthesis, enhancement of immune response (Carver *et al.,* 1991; Schaller *et al.,* 2004), influence metabolism of fatty acids, contribute to iron absorption in the gut and improve gastrointestinal tract repair after damage (Carver and Walker, 1995; Sanchez-Pozo and Gil, 2002).

Nucleotides are *O*-phosphoric acid esters of nucleosides (Schlimme *et al.,* 2002). The concentration of nucleotides in colostrum is higher than in milk (Schlimme *et al.,* 2002). Initially, the concentration of nucleotides in colostrum is very low, but

reaches a maximum 24-48 h after parturition, followed by a gradual decrease as lactation continues, up until the third week when levels stabilise (Gil and Sanchez-Medina, 1981a,b; Sugawara, 1995**;** Gil and Indyk, 2007a). This pattern is seen for nucleoside-5'-monophosphates; 5'-AMP, 5'CMP and 5'UMP, as well as nucleoside-5'-, di- and tri-phosphates, UDP galactose and UDP glucose (Gil and Sanchez-Medina, 1981). Generally, the concentration of nucleotides in milk is about one or two orders of magnitude higher than the concentration of nucelosides (Gil and Sanchez-Medina 1981a; Raezke and Schlimme, 1990; Schlimme *et al.,* 1991).

Nucleosides are *N*-glycosides of pyrimidines and purines (Schlimme *et al.,* 2002). The concentration of nucleosides is higher in colostrum than in milk; however, unlike nucleotides, no distinct maximum is observed during the first two days after parturition (Raezke and Schlimme, 1990; Schlimme *et al.,* 1991). The nucleoside concentration decreases during the colostral phase and reaches a constant level approximately three weeks *post partum* (Schlimme *et al.,* 2000). The levels of pyrimidine nucleosides in milk are higher than for purine nucleosides. The concentration of uridine is relatively high in early colostrum but decreases by approximately two orders of magnitude during the first few hours *post partum*, whereas the concentration of cytidine reaches a maximum on the second day of lactation (Gil and Indyk, 2007b).

1.2.7 *Cytokines*

Cytokines are a diverse group of proteins, peptides or glycoproteins which have profound biological effects at minute concentrations (10 to 1000 pg/mL) (Gauthier *et al.,* 2006). These molecules are principally responsible for modulating the immune system (Biswas *et al.,* 2007) and include the interleukins (IL) series, tumour necrosis factors (TNF) and interferons (INF). Sacerdote *et al.* (2013) reported the presence of a wide range of cytokines in colostrum, i.e., IL-1β, IL-2, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, IFN-γ and TNF; however, the equivalent concentrations in milk were not measured. Hagiwara *et al.* (2000) reported that the concentrations of IL-1β, IL-6, TNF-α, INF-γ and IL-1ra are significantly higher in colostrum than in

milk, i.e., mean concentrations of 844, 77, 927, 261 and 5206 ng/mL in the first milking *post partum*, respectively, and 3.4, 0.22, 4.58, 0 and 26 ng/mL in milk, respectively. These results are in agreement with the study of Sobczuk-Szul *et al.* (2013), who observed increased levels of IL-1β, IL-6 and TNF-α in colostrum compared with milk. Similarly, Goto *et al.* (1997) reported increased concentrations of IL-1β in colostrum compared with milk.

1.2.8 *Lipids*

The lipids in milk are present in microscopic globules as an oil-in-water emulsion. Both the fat content of milk and its fatty acid composition vary considerably depending on factors such as individuality of the animal, diet and stage of lactation, e.g., the fat content can vary from 3.0 to 6.0% but typically is in the range 3.5 to 4.7% (MacGibbon and Taylor, 2006). Triacylglycerols (TAG) represent 97-98% of the fat in milk; however, there are also minor amounts of diacylglycerols, monoacylglycerols, free (unesterified) fatty acids, phospholipids, sterols and other components (Fox and McSweeney, 1998). The composition and structure of milk fat has been reviewed extensively (Jensen and Newberg, 1995; Jensen, 2002; Vanhoutte and Huyghebaert, 2003; MacGibbon and Taylor, 2006; Palmquist, 2006); however, relatively little work has been carried out on changes in the composition and structure of milk fat during the transition from colostrum to milk. Generally, but not always, the fat content of colostrum is higher than that of milk (Foley and Otterby, 1978; Marnila and Korhonen, 2002). Parrish *et al.* (1950) reported a mean fat content of 5.7% in the first milking, followed by a gradual decrease to 4.6% in the sixth milking. The same author observed significant variability in the fat content of colostrum, not only among different breeds, but also between individual cows of the same breed, i.e., the fat content of the first milking *post partum* ranged from 0.3% to 18.0%. Tsioulpas *et al.* (2007) reported that the fat content varied throughout the sampling period (0-90 days), but no particular trend was observed. Kehoe *et al.* (2007) reported that the average fat content of the first milking *post partum* ranged from 2.0 to 26.5%, with an average value of 6.7%. Morrill *et al.*

(2012) observed a similar trend, i.e., the fat content of colostrum varied from 1.0 to 21.7%, with an average value of 5.6%. Abd El-fattah *et al.* (2012) reported a decrease in the fat content of colostrum from 8.04% at parturition to 3.9% after 5 days.

Approximately 400 different fatty acids have been identified in bovine milk fat, but only about 15 of these are present at or above 1.0% of total lipids (MacGibbon and Taylor, 2006). Fatty acids in milk arise from two sources: synthesis *de novo* in the mammary gland and plasma lipids which originate from the feed (MacGibbon and Taylor, 2006). Laakso *et al.* (1996) reported that, during the first week *post partum*, the proportions of stearic acid, oleic acid and short chain fatty acids (C4-C10) in colostrum were low and increased thereafter. Similarly, Palmquist *et al.* (1993) observed that the proportion of short-chain fatty acids, with the exception of C4, are low in colostrum, but that these fatty acids increase, reaching >90% of maximum levels by 8 wk of lactation. Laakso *et al.* (1996) also reported that the relative amounts of C12-C16, in particular myristic and palmitic acid, in colostrum were high initially and decreased with time *post partum*. Several authors have reported that colostrum contains high levels of C18:0 and C18:1 (Syrstad *et al.,* 1982; Lynch *et al.,* 1992; Palmquist *et al.,* 1993). High levels of long-chain fatty acids are found in colostrum because, at parturition, cows are in a negative energy balance, resulting in mobilisation of adipose fatty acids which are incorporated into milk fat (Belyea and Adams, 1990). Concomitantly, high levels of long-chain fatty acids inhibits *de novo* synthesis of short-chain fatty acids (Bauman and Davis, 1974). Laakso *et al.* (1996) observed changes in the TAG distribution during the colostral period, i.e., the proportion of molecules with acyl carbon number (ACN) 38-40 increased and those with ACN 44-48 decreased. Paszczyk *et al.* (2005) reported that colostrum contains a lower content of *trans* fatty acids and *cis*-9 trans-11 C18:2 (CLA) than milk.

The phospholipids are minor constituents of milk fat and constitute about 1% of total lipids in milk (Fox and McSweeney, 1998). They are important structural components of the MFGM and are present in five major subclasses: sphingomyelin, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and phosphatidyl

enthanolamine (Palmquist, 2006). Bitman and Wood (1990) reported that the total phospholipid content of milk increases from 0.72 to 1.06% of total lipids from the third to seventh day of lactation, respectively. The same authors observed that the five major subclasses of phospholipids mentioned above are all present at significantly lower concentrations in colostrum than in milk.

Sterols are minor components of milk lipids, at approximately 0.3% of total lipids. Cholesterol is the main component of sterols, accounting for approximately 95% (MacGibbon and Taylor, 2006). Small amounts of other sterols have also been identified in milk, namely campesterol, stigmasterol and β-sitosterol (Minicione *et al.,* 1977). Shope and Gowen (1928) reported that the cholesterol content of colostrum was highest initially and decreased progressively over 48 h at which point a plateau was reached. Precht (2001) observed that the cholesterol content of colostrum is significantly higher than of milk, i.e., 327 and 285 mg/100g fat, respectively, with colostrum from one cow registering a cholesterol content of approximately 600 mg/100g fat immediately after calving.

1.2.9 *Minerals*

The mineral components, or milk salts, include the citrates, phosphates and chlorides of H^+, K^+, Na^+, Mg^{2+} and Ca^{2+}, which are present as either as ions in solution or as colloidal species complexed with the caseins (Lucey and Horne, 2009). Potassium, sodium and chloride ions are essentially soluble in milk, while calcium, inorganic phosphate and magnesium are partly associated with the casein micelles (Gaucheron, 2005). The principal colloidal salt in milk is calcium phosphate; the colloidal salts are thus often referred to collectively as colloidal calcium phosphate (CCP) (Fox and McSweeney, 1998). Several factors affect the concentration of salts in milk, i.e., stage of lactation, mastitis, diet and season, whereby the most pronounced changes occur around parturition (Holt, 1985).

Milk is saturated with respect to calcium and phosphate ions, which exist in a dynamic equilibrium with the colloidal forms (Lucey and Horne, 2009). The

concentration of calcium and phosphorus in milk is approximately 1200 and 950 mg/kg, respectively (Fox and McSweeney, 1998). Several authors have reported high concentrations of calcium and phosphorus in colostrum; Tsioulpas *et al.* (2007) reported that the concentrations of calcium and phosphorus decreased from 2168 and 1635 mg/kg, respectively, at parturition, to 1342 and 929 mg/kg, respectively, after a 15 days. A similar trend was observed by Garret and Overman (1940), Rook and Campling (1965) and Jeong *et al.* (2009). Abd El-Fattah (2012) observed a similar trend for the calcium concentration during the transition from colostrum to milk, but, the concentration of phosphorus in colostrum and milk was significantly lower than previous reports, i.e., 530 and 520 mg/kg, respectively. On the other hand, Kehoe *et al.* (2007) reported mean concentrations for calcium and phosphorus in colostrum of 4716 and 4452 mg/kg, respectively, which are approximately 4- and 5-fold higher than the concentrations found in milk. White and Davies (1958) reported that the average concentrations of colloidal inorganic calcium and colloidal inorganic phosphorus were lower in colostrum than in milk. Colloidal calcium and colloidal phosphorus are associated predominantly with casein, which, as mentioned earlier, is present in higher concentrations in colostrum than in milk (Cerbulis and Farrell, 1975). However, during the colostral period, casein micelles are not as mineralised as later in lactation, and so the concentrations of colloidal calcium and colloidal phosphorus are not as high as expected.

The concentrations of magnesium and sodium in milk are approximately 120 and 500 mg/kg, respectively (Fox and McSweeney, 1998). Several authors have reported increased concentrations of these elements in colostrum. Jeong *et al.* (2009) reported that the concentrations of magnesium and sodium decreased from 287 and 761 mg/kg, respectively, at parturition, to 120 and 430 mg/kg, respectively, after 4 days. A similar trend was observed by Garrett and Overman (1940), Tsioulpas *et al.* (2007) and Abd El-Fattah *et al.* (2012). Kehoe *et al.* (2007) reported that the concentrations of magnesium and sodium in colostrum ranged from 230 to 1399 and 330 to 2970 mg/kg, with mean values of 733 and 1059 mg/kg, respectively.

The concentration of potassium in milk is approximately 1450 mg/kg (Fox and McSweeney, 1998). Unlike for calcium, phosphorus, magnesium and sodium, there are mixed reports in the literature regarding the concentration of potassium in colostrum. Garret and Overman (1939), Toshiyoshi *et al.* (1982) and Kilmes *et al.* (1986) reported low levels of potassium in colostrum at parturition but a gradual increase thereafter. Abd El-Fattah (2012) reported that the concentration of potassium in colostrum decreased from 1795 mg/kg at parturition to 650 mg/kg after 14 days, which is significantly lower than typically found in milk. No particular trends were observed for potassium concentration in the studies of Rook and Campling (1965), Ontsouka *et al.* (2003), Tsioulpas *et al.* (2007) and Jeong *et al.* (2009). Kehoe *et al.* (2007) reported that the concentration of potassium in colostrum ranges from 983 to 5511 mg/kg, with a mean value of 2845 mg/kg, which is significantly higher than other reports in the literature.

In addition to the macro-elements mentioned above, approximately 20 other elements are found in milk in trace amounts, including copper, iron, zinc and manganese, which are present in milk at approximately 200, 500, 3500 and 30 μg/kg, respectively (Fox and McSweeney, 1998). The presence of these trace elements in milk has been studied by several authors (Coni *et al.,* 1994, 1995; Ataro *et al.,* 2008; Pechova *et al.,* 2008; Pilarcyzk *et al.,* 2013), but, relatively little work has been carried out on their concentrations in colostrum and milk during the early *post partum* period. Kehoe *et al.* (2007) reported that the average concentrations of copper, iron, zinc and manganese in colostrum were 340, 5330, 38100 and 100 μg/kg, respectively, with values ranging from 130 to 640, 1700 to 17500, 11200 to 83600 and 0 to 360 μg/kg, respectively. The mean values reported by Kehoe *et al.* (2007) are 1.7, 10.7, 10.9 and 3.3 fold higher than values for normal milk. Jeong *et al.* (2009) observed no particular trends in the concentrations of copper and iron in colostrum over the first 108 h *post partum*, with values ranging from 1000 to 1700 μg/kg for both elements. In the same study, the concentration of zinc was highest in the first milking, at 14300 μg/kg, but decreased sharply thereafter, while no manganese was detected in colostrum. Abd El-Fattah (2012) reported a general decrease in the levels of copper, iron and zinc in colostrum over the first 336 h *post*

partum. The concentration of copper fluctuated during the first 24 h from 1780 to 2040 µg/kg, but then decreased gradually to 600 µg/kg after 336 h. Similarly, the concentrations of iron and zinc decreased from 22100 and 2700 µg/kg, respectively, at calving, to 4600 and 1700 µg/kg at 336 h *post partum*.

1.2.10 *Vitamins*

Vitamins are a heterogeneous group of organic chemicals required in extremely small amounts for growth and maintenance of normal cells and body function (Morrissey and Hill, 2009). Vitamins are generally not synthesised in the body and are classified based on their solubility in either water or fat. Water-soluble vitamins include the B group vitamins and vitamin C, while the fat-soluble vitamins are A, D, E and K (Fox and McSweeney, 1998).

1.2.10.1 *Fat-soluble vitamins*

Vitamin A is present in milk as a variety of substances including retinol, retinal, retinoic acid, retinyl esters as well as provitamin A carotenoids such as β-carotene (Morrissey and Hill, 2009). Retinol and its derivatives are found only in animal tissues, whereas β-carotene is found principally in plants (Blomhoff, 1994; Basu and Dickerson, 1996). The concentrations of vitamin A and β-carotene in milk is approximately 40 µg/100g and 20 µg/100g, respectively (Walstra and Jenness, 1984). The concentration of vitamin A and carotenoids in milk is strongly influenced by the amount of carotenoids in the feed (Jensen *et al.,* 1999), as carotenoids with a β-ionone ring (e.g., β-carotene) serve as vitamin A precursors (Fox and McSweeney, 1998). The concentration of vitamin A in colostrum and milk is also influenced by parity, i.e., primiparous cows exhibit significantly higher vitamin A concentrations than multiparous cows (Franklin *et al.,* 1998; Kumagai *et al.,* 2001). Several authors have reported increased concentrations of vitamin A in colostrum, with values ranging from 233 to 369 µg/100g (Drummond *et al.,* 1921; Dann, 1933; French, 1943; Franklin *et al.,* 1998; Jensen *et al.,* 1999; Debier *et al.,* 2005). The

concentration of vitamin A and carotenoids decreases drastically during the first few days of lactation and stabilises beyond approximately day 5 (Parrish *et al.,* 1948; Calderon *et al.,* 2007; Abd El-fattah *et al.,* 2012).

Vitamin E includes two main groups: tocopherols (α-, β-, γ- and δ-) and tocotrienols (α-, β-, γ- and δ-) (Morrissey and Hill, 2009). The transfer of vitamin E into colostrum does not appear to occur via a passive mechanism associated with the transfer of lipids (Debier *et al.,* 2005) but rather by a mechanism involving low-density lipoproteins (Schweigert, 1990). The concentration of vitamin E in milk is approximately 90 µg/100g (Fox and McSweeney, 1998), while its concentration in colostrum is much higher (Hidiroglou, 1989; Schweigert, 1990; Debier *et al.,* 2005; Calderon *et al.,* 2007). Kehoe *et al.* (2007) reported that the concentration of vitamin E in colostrum ranges from 60 to 1040 µg/100g, with a mean value of 292 µg/100g. Parrish *et al.* (1949) reported that the concentration of vitamin E decreased from 84 to 31 µg/g fat over the course of the first six milkings *post partum*, compared with an average vitamin E concentration in milk of 15 µg/g fat. The concentration of α-tocopherol in milk ranges from 0.2 to 0.7 mg/L (Renner *et al.,* 1989; Jensen *et al.,* 1995). Hidiroglou (1989) reported that the concentration of α-tocopherol in colostrum decreased from 1.9 to 0.3 mg/L over the first four days *post partum*. γ-Tocopherol and α-tocotrienol have also been detected in trace amounts in colostrum and milk (Barrefors *et al.,* 1995).

The two major forms of vitamin D are cholecalciferol (vitamin D_3), which is synthesised by the skin of the cow following exposure to ultraviolet radiation, and ergocalciferol (vitamin D_2), which is produced by plants following exposure to UV radiation (Bulgari *et al.,* 2013). The concentration of vitamin D in milk is quite low, ranging from 0.01 to 0.15 µg/100g (Reeve *et al.,* 1982). The most important factor affecting the vitamin D content of milk is exposure to sunlight, with higher values found in summer (approx. 0.035 µg/100g) than in winter (approx. 0.025 µg/100g) (Scott *et al.,* 1984). Henry and Kon (1937) reported a decrease in the vitamin D content of colostrum from 1.2 to 0.36 IU/g fat during the first 5 days *post partum*, compared with an average vitamin D content of 0.41 IU/g fat in milk. These results are in agreement with the study of Eaton *et al.* (1947) who reported that the

concentration of vitamin D in colostrum ranged from 0.83 to 1.81 IU/g fat. Yan *et al.* (1993) also observed an higher concentration of vitamin D in colostrum compared with milk.

Vitamin K exists in two forms; phylloquinone (vitamin K_1) and menaquinones (vitamin K_2) (Morrissey and Hill, 2009). The concentration of vitamin K in milk is quite low, ranging from 0.35 to 1.8 µg/100g (Haroon *et al.,* 1982). Indyk and Woolard (1995) reported that the concentration of phylloquinone was higher in colostrum than in milk, decreasing from approximately 2 µg/100g in the initial colostrum to normal levels (0.4 to 0.66 µg/100g) after five days of lactation.

1.2.10.2 *Water-soluble vitamins*

Ascorbic acid (vitamin C) is synthesized in the liver of cows (Chatterjee, 1978), but calves do not begin to synthesize endogenous vitamin C until approximately 3 weeks of age and so are reliant on vitamin C from milk during this period (Palladun and Wegger, 1984). The vitamin C content of milk ranges from 1.65 to 2.75 mg/100g, with a mean concentration of 2.11 mg/100g (Walstra and Jenness, 1984). Kon and Watson (1936) reported that the concentration of vitamin C in colostrum is slightly higher than that in milk, with vitamin C content in first *post partum* milkings ranging from 1.6 to 3.2 mg/100g and 1.97 to 2.15 mg/100g after 6 days. Hidiroglou *et al.* (1995) reported no differences in the concentration of vitamin C between the initial colostrum and milk, but, the concentration of vitamin C was twice as high in colostrum (16 µg/mL) as in milk at 2 days after calving (8 µg/mL).

The B group vitamins are thiamine, riboflavin, niacin, biotin, pantothenic acid, folate, pyridoxine (and related substances, such as vitamin B_6) and cobalamin (and its derivative, vitamin B_{12}). The concentration of thiamine, riboflavin, folate, vitamin B6 and B12 are higher in colostrum than in milk, while the levels of pantothenic acid and biotin are lower in colostrum and that of niacin approximately the same as in milk (Marnila and Korhonen, 2002). Pearson and Darnell (1946) reported that the concentrations of thiamin and riboflavin in colostrum were 0.62 and 6.1 µg/mL,

respectively, compared with 0.38 and 1.77 μg/mL in milk. These values are in agreement with those reported by Kehoe *et al.* (2007), who reported that the concentration of thiamin and riboflavin in colostrum ranged from 0.3 to 2.1 μg/mL and 2.4 to 9.2 μg/mL, respectively, with mean values of 0.9 and 4.5 μg/mL. Sutton *et al.* (1947) reported that the concentration of riboflavin in colostrum was 3.3-fold higher than in milk, with values decreasing sharply from 6.2 to 3.7 μg/mL between the first and second milking, followed by a more gradual decrease from 2.4 to 1.8 μg/mL from the second to tenth milking. Pearson and Darnell (1946) also reported that the concentration of pantothenic acid increased from 2.24 to 3.67 μg/mL during the transition from colostrum to milk, while the niacin content of colostrum was approximately the same as in milk, i.e., 0.96 and 0.91 μg/mL in colostrum and milk, respectively. Both of these trends were also reported by Lawrence *et al.* (1946). In contrast to this, Kehoe *et al.* (2007) observed that the concentration of niacin was lower in colostrum (0.34 μg/mL) than in milk. The level of biotin in milk ranges from 21 to 43 ng/ml (Gregory *et al.,* 1958; Causeret, 1971; Higuchi *et al.,* 2003). Hirano *et al.* (1991) reported that the biotin content in colostrum was low initially (5.1 ng/mL) but increased with the progression of lactation, which is in agreement with the findings of Lawrence *et al.* (1946) and Indyk *et al.* (2014). Cobalamin is present in milk at <1 μg/100g and its level is fairly constant throughout lactation, except during the colostral period during which it is very high (Nohr and Biesalski, 2009). Anthony *et al.* (1951) observed that the concentration of cobalamin in colostrum decreased from 4.9 to 2.3 μg/mL during the first 24 h after parturition, followed by a more gradual decrease thereafter. Kehoe *et al.* (2007) reported that the concentration of cobalamin in colostrum ranged from 0.2 to 1.1 μg/mL, with a mean value of 0.6 μg/mL, which is significantly lower than reported by Anthony *et al.* (1951). The concentration of pyridoxine in milk is approximately 0.36 μg/g (Nohr and Biesalski, 2009). Kehoe *et al.* (2007) reported that the concentration of pyridoxine in colostrum ranges from 0 to 0.2 μg/mL, with a mean value of 0.04 μg/mL, which is lower than the level found in milk and is not in agreement with Marnila and Korhonen (2002). Collins *et al.* (1950) reported that the concentration of folic acid in colostrum decreases abruptly from 0.75 μg/100g at parturition to 0.2 μg/100g after 24 h, at which point a plateau was reached.

1.3 Physical properties

1.3.1 *pH*

The pH of milk is strongly influenced by temperature (Walstra *et al.,* 2006). At 25°C, the pH of milk is usually in the range 6.5 to 6.7, with a mean value of 6.6 (Fox and McSweeney, 1998). The main factor influencing the pH of milk is stage of lactation; the pH of colostrum is lower and that of late-lactation milk is higher than the pH of normal mid-lactation milk (McCarthy and Singh, 2009). McIntyre *et al.* (1952) reported that the pH of colostrum at parturition ranged from 6.0 to 6.61, with an average value of 6.32. This value increased with time and reached pH 6.5 after 2 weeks. Several authors have reported similar trends, whereby the pH of colostrum is low initially and increases with time *post partum* (Klimes *et al.,* 1986; Madsen *et al.,* 2004; Tsioulpas *et al.,* 2007; Jeong *et al.,* 2009). The precise reason for the low pH of colostrum is unknown. During the *pre partum* period, there is increased permeability of the mammary gland membranes, and thus more blood constituents gain access to the milk. Given that colostrum contains significantly more blood components than milk, a pH closer to that of blood (pH 7.35 to 7.45) (Nappert and Taylor, 2001) should be expected. Sebela and Klicnik (1977) reported that the low pH of colostrum is caused by the increased concentration of protein, dihydrogen phosphate, citrate and carbon dioxide. Another possible explanation for the low pH is the high ash content, i.e., hydrogen ions are released during the formation of colloidal calcium phosphate, which may cause a drop in pH.

1.3.2 *Buffering capacity*

The buffering capacity of milk is defined as the resistance to changes in pH on addition of acid or base (Fox and McSweeney, 1998). The principal buffering components of milk are soluble phosphate, colloidal calcium phosphate, citrate, carbonate and proteins (Lucey *et al.,* 1993). McIntyre *et al.* (1952) reported that the buffering capacity of colostrum was greater than that of milk and decreased rapidly during the first four milkings. Klimes *et al.* (1986) also reported a markedly higher

buffering capacity of colostrum compared with milk which decreased with advanced lactation. Titratable acidity is defined as the buffering capacity of milk between its own pH and pH 8.3 (Walstra *et al.,* 2006). Kilmes *et al.* (1986) reported that the titratable acidity of colostrum is approximately 2 to 2.5 times higher than that of milk. This was supported by Tsioulpas *et al.* (2007) and Jeong *et al.* (2009). Tsioulpas *et al.* (2007) observed a logarithmic relationship between titratable acidity and total protein, while Nardone *et al.* (1997) also observed a higher titratable acidity for colostrum than milk, and reported a positive correlation with protein content.

1.3.3 *Colour*

The colour of milk is typically dominated by the presence of casein micelles and fat globules, which scatter light and are responsible for its white/opaque appearance. The serum phase of milk is greenish due to the presence of riboflavin, while the colour of the lipid phase is dependent on the presence and concentration of fat-soluble pigments, in particular carotenoids, which are not synthesised by the animal but are obtained from plant sources in the animals diet (Fox and McSweeney, 1998). Colostrum has a reddish-yellow colour (Edelsten, 1988). This yellow colour is due largely to the presence of carotenoids (Edelsten, 1988). It has been observed that levels of carotenoids are high in the initial colostrum, in particular the fat fraction, but decrease rapidly as the mammary secretions change to normal milk (Parrish *et al.,* 1948). Calderon *et al.* (2007) reported that the concentration of the carotenoids lutein, all-trans β-carotene and cis-13 β-carotene in colostrum were highest initially and decreased drastically during the first week of lactation. The same authors reported a linear relationship between the concentration of β-carotene and the colour index and stated that β-carotene accounted for 65% of variations in the colour index in colostrum. Kehoe *et al.* (2007) reported that the concentration of β-carotene in colostrum ranges from 0.1 to 3.4 μg/g, with an average value of 0.68 μg/g. This is significantly higher than the average concentration of β-carotene in milk, i.e., 0.2 μg/g (Renner *et al.,* 1989). The reddish

colour of colostrum is due to the presence of red blood cells. During the *pre partum* period there is increased permeability of the mammary gland membranes, and more blood constituents gain access to the milk. Linzell and Peaker (1975) observed that as little as 0.1 mL blood/L can be detected visually and that 0.4 mL/L turns milk very pink. Using the CIELAB colour space, Madsen *et al.* (2004) reported that the colour of colostrum changed with time after calving; it became lighter (lightness, L, increased over the first six milkings), less red (redness, a, decreased and stabilized after the second milking) and less yellow (yellowness, b, decreased over the first 12 milkings). The same authors reported that the presence of blood in colostrum significantly affected the colour, causing the sample to be darker, less yellow and more red. These results are in agreement with the study of Espada and Vijverberg (2002), who reported that colostrum has a less blue colour than milk.

1.3.4 *Density/ Specific gravity*

The density of milk is the mass per unit volume and is usually expressed as kg/m^3. The density of whole milk at 20°C is about 1030 kg/m^3 and ranges from 1027 to 1033 kg/m^3 (Walstra and Jenness, 1984). Density varies with the composition of milk, i.e., the density increases with increasing content of solids-not-fat and decreases with increasing fat content (Walstra *et al.,* 2006). Madsen *et al.* (2004) reported that the density of colostrum decreased rapidly from 1048 to 1034 kg/m^3 during the first 2 d *post partum*, followed by a more gradual decrease to 1030 kg/m^3 by 6 d *post partum*. Strekozov *et al.* (2008) observed variations in the density of colostrum according to parity and season of calving. On average, the density of colostrum from first-calf heifers was 1059 kg/m^3, compared with 1068 kg/m^3 for third- and fourth-calf cows. Within these groups, the density of colostrum from cows which calved in fall was highest, followed by winter and then spring.

The density of milk should be distinguished from its specific gravity, i.e., the ratio of the density of milk to the density of water, at a specific temperature. Specific gravity is a dimensionless quantity and is much less temperature-dependant than density. The specific gravity of whole milk at 15.5°C ranges from 1.030 to 1.035,

with a mean value of 1.032 (Jenness and Patton, 1959). Parrish *et al.* (1950) reported that the specific gravity of colostrum is highest initially, decreases rapidly between the first and fourth milkings and continues to decrease after the fourth milking, but at a slower rate. The same authors observed that the lowest specific gravity is usually found in secretions from Holstein cows, while marked variations were also found in Ayrshire, Jersey and Guernsey cows. Quigley *et al.* (1994) reported that the specific gravity of colostrum is in the range 1.028 to 1.074, with a mean value of 1.052. The same authors reported that correlations of total Igs and IgG with specific gravity were lower than reported by Mechor *et al.* (1992) but were highly related to total N and protein N. Jeong *et al.* (2009) observed a similar trend to that seen by Parrish *et al.* (1950), i.e., the specific gravity of colostrum decreases rapidly between the first and fourth milking from 1.055 to 1.034 and continues to decrease but at a less rapid rate. Similarly, Kertz (2008) reported that the specific gravity of colostrum decreases from 1.056 to 1.033 during the first 5 milkings *post partum*.

1.3.5 *Thermal properties*

Heat treatment of milk and dairy products is mainly aimed at killing of microorganisms and inactivation of enzymes. Pathogens that may be transmitted to dairy calves in colostrum include *Mycobacterium avium* subsp. *paratuberculosis*, *Salmonella* spp., *Mycoplasma* spp., *Listeria monocytogenes*, *Campylobacter* spp., *Mycobacterium bovis* and *Escherichia coli* (Elizondo-Salazar and Heinrichs, 2008). One strategy to prevent the transmission of infectious diseases to calves is heat treatment of colostrum. However, pasteurisation of colostrum using the same time temperature combinations as used for milk reduces Ig concentration and increases viscosity. Meylan *et al.* (1996) investigated the effects of batch pasteurisation (63°C for 30 min) on colostrum and reported a mean loss of native Ig after pasteurisation of 12.3 ± 8.7%. In a similar study, Godden *et al.* (2003) investigated the effects of batch pasteurisation on native IgG concentrations of colostrum and reported that pasteurisation reduced native IgG concentration by 58.5 and 23.6% for 95- and 57-L

batches, respectively. The same authors observed that the consistency of colostrum was mildly thickened following pasteurisation. McMartin *et al.* (2006) found that heating colostrum to 63°C for 120 min resulted in an estimated 34% decrease in native IgG concentration and a 33% increase in viscosity. The same authors reported no differences in native IgG concentration and viscosity after heating colostrum to 60°C for 120 min and, in doing so, identified the critical temperature at or below which heating would produce no significant changes in native IgG concentration and viscosity. Godden *et al.* (2006) reported that heating of colostrum to 60°C for 120 min was sufficient to reduce the level of viable *Mycoplasma bovis*, *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella* enteritidis and *Mycobacterium avium* subspecies *paratuberculosis* below detectable limits. Similarly, Donahue *et al.* (2012) found that heating colostrum at 60°C for 60 min decreased total plate counts and coliform counts, and did not affect native IgG concentration. Johnson *et al.* (2007) described increased efficiency of IgG absorption and, consequently, higher serum IgG concentration in calves fed heat-treated (60°C for 60 min) compared to raw colostrum. These results are consistent with those of Godden *et al.* (2012) who, in addition, reported that calves fed heat-treated (60°C for 60 min) colostrum were at significantly lower risk for treatment of any illness in the preweaning period compared with calves fed unheated colostrum. The precise reason for this remains unclear, but it was hypothesised that the presence of bacteria in colostrum could interfere with systemic absorption of Ig molecules in the small intestine. Green *et al.* (2003), using HTST (high-temperature short-time) pasteurisation (72°C for 15 s) of colostrum, reported an average 28.4% loss of native IgG, and that all samples congealed into a thick, pudding-like consistency during or immediately after pasteurisation. These results are in agreement with those of Stabel *et al.* (2004), who reported a 25% reduction in native IgG concentration and gelling of the colostrum after HTST pasteurisation.

1.3.6 *Casein micelle properties*

Casein exists in milk as large colloidal aggregates referred to as casein micelles. Micelles have a spherical shape, a diameter ranging from 50 to 500 nm (average 150 nm) and molecular weight of 10^6 to > 10^9 Da (average 10^8 Da) (Fox, 2003). Brooker and Holt (1978) reported that colostrum contains casein micelles several micrometres in diameter. These micelles contain closed cavities, suggesting that they were formed by aggregation of particles comparable in size to normal casein micelles. Walstra *et al.* (2006) reported that colostrum and early-lactation milk contain a small number of very large micelles, reaching diameters of 600 nm. Tsioulpas *et al.* (2007) reported that the average diameter of casein micelles was almost constant throughout the first 90 days of lactation, except for the transition from day 1 to 2, during which it decreased from 227 to 189 nm. The same authors attributed the increased size of casein micelles in colostrum to the high protein (casein) and Ca (especially Ca^{2+}) concentrations. The relative proportions of the individual caseins in the micelles vary with micelle size. α_s- and β-casein decrease with decreasing micelle size, while κ-casein increases (McGann *et al.,* 1980; Donnelly *et al.,* 1983). The relative proportion of glycosylated κ-casein is inversely related to micelle size (Zbikowska *et al.,* 1992). Kroeker *et al.* (1985) reported that, in terms of the relative percentage of casein, the percentage of α_s-casein in colostrum fell initially after parturition and remained constant thereafter. The same authors observed that the relative percentage of β-casein was low initially and increased during the first two months of lactation, while the relative percentage of κ-casein was unaffected by stage of lactation. Barry and Donnelly (1980) reported that the relative percentage of α_s-casein was low in colostrum and increased in early lactation, which was not supported by the findings of Kroeker *et al.* (1985), while the relative percentage of β- and γ-caseins varied in a directly inverse relationship. Sobczuk-Szul *et al.* (2013) investigated the relative percentage of individual caseins in colostrum over the first 8 milkings *post partum* and reported that the relative percentage of α_s-casein increased, the relative percentage of β-casein remained stable and the relative percentage of κ-casein decreased over time

after calving. A decrease in κ-casein content and increase in α_s-casein content was also reported by Wielgosz-Groth (2004).

1.3.7 *Ethanol stability*

Ethanol stability is defined as the minimum concentration of added aqueous ethanol that causes milk coagulation (Horne and Parker, 1979). Serum phase components, in particular ionic calcium, govern the sigmoidal shape and position of the ethanol stability/pH profile (Horne and Parker, 1981a,b). Other factors which influence the ethanol stability of milk include salts (calcium, magnesium, phosphorus and citrate) (Donnelly and Horne, 1986), ionic strength (Horne, 1987) and pH (Horne, 1992). Chavez *et al.* (2004) reported that milk samples that were unstable to ethanol (72%, v/v) had a lower pH, SCC, casein and non-fat-solids relative to ethanol stable samples (ethanol at 78%, v/v or more), whereas freezing point, chloride, sodium and potassium concentrations were higher in the unstable group. Mitamura (1937) observed that colostrum reacts strongly to an equal volume of 70% ethanol immediately after parturition but that the degree of coagulation weakens gradually and disappears after approximately 10 days. Tsioulpas *et al.* (2007) investigated the stability of colostrum to equal volumes of ethanol ranging from 40 to 100% and found that the ethanol stability of colostrum was low for the first four days following parturition (54% on average) and only achieved 70% by day 5. The same author observed that samples which had poor ethanol stability also had low pH values and high ionic calcium concentrations.

1.3.8 *Rennet coagulation*

Rennet coagulation of milk occurs in two stages, i.e., enzymatic modification of casein micelles and Ca^{2+}-induced aggregation of the rennet-altered micelles (Horne and Banks, 2004). In milk, rennet coagulation time increases with increasing pH and decreases with increasing protein content and Ca^{2+} concentration (Fox and McSweeney, 1998; Madsen, 2004). Sebela and Klicnik (1975) observed a shorter

rennet coagulation time (RCT) for colostrum than for milk and recorded the lowest RCT on day 3 following parturition. A similar trend was reported by Klimes *et al.* (1986). Kvapilik *et al.* (1975) found that the RCT of colostrum from days 2 to 7 was more rapid than in subsequent days. Madsen *et al.* (2004) observed a decrease in the RCT of colostrum during the first six milkings, followed by an increase at a slower rate. However, curd firmness increased during the first six milkings and then decreased. With the exception of the first six milkings, Madsen *et al.* (2004) attributed changes in RCT primarily to changes in pH and casein content. Additionally, an elevated SCC was associated with a significant increase in RCT, which is in agreement with the study of Politis and Ng-Kwai-Hang (1988a). This is most likely due to the lower fat and casein contents of milk with a high SCC (Politis and Ng-Kwai-Hang, 1988b). Tsioulpas *et al.* (2007) reported that the RCT of colostrum was high on day 1, decreased sharply on day 2, remained constant for the next 3 days, and then increased steadily for the remainder of the study (90 days). With the exception of day 1, RCT followed a similar pattern to pH. The high RCT on day 1 could not be explained, as the colostrum had a low pH and high protein content. A possible explanation proposed by Tsioulpas *et al.* (2007) was a delay in the enzymatic phase of coagulation as a result of the high protein to chymosin ratio.

1.3.9 *Somatic cell count (SCC)*

Somatic cells in milk are of two types: sloughed epithelial cells from the udder and leukocytes form the blood (Singh and Ludri, 2001). Several authors have reported that the SCC for colostrum is much higher than that of milk (Emanuelson and Persson, 1984; Hallberg *et al.,* 1995; Andrew, 2001). In most cases, a high SCC in colostrum is not due to a mastitic infection but is of a physiological nature and is most likely due to penetration of cells through leaky tight junctions between the mammary epithelial cells (Nguyen and Neville, 1998). Miller *et al.* (1991) reported that the SCC was highest during the first 2 wk after calving, with smaller changes thereafter. These results were in agreement with Bodoh *et al.* (1976) who also

reported elevated SCC in early lactation. Ontsouka *et al.* (2003) reported that the SCC of colostrum on d 2 was 1,479,000 cells/mL compared with 274,000 cells/mL in milk. Madsen *et al.* (2004) found that the SCC of colostrum decreased from approximately 1,000,000 cells/mL for milkings 1 and 2 to < 100,000 at milking 12. Jeong *et al.* (2009) reported that the SCC of colostrum was highest initially and decreased gradually from approximately 2,454,708 cells/mL at parturition to 645,654 cells/mL after 132 h. The same author observed no differences in the SCC of colostrum from cows of different parities.

1.3.10 *Osmotic pressure*

The osmotic pressure of milk is a colligative property which is closely related to its freezing and boiling point. Natural variation in the osmotic pressure of milk (and hence freezing point) is limited by the physiology of the mammary gland and so remains relatively constant (Fox and McSweeney, 1998). Very little work has been carried out on the osmotic pressure of colostrum. McIntyre *et al.* (1952) reported that the average osmotic pressure of colostrum decreased rapidly in the first four milkings *post partum* and only relatively small changes were noted thereafter. The osmotic pressure of the 28th milking was about two-thirds of the initial colostrum. The high osmotic pressure of colostrum was attributed to its high mineral content. In contrast to this, Ontsouka *et al.* (2003) reported that the osmolarity of colostrum on d 2 was numerically, but not statistically, higher than milk.

1.3.11 *Other properties*

In addition to the properties discussed above, other physical properties of milk include ionic strength, redox potential, interfacial tension and various rheological properties, which, to the best of the author’s knowledge, have not been studied in bovine colostrum.

1.4 Conclusions

Colostrum is a secretion of the mammary gland produced immediately after parturition which is tailored to meet the unique nutritional and physiological requirements of the neonate. In particular, colostrum confers passive immunity to the newborn calf through its high concentration of IgG. The composition of colostrum differs markedly from milk, reflecting a difference in the biological function of the two materials. Its composition and physical characteristics vary with a number of factors including individuality, breed, parity, prepartum ration, length of dry period of cows and time *post partum*.

Colostrum has a much higher total solids content than milk, reflected mainly by a significantly higher protein content. The concentration of casein is higher in colostrum than in milk and the relative proportions of the individual caseins vary, i.e., colostrum contains reduced proportions of α_s-casein and elevated proportions of κ-casein, while the proportion of β-casein is constant. The proportion of whey proteins is substantially higher in colostrum than in milk. The predominant protein fraction in colostrum is the immunoglobulins (IgG, IgM, IgA), but colostrum also contains elevated levels of β-lg, α-la, serum albumin and lactoferrin. Additionally, certain proteins associated with the milk fat globule membrane are present in higher concentration in colostrum than in milk, i.e., apolipoprotein A1, apolipoprotein E and clusterin. The concentration of certain growth factors (IGF-I, IGF-II, EGF, TGF-β1, TGF-β2), antioxidants (lactoperoxidase, catalase, superoxide dismutase), proteinases (plasmin, cathepsin D), lipases (lipoprotein lipase) and esterases (arylesterase, carboxylesterase) are higher in colostrum than in milk, as are the concentrations of other enzymes including alkaline phosphatase, ribonuclease, lysozyme and γ-glutamyl transferase. Similarly, the concentrations of various enzyme inhibitors are higher in colostrum than in milk, i.e., cysteine protease inhibitors, α2-macroglobulin, α2-antiplasmin, antithrombin III, C1-inhibitor, inter-α-trypsin inhibitor, bovine plasma elastase inhibitor and bovine plasma trypsin inhibitor. The concentrations of nucleotides, nucleosides and cytokines are higher in colostrum than in milk. Lactose concentration is reduced in colostrum and is inversely related to other constituents such as solids, protein and

ash; a lactose concentration as low as 1.2% has been reported in colostrum. The concentrations of oligosaccharides, mostly acidic but also neutral, are higher in colostrum than in milk. Generally, but not always, the fat content of colostrum is higher than that of milk. Reported values range from 0.3 to 26.5%, with a mean value of approximately 6.5%. The proportion of short-chain fatty acids is low in colostrum, while the proportion of long chain-fatty acids in colostrum is high initially and decreases with time *post partum*. The concentration of phospholipids is significantly lower in colostrum than in milk, while the concentration of cholesterol is higher. Colostrum contains higher concentrations of calcium, phosphorus, magnesium, sodium, copper, iron, zinc and manganese than milk, while there are mixed reports in the literature regarding the concentration of potassium. For the most part, the concentrations of both fat-soluble (vitamin A, E, D, K) and water-soluble (vitamin C, B) vitamins are higher in colostrum than in milk.

The high compositional variability of colostrum has pronounced effects on its physical properties. The pH of colostrum is low initially and increases with time *post partum*, the precise reason for which is unknown. The buffering capacity and titratable acidity of colostrum are greater than that of milk due to increased levels of protein, soluble phosphate, colloidal calcium phosphate, citrate and carbonate. Colostrum has a reddish-yellow colour due to the presence of carotenoids and red blood cells. The density and specific gravity of colostrum are greater than that of milk due to higher a higher content of solids-not-fat. Colostrum has poor heat stability to typical pasteurisation or UHT treatments, but can be heated at 60°C for up to 120 min without affecting native IgG concentration or viscosity. The casein micelles are larger in the initial colostrum than in subsequent milkings. Colostrum has poor ethanol stability due to low pH, high SCC and increased concentrations of casein and non-fat-solids. There are mixed reports in the literature relating to the RCT of colostrum but, in general, the RCT of colostrum is lower than that of milk. The SCC of colostrum is much higher than that of milk, most likely due to penetration of cells through leaky tight junctions between mammary epithelial cells.

1.5 References

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Chapter 2

Composition and physico-chemical properties of bovine colostrum during the first 3 days *post partum*

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Declaration: Properties, including pH, casein micelle size, viscosity, colour and heat coagulation time were measured by Brian McGrath at University College Cork. Total protein, casein protein and whey protein, isoelectric precipitability and ultracentrifugal sedimentability were analysed by Brian McGrath and Thom Huppertz at University College Cork and NIZO Food Research Centre. Rennet coagulation time, acid-induced gelation and rheology of rennet- and acid-induced gels were analysed by Therese Uniacke-Lowe at University College Cork. Minerals were measured by Bernard Corrigan and Brian McGrath at Teagasc, Moorepark Food Research Centre. Carbohydrates were analysed by Thom Huppertz at NIZO Food Research Centre. Statistical analysis was carried out by Therese Uniacke-Lowe and Brian McGrath at University College Cork. All experimental data/results were analysed and the chapter written by Brian McGrath.

Abstract

The aim of this study was to analyse the secretions from the first six *post partum* milkings from six individual Holstein-Friesian cows to investigate how composition and physico-chemical properties change throughout the early lactation period. Properties, including pH, casein micelle size, viscosity, colour, rennet coagulation time (RCT), acid-induced gelation, rheology of rennet- and acid-induced gels, heat coagulation time, isoelectric precipitability, and ultracentrifugal sedimentability were analysed, together with some compositional factors, such as carbohydrates, minerals, total protein, casein protein and whey protein. In general, pH increased with increasing time *post partum*, while total protein content decreased with time, due to a decrease in the level of whey proteins. Casein micelles in all first milkings were larger than at subsequent milkings. Heat stability increased with increasing time *post partum*, most likely due to decreasing levels of whey proteins. Viscosity and RCT of colostrum samples and G’ values of rennet gels decreased with increasing time *post partum*. G’ values for acid gels from colostrum were low compared with those from mature milk, but no particular trend was observed. Total Na, Mg, P, K and Ca were high initially and decreased with time *post partum*. The concentration of lactose was low initially and increased with time *post partum*. Colostrum samples contained elevated levels of oligosaccharides and low levels of monosaccharides. The colour of colostrum samples was highly variable, presumably due to the presence of carotenoid and blood pigments. Caseins did not precipitate at pH 4.6 in several colostrum samples; altering the serum mineral composition and increasing the level of certain whey proteins had no effect on isoelectric precipitation, but reducing the pH below 4.6 restored precipitability, as did dilution with distilled water. Reversing the serum phase of colostrum and mature milk also resulted in precipitation of the caseins at pH 4.6. The results described in this study expand on current knowledge of the composition and physico-chemical properties of bovine colostrum.

2.1 Introduction

Colostrum is usually defined as the mammary secretion produced in the first five to six *post partum* milkings, the composition of which changes drastically during the transition from colostrum to early lactation milk (Christiansen *et al.,* 2010). Colostrum is a complete source of protein, carbohydrates, fat, vitamins and minerals, and is also rich in biologically active constituents. Colostrum plays an important role in development of immunological, metabolic, hormonal and haematological traits, and in growth performance in neonatal calves (Rauprich *et al*., 2000; Blattler *et al.,* 2001). Its composition and physical properties depend on various factors, including individuality, breed, parity, *pre partum* ration, length of dry period of cows, and time *post partum* (Foley and Otterby, 1978).

Bovine milk is classified as mature milk 72 h after parturition (Sgarbieri, 2004). Mature milk contains about 3.5% protein, of which approximately 80% are caseins, a heterogeneous group of proteins which are insoluble at their isoelectric point (pH 4.6). The casein fraction can be subdivided into four groups: α_{S1}, α_{S2}, β and κ, which occur in milk as a micellar complex in the approximate proportions 4:1:4:1, respectively (Visser *et al*., 1991). The remaining 20% of milk proteins are the whey proteins (soluble at pH 4.6), of which there are over 200 different types, with β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins (Igs) being the major constituents. The natural function of milk proteins is to provide young mammals with amino acids and calcium, both of which are necessary for growth. Casein micelles serve both functions as, in addition to protein, they provide the neonate with high levels of calcium and phosphate, which are required for bone growth (Fox and McSweeney, 1998). Madsen *et al*. (2004) found that colostrum contains a higher concentration of casein than mature milk. Colostrum has a distinctive whey protein composition, which includes elevated levels of IgG, IgA and IgM (Smolenski *et al*., 2007). The composition and physical properties of Igs in colostrum have been studied extensively (Mechor *et al*., 1992; Quigley *et al*., 1993; Levieux and Ollier, 1999; Elfstrand *et al*., 2002; Gapper *et al*., 2007; Yonghao *et al*., 2008; Zang *et al*., 2011; Golinelli *et al.,* 2011). For ruminants, Igs in colostrum are of particular importance to the neonate, as the transfer of

passive immunity from the mother to the neonate occurs through colostrum and not *via* the placenta. Passive transfer of Igs is important in protecting the neonate from infectious disease (Elizondo-Salazar and Heinrichs, 2008). Colostrum has a high concentration of bioactive components, e.g., growth factors, especially insulin-like growth factor-1 (IGF-1), transforming growth factor beta-2 (TGF-β2) and growth hormone (GH), as well as lysozyme and lactoperoxidase (Elfstrand *et al*., 2002). The concentration of growth factors in colostrum is highest during the first hours after calving and generally then declines sharply in a time-dependant manner (Gauthier *et al*., 2006). Growth factors control some fundamental life processes such as cell division, cell differentiation or apoptosis, and stimulate the growth and development of the gastrointestinal tract of new-born animals (Elfstrand *et al*., 2002). Proteins secreted by the mammary gland, such as β-Lg, α-La, lactoferrin (LF), proteinase inhibitors and some enzymes, are present at higher concentrations in colostrum than in mature milk (Levieux and Ollier, 1999). Proteins derived from the blood, such as albumin, α_2-macroglobulin, and transferrin are also present at high concentrations in colostrum (Levieux and Ollier, 1999).

The predominant carbohydrate in bovine milk is lactose (Nakamura *et al.,* 2003). Lactose concentration is low in colostrum and changes inversely to other constituents, such as total solids, protein, fat and ash (Kehoe *et al*., 2007; Tsioulpas *et al*., 2007a). In addition to lactose, the carbohydrate and carbohydrate-containing components of bovine colostrum include oligosaccharides, glycoproteins and glycolipids. The oligosaccharide content of bovine colostrum is lower and less complex than that of human milk, but there is a close homology between the major acidic oligosaccharides in both species (Gopal and Gill, 2000; Urashima *et al*., 2009). Oligosaccharides are considered to be some of the most important bioactive components in milk, and their primary role seems to be to provide protection against pathogens by acting as competitive inhibitors for binding sites on the epithelial surfaces of the intestine (Gopal and Gill, 2000). Colostrum contains much higher concentrations of oligosaccharides and other glycoconjugates than mature milk (Nakamura *et al*., 2003; Urashima *et al.,* 2013).

Bovine milk typically contains 3.5% fat, but the level varies widely. Of the common European breeds, milk from Holstein/Friesian cows, which were used in the current study, contain the lowest level of fat (Fox and McSweeney, 1998). Parrish *et al*. (1950) and Kehoe *et al*. (2007) reported an elevated fat level in colostrum compared to mature milk. Differences between the lipids in bovine colostrum and milk have not been studied in great detail, but Precht (2001) reported a higher cholesterol content in bovine colostrum than in milk.

Kilmes *et al*. (1986) and Tsioulpas *et al*. (2007a) found that concentrations of Ca, Mg, P, Na and K were much higher in colostrum than mature milk. Elevated levels of minerals have a pronounced effect on the technological properties of milk, e.g., renneting, fouling on heat exchangers, gelation and sedimentation (Tsioulpas *et al.,* 2007b).

Several authors have studied changes in the physico-chemical properties of bovine milk from the colostrum period to early lactation (Ontsouka *et al*., 2003; Madsen *et al*., 2004; Tsioulpas *et al*., 2007a), but relatively little work has been carried out on the composition of bovine colostrum. Foley and Otterby (1978) reviewed the literature, but no comprehensive research on colostrum composition has been published since. Most research on colostrum focuses narrowly on IgG and ignores other Igs and nutrients.

The aim of this study was to investigate the composition and physico-chemical properties of bovine colostrum during the early *post partum* period.

2.2 Materials and Methods

2.2.1 Collection of milk samples

Milk samples were obtained from Teagasc – Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork. The first six *post partum* milkings from six individual Holstein-Friesian cows, referred to as samples 1 to 6 from cows A to F, were obtained. Of the six cows, one was a heifer, four were in second lactation, and one was in third lactation. Three bulk mature milk samples (bulk 1 to 3) from a herd of cows in mid-lactation were also obtained for purposes of comparison.

2.2.2 Carbohydrate and protein analysis

The lactose, glucose, galactose and oligosaccharide content of milk samples was determined by HPLC using a method modified from those of Biggs and Szijarto (1963), Brons and Olieman (1983), and Koops and Olieman (1985). The HPLC system was equipped with an ERC-7510 refractive index detector (Erma Optical Works, Ltd., Tokyo, Japan), a mixed-bed guard column and an Aminex HPX-87P column (300 x 7.8mm, Bio-Rad, Hercules, CA, USA) in series, an LC-10ATvp pump, a SIL-10ADvp autosampler and a CTO 6A column oven set at 80°C (Shimadzu, Kyoto, Japan). A 25 µL sample was injected and sugars were eluted at a flow rate of 0.40 mL/min using an isocratic mobile phase of ultrapure MilliQ water (Millipore Corporation). Lactose, glucose and galactose were identified using appropriate standards.

Protein content was determined by the macro-Kjeldahl method (IDF, 1986). The total nitrogen content was converted to protein content using a conversion factor of 6.38.

2.2.3 Viscosity

The viscosity of whole milk and colostrum samples was measured using a rotational viscometer (Haake Roto Visco 1, Thermo Fisher Scientific, Waltham, MA, USA). For each sample, the shear rate was increased from 0 to 100/s, spaced at equal logarithmic intervals, over 2 min. Torque measurements were taken at a constant shear rate of 100/s over 2 min. The shear rate was then decreased from 100 to 0/s, spaced at equal logarithmic intervals, over 2 min. The sample cup (DG43 Series 1) was maintained at either 5°C or 20°C using a circulating waterbath (Haake K15 with SC150 temperature controller; Haake Mess-Technik GmbH, Karlsruhe, Germany). The instrument was checked prior to analysis using Milli-Q® water.

2.2.4 pH

The pH of skimmed milk and colostrum samples was measured at room temperature using a Meterlab standard pH meter PHM210 (Radiometer, Copenhagen, Denmark). The meter was calibrated using standard phosphate buffers (pH 4.01 and 7.00, Radiometer, Copenhagen, Denmark) and the electrode was washed with distilled water between measurements.

2.2.5 Colour

The colour of whole milk and colostrum samples was measured on three replicates of each sample at 20°C using a Minolta CR-400 colorimeter (Minolta Corp., Osaka, Japan). The CIE L*a*b* (CIE, 1976) standard measurement system, which measures colour using 3 coordinates, L*, a*, and b*, was used. The instrument was calibrated with a white tile (standard tristimulus values: Y = 92.4; x = 0.3161; y = 0.3325) supplied by Minolta, prior to measurement and at regular intervals throughout. Experimental error was +/- 0.1 units and each sample was assessed five times.

2.2.6 Casein micelle size

Dynamic light scattering was used to determine the average size of the casein micelles using a Zetasizer Malvern System ZS (Malvern Instruments Ltd., Malvern, UK). Samples were skimmed by centrifugation at 3,000 x g for 30 min at 4°C before analysis. Skimmed milk was diluted 50-fold with synthetic milk ultrafiltrate prepared according to the method of Jenness and Koops (1962), placed in a plastic cuvette and analysed.

2.2.7 Rennet coagulation

2.2.7.1 Rennet coagulation time

The rennetability of milk samples was studied in triplicate using a modification of the Berridge method (IDF, 1987). Two mL of skim milk (at either natural pH or at pH 6.6) were placed in thin-walled glass tubes and tempered for 15 min at 30°C in a thermostatically controlled waterbath. Ten µL/mL of a 1:10 (v/v) aqueous dilution of fermentation-produced chymosin (Maxiren 180, 180 international milk-clotting units (IMCU) per mL; DSM Food Specialists, Delft, the Netherlands) were added and the tubes were oscillated gently on a rocking platform in the waterbath. Rennet coagulation time was recorded as the first sign of flocculation, i.e., when visible curd flocs appeared on the wall of the tube.

2.2.7.2 Rheology of rennet-induced gels

Milk gels are viscoelastic and their rheological properties can be characterised by measuring both their elastic (G’) and viscous (G’’) moduli (Lucey, 2002). Dynamic oscillatory analysis (low-amplitude oscillatory measurement) of renneted skim milk samples was performed using a controlled shear stress AR-G2 rheometer equipped with a peltier concentric cylinder system and a conical rotor (diameter 28.02 mm x length 42.01 mm; TA Instruments, Waters LLC, Leatherhead, Surrey, UK). Twenty five mL of each milk sample were pre-warmed in a waterbath at 30°C for 15 min

and 250 µL of a 1:10 (v/v) dilution of Maxiren 180 were added. The fifth milking from cow C and the first milking from cow F did not coagulate under these conditions, and so, 750 µL of a 1:10 (v/v) dilution of Maxiren 180 were added. The storage modulus, G', of the sample was recorded continuously at a low-amplitude shear strain (0.01) over 90 min at a frequency of oscillation of 0.6283 rad/s. Analysis was carried out in triplicate.

2.2.8 Glucono- δ-lactone-induced acidification of colostrum and mature milk

Skimmed milk and colostrum were acidified using varying levels of glucono-δ-lactone (GDL). GDL converts to gluconic acid in water and slowly releases H^+ over time, lowering the pH of milk and providing a simplified and controlled model of milk fermentation (Lucey, 2002; Girard and Schaffer-Lequart, 2007). The concentration of GDL required to reach pH 3.9 ranged from 2.2 to 3.5%, depending on the protein content and buffering capacity of each sample. Addition of sufficient GDL to milk ensures that all the colloidal inorganic phosphate is solubilised in 90 min and that calcium is completely removed from the casein micelles (Heertje *et al.,* 1985). Acidification to pH 3.9 took ~90 min. GDL was added to milk samples, previously warmed to 30°C, maintained at this temperature for 20 min, and stirred vigorously for 1 min to ensure complete dissolution. In tandem with rheological measurements, the pH of a sub-sample of GDL-treated milk was monitored and recorded continuously. Dynamic oscillatory analysis (small amplitude oscillatory measurement) of acidified milk was performed as described in Section 2.2.7.2, with some modifications; 25 mL of each milk sample were pre-warmed in a waterbath at 30°C for 15 min, GDL was added, and the sample was placed immediately in the pre-heated cup (30°C) of the rheometer.

2.2.9 Heat stability

The heat stability of skimmed milk and colostrum was determined in an oil bath at temperatures ranging from 70 to 140°C by the subjective method of Davies and

White (1966). Three mL aliquots were placed in glass tubes (length, 130 mm; internal diameter, 7.5 mm; wall thickness, 1.6 mm), sealed with silicone rubber stoppers; tubes were heated in an oscillating oil bath until visual coagulation of protein particles was observed.

2.2.10 Ultracentrifugal sedimentation

The level of sedimentable and non-sedimentable protein in skimmed colostrum samples was measured by ultracentrifugation at 100,000 g for 60 min at 4 or 20°C in a Beckman Optima LE-80K Ultracentrifuge and the associated Beckman 50.2 Ti rotor (Beckman Instruments Inc., Palo Alto, CA, USA). Aliquots of skimmed milk were transferred to tared centrifuge tubes (Optiseal, polyallomer centrifuge tubes, Beckman Coulter Inc., Brea, CA, USA) and ultracentrifuged. The supernatant was removed carefully from the pellets, weighed, and its protein content determined. The supernatants were also analysed by RP-HPLC, as described in Section 2.2.12. Samples were diluted before analysis, using a suitable dilution factor based on protein concentration. These dilution factors were taken into account when correcting for peak areas.

2.2.11 Isoelectric precipitation of casein

Skimmed milk and colostrum samples were adjusted to pH 4.6 according to the method of Kuchroo and Fox (1982) with slight modifications. Prior to pH adjustment, samples were diluted 1:1, 1:2, 1:3 or 1:4 with distilled water. Undiluted samples were also analysed. Samples were adjusted to pH 4.6 with 1 N HCl and held at room temperature. After 30 min, pH was re-checked and re-adjusted if necessary. Samples were placed in a waterbath at 40°C for 1 h followed by centrifugation at 3000 g for 30 min at 4°C. The resulting supernatant was filtered through glass wool and Whatman no. 113 filter paper. The filtrate was stored at -20°C for further analysis.

2.2.11.1 Effect of varying pH on isoelectric precipitation of casein

The pH of skimmed colostrum from cow F, 1st and 2nd milking, was adjusted to different values ranging from 4.0 to 5.0 according to the method of Kuchroo and Fox (1982) with slight modifications. Briefly, the pH of the colostrum was adjusted to different values ranging from 4.0 to 5.0 using 1 N HCl and held at room temperature. After 30 min, the pH was re-checked and re-adjusted if necessary. Samples were placed in a waterbath at 40°C for 1 h followed by centrifugation at 3000 g for 30 min at 4°C. The resulting supernatant was filtered through glass wool and Whatman no. 113 filter paper. The filtrate was stored at -20°C for further analysis.

2.2.11.2 Interchange of serum phase minerals between colostrum and mature milk and its effect on isoelectric precipitation of casein

The serum phase minerals of skimmed milk and colostrum were interchanged by equilibrium dialysis. Briefly, 1 volume of skimmed colostrum was dialysed against 6.5 volumes of skimmed milk at 4°C for 24 h using a 3500 Da dialysis membrane (Medicell International, London). This was carried out in triplicate to bring the total dialysis time to 72 h. The same was done for the skimmed milk where 1 volume was dialysed against 6.5 volumes of skimmed colostrum under the same conditions as above. The dialysed colostrum and mature milk were then adjusted to pH 4.6 according to the method of Kuchroo and Fox (1982), as described in Section 2.2.11.

2.2.11.3 Addition of lactoferrin to mature milk and its effect on isoelectric precipitation of casein

The lactoferrin content of skimmed milk was modified by direct addition of 0 to 25.6 mg/mL lactoferrin (NIZO Food Research, Ede, the Netherlands). The addition was made at ambient temperature with stirring. Modified milk samples were

equilibrated overnight at 4°C and adjusted to pH 4.6 according to the method of Kuchroo and Fox (1982), as described in Section 2.2.11.

2.2.11.4 Purification of immunoglobulins from colostrum

Colostrum was defatted by centrifugation at 3,000 g for 30 min at 4°C. Skimmed colostrum was adjusted to pH 4.6 according to the method of Kuchroo and Fox (1982), as described in Section 2.2.11, with the slight modification in which samples were diluted 1:2 with distilled water before pH adjustment. Equal volumes of the pH 4.6-soluble fraction of colostrum and saturated ammonium sulphate were mixed and allowed to equilibrate for 30 min. Addition of ammonium sulphate compressed the solvation layer and increased protein-protein interactions. As the ammonium sulphate concentration of the solution was increased, the charges on the surface of the protein interacted with the ammonium sulphate, not the water, thereby exposing hydrophobic patches on the protein surface and causing the protein to aggregate and precipitate. The solution was centrifuged at 3,000 g for 30 min at 20°C. The precipitate was taken, dissolved in 0.01 M NaCl and allowed to hydrate overnight at 4°C. The solution was dialysed for three 24 h periods against 20 volumes of distilled water using a 12,000 – 14,000 Da dialysis membrane (Medicell International, London), and dialysis retentate was lyophilised.

2.2.11.5 Addition of a colostral Ig preparation to mature milk and its effect on isoelectric precipitation of casein

The Ig content of skim milk was modified by addition of 25, 50, 100 or 150 mg/mL of the Ig preparation, prepared as described in Section 2.2.11.4. The addition was performed at ambient temperature with stirring. Modified milk samples were equilibrated overnight at 4°C and adjusted to pH 4.6 according to the method of Kuchroo and Fox (1982), as described in Section 2.2.11.

2.2.11.6 Reversal of serum phase of colostrum and mature milk and its effect on isoelectric precipitation of casein

Casein from bulk mature milk samples, 1 to 3, was sedimented by ultracentrifugation at 100,000 g x 1 h at 20°C and lyophilised. The serum phase of the first milking from cow E and bulk mature milk sample 1 were prepared by ultracentrifugation at 100,000 g x 1 h at 20°C to remove casein. Sedimented mature milk casein was dispersed (3%, w/v) in the serum phase of the first milking from cow E or bulk mature milk 1, at ambient temperature with stirring. Samples were equilibrated overnight at 4°C and adjusted to pH 4.6 according to the method of Kuchroo and Fox (1982), as described previously in Section 2.2.11.

2.2.12 Reversed phase-high performance liquid chromatography

Clarified samples were obtained by mixing skimmed milk or colostrum (1:1, v/v) with a buffer containing 0.1 M bis(2-hydroxymethyl)amino-tris(hydroxymethyl)methane, 8 M urea, 0.3% mercaptoethanol (ME) and 1.3% trisodium citrate dehydrate (pH 7); in some experiments, ME was omitted from the buffer. After standing at room temperature for 1 h, the mixture was diluted with solvent A (acetonitrile-water-trifluoroacetic acid, 100:900:1 v/v/v) containing 6 M urea.

The HPLC equipment consisted of two M 6000A pumps (Waters Association, Milford, MA, USA), an ISS-100 automatic sample injector (Perkin-Elmer, Waltham, MA, USA), a Kratos Model 783G UV detector and a Waters Type 680 automated gradient controller. The equipment was linked to a data acquisition and processing system (Waters Maxima 820). A 250 mm x 4.6 mm I.D. HiPore RP-318 column (Bio-Rad, Hercules, California, USA) was used with a C_{18} cartridge (Bio-Rad, Hercules, California, USA) as a guard column. Solvent A was acetonitrile-water-trifluoroacetic acid (100:900:1 v/v/v) and solvent B had the same composition with the proportions 900:100:0.7 (v/v/v). The solvent gradient reported for casein separation (Visser *et al.,* 1986) was adapted in the current study for genetic casein

variants and whey proteins: starting from 26% of solvent B (equilibration buffer) a gradient was generated immediately after injection by increasing this proportion at 0.60%/min (15 min), 0%/min (7 min), 0.67%/min (3 min), 0%/min (12 min), 0.44%/min (18 min), 12.5%/min (2 min) and 0%/min (5 min), before returning to starting conditions in 5 min. The column temperature was 30°C, peak detection was at 220 nm, the flow-rate was 0.8 mL/min, and the system pressure was 10.3 MPa.

2.2.13 SDS- polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Laemmli (1970) using a 15%, w/v, acrylamide separating gel and a 3%, w/v, stacking gel, both containing 0.1%, w/v, SDS. Milk samples were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2%, w/w, SDS, 5%, w/w, mercaptoethanol and 0.012%, w/w, bromophenol blue and heated at 95°C for 4 min prior to loading into gel slots. Electrophoresis was carried out at a constant voltage (200 V) for 8 h using a tris-glycine buffer (pH 8.3) containing 0.125%, w/w, SDS. The gel was removed from the electrophoresis unit, fixed in 50:10:40 methanol:acetic acid:water; and stained with Coomassie Brilliant Blue R-250 to visualize the proteins. Images of gels were captured using the Epson Perfection 4180 Scanner (Epson, Suwa, Nagano, Japan) with Total Lab Quant software (Newcastle, UK). Image resolution was set at 600 dots per inch (dpi).

2.2.14 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

Colostrum and mature milk samples were standardised to approximately equal total solids in order to avoid matrix effects between samples. Samples were then digested in 70% nitric acid in closed digestion vessels using a MARSXpress microwave digester (CEM, Matthews, NC, USA). When the samples were fully digested, the contents were carefully washed into a 100 ml volumetric flask and brought up to volume with ultrapure water from a Milli-Q system (Millipore,

Bedford, MA, USA). Further dilutions were made as necessary. All samples were run on an Agilent 7700x series (Agilent Technologies, Tokyo, Japan) with Octopole Reaction System (ORS) and MassHunter software. Argon was used as the carrier gas and helium as the collision gas. External standards of known mineral composition (Reagecon, Co. Clare, Ireland) were run and a multipoint calibration graph was prepared for each element. R^2 was >0.99% in all cases. Internal standards were run for all elements (Agilent Technologies, Santa Clara, CA, USA). A certified standard of known composition (BCR -IRMM, Geel, Belgium) was run as a QC check for each batch.

2.2.15 Statistical analysis

Statistical analysis was carried out using Minitab version 16 (Minitab Inc., State College, PA, USA). Five separate statistical tests were carried out.

General linear model analysis of variance (GLM ANOVA) was used to assess the effect of cow to cow variation (A to F) and time of milking (1st to 6th milking *post partum*) on the composition and physico-chemical properties of colostrum, of which 22 parameters were measured (referred from herein as variables): L*, a*,b*, pH, protein concentration, non-sedimentable protein at 4 and 20°C, sedimentable protein at 4 and 20°C, minerals (Na, Mg, P, K, Ca), casein micelle diameter, viscosity at 5 and 20°C, soluble protein at pH 4.6 (undiluted, 1:1 dilution, 1:2 dilution), rennet coagulation time and storage modulus (G') after rennet addition. Plots of residuals from GLM ANOVA were examined initially to satisfy the requirement that they be normally distributed and thus confirm the validity of the statistical test. Plots of residuals *versus* fits and residuals *versus* order were examined to identify potential patterns in the residual error and highlight any outliers in the data from each parameter. Data from Sections 2.3.2, 2.3.3, 2.3.4, 2.3.5, 2.3.7 and 2.3.10 were not included in the statistical analysis as replication of analysis was not consistent with other variables measured. The assumption in this study was that the six cows selected represented variability across a herd and, thus, individual cows were treated as random factors. GLM ANOVA allows the use of random factors, unlike

two-way ANOVA, which only allows the use of fixed factors. Initial models for GLM ANOVA were identified by graphically plotting the responses for the six cows over time. Scatter-plots helped to identify possible quadratic time effects in several cases. Where linear or quadratic effects seemed to be appropriate functions for the response over the effects of milking number, sample milking numbers were included as covariates. In a few models, the milking numbers did not fit a linear effect, and thus milking number was considered a separate level for GLM ANOVA.

Because GLM ANOVA does not run multiple comparison tests on factors that interact with random factors, or on covariates, principal component analysis (PCA) was carried out to reduce and interpret the large multivariate dataset using underlying linear structures, and to determine if there were any interactions between samples over time, based on the 22 variables described above. For PCA computation, measurements were standardized using a correlation matrix as variables were measured on different scales. Outliers were determined as Mahalanobis distances for each observation, which took into account the variance of each variable and the covariance between variables, and was useful in identifying and gauging similarities in the dataset and pinpointing any obvious outliers. For clarity of presentation and interpretation of results, loading and score plots were plotted separately. The former is a plot of the loadings of PC2 (y-axis) *versus* the loadings of PC1 and a line is drawn from each loading to the (0,0) point. The latter is a plot of the scores for PC2 (y-axis) *versus* the scores for PC1 (x-axis). Finally, a biplot was constructed which is an overlay of the score and loading plots for PCs 1 and 2.

GLM ANOVA, using the 'comparisons with a control function', was repeated on the dataset for the 22 measured variables, but milking number was excluded, while results for bulk mature milk (which were not measured at each time point) were included in the analysis. Dunnett's multiple comparison test was used to compare the mean of each factor level (i.e., measured parameter) with that of the control bulk mature milk samples. This test resulted in a grouping table and a set of confidence intervals for the difference between the mean of the control level and

the other sample level means. Unusual observations were noted for observations where standardized residuals differed from zero by more than 2.00.

One-way ANOVA was used in Section 2.3.11.3 to determine if there were any significant differences between mean values for protein solubility at pH 4.6 of colostrum and mature milk after interchanging the serum salts by equilibrium dialysis. One-way ANOVA was also carried out on data investigating the effect of addition of lactoferrin to mature milk on the isoelectric precipitation of casein (Section 2.3.11.4). Initially, the data for all parameters measured were examined for normality using the Anderson-Darling normality test at a significance level, α, of 0.1; when calculated p values were found to be > 0.1, it was assumed that the data were normally distributed. As all data were normally distributed ($p > 0.1$), one-way ANOVA was carried out using a significance level, α, of 0.05. Where significant differences occurred between sample means, Tukey's paired multiple comparison test was used to compare all possible pairs of level means for the factors specified at a significance level, α, of 0.05. The grouping information table generated by Tukeys test was used to group factor level means that were not significantly different.

Two-way ANOVA at a significance level, α, of 0.05 was used to assess the effect of pH on the precipitation of casein for the first and second milking of cow F (Section 2.3.11.3). Unusual observations in the statistical analysis were recorded where standardized residuals differed from zero by > 2.00.

2.3 Results and Discussion

2.3.1 Physical properties and composition

Average values for physical properties and composition of the first six milkings *post partum* from six individual cows and three bulk mature milk samples are presented in Table 2.1. In general, changes were substantial over the first six milkings, most notably during the first two milkings.

The pH of mature milk at 25°C is between 6.5 and 6.7, with pH 6.6 being the most commonly reported value (McCarthy and Singh, 2009). The main factor influencing the pH of milk is stage of lactation; the pH of colostrum is lower, and that of late-lactation milk is higher (may be up to 7.5), than the pH of normal mid-lactation milk (McCarthy and Singh, 2009). The pH of colostrum samples in the current study was very low initially and increased with time *post partum*; in almost all cases, the pH did not reach the normal value by the sixth milking (on average, pH increased from 6.35 to 6.49 during the first six milkings *post partum*). Klimes *et al*. (1986) and Tsioulpas *et al*. (2007a) observed a similar trend for pH values, although their average pH value for the first milking was slightly lower than that found in the current study. The pH of the initial colostrum ranged from 6.28 to 6.39, while the pH of mature milk samples 1 to 3 varied from 6.62 to 6.66. The lowest pH was observed in the first milkings from cows A and D (6.31 and 6.28, respectively). McIntyre *et al*. (1951) reported that the pH of colostrum at the first milking ranged from 6.00 to 6.61, with a mean value of 6.32.

The reason for the low pH value of early colostral milkings is unknown. During the *pre partum* period, there is increased permeability of the mammary gland membrane and thus more blood constituents gain access to the milk. Given that colostrum contains significantly more blood components than mature milk, a pH value closer to that of blood (pH 7.35 to 7.45) (Nappert and Naylor, 2001) would be expected. A possible explanation for the low pH is the high mineral content of

colostrum (see Section 2.3.8), i.e., hydrogen ions are released during the formation of colloidal calcium phosphate (CCP), which may cause a drop in pH.

By the sixth milking, pH values ranged from 6.37 to 6.67, with a mean value of 6.49. According to Edelsten (1988), a pH value less than 6.5 in milk indicates the presence of colostrum, although a low pH value may also be caused by bacterial contamination (Tsioulpas *et al*., 2007a), while a value greater than 6.7 is usually observed in late-lactation milk or denotes a mastitis infection. Therefore, results from the present study indicate that, at the sixth milking *post partum*, milk may still be considered as colostrum and may be unsuitable for certain dairy processing operations.

Mature bovine milk contains ~3.5% (w/v) protein, which is made up of approximately 80% casein and 20% whey protein (Golinelli *et al*., 2011). The protein concentration in colostrum samples was highly variable and ranged from 4.0 to 24.9% over the first six milkings *post partum* (Table 2.1). The average value for total protein decreased rapidly, from 14.4% at the first milking to 8.3% at the second milking and then continued to decrease until the sixth milking, at which time it still had not reached normal levels. Foley and Otterby (1978) and Tsioulpas *et al*. (2007a) observed a similar trend for protein concentration; however, average values in those studies did not reflect the high variability in protein concentration between individual cows. The total protein level at the first milking from cows A to F ranged from 6.6 to 24.9%. This variability decreased with time *post partum* and, by the sixth milking, total protein from cows A to F ranged from 4.0 to 5.2%. Samples from cow D had the highest concentration of total protein over the first six milkings *post partum*: 24.9% at the first milking, 15.0% at the second milking and 5.2% at the sixth milking. In contrast, total protein concentration at the first six milkings *post partum* from cow C were much lower, albeit still higher than mature milk: 6.6% at the first milking, 5.3% at the second milking, and 4.4% at the sixth milking. High levels of total protein in colostrum have been reported by several authors (Oyeniyi and Hunter, 1977; Levieux and Ollier, 1999; Madsen *et al.,* 2004; Tsioulpas *et al.,* 2007a; Abd El-Fattah *et al.,* 2012); this high level of total protein is due mainly to elevated levels of whey proteins, in particular Ig. The major Ig in

colostrum is IgG, with IgG_1 representing more than 90% of Igs (Elfstrand *et al*., 2002). Proteins secreted by the mammary gland, such as β-Lg, α-La, LF, proteinase inhibitors and some enzymes, are present at high concentrations in colostrum compared to mature milk (Levieux and Ollier, 1999). Proteins derived from the blood such as serum albumin, α_2-macroglobulin, and transferrin are also present at higher concentrations in colostrum (Levieux and Ollier, 1999).

Large variations were seen in the level of protein sedimentable at 100,000 g (casein) and, in particular, non-sedimentable protein in colostrum samples. The concentration of non-sedimentable protein ranged from 8.2 to 171.3 g/L. Average levels of non-sedimentable protein decreased rapidly from 89.6 g/L at the first milking to 39.3 g/L at the second milking and then continued to decrease until the sixth milking, at which time they still had not reached normal levels, i.e., average value of 8.45 g/L for bulk mature milk samples. Variations were also seen in the level of sedimentable protein, which ranged from 31.8 – 77.7 g/L. Madsen *et al*. (2004) reported average values for casein concentration in first colostral milkings of 92.4 g/L, which is higher than that found in the current study. However, as discussed in Section 2.3.6., a significant proportion of casein was non-sedimentable at 100,000 g, which may have caused the underestimation of the concentration of casein in colostrum. A similar trend to that seen for changes in the level of non-sedimentable protein in colostrum samples was observed for sedimentable protein, which decreased from an average value of 54.8 g/L at the first milking to 43.5 g/L at the second milking and then continued to decrease until the sixth milking, at which time they still had not reached normal levels. These results agree with those of Parrish *et al*. (1948), who reported that the concentration of protein components in colostrum, including casein, albumins and globulins, decrease as it changes to normal milk.

Casein exists in milk as large colloidal aggregates referred to as casein micelles, which have a spherical shape, a diameter ranging from 50 to 500 nm (average 150 nm) and molecular mass of 10^6 to > 10^9 Da (average 10^8 Da) (Fox, 2003). The average diameter of casein micelles in colostrum was found to be almost constant throughout the study, except for the transition from the first to second milking,

during which time it decreased, on average, from 235.5 to 192.8 nm (Table 2.1). No particular trends were observed in casein micelle diameter from the second milking onwards. Tsioulpas *et al*. (2007a) reported a similar result, where the average diameter of casein micelles was almost constant throughout the first 90 days of lactation except for the transition from day 1 to day 2, during which time it decreased from 227 to 189 nm. Casein micelle diameter across all colostrum samples ranged from 173.3 to 266.9 nm. The average diameter of casein micelles in bulk mature milk samples was 195 nm, which is in agreement with reported values (Walstra, 1990). It is known that micelles of different sizes contain different proportions of the individual caseins, with smaller micelles containing a larger proportion of κ-casein (Dalgleish *et al.,* 1981), and the relative amounts of β-casein and α_{S2}-casein decrease with decreasing micelle size (Davies and Law, 1983). This disagrees with the results of the current study, in which the proportion of κ-casein as a function of total casein was found to be high in early milkings (see Table 2.3).

The viscosity of milk is affected by the state and concentrations of fat and protein, temperature, pH and age of the milk (Jenness and Patton, 1959). Under most conditions, milk behaves as a Newtonian liquid with shear stress proportional to shear rate (Walstra *et al.,* 1999). In general, large variations in the viscosity of colostrum samples were observed. This was most evident during the transition from the first to second milking, in particular for cows D, E and F. This is most likely due to high levels of total solids, of which protein was the major constituent, in early colostral milkings. Alcantara *et al*. (2012) reported that the viscosity of milk decreased as temperature increased and total solids decreased, and increased as fat, lactose, protein and minerals increased. This is consistent with the results of the current study, as early colostral milkings with the highest viscosities also had the highest concentrations of protein and minerals. There may also be a correlation between the viscosity of colostrum and the lactose concentration. Lactose in milk acts as an osmoregulator, and thus a low level of lactose causes the production of milk which is extremely viscous (Bleck *et al.,* 2009). In the current study, colostrum samples with the highest viscosity (Table 2.1) also contained the lowest concentrations of lactose (see Table 2.4). The average viscosity of bulk mature milk

at 20°C was found to be 3.2 mPa s. This value was higher than the value of 2.1 mPa s reported by Jenness and Patton (1959). In general, the viscosity of colostrum from the first 3 milkings of cows A to F were greater than that of the bulk mature milk samples; these values decreased with time *post partum* and fell below the value for mature milk by the sixth milking.

Table 2.1 Mean values (± S.D., n=3) of pH, total protein, non-sedimentable protein, sedimentable protein, viscosity, and casein micelle diameter of colostrum samples from the first six milkings of cows A to F and bulk mature milk samples 1 to 3. Values without an asterisk (*) were significantly different ($p < 0.05$) from control bulk milk samples.

Sample	pH	Protein (mg/mL)	Non-sedimentable protein (mg/mL)	Sedimentable protein (mg/mL)	Micelle Size d.nm	Viscosity (mPa s) *5°C*	Viscosity (mPa s) *20°C*
A1	6.31 ± 0.01	115.00 ± 1.27	70.21 ± 0.71	44.79 ± 0.71	219.6 * ± 7.89	14.72 ± 0.32	6.52 ± 0.21
A2	6.37 ± 0.01	71.00 ± 0.71	34.39 ± 0.30	36.61 ± 0.30	187.8 * ± 2.75	10.73 ± 0.32	5.17 * ± 0.19
A3	6.40 ± 0.01	50.00 ± 0.42	16.86 ± 0.17	33.14 ± 0.17	186.0 * ± 3.75	3.88 * ± 0.18	3.29 * ± 0.28
A4	6.47 ± 0.02	48.00 ± 0.99	13.55 ± 0.45	34.45 ± 0.45	187.3 * ± 4.69	4.84 * ± 0.43	2.19 * ± 0.17
A5	6.44 ± 0.01	46.00 * ± 1.41	11.29 ± 0.18	34.71 ± 0.18	184.4 * ± 5.40	3.72 * ± 0.20	2.77 * ± 0.16
A6	6.46 ± 0.00	40.00 * ± 0.64	8.17 * ± 0.12	31.83 ± 0.12	183.9 * ± 2.34	3.92 * ± 0.16	2.47 * ± 0.19
B1	6.37 ± 0.01	78.00 ± 0.21	28.34 ± 0.14	49.66 ± 0.14	182.9 * ± 4.36	6.74 * ± 0.21	3.35 * ± 0.17
B2	6.32 ± 0.00	58.00 ± 0.71	13.12 ± 0.03	44.88 ± 0.03	179.4 ± 5.02	4.09 * ± 0.22	3.07 * ± 0.16
B3	6.32 ± 0.01	57.00 ± 0.85	13.58 ± 0.12	43.42 ± 0.12	181.6 * ± 4.45	5.02 * ± 0.19	3.29 * ± 0.37
B4	6.36 ± 0.00	59.00 ± 2.40	13.25 ± 0.01	45.75 ± 0.01	183.3 * ± 3.21	8.54 * ± 0.30	6.28 ± 1.14
B5	6.40 ± 0.03	51.00 ± 0.99	11.19 ± 0.20	39.81 ± 0.20	181.6 * ± 3.02	6.50 * ± 0.49	2.31 * ± 0.51
B6	6.47 ± 0.01	52.00 ± 1.84	10.99 ± 0.09	41.01 ± 0.09	173.3 ± 1.68	4.67 * ± 0.19	2.26 * ± 0.64
C1	6.39 ± 0.01	66.00 ± 0.99	27.18 ± 0.17	38.82 ± 0.17	265.8 ± 13.56	5.03 * ± 0.21	2.48 * ± 0.29
C2	6.44 ± 0.01	53.00 ± 0.71	16.34 ± 0.39	36.66 ± 0.39	188.4 * ± 3.86	5.51 * ± 0.83	3.11 * ± 0.19
C3	6.51 ± 0.01	51.00 ± 0.28	16.59 ± 0.09	34.41 ± 0.09	192.0 * ± 4.27	5.05 * ± 0.16	3.91 * ± 0.22
C4	6.54 ± 0.03	48.00 ± 0.21	12.67 ± 0.06	35.33 ± 0.06	199.1 * ± 4.01	4.18 * ± 0.20	2.94 * ± 0.18
C5	6.48 ± 0.01	46.00 ± 0.64	12.79 ± 0.15	33.21 ± 0.15	195.9 * ± 5.67	3.19 * ± 0.15	1.37 * ± 0.17
C6	6.50 ± 0.03	44.00 * ± 0.07	10.98 ± 0.15	33.02 ± 0.15	195.3 * ± 3.56	3.60 * ± 0.15	2.60 * ± 0.15
D1	6.28 ± 0.01	249.00 ± 0.35	171.34 ± 0.71	77.66 ± 0.08	240.6 ± 12.21	271.20 ± 3.10	65.52 ± 0.50
D2	6.25 ± 0.00	150.00 ± 1.84	96.63 ± 0.09	53.37 ± 0.09	215.6 * ± 6.62	29.81 ± 0.50	14.25 ± 0.29
D3	6.39 ± 0.00	107.00 ± 0.07	58.07 ± 0.01	48.93 ± 0.01	200.1 * ± 6.47	11.56 ± 0.20	8.87 ± 0.19
D4	6.51 ± 0.01	71.00 ± 0.21	30.06 ± 0.03	40.94 ± 0.03	179.9 ± 7.94	8.24 * ± 0.18	4.84 * ± 0.20
D5	6.68 * ± 0.00	58.00 ± 2.33	19.07 ± 0.19	38.93 ± 0.19	187.4 * ± 3.77	7.06 * ± 0.21	1.87 * ± 0.18
D6	6.67 * ± 0.01	52.00 ± 0.21	19.98 ± 1.41	32.02 ± 0.45	189.5 * ± 4.55	5.86 * ± 0.16	2.51 * ± 0.16

(continued)

Table 2.1 (continued)

Sample	pH	Protein (mg/mL)	Non-sedimentable protein (mg/mL)	Sedimentable protein (mg/mL)	Micelle Size d.nm	Viscosity (mPa s) *5°C*	Viscosity (mPa s) *20°C*
E1	6.39 ± 0.02	185.00 ± 8.98	130.62 ± 0.59	54.38 ± 0.59	266.9 ± 11.25	107.13 ± 2.08	37.67 ± 0.63
E2	6.47 ± 0.02	81.00 ± 0.07	38.21 ± 0.21	42.79 ± 0.21	185.1* ± 2.57	7.83* ± 0.18	4.30* ± 0.18
E3	6.42 ± 0.00	60.00 ± 0.49	19.69 ± 2.33	40.31 ± 0.13	191.4* ± 3.40	8.68* ± 0.35	3.87* ± 0.20
E4	6.53 ± 0.01	54.00 ± 0.21	15.82 ± 0.01	38.18 ± 0.01	191.8* ± 5.70	7.51* ± 0.18	3.41* ± 0.22
E5	6.52 ± 0.00	46.00 ± 1.34	11.20 ± 0.19	34.80 ± 0.19	173.8 ± 0.83	3.91* ± 0.18	3.38* ± 0.44
E6	6.44 ± 0.01	48.00 ± 0.35	10.45 ± 0.10	37.55 ± 0.10	190.3* ± 2.54	6.34* ± 0.19	2.55* ± 0.27
F1	6.35 ± 0.00	173.00 ± 2.40	109.76 ± 0.49	63.24 ± 0.49	237.1 ± 7.46	66.49 ± 0.91	25.36 ± 0.49
F2	6.33 ± 0.00	84.00 ± 0.28	37.30 ± 0.39	46.70 ± 0.39	200.5* ± 4.22	6.08* ± 0.16	4.14* ± 0.17
F3	6.26 ± 0.00	60.00 ± 0.92	21.57 ± 0.28	38.43 ± 0.28	193.2* ± 4.04	4.44* ± 0.25	2.19* ± 0.19
F4	6.34 ± 0.00	52.00 ± 1.41	13.17 ± 0.10	38.83 ± 0.10	185.9* ± 3.12	7.86* ± 0.25	2.85* ± 0.30
F5	6.35 ± 0.01	45.00* ± 1.41	9.38* ± 0.23	35.62 ± 0.23	187.8* ± 6.35	3.84* ± 0.23	2.79* ± 0.16
F6	6.37 ± 0.00	43.00* ± 1.41	8.70* ± 0.01	34.30 ± 0.13	191.1* ± 3.85	6.48* ± 0.17	4.34* ± 0.15
Bulk 1	6.66 ± 0.00	39.00 ± 0.42	8.26 ± 0.07	30.74 ± 0.17	192.3 ± 2.06	5.78 ± 0.24	4.80 ± 0.15
Bulk 2	6.62 ± 0.03	38.00 ± 0.71	7.87 ± 0.14	30.13 ± 0.31	198.3 ± 7.82	6.85 ± 0.27	1.95 ± 0.27
Bulk 3	6.66 ± 0.01	40.00 ± 2.19	9.23 ± 0.00	30.77 ± 0.13	194.5 ± 6.29	5.20 ± 0.29	2.95 ± 0.15
1st milking (average)	6.35 ± 0.04	144.33 ± 70.47	89.57 ± 57.96	54.76 ± 13.97	235.5 ± 31.47	78.55 ± 102.68	23.48 ± 24.92
2nd milking (average)	6.36 ± 0.08	82.83 ± 35.11	39.33 ± 30.10	43.50 ± 6.39	192.8 ± 13.13	10.67 ± 9.65	5.67 ± 4.28
3rd milking (average)	6.38 ± 0.09	64.17 ± 21.42	24.39 ± 16.72	39.77 ± 5.87	190.7 ± 6.35	6.44 ± 3.02	4.24 ± 2.35
4th milking (average)	6.46 ± 0.09	55.33 ± 8.71	16.42 ± 6.77	38.91 ± 4.10	187.9 ± 6.78	6.86 ± 1.87	3.75 ± 1.52
5th milking (average)	6.48 ± 0.12	48.67 ± 5.05	12.48 ± 3.40	36.18 ± 2.60	185.2 ± 7.34	4.70 ± 1.64	2.42 ± 0.72
6th milking (average)	6.49 ± 0.10	46.50 ± 4.97	11.54 ± 4.30	34.96 ± 3.63	187.2 ± 7.74	5.14 ± 1.25	2.79 ± 0.77

2.3.2 Reversed phase-high performance liquid chromatography of protein

RP-HPLC chromatograms of the colostrum from the first six milkings from cows A to F and bulk mature milk samples 1 to 3 are shown in Figures 2.1 and 2.2. Samples were loaded on an equal protein basis. The proportion of each individual protein, as a percentage of total casein or whey protein, is shown in Table 2.2. Quantification of α_{S2}-casein was not possible in the current study, as complete elution of α_{S2}-casein could not be achieved (Thom Huppertz, personal communication).

It was found that, in early colostrum, the proportion of κ-casein as a percentage of total casein (excluding α_{S2}-casein) was high (Table 2.2). In colostrum, κ-casein accounted for between 18.7 and 21.6% of total casein in first milkings from cows A to F. In all cases, these values decreased with time *post partum,* but did not reach normal levels by the sixth milking. On average, κ-casein in mature milk accounted for 12.7% of total casein, which is significantly lower than was found for colostrum. κ-Casein is a phosphoglycoprotein, containing up to 15% (w/w) carbohydrate (Robitaille *et al.,* 1991). In milk, a significant proportion of κ-casein molecules are carbohydrate-free, with the remainder being glycosylated to varying degrees (Robitaille *et al.,* 1990). Variations in the degree of glycosylation of κ-casein were observed in the current study by comparison of RP-HPLC chromatograms. κ-Casein eluted within the 8 to 11 min region in the chromatograms (Figure 2.1 and 2.2). The first-eluting protein peaks represent a mixture of carbohydrate-rich κ-casein A and B. Following this, κ-casein elutes in order of decreasing degree of glycosylation. The genetic variants of carbohydrate-free κ-casein elute at different positions and can be distinguished from each other. Minor peaks at intermediate positions originate from partly glycosylated κ-casein A or B. In early colostrum, the proportion of glycosylated κ-casein as a percentage of total κ-casein was high and decreased with time *post partum*. The degree of glycosylation of κ-casein has been shown to affect the technological properties of milk in several ways. Cases *et al*. (2006) reported that removal of sialic acid groups from the carbohydrate moieties of κ-casein enhances the acid coagulability of milk by reducing the net negative charge of the

casein micelle. Glycosylation of κ-casein is inversely correlated with micelle size, which, during rennet coagulation of milk, can potentially increase the rate of firming and curd firmness, as reported by Robitaille *et al*. (1993).

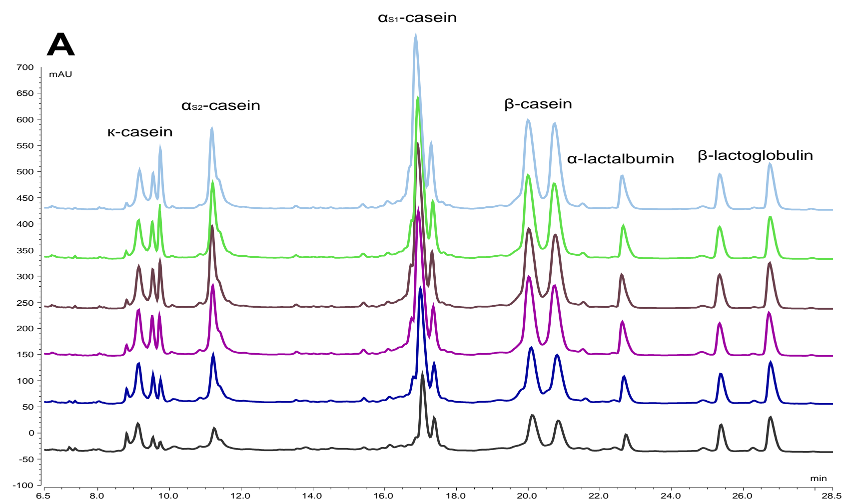
In general, the proportion of α_{S1}-casein as a percentage of total casein (excluding α_{S2}-casein), was low in early colostrum and increased with time *post partum* (Table 2.2). There are eight genetic variants of α_{S1}-casein, the two most common being B and C (Luhken *et al.,* 2009). The α_{S1}-casein B variant and to a lesser extent the C variant were predominant in samples analysed in the current study. No separation of the B/C variant (Glu – Gly at position 192) was achieved, as they gave rise to a double peak, due to a difference in the degree of post-translational phosphorylation.

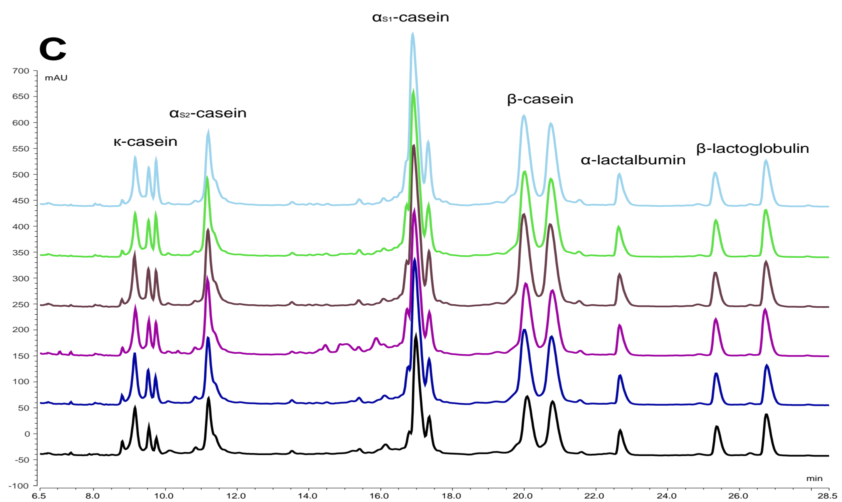
The proportion of β-casein, as a percentage of total casein, showed no particular trend over the first six milkings, although values were slightly lower than those obtained for mature milk samples (Table 2.2). There are 12 genetic variants of β-casein (Martin *et al.,* 2013) which occurs mainly as its A1, A2 and, to a lesser extent, its B variant (Farrell *et al.,* 2004). The A1 and A2 variants of β-casein differ at position 67, where proline in variant A2 is substituted by histidine in variant A1 (Kaminski *et al.,* 2007). In the current study, three cows (A, C and F) were found to contain variants A1 and A2, while cows B, D and E were found to contain the A2 variant only. Mature milk samples contained variants A1 and A2, but at a differing ratio to that in cows A, C and F.

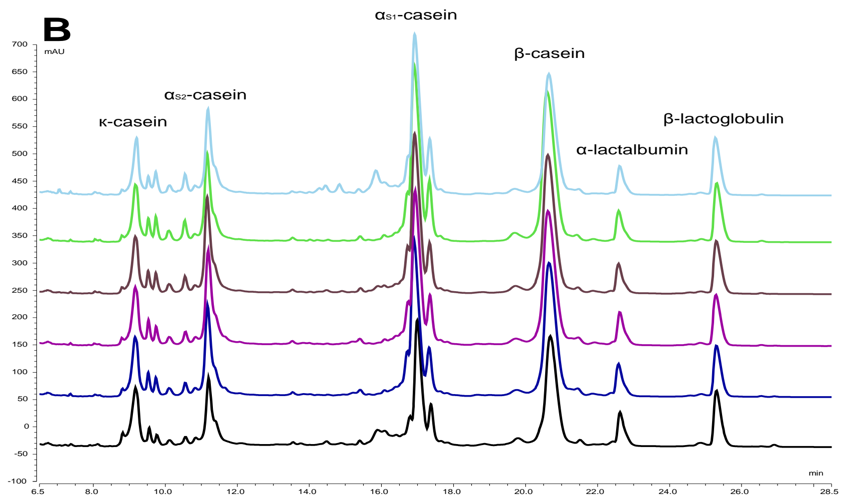
There are eleven known genetic variants of bovine β-Lg (Ganai *et al*., 2009). The most abundant are variants A and B (Farrell *et al.,* 2004), which differ by two amino acid substitutions: Asp-64-Gly and Val-118-Ala, respectively. Colostrum samples were found to contain differing levels of variants A and B. Four cows (A, C, E and F) contained both variants. One cow (B) contained the A variant only and one cow (D) contained the B variant only. Mature bulk milk samples contained both variants. β-Lg variants A and B are associated with different amounts of β-Lg in milk: milk containing variant A has a higher β-Lg content than variant B (Hill, 1993). This

correlation could not be investigated in colostrum samples as the protein concentration was highly variable.

The level of α-La, as a percentage of total β-Lg and α-La, was found to be very low in certain colostrum samples (D1, D2, D3, E1, F1), as shown in Table 2.2. This has important implications for the carbohydrate profile of colostrum samples, as shown in Table 2.4, as α-La is an important component in the lactose synthesis pathway. This will be discussed in Section 2.3.4.







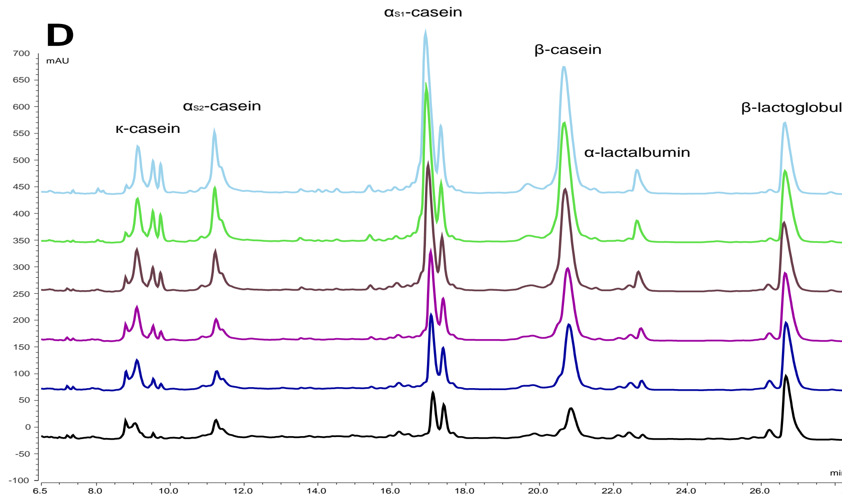
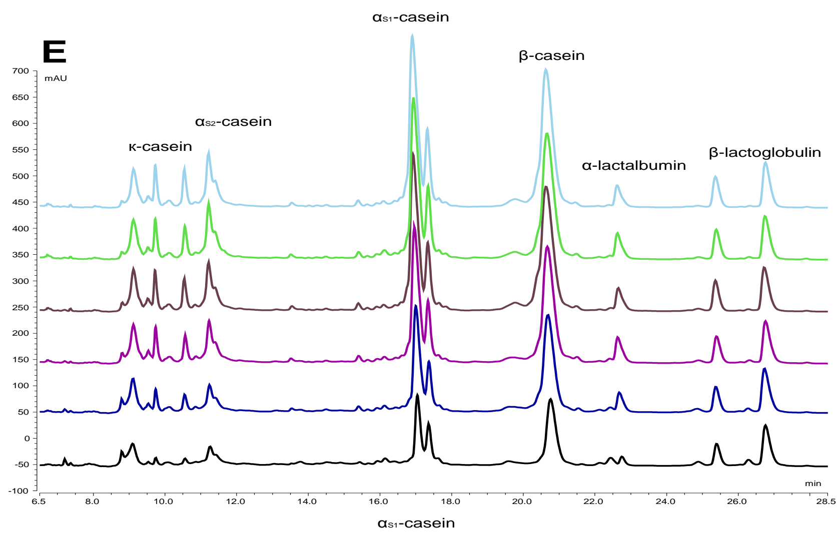
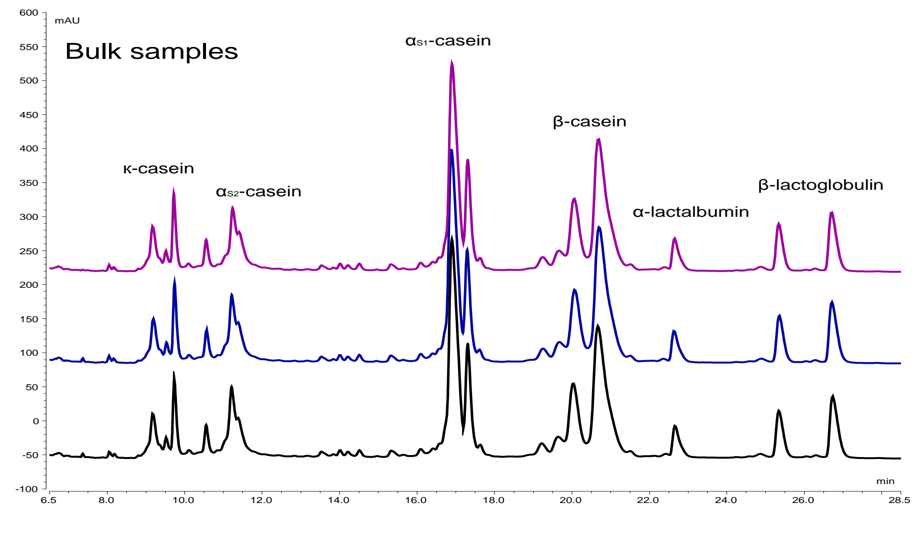


Figure 2.1 RP-HPLC chromatograms of skimmed samples of colostrum from the first (▬), second (▬), third (▬), fourth (▬), fifth (▬) and sixth (▬) milkings from cows A to D.





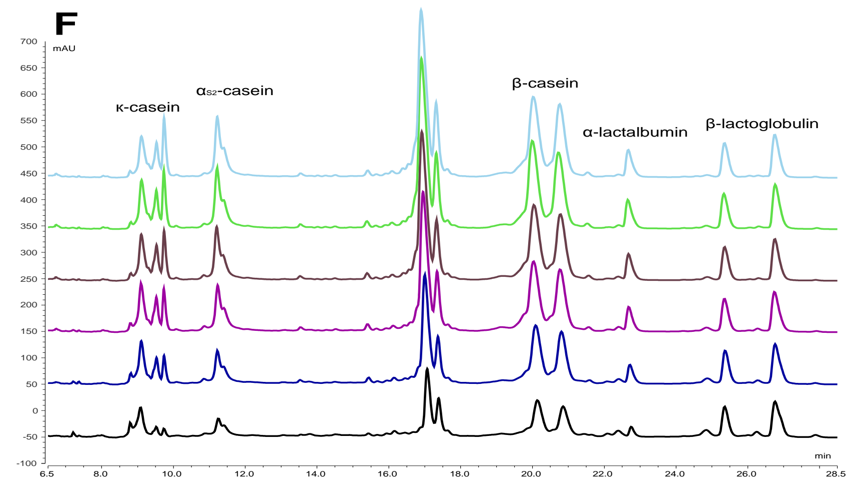


Figure 2.2 RP-HPLC chromatograms of skimmed samples of colostrum from the first (▬), second (▬), third (▬), fourth (▬), fifth (▬) and sixth (▬) milkings from cows E and F and bulk mature milk samples 1 to 3.

Table 2.2 Calculated levels of α_{S1}-, β-, and κ-casein as a % of total casein (excluding α_{s2}-casein) and levels of α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) as a % of total α-La and β-Lg for the first six milkings from cows A to F and bulk mature milk samples 1 to 3.

Sample/	% of total casein			% of total α-La and β-Lg	
Milking	α_{S1}-casein	β-casein	κ-casein	α-La	β-Lg
A1	39.1	39.3	21.6	18.3	81.7
A2	40.5	39.8	19.7	26.1	73.9
A3	41.7	40.7	17.6	31.7	68.3
A4	43.2	40.4	16.4	29.3	70.7
A5	43.2	40.8	16.0	30.0	70.0
A6	43.7	41.0	15.4	29.9	70.1
B1	41.6	39.6	18.8	35.9	64.1
B2	42.0	41.5	16.5	37.3	62.7
B3	41.5	42.0	16.5	37.3	62.7
B4	42.4	41.9	15.8	34.1	65.9
B5	42.7	42.9	14.4	33.0	67.0
B6	47.5	36.5	16.0	31.6	68.4
C1	39.3	41.4	19.3	24.1	75.9
C2	42.1	41.8	16.1	28.0	72.0
C3	44.6	39.2	16.2	25.0	75.0
C4	42.5	42.6	14.8	28.3	71.7
C5	44.2	41.6	14.2	24.7	75.3
C6	44.0	41.6	14.4	27.9	72.1
D1	41.7	37.5	20.8	3.7	96.3
D2	38.6	41.3	20.1	7.0	93.0
D3	39.3	41.3	19.4	10.0	90.0
D4	41.9	40.4	17.8	15.9	84.1
D5	42.5	41.1	16.4	17.2	82.8
D6	43.5	40.8	15.6	19.9	80.1
E1	39.3	42.0	18.7	9.8	90.2
E2	38.7	43.4	17.9	20.2	79.8
E3	40.9	42.3	16.9	26.8	73.2
E4	42.2	42.4	15.4	22.9	77.1
E5	44.7	40.5	14.8	26.1	73.9
E6	44.6	42.0	13.5	21.8	78.2
F1	38.2	42.0	19.8	9.8	90.2
F2	38.4	41.9	19.7	17.5	82.5
F3	40.5	40.7	18.8	22.5	77.5
F4	41.5	40.5	17.9	24.5	75.5
F5	43.3	39.7	17.0	25.4	74.6
F6	44.4	39.1	16.5	25.0	75.0
Bulk 1	42.6	44.6	12.8	21.6	78.4
Bulk 2	41.9	45.4	12.7	22.1	77.9
Bulk 3	41.9	45.1	12.9	22.4	77.6

2.3.3 Gel electrophoresis

SDS-PAGE electrophoretograms of the first six milkings from cows A to F and bulk mature milk samples 1 to 3 are shown in Figure 2.3. Samples were loaded on an equal protein basis under reducing conditions. In colostrum samples, the levels of α_{S1}-, α_{S2}-, β- and κ-caseins, as a percentage of total protein, were low initially but increased with time *post partum.* This was due to elevated levels of whey proteins, and not reduced levels of casein. By the sixth milking, the casein:whey protein ratio had reached normal levels of approximately 80:20; however, it should be noted that total protein concentration remained elevated at this point. The caseins are all in the approximate size range 20 – 25 kDa and so do not separate well by SDS-PAGE: For this reason, densitometric analysis was not carried out on the individual caseins, however, proportions of the individual caseins were studied by RP-HPLC, as described in Section 2.3.2. As already discussed, the major Ig present in colostrum is IgG, in particular IgG_1, but IgA and IgM are also found. According to Elfstrand *et al*. (2002), IgG_1 represents approximately 90% of total Ig found in bovine milk. IgG is a monomeric glycoprotein consisting of two heavy and two light polypeptide chains that are linked by disulfide bonds (Gapper *et al.,* 2007). Under reducing conditions, the disulfide bonds linking the heavy and light chains are broken. The heavy chains are approximately 50 kDa and are well resolved on the SDS gel in Figure 2.3. The light chains are approximately 25 kDa in mass and so are located in the same region as the caseins, which makes analysis difficult.

Quantitation of Lf, Ig, β-Lg and α-La was carried out by densitometric analysis of representative SDS profiles (Table 2.3). Briefly, the intensities of specific bands, corresponding to the proteins of interest, were measured and expressed as a percentage of total band intensity, which was then converted to mg/mL protein. The concentrations of LF, Ig and β-Lg were high initially, but decreased with time *post partum*.

On average, the concentration of IgG_1 (heavy chain) in mature milk was 0.64 mg/mL (Table 2.3). The concentration of IgG_1 (heavy chain) in first colostrum from cows A

to F ranged from 9.88 to 41.59 mg/mL, which is between 15 and 65 times that found in mature milk (Table 2.3). These values decreased with time *post partum*, but did not reach normal levels by the sixth milking. Similarly, elevated levels of lactoferrin were found in colostum. Lactoferrin is an 80 kDa iron-binding glycoprotein of the transferrin family which has anti-microbial, anti-inflammatory and anti-cancer activities (Lonnerdal and Suzuki, 2013). On average, the concentration of LF in mature milk was found to be 0.69 mg/mL (Table 2.3). The concentration of LF in first colostrum from cows A to F ranged from 3.43 to 14.29 mg/mL, which is between 5 and 21 times that found in mature milk. These values decreased with time *post partum,* but did not reach normal levels by the sixth milking (with the exception of cows B and C). The concentration of β-Lg was high initially, ranging from 6.9 to 29.7 mg/mL in first colostral milkings. These values decreased with time *post partum* and reached normal levels by the sixth milking (with the exception of cow D). In general, colostrum samples were found to contain elevated levels of α-La compared to mature milk, which contained, on average, 1.89 mg/mL. No particular trend for α-La concentration was observed in the first six milkings from cows A to F, but some initial milkings were found to contain the highest concentrations, in particular the first milkings from cows B and E (4.07 and 4.36 mg/mL. respectively). It is important to note that the values above were obtained by densitometric analysis and so are relative and not absolutely quantitative. These values should not be confused with those described in Section 2.3.2, whereby the level of α-la, as a percentage of total α-la and β-lg, was calculated, as quantitation by RP-HPLC was not possible. In some colostrum samples and all mature milk samples, a band was evident in the molecular mass range 12-13 kDa, migrating just faster than α-La; however, this band was not identified.

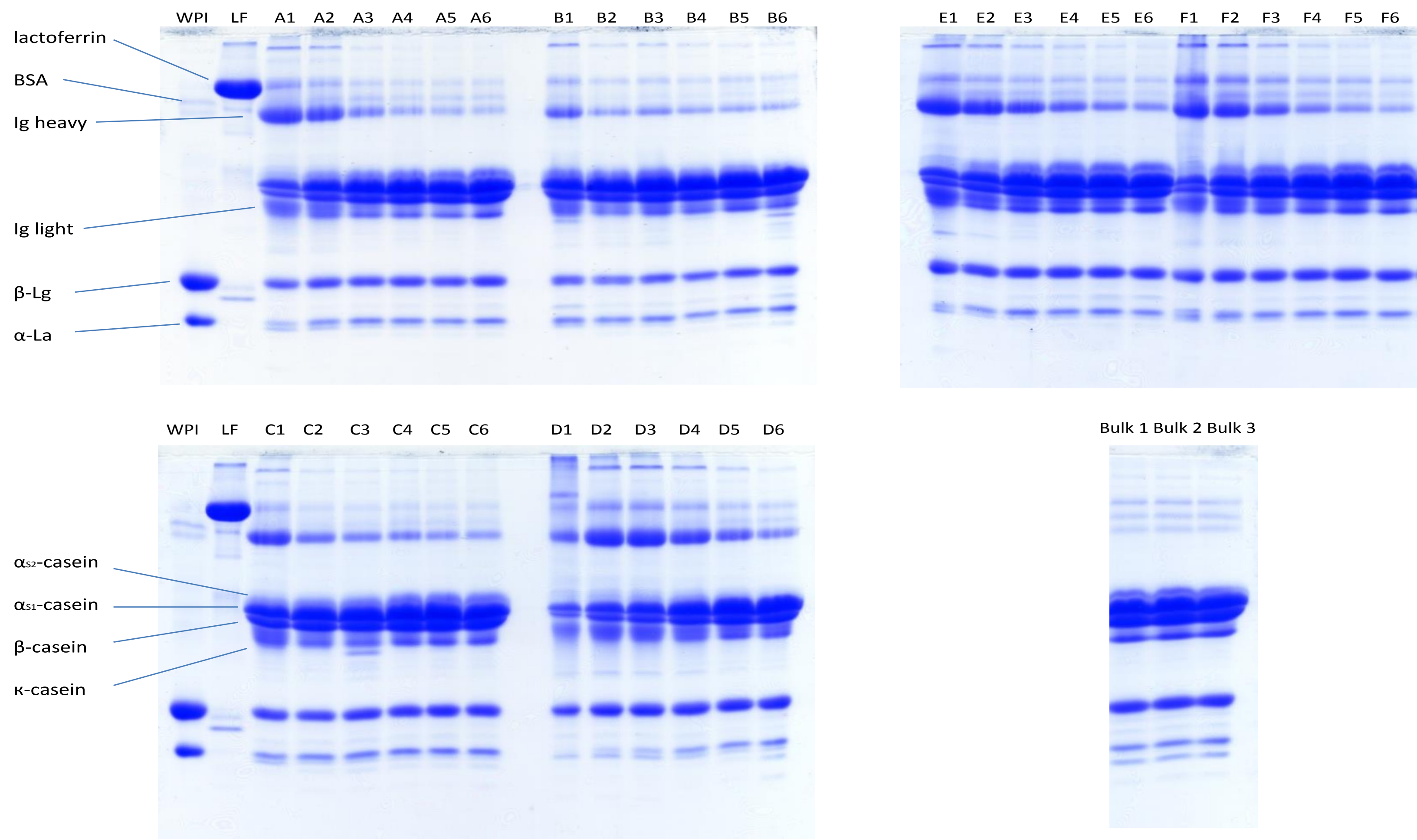


Figure 2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoretograms of colostrum from the first six milkings from cows A to F and bulk mature milk samples 1 to 3. Fifty μg protein was loaded for each sample. Also shown are whey protein isolate (WPI) and lactoferrin (Lf) standards.

Table 2.3 Concentration of lactoferrin (Lf), immunoglobulin G heavy chain (Ig), beta-lactoglobulin (β-Lg), and alpha-lactalbumin (α-La) in the first six milkings from cows A to F and bulk mature milk samples 1 to 3, as calculated using densitometric analysis (results shown are based on a single analysis). Total protein values are taken from Table 2.1.

Sample	total protein mg/ml	Lf mg/ml	Ig mg/ml	β-Lg mg/ml	α-La mg/ml
A1	115	7.99	27.54	10.98	2.26
A2	71	3.10	12.55	7.82	2.69
A3	50	1.32	4.59	6.14	2.97
A4	48	1.05	2.46	6.42	2.78
A5	46	1.03	2.13	5.99	2.55
A6	40	0.87	1.43	5.19	2.22
B1	78	3.43	9.88	6.89	4.07
B2	58	1.45	3.42	5.57	3.23
B3	57	1.44	3.60	5.68	3.30
B4	59	1.15	3.10	6.14	2.70
B5	51	1.12	2.22	6.12	2.70
B6	52	0.77	1.94	5.82	2.49
C1	66	3.47	11.81	7.16	2.12
C2	53	0.94	5.61	6.70	2.72
C3	51	0.74	3.84	7.02	2.91
C4	48	0.89	3.50	6.61	2.82
C5	46	0.73	2.64	6.64	2.66
C6	44	0.68	2.29	6.02	2.40
D1	249	13.45	32.26	29.69	1.73
D2	150	10.21	32.79	19.17	1.36
D3	107	6.48	22.03	14.15	1.05
D4	71	3.35	11.91	10.19	2.18
D5	58	1.59	6.84	8.72	2.22
D6	52	1.02	4.21	7.95	2.02
E1	185	12.73	41.59	19.17	4.36
E2	81	4.26	15.22	9.15	2.49
E3	60	2.46	7.79	7.15	3.18
E4	54	1.95	5.17	6.69	2.56
E5	46	1.48	3.44	5.88	2.60
E6	48	1.42	2.99	6.03	2.05
F1	173	14.29	30.06	16.53	3.86
F2	84	5.32	12.32	9.25	3.59
F3	60	2.82	6.86	6.87	2.75
F4	52	1.86	4.04	6.55	2.55
F5	45	1.58	2.51	6.03	2.35
F6	43	1.17	1.83	6.02	2.29
Bulk 1	39	0.77	0.69	6.21	1.90
Bulk 2	38	0.67	0.64	6.01	1.85
Bulk 3	40	0.64	0.58	6.07	1.93

2.3.4 Carbohydrate analysis

Average levels of lactose, glucose and galactose for the first six milkings from cows A to F and bulk mature milk samples 1 to 3 are shown in Table 2.4. Lactose is a reducing disaccharide, composed of galactose and glucose linked by a β (1-4) glycosidic bond, the concentration of which in mature bovine milk is approximately 4.8% (Fox, 2009). Several factors influence the lactose content of milk, i.e., breed of cow, individuality factors, mastitis and, in particular, stage of lactation (Fox and McSweeney, 1998). Lactose concentration is reduced in colostrum and is inversely correlated with other constituents such as solids, protein, and ash (Kehoe *et al.,* 2007). In the current study, the level of lactose was found to be low initially and increased with time *post partum* (Table 2.4). Several authors reported a similar trend (Parrish *et al.,* 1950; Klimes *et al*., 1986; Kehoe *et al*., 2007; Tsioulpas *et al*., 2007a). In the current study, the concentration of lactose reached normal levels by the sixth milking *post partum*. This is in agreement with Klimes at al. (1986), but Tsiuolpas *et al*. (2007a) reported that the lactose concentration in colostrum and milk did not reach normal levels until 60 d *post partum*. Lactose is important in maintaining osmotic pressure in the mammary system and thus any increase or decrease in lactose is correlated with an increase or decrease in soluble salts (Holt, 1985). This is consistent with the current study, i.e., colostrum samples with a low level of lactose contained an elevated level of certain minerals (Table 2.9). In the mammary gland, α-La forms a complex with the enzyme galactotransferase to form lactose synthase, which is responsible for lactose production (Neville, 2009). Decreasing levels of α-La during lactation have been correlated with decreasing levels of lactose (Larson, 1985). A positive correlation between the concentration of lactose and the relative proportion of α-La (as a percentage of total β-Lg and α-La) was found in the current study (Table 2.4).

Milk contains several carbohydrates in addition to lactose. In the current study, large variations in the levels of free glucose and galactose were found. In general, no apparent trends were observed for glucose concentration in colostrum;

however, values were significantly lower than those found for mature milk (Table 2.4). On average, mature milk samples contained 225.8 mg/L glucose (Table 2.4); Walstra (1999) and Cataldi *et al*. (2003) reported that mature bovine milk contains 18.0 and 25.2 mg/L glucose, respectively, which is much lower than was found in the current study. The glucose content of colostrum samples was also elevated, compared to levels in mature milk, as reported by Walstra (1999) and Cataldi *et al*. (2003); however, they were not as high as those in mature milk samples. Galactose levels were low compared to mature milk samples, with the exception of the first milking of cows D and F. The galactose content of colostrum samples was high initially and decreased with time *post partum*. On average, the mature milk contained 133.7 mg/L galactose (Table 2.4). Walstra (1999) and Cataldi *et al*. (2003) reported that mature bovine milk contains 36.0 and 40.4 mg/L galactose, respectively, which, as for glucose, was a significantly lower level than was found in the current study.

Table 2.4 Concentrations of lactose, glucose and galactose for the first six milkings from cows A to F and bulk mature milk samples 1 to 3, as determined by HPLC (results shown are based on a single analysis). α-La (as a % of total α-la and β-lg) values are taken from Table 2.2.

Sample	Lactose [g/L]	Glucose [mg/L]	Galactose [mg/L]	α-La (% of total α-La & β-Lg)
A1	29.8	49.7	116.1	18.28
A2	39.1	33.5	56.3	26.10
A3	42.4	34.2	36.5	31.71
A4	45.5	30.1	25.5	29.25
A5	45.3	34.3	27.9	30.03
A6	45.4	45.0	30.4	29.89
B1	35.8	62.1	119.7	35.89
B2	40.8	39.3	52.4	37.26
B3	44.2	51.3	55.6	37.29
B4	44.5	71.8	48.4	34.11
B5	47.9	73.9	39.9	32.98
B6	45.9	71.9	38.6	31.61
C1	21.9	16.1	24.3	24.10
C2	43.7	28.8	28.8	27.98
C3	44.7	33.5	28.3	24.99
C4	45.0	34.7	32.4	28.27
C5	45.6	35.1	22.1	24.69
C6	46.8	60.5	52.9	27.90
D1	8.1	19.1	248.4	3.74
D2	28.0	21.7	91.9	7.03
D3	34.3	29.3	86.7	9.98
D4	39.9	29.1	54.9	15.94
D5	40.8	23.3	35.1	17.22
D6	45.1	28.4	31.2	19.95
E1	22.7	61.9	136.4	9.76
E2	38.6	23.8	54.6	20.23
E3	40.9	19.0	42.0	26.82
E4	41.7	16.9	27.2	22.89
E5	45.4	8.4	23.0	26.15
E6	44.4	24.4	27.1	21.78
F1	23.1	45.6	155.0	9.78
F2	37.6	18.9	52.2	17.53
F3	43.3	21.0	44.6	22.49
F4	47.0	22.9	46.2	24.46
F5	48.1	51.7	45.9	25.41
F6	48.5	64.7	40.4	24.99
Bulk 1	48.4	226.6	132.8	21.58
Bulk 2	48.8	229.9	135.2	22.11
Bulk 3	48.6	221.0	133.0	22.45

Lactose is the predominant carbohydrate in mature bovine milk, but colostrum contains less lactose and much higher concentrations of oligosaccharides than mature milk (Nakamura *et al.,* 2003; Urashima *et al.,* 2009). The oligosaccharides in milk can be divided into two broad classes, neutral and acidic. Nearly 40 oligosaccharides are present in bovine milk, of which 70% are sialylated (Tao *et al.,* 2008). The concentration of oligosaccharides in bovine colostrum is approximately 0.7 to 1.2 g/L (Veh *et al.,* 1981), whereas mature milk contains only trace amounts (Gopal and Gill, 2000). In the current study, the level of oligosaccharides in colostrum varied with time *post partum* (Figure 2.4). Early colostral milkings contained significantly higher levels of oligosaccharides than later milkings and mature milk samples. This is in agreement with the studies of Gopal and Gill (2000), Nakamura *et al*. (2003) and McJarrow and Van Amelsfort-Schoonbeek (2004).

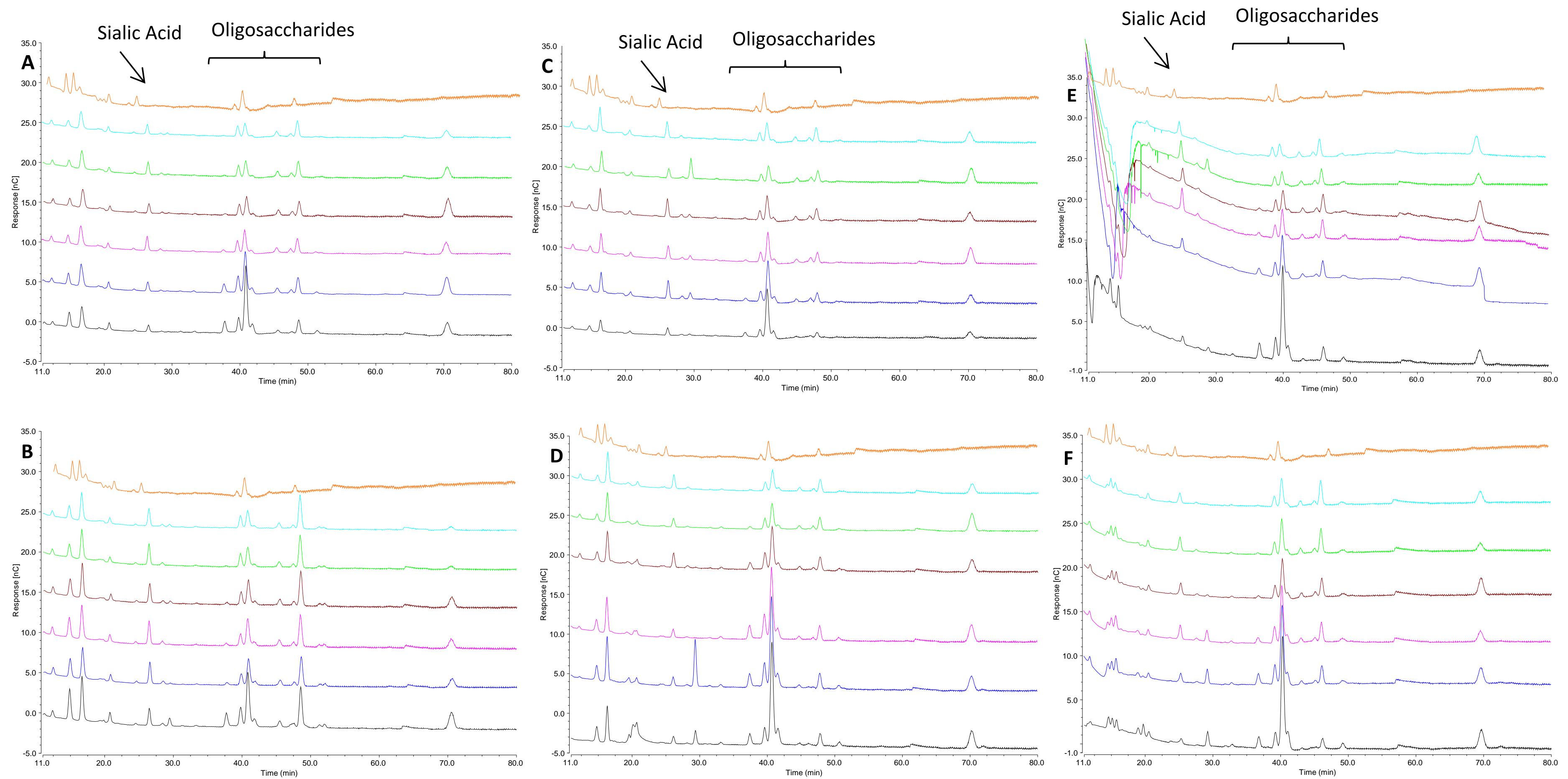


Figure 2.4 RP-HPLC chromatograms of the change in peak area of sialic acid and oligosaccharides in the first (black), second (blue), third (magenta), fourth (brown), fifth (green) and sixth (cyan) milkings from cows A to F. Also shown in each chromatogram is a representative bulk mature milk sample (orange).

2.3.5 Colour

Table 2.5 presents L*a*b* values for the first six *post partum* milkings from cows A to F and bulk mature milk samples 1 to 3. The colour of milk is typically dominated by the presence of casein micelles and fat globules, which scatter light and are the reason for the white/opaque appearance of milk (Fox and McSweeney, 1998). Colostrum has a reddish-yellow colour (Edelsten, 1988). The yellow colour is due to the presence of carotenoid pigments (Edelsten, 1988), which are not synthesised by the animal but are obtained from feed sources. It has been reported that levels of carotenoid pigments are high in the initial colostrum, in particular the fat fraction, but decrease rapidly as the mammary secretions change to normal milk (Parrish *et al.,* 1948). The red colour is due to the presence of red blood cells. During the *pre partum* period there is increased permeability of the mammary gland membranes, and thus more blood constituents gain access to the milk. Linzell and Peaker (1975) observed that as little as 0.1 mL blood/L of milk can be detected visually and that 0.4 mL/L turns milk very pink.

As can be seen from Table 2.5, large variations in L*a*b* values were found. In general, colostrum samples were darker (lightness, L*, was low initially and increased over the six milkings), more red (redness, a*, high initially and decreased over the six milkings) and more yellow (yellowness, b*, was high throughout the first six milkings), compared to mature milk samples. Madsen *et al*. (2004) analysed the colour of colostrum and mature milk over 14 d. Results of the two studies are not comparable, as Madsen *et al*. (2004) used a black capsule when measuring colour, which greatly affected L* values, but the trends in L*a*b* values over the first 6 milkings were consistent with those of the current study: L* values were low initially but increased over time, while a* and b* values decreased over time. The initial colostrum from cow E, and in particular cow F, had a very low L* value (71.03 and 53.24 for the first milking from cow E and F, respectively) and high a* value (-1.75 and 14.72 for the first milking from cows E and F, respectively). This suggests that colostrum contained relatively high levels of red blood cells. The first milkings

from cows A, D and, in particular cow E, were very yellow, which is reflected by very high b* values (35.95, 37.22, 31.91, respectively). This suggests that these colostrum samples contained high levels of carotenoid pigments.

Table 2.5 Mean values (± S.D., n=3) of colour (lightness-blackness, L; greenness-redness, a; and blueness-yellowness, b) for the first six milkings from cows A to F and bulk mature milk samples 1 to 3. Values without an asterisk (*) were significantly different ($p < 0.05$) from control bulk milk samples.

Sample	L*		a*		b*	
A1	75.49	± 0.03	-3.45 *	± 0.01	35.95	± 0.09
A2	78.28 *	± 0.05	-1.57	± 0.04	35.95	± 0.01
A3	80.44	± 0.03	-3.85	± 0.02	18.29	± 0.04
A4	81.67 *	± 0.29	-3.37 *	± 0.03	18.94	± 0.01
A5	81.75 *	± 0.07	-3.90	± 0.01	10.70 *	± 0.13
A6	81.75 *	± 0.11	-3.83	± 0.01	9.96	± 0.01
B1	79.94	± 0.07	-4.30	± 0.01	18.79	± 0.03
B2	80.79	± 0.08	-4.90	± 0.03	17.56	± 0.17
B3	80.86	± 0.02	-4.90	± 0.01	17.43	± 0.04
B4	81.22	± 0.03	-3.30	± 0.03	25.60	± 0.01
B5	80.68	± 0.01	-4.38	± 0.02	16.21	± 0.05
B6	81.82 *	± 0.04	-3.60	± 0.02	19.95	± 0.03
C1	79.28	± 0.15	-3.83	± 0.02	21.01	± 0.15
C2	80.92	± 0.09	-3.69	± 0.02	19.31	± 0.12
C3	81.56 *	± 0.47	-3.38 *	± 0.02	13.39	± 0.02
C4	81.47 *	± 0.05	-3.28	± 0.01	20.66	± 0.06
C5	81.84 *	± 0.02	-3.33	± 0.01	12.69	± 0.01
C6	82.01 *	± 0.00	-3.44 *	± 0.00	10.59 *	± 0.01
D1	73.43	± 0.03	-1.02	± 0.04	37.22	± 0.08
D2	76.25	± 0.09	-3.25	± 0.03	27.19	± 0.10
D3	79.63	± 0.01	-3.03	± 0.06	26.45	± 0.03
D4	79.99	± 0.11	-3.47 *	± 0.04	23.00	± 0.29
D5	80.49	± 0.09	-3.43 *	± 0.04	19.00	± 0.07
D6	81.15	± 0.02	-3.84	± 0.02	14.74	± 0.03
E1	71.03	± 0.01	-1.75	± 0.03	31.91	± 0.06
E2	73.54	± 0.09	3.80	± 0.01	18.69	± 0.11
E3	77.43	± 0.21	0.27	± 0.03	21.02	± 0.10
E4	79.50	± 0.02	-2.46	± 0.07	19.83	± 0.02
E5	80.21	± 0.03	-3.46 *	± 0.03	15.04	± 0.09
E6	81.15	± 0.32	-3.26	± 0.03	13.78	± 0.08
F1	53.24	± 0.04	14.72	± 0.01	18.67	± 0.03
F2	64.26	± 0.05	6.32	± 0.04	14.21	± 0.03
F3	71.92	± 0.16	0.70	± 0.02	14.39	± 0.03
F4	76.51	± 0.06	0.95	± 0.02	16.93	± 0.03
F5	79.44	± 0.06	-2.44	± 0.01	13.96	± 0.03
F6	74.90	± 0.02	0.04	± 0.03	15.94	± 0.02
Bulk 1	81.84	± 0.01	-2.91	± 0.01	8.82	± 0.01
Bulk 2	77.89	± 0.08	-3.14	± 0.02	10.26	± 0.00
Bulk 3	82.31	± 0.10	-3.42	± 0.01	10.60	± 0.04

2.3.6 Ultracentrifugal sedimentation

Casein exists in milk as large colloidal micelles, most (90-95%) of which may be sedimented by centrifugation at 100,000 g for 1 h (O'Mahony and Fox, 2013). Mean values for non-sedimentable protein after ultracentrifugation (100, 000 g x 1 h) of the first six milkings form cows A to F and bulk mature milk samples 1 to 3 are shown in Table 2.6. The concentration of sedimentable and non-sedimentable protein has been discussed in Section 2.3.1. Briefly, the level of sedimentable, and, in particular, non-sedimentable protein, were very high initially and decreased with time *post partum,* but in general did not reach normal levels by the sixth milking. The non-sedimentable material was analysed by RP-HPLC (Section 2.3.6) and the proportions of individual caseins (κ-, α_{S1}- and β-caseins) in ultracentrigugal supernatants, as a percentage of total casein (excluding α_{S2}-casein), was calculated (Table 2.7). It is clear from Table 2.7 that a significant proportion of the casein, in particular in early milkings, was non-sedimentable. The range of non-sedimentable casein, as a percentage of total casein, for the first milkings of cows A to F at 20°C, was 11.3 to 86.8%. These values decreased with time *post partum* and in all cases reached normal levels by the sixth milking. The most likely explanation for the poor sedimentability of the casein micelles in colostrum was the high viscosity of certain samples (Table 2.1). The relationship between sedimentation of particles and viscosity is described by the equation:

$$V = (2r^2 [\rho^1-\rho^2]/9\eta) \times \omega^2 r$$

where V is sedimentation velocity (svedberg), r is the radius of the particle (m), ρ^1 is the density of the continuous phase (kg/m^3), ρ^2 is the density of the dispersed phase (kg/m^3), η is the viscosity (Pa s) and $\omega^2 r$ is centrifugal acceleration. A correlation existed between the high viscosity of some early milkings (Table 2.1) and poor sedimentability of casein micelles in colostrum (Table 2.6 and 2.7). However, this was not true in all cases, e.g., the first milking from cow C had a viscosity of 2.5 mPa s (Table 2.1) and 11.3% of casein did not sediment at 20°C when centrifuged at 100,000 g x 1 h (Table 2.7) whereas, for bulk mature milk

sample 1, which had a higher viscosity than the first milking from cow C (4.8 mPa s), only 3.42% of casein did not sediment at 20°C when centrifuged at 100, 000 g x 1 h.

Of the individual caseins which did not sediment at 100,000 g, κ-casein was present in the highest proportion, relative to the initial concentration of each individual casein. The stability of casein micelles in an aqueous system is due primarily to steric and electrostatic interactions, caused by the hairs of κ-casein (Walstra, 1990); De Kruif (1999) described κ-casein as a polyelectrolyte brush which sterically stabilises the casein micelles and maintains solvency. Another possible explanation for the poor sedimentability of casein micelles in certain colostrum samples is the high level of glycosylated κ-casein, which may lead to enhanced steric and electrostatic interactions, thereby increasing solvency. Also, salts in the serum phase of milk have the ability to increase micellar solubility by stabilisation of the hydrophilic end of κ-casein through ionic bonding (Roach and Harte, 2008). This may also be a contributory factor to the poor sedimentability of casein micelles, as colostrum samples contained high salt levels (Table 2.9). The same trend was observed for non-sedimentable protein at 4°C, but significantly more casein was non-sedimentable at 4°C than at 20°C. This is because, at low temperatures, casein dissociates from the micelles, and β-casein is the predominant protein released (Post *et al.,* 2012; Liu *et al.,* 2013). Several authors have reported that the solubility of β-casein increases as the temperature is reduced (Bingham, 1971; Post *et al.,* 2012). The range of non-sedimentable casein, as a percentage of total casein, for the first milkings of cows A to F at 4°C, was 24.9 to 99.3%. Once again, these values decreased with time *post partum* and in all cases reached normal levels by the sixth milking (Table 2.7). The average level of non-sedimentable casein, as a percentage of total casein, in bulk mature milk samples at 4°C was 19.8%, compared to 4.4% at 20°C.

Table 2.6 Mean levels (± S.D., n=3) of protein not sedimented by ultracentrifugation at 100,000 g x 1h at 4°C or 20°C for the first six milkings from cows A to F and bulk mature milk samples 1 to 3. Values without an asterisk (*) were significantly different ($p < 0.05$) from control bulk milk samples.

Sample	4°C		20°C	
	non-sedimentable protein (g/L)	soluble protein (as a % of total protein)	non-sedimentable protein (g/L)	soluble protein (as a % of total protein)
A1	77.46 ± 0.87	67.35 ± 0.75	70.21 ± 0.71	61.05 ± 0.62
A2	41.58 ± 0.30	58.57 ± 0.42	34.39 ± 0.30	48.44 ± 0.42
A3	19.40 ± 0.11	38.80 ± 0.23	16.86 ± 0.17	33.72 ± 0.35
A4	17.29 ± 0.05	36.02 ± 0.11	13.55 ± 0.45	28.24 ± 0.93
A5	13.89 ± 0.07	30.20 * ± 0.15	11.29 ± 0.18	24.54 * ± 0.40
A6	11.59 * ± 0.05	28.97 * ± 0.12	8.17 * ± 0.12	20.43 * ± 0.31
B1	35.37 ± 0.65	45.35 ± 0.83	28.34 ± 0.14	36.34 ± 0.17
B2	18.93 ± 0.27	32.64 * ± 0.47	13.12 ± 0.03	22.62 * ± 0.05
B3	18.97 ± 0.23	33.29 * ± 0.40	13.58 ± 0.12	23.82 * ± 0.22
B4	20.12 ± 0.14	34.11 * ± 0.23	13.25 ± 0.01	22.46 * ± 0.01
B5	16.09 ± 0.58	31.54 ± 1.15	11.19 ± 0.20	21.94 * ± 0.40
B6	16.04 ± 0.27	30.85 ± 0.52	10.99 ± 0.09	21.13 * ± 0.18
C1	31.27 ± 0.29	47.38 ± 0.44	27.18 ± 0.17	41.17 ± 0.25
C2	19.59 ± 0.44	36.97 ± 0.84	16.34 ± 0.39	30.83 ± 0.73
C3	19.72 ± 0.08	38.67 ± 0.15	16.59 ± 0.09	32.53 ± 0.18
C4	15.78 ± 0.16	32.88 * ± 0.34	12.67 ± 0.06	26.40 ± 0.12
C5	17.05 ± 0.35	37.06 ± 0.76	12.79 ± 0.15	27.80 ± 0.33
C6	14.69 ± 0.33	33.38 ± 0.75	10.98 ± 0.15	24.94 ± 0.34
D1	180.26 ± 1.15	72.39 ± 0.46	171.34 ± 0.71	68.81 ± 1.06
D2	106.25 ± 0.15	70.83 ± 0.10	96.63 ± 0.09	64.42 ± 0.06
D3	67.88 ± 0.34	63.44 ± 0.32	58.07 ± 0.01	54.27 ± 0.01
D4	35.36 ± 0.52	49.80 ± 0.74	30.06 ± 0.03	42.34 ± 0.04
D5	22.94 ± 0.19	39.56 ± 0.33	19.07 ± 0.19	32.88 ± 0.33
D6	26.53 ± 0.94	51.02 ± 1.81	19.98 ± 1.41	38.43 ± 1.56
E1	132.51 ± 0.64	71.63 ± 0.35	130.62 ± 0.59	70.61 ± 0.32
E2	44.19 ± 0.48	54.56 ± 0.59	38.21 ± 0.21	47.17 ± 0.26
E3	26.06 ± 0.73	43.43 ± 1.22	19.69 ± 2.33	32.82 ± 0.39
E4	21.46 ± 0.03	39.74 ± 0.05	15.82 ± 0.01	29.30 ± 0.03
E5	14.66 ± 0.13	31.86 * ± 0.27	11.20 ± 0.19	24.34 ± 0.40
E6	16.17 ± 0.05	33.69 * ± 0.11	10.45 ± 0.10	21.76 * ± 0.21
F1	124.19 ± 0.71	71.79 ± 0.41	109.76 ± 0.49	63.44 ± 0.28
F2	43.20 ± 0.03	51.43 ± 0.04	37.30 ± 0.39	44.41 ± 0.46
F3	24.52 ± 0.39	40.86 ± 0.65	21.57 ± 0.28	35.96 ± 0.46
F4	16.67 ± 0.00	32.06 * ± 0.00	13.17 ± 0.10	25.34 ± 0.19
F5	12.90 * ± 0.43	28.66 * ± 0.95	9.38 * ± 0.23	20.84 * ± 0.51
F6	14.02 ± 0.00	32.60 * ± 0.01	8.70 * ± 0.01	20.23 ± 0.10
Bulk 1	10.30 ± 0.01	26.41 ± 0.02	8.26 ± 0.07	21.18 ± 0.13
Bulk 2	12.73 ± 0.06	33.50 ± 0.15	7.87 ± 0.14	20.71 ± 0.03
Bulk 3	11.33 ± 0.20	28.33 ± 0.49	9.23 ± 0.00	23.06 ± 0.42

Table 2.7 Percent of total casein in skimmed colostrum which was non-sedimentable on ultracentrifugation at 100, 000 g x 1 h at 4°C or 20°C for the first six milkings from cows A to F and bulk mature milk samples 1 to 3. Also shown is the proportion of individual caseins (κ-, α_{S1}- and β-caseins) present in ultracentrifugal supernatants. Values were calculated based on peak areas from RP-HPLC chromatograms (not shown).

Sample	Ultracentrifugal supernatant (4°C)				Ultracentrifugal supernatant (20°C)			
	non-sedimentable casein as a % of total casein	α_{S1}-casein	β-casein	κ-casein	non-sedimentable casein as a % of total casein	α_{S1}-casein	β-casein	κ-casein
		as a % of non-sedimentable casein				as a % of non-sedimentable casein		
A1	45.73	32.92	34.49	32.59	36.44	30.38	33.45	36.17
A2	48.50	33.63	39.90	26.47	26.59	28.13	37.70	34.17
A3	27.00	30.84	41.30	27.87	14.28	25.64	38.04	36.32
A4	26.65	31.65	45.03	23.32	10.93	24.96	41.78	33.27
A5	19.84	32.44	44.24	23.32	6.19	18.85	45.10	36.05
A6	13.33	29.92	48.11	21.96	0.89	10.81	34.36	54.83
B1	45.92	37.91	32.56	29.52	20.63	34.88	24.78	40.34
B2	23.84	31.68	39.21	29.12	7.67	22.60	34.77	42.63
B3	25.33	31.51	40.84	27.65	8.87	22.75	35.53	41.72
B4	31.12	34.26	42.81	22.94	8.94	25.06	37.75	37.20
B5	21.31	31.48	46.75	21.78	5.26	22.07	43.17	34.77
B6	19.14	42.32	33.75	23.93	4.01	55.87	13.94	30.18
C1	24.85	32.82	33.71	33.47	11.31	26.32	33.41	40.27
C2	23.62	28.10	44.14	27.76	7.11	24.12	27.82	48.06
C3	25.13	32.44	39.71	27.85	4.97	32.88	15.01	52.11
C4	18.69	28.24	47.72	24.05	6.21	21.46	44.21	34.33
C5	25.79	31.94	47.34	20.72	7.13	27.94	36.01	36.06
C6	20.11	32.22	45.09	22.69	5.29	21.85	36.99	41.16
D1	86.44	39.58	36.70	23.72	86.79	42.37	33.82	23.82
D2	67.27	35.01	39.15	25.85	46.68	36.23	39.33	24.44
D3	55.82	32.99	39.81	27.19	25.44	29.85	34.09	36.07
D4	29.67	32.56	39.15	28.30	11.33	31.36	30.24	38.40
D5	28.17	30.13	45.14	24.72	9.36	26.59	35.71	37.70
D6	15.55	28.68	43.73	27.59	3.64	32.51	22.60	44.90
E1	99.30	37.97	37.17	24.86	62.88	38.86	36.00	25.13
E2	47.24	31.46	40.69	27.85	15.40	29.76	33.72	36.52
E3	28.54	30.90	38.62	30.48	9.18	27.57	26.50	45.93
E4	31.81	30.32	43.70	25.98	7.09	27.54	30.00	42.46
E5	16.11	33.98	36.82	29.20	3.88	29.02	21.38	49.59
E6	25.86	30.17	47.03	22.80	1.87	18.53	14.93	66.54
F1	80.30	34.71	39.71	25.58	40.02	35.06	35.67	29.27
F2	36.46	29.36	39.79	30.84	16.27	28.98	34.18	36.84
F3	26.06	29.87	40.56	29.57	13.34	31.84	31.31	36.86
F4	19.45	26.30	47.88	25.82	3.39	26.62	20.02	53.36
F5	11.86	28.20	44.59	27.21	0.74	0.00	0.00	100.00
F6	23.57	34.01	43.18	22.81	0.86	0.00	12.71	87.29
Bulk 1	16.34	24.91	50.84	24.25	3.42	24.31	27.08	48.62
Bulk 2	24.36	30.18	48.94	20.88	2.93	21.44	30.11	48.45
Bulk 3	18.75	25.86	49.54	24.60	6.78	29.55	35.58	34.87

2.3.7 Heat-induced coagulation

The heat stability of milk refers to the ability of milk to withstand high processing temperatures without visual signs of coagulation or gelation (Singh, 2004). Mature milk is a very heat-stable system, which allows it to be subjected to severe heat treatments with relatively minor changes (Fox, 1982). The heat coagulation time (HCT) of mature milk is affected by a number of factors, pH being the most important (Singh, 2004). Generally, in mature milk, HCT increases with increasing pH from 6.4 to 6.7 but decreases sharply below pH 6.4 (Fox and McSweeney, 1998). The proteins in milk are probably the constituents most affected by heating. This is especially true for colostrum, due to its elevated whey protein content. The whey proteins, which in colostrum represent up to 70% of total protein (Table 2.1), are globular proteins with high levels of secondary and tertiary structures, and are therefore susceptible to denaturation by heating (Fox and McSweeney, 1998).

Table 2.8 presents the heat coagulation times of the first six milkings from cows A to F and bulk mature milk samples 1 to 3, measured at the natural pH of each sample, at temperatures ranging from 70 to 140°C. It was found that mature milk samples did not coagulate in 15 min, even when heated to 140°C, while early colostrum samples were the most susceptible to heat-induced coagulation. This is most likely due to a combination of early colostrum samples containing the highest concentrations of whey proteins (Table 2.1) and also having the lowest pH (Table 2.1). Oldfield *et al*. (2005) observed a similar trend where the rate of whey protein denaturation and aggregation in mature milk increased with increasing whey protein concentration. In all cases, there was an increase in the heat coagulation time of colostrum with time *post partum*. This was due to decreasing levels of whey proteins and increasing pH with time *post partum*. The first milking from cows A to F coagulated at 70°C. Large variations in HCT between individual cows were observed, for reasons discussed above. For cow D, colostrum from the first four milkings coagulated at 70°C. In contrast to this, the second to fifth milkings from cow B required heating to 140°C for coagulation. It is clear from the current study

Table 2.8 Heat stability of samples from the first six milkings from cows A to F and bulk mature samples 1 to 3 measured at natural pH values

	Heat Coagulation Time (mins)					
Sample	*70 °C*	*80 °C*	*90 °C*	*100 °C*	*120 °C*	*140 °C*
A1	3.98					
A2	6.16					
A3	x	x	9.35			
A4	x	x	x	x	x	5.13
A5	x	x	x	x	x	12.22
A6	x	x	x	x	x	12.78
B1	8.37					
B2	x	x	x	x	x	9.50
B3	x	x	x	x	x	10.07
B4	x	x	x	x	x	8.85
B5	x	x	x	x	x	14.62
B6	x	x	x	x	x	14.98
C1	11.32					
C2	x	x	4.95			
C3	x	x	10.25			
C4	x	x	x	x	x	7.50
C5	x	x	x	x	x	4.50
C6	x	x	x	x	x	11.15
D1	2.52					
D2	4.05					
D3	3.27					
D4	5.66					
D5	x	12.40				
D6	x	x	x	x	x	5.63
E1	3.58					
E2	3.62					
E3	x	2.25				
E4	x	4.48				
E5	x	x	x	x	10.22	
E6	x	x	x	x	3.08	
F1	2.12					
F2	3.17					
F3	6.17					
F4	x	5.73				
F5	x	x	x	11.10		
F6	x	x	x	x	6.66	
Bulk 1	x	x	x	x	x	x
Bulk 2	x	x	x	x	x	x
Bulk 3	x	x	x	x	x	x

x indicates samples did not coagulate in 15 min

that, even at the sixth milking, colostrum samples are relatively heat-labile compared to mature milk.

2.3.8 Minerals

Average values for the mineral composition of colostrum from the first six milkings of cows A to F and bulk mature milk samples 1 to 3 are shown in Table 2.9. The salts in milk exist in a dynamic equilibrium between the soluble and colloidal phases, and play an important role in the stability of the proteins. The mineral content has a profound effect on the technological properties of milk, as it affects its heat stability and susceptibility to renneting, gelation and sedimentation (Tsioulpas *et al.,* 2007b). The composition of milk salts is influenced by several factors, especially by the stage of lactation (Holt, 1985). Klimes *et al*. (1986) and Tsioulpas *et al*. (2007a) found that concentrations of Ca, Mg, P, Na and K were highest in colostrum at the first milking *post partum*. In the current study, large variations in mineral concentrations were observed but, in general, colostrum samples contained elevated levels of Na, Mg, P, K and Ca, which decreased with time *post partum* (Table 2.9).

Calcium partitions between the colloidal and serum phases and is in electrochemical equilibrium with several major milk components (Silanikove *et al.,* 2003). The distribution of Ca between the colloidal and serum phases in milk is governed by the level of casein in the milk (Holt and Jenness, 1984). The mature milk samples contained, on average, 1396 mg/L Ca (Table 2.9), which is in agreement with reported values (Fox and McSweeney, 1998). In general, the total Ca content was very high in colostrum samples. This was most evident for the first milkings from cows D, E and F. Cow F contained the highest level of total Ca; the concentration of Ca at the first milking was 2991 mg/L, and declined to 2157 mg/L at the second milking, and to 1667 mg/L by the sixth milking. Tsioulpas *et al*. (2007a) observed a similar trend over the first 3 days *post partum*, at which time total Ca varied from 2172 to 1551 mg/L. Similar trends were seen for the first six milkings from cows D and E, in which the Ca concentration decreased from 2300 to 1412 mg/L and 2964 to 1513 mg/L, respectively. The high concentration of Ca in colostrum is most likely due to elevated levels of casein (Table 2.1), which act as a

Ca carrier in milk (Little and Holt, 2004). The colostrum from Cow B contained an elevated level of Ca, especially in early milkings, but this figure reached normal levels by the sixth milking. All milkings from cows A and C, with the exception of the first milking from cow A, contained average concentrations of calcium, i.e. approximately 1200 mg/L. Tsioulpas *et al*. (2007a) reported that the Ca^{2+} levels in milk increased as the pH decreased. Ca^{2+} was not measured in the current study, but it is speculated that colostrum samples would contain high levels of Ca^{2+}, due to the combined effect of low pH (Table 2.1) and high Ca level (Table 2.9).

Phosphorus in milk exists in several forms, e.g., free inorganic orthophosphate in solution, colloidal phosphate associated with Ca in micellar Ca phosphate (CCP) and the ester phosphate of various proteins and phospholipids (Lucey and Horne, 2009). Total phosphorus was measured in the current study (Table 2.9). Mature milk samples were found to contain, on average, 1029 mg/L P, which is in agreement with reported values (Fox and McSweeney, 1998). In general, the total P content was very high in colostrum. This was most evident for the early milkings from cows B, E and F. Cow E contained the highest levels of total P, i.e., 2564 mg/L at the first milking, declined to 1968 mg/L at the second milking and 1865 mg/L by the sixth milking. Similar trends were seen for the first six milkings from cows B and F, in which the P concentration over the first six milkings ranged from 1804 to 1133 mg/L and 2127 to 1345 mg/L, respectively.

The concentration of Na in colostrum was not as elevated as that of Ca and P. In the current study, the average concentration of Na in mature milk was 500 mg/L (Table 2.9), which is in agreement with reported values (Fox and McSweeney, 1998). Colostrum from cows D, E and F contained the highest concentrations (mg/L) of Na, ranging from 713-557, 914-549 and 935-527 mg/L, respectively. Na concentrations in the first six milkings from cows A, B and C were slightly lower than average levels found in mature milk.

The concentration of Mg in colostrum samples was found to be very high, especially in early milkings. The average concentration of Mg in mature milk in the current study was found to be 131 mg/L (Table 2.9), which is in agreement with reported

values (Fox and McSweeney, 1998). Colostrum from cows D, E and F contained the highest concentrations of Mg, ranging from 527-132, 610-143 and 477-150 mg/L, respectively. Mg concentrations in milkings from cows A, B and C were found to be high initially, but decreased rapidly and fell below average levels by the third milking.

Mature milk contains approximately 1450 mg/L K (Fox and McSweeney, 1998). The average concentration of K in mature milk samples in the current study was found to be 1773 mg/L (Table 2.9), which is higher than reported values. No particular trend was found regarding K concentration, which ranged from 2991 to 941 mg/L across all colostrum samples. This is in agreement with the study of Tsioulpas *et al*. (2007a) who also found no trend in K concentration over the first 90 days *post partum*.

Table 2.9 Mean values (± S.D., n=3) for concentration of Na, Mg, P, K and Ca for the first six milkings from cows A to F and bulk mature milk samples 1 to 3. Values without an asterisk (*) were significantly different ($p < 0.05$) from control bulk milk samples.

Sample	Na mg/L	Mg mg/L	P mg/L	K mg/L	Ca mg/L
A1	439* ± 10	237 ± 7	1443 ± 26	1532* ± 42	1508* ± 23
A2	498* ± 17	160* ± 5	1434 ± 40	1834* ± 83	1280* ± 44
A3	365* ± 18	99* ± 4	1077* ± 49	1411 ± 53	995 ± 51
A4	359* ± 7	94* ± 2	1008* ± 12	1387 ± 24	941 ± 12
A5	412* ± 34	112* ± 8	1199* ± 98	1691* ± 16	1167 ± 103
A6	378* ± 10	116* ± 2	1186* ± 35	1726* ± 43	1233* ± 49
B1	488* ± 3	192 ± 1	1804 ± 20	1785* ± 9	1882 ± 13
B2	506* ± 23	148* ± 5	1798 ± 89	2096 ± 87	1795 ± 54
B3	478* ± 2	141* ± 0	1712 ± 22	1969* ± 16	1708 ± 8
B4	419* ± 7	121* ± 1	1357 ± 14	1604* ± 17	1378* ± 13
B5	410* ± 4	121* ± 0	1316 ± 14	1638* ± 4	1411* ± 3
B6	343 ± 3	103* ± 0	1133* ± 29	1433 ± 7	1286* ± 20
C1	437* ± 6	158* ± 2	1465 ± 28	1879* ± 32	1288* ± 15
C2	411* ± 5	116* ± 0	1293 ± 10	1749* ± 18	1199 ± 3
C3	428* ± 4	114* ± 1	1355 ± 10	1751* ± 7	1110 ± 6
C4	472* ± 18	123* ± 4	1524 ± 55	2136 ± 101	1373* ± 48
C5	358* ± 77	89* ± 19	1044* ± 247	1446 ± 34	965 ± 21
C6	386* ± 15	104* ± 4	1224* ± 41	1902* ± 54	1165 ± 33
D1	670 ± 31	527 ± 22	1694 ± 81	1134 ± 66	2085 ± 95
D2	592* ± 5	387 ± 5	2049 ± 45	2028* ± 37	2300 ± 40
D3	557* ± 23	261 ± 9	1648 ± 44	1900* ± 80	1682 ± 51
D4	713 ± 16	210 ± 5	1715 ± 42	2291 ± 54	1799 ± 41
D5	682 ± 61	136* ± 12	1316 ± 15	1930* ± 18	1412* ± 13
D6	667 ± 1	132* ± 1	1356 ± 20	1924* ± 7	1462* ± 12
E1	914 ± 49	610 ± 32	2564 ± 159	1723* ± 104	2964 ± 13
E2	645 ± 9	264 ± 4	1968 ± 41	2005* ± 38	2106 ± 35
E3	651 ± 57	220 ± 18	2126 ± 23	2357 ± 29	2060 ± 18
E4	637* ± 57	183* ± 15	1771 ± 17	2017* ± 17	1730 ± 14
E5	549* ± 12	143* ± 4	1435 ± 48	1832* ± 61	1513* ± 45
E6	604* ± 2	162* ± 1	1815 ± 8	2177 ± 15	1812* ± 16
F1	935 ± 133	477 ± 62	2127 ± 31	1538* ± 23	2991 ± 41
F2	645 ± 54	279 ± 22	1984 ± 17	2039* ± 18	2157 ± 18
F3	623* ± 21	226 ± 6	1844 ± 61	2099 ± 58	2085 ± 60
F4	576* ± 12	173* ± 3	1513 ± 35	1779* ± 36	1728 ± 33
F5	527* ± 74	151* ± 19	1391 ± 21	1752* ± 25	1702 ± 23
F6	540* ± 21	150* ± 6	1345 ± 35	1798* ± 69	1667 ± 70
Bulk 1	468 ± 10	126 ± 27	976 ± 24	1652 ± 39	1345 ± 30
Bulk 2	519 ± 8	136 ± 2	1068 ± 4	1775 ± 13	1486 ± 16
Bulk 3	512 ± 6	131 ± 16	1044 ± 53	1893 ± 36	1357 ± 57

2.3.9 Rennet coagulation time (RCT) and rheology of rennet-induced gels

RCT values, measured at both the natural pH and at pH 6.6, for the first six milkings from cows A to F and bulk mature milk samples 1 to 3 are shown in Table 2.10. The main factors affecting the rennet clotting of milk are enzyme concentration, temperature, pH and concentration of Ca^{2+} (Najera *et al.,* 2003). The enzyme concentration and temperature used in the current study did not vary and so will not be discussed. It is well known that reducing the pH of milk from 6.7 to 5.8 causes a decrease in clotting time (Dalgleish., 1993; Fox and McSweeney, 1998; Daviau *et al*., 2000; Madsen *et al.,* 2004), due to a combination of factors, including increased rennet activity as the enzyme moves towards its pH optimum, a reduction in the electrostatic repulsion between micelles as a result of increased Ca^{2+} activity, and flocculation at a lower degree of κ-casein hydrolysis (Walstra, 1993). In general, it was found that the RCT of colostrum, measured at its natural pH, decreased with time *post partum*. This decrease in RCT was inversely related to an increase in the natural pH of colostrum, which is not in agreement with the behaviour of mature milk, as reported by Dalgleish (1993), and cannot be explained; an increase in RCT with increasing pH was expected. Colostrum samples, in particular early milkings, contained high concentrations of calcium (Table 2.9), which decreased with time *post partum*. Addition of Ca^{2+} causes a decrease in RCT due to the neutralization of negatively charged residues on caseins, which increases the aggregation of renneted micelles (Lucey and Fox, 1993). In general, the opposite effect was seen here, where RCT decreased with decreasing calcium concentration. Madsen *et al*. (2004) reported that RCT decreases with increasing protein concentration. The opposite effect was found in the current study, where RCT values decreased with decreasing protein concentration. The exception to these irregularities was cow C, where RCT values increased with increasing pH and decreasing protein concentration.

In most cases, increasing the natural pH of colostrum to pH 6.6 resulted in a significant increase in RCT. At pH 6.6, RCT of colostrum from cows A and B was high

initially and decreased with time *post partum.* In general, at pH 6.6, the RCT of colostrum, in particular in early milkings, was higher than that of bulk mature milk. Other factors which may have affected the RCT of colostrum were viscosity and the presence of protease inhibitors. The high viscosity of colostrum may have caused an increase in RCT by limiting diffusion of *para*-κ-casein resulting in slower aggregation of casein micelles. Colostrum contains a number of enzyme inhibitors, all of which originate from the blood (Kelly *et al*., 2006), the concentration of which is highest initially and decreases with time *post partum* (Georgiev, 2008). Huppertz et al. (2006) reported that protease inhibitors present in blood serum can significantly reduce chymosin activity, which could lead to an increase in the RCT of milk.

Table 2.10 Mean values (± S.D., n = 3) for pH, RCT at natural pH, and RCT at pH 6.6 for colostrum from the first six milkings from cows A to F and bulk mature milk samples 1 to 3. Values without an asterisk (*) were significantly different (p < 0.05) from control bulk milk samples.

Sample	pH	RCT natural pH	RCT pH 6.6
A1	6.31	5.10 ± 0.09	7.89 ± 0.02
A2	6.37	4.56 ± 0.06	7.26 ± 0.05
A3	6.40	4.73 ± 0.04	8.07 ± 0.14
A4	6.47	4.40 ± 0.03	5.10 ± 0.04
A5	6.44	3.20 * ± 0.02	4.35 ± 0.02
A6	6.46	3.28 * ± 0.03	4.20 ± 0.06
B1	6.37	2.89 ± 0.02	4.33 ± 0.03
B2	6.32	2.57 ± 0.02	4.16 ± 0.01
B3	6.32	3.50 * ± 0.01	4.15 ± 0.01
B4	6.36	2.43 ± 0.03	3.30 * ± 0.01
B5	6.40	3.10 ± 0.01	3.08 ± 0.04
B6	6.47	3.27 * ± 0.02	3.02 ± 0.01
C1	6.39	2.36 ± 0.03	
C2	6.44	4.18 ± 0.01	
C3	6.51	4.10 ± 0.02	
C4	6.54	5.68 ± 0.02	
C5	6.48	7.57 ± 0.01	
C6	6.50	5.10 ± 0.01	
D1	6.28	0.46 ± 0.12	
D2	6.25	4.04 ± 0.11	6.92 ± 0.07
D3	6.39	1.50 ± 0.04	
D4	6.51	1.83 ± 0.08	
D5	6.68	3.20 * ± 0.02	
D6	6.67	2.97 ± 0.02	
E1	6.39	3.90 * ± 0.21	
E2	6.47	3.58 * ± 0.20	
E3	6.42	2.80 ± 0.08	
E4	6.53	1.21 ± 0.04	3.41 * ± 0.02
E5	6.52	1.64 ± 0.02	
E6	6.44	3.92 * ± 0.02	
F1	6.35	8.20 ± 0.01	
F2	6.33	3.90 * ± 0.01	5.63 ± 0.03
F3	6.26	3.00 ± 0.02	
F4	6.34	2.30 ± 0.01	4.20 ± 0.03
F5	6.35	2.72 ± 0.02	3.23 ± 0.04
F6	6.37	2.22 ± 0.02	
Bulk 1	6.66	3.79 ± 0.01	
Bulk 2	6.62	3.37 ± 0.01	
Bulk 3	6.66	3.62 ± 0.03	

Small-amplitude oscillatory rheology was used to characterise the rheological properties of rennet-induced gels without damaging the gel network (Table 2.11 and Figure 2.5). Gel properties were studied for 90 min after rennet addition; however, it should be noted that G′ values presented in Table 2.11 were not the final G′ values in all cases, i.e., for some samples, the G′ value was still increasing after 90 min (Figure 2.5). In general, G′ of gels formed at the natural pH value was very high initially and decreased with time *post partum*, but, in most cases, did not reach normal levels by the sixth milking. On average, the G′ value of bulk mature milk was found to be 87.1 Pa. G′ values of the first milkings from cows D and E were over five times greater than this, i.e., 495.9 and 495.8 Pa, respectively. The high G′ values in colostrum samples are probably due to a combination of factors, e.g., low pH, high levels of casein and calcium. As a result, both bond formation between casein particles, and particle fusion, are likely to proceed faster, resulting in higher G′ values. Daviau *et al*. (2000) observed that increasing the concentration of casein in skim milk led to a reduction in RCT and an increase in G′. The same authors found that decreasing pH in the range 6.4 to 6.0 resulted in an increased firming rate. In general, the opposite was seen for G′ values, measured at a fixed pH value of 6.6, where the G′ value of colostrum samples increased with time *post partum.*

The effect of interchanging the serum phase of colostrum and bulk mature milk on the rheology of rennet-induced gels was investigated (Table 2.11). The serum phase of colostrum and bulk mature milk was prepared by ultracentrifugation at 100,000 g x 1 h at 20°C. The average G′ value of bulk mature milk 3 was 90.27 Pa; however, interchanging the serum phase of this sample with either the first milking from cow B or the second milking from cow F resulted in an increase in the G′ value of bulk mature milk 3 (138.71 and 122.99 Pa, respectively). This increase in G′ was most likely because of the high proportion of casein in colostrum which was non-sedimentable at 100,000 g x 1 h (Table 2.7), thereby increasing the concentration of casein in bulk mature milk 3. Another explanation for the increase in G′ was the effect of pH; the pH of colostrum was lower than that of bulk mature milk (Table 2.1), and so, interchanging the serum phase of colostrum with bulk mature milk would reduce the pH of bulk mature milk, thereby reducing the RCT and increasing

G'. The opposite effect was observed after interchanging the serum phase of the first milking from cows B or F with that of bulk mature milk 3, i.e., G' values decreased from 406.61 and 111.11 Pa to 143.99 and 47.93 Pa, respectively. As before, this decrease in G' was most likely because of the high proportion of casein in colostrum which was non-sedimentable at 100,000 g x 1 h (Table 2.7), thereby reducing the concentration of casein in the first milkings of cows B and F. Also, interchanging the serum phase of colostrum with bulk mature milk would increase the pH of colostrum, thereby increasing the RCT and reducing G' values.

Table 2.11 Mean values (± S.D., n = 3) for pH, G' values at the natural pH, and G' values at pH 6.6, of rennet-induced gels for colostrum from the first six milkings from cows A to F and bulk mature milk samples 1 to 3. Values without an asterisk (*) were significantly different ($p < 0.05$) from control bulk milk samples.

Sample	pH	G' natural pH	G' pH 6.6
A1	6.31	112.56 * ± 8.33	29.04 ± 1.87
A2	6.37	16.30 ± 2.11	
A3	6.4	139.90 ± 7.90	47.72 ± 3.34
A4	6.47	149.96 ± 4.13	
A5	6.44	153.54 ± 6.11	132.32 ± 3.98
A6	6.46	142.66 ± 5.97	
B1	6.37	406.61 ± 11.49	
B2	6.32	301.61 ± 13.02	191.70 ± 9.22
B3	6.32	257.71 ± 12.68	
B4	6.36	281.10 ± 12.00	226.06 ± 10.13
B5	6.4	271.75 ± 8.78	
B6	6.47	268.66 ± 6.33	
C1	6.39	204.49 ± 10.22	
C2	6.44	118.61 * ± 7.18	
C3	6.51	99.88 * ± 5.49	
C4	6.54	59.98 * ± 4.19	
C5	6.48	14.03 ± 2.06	
C6	6.5	77.24 * ± 6.20	
D1	6.28	495.92 ± 21.21	
D2	6.25	405.09 ± 18.98	212.90 ± 9.26
D3	6.39	260.56 ± 4.54	
D4	6.51	134.16 * ± 6.13	
D5	6.68	107.34 * ± 7.22	
D6	6.67	140.50 ± 9.01	
E1	6.39	495.76 ± 33.16	
E2	6.47	260.03 ± 5.29	
E3	6.42	202.39 ± 4.11	
E4	6.53	124.78 * ± 5.05	
E5	6.52	130.79 * ± 5.82	
E6	6.44	187.64 ± 6.11	
F1	6.35	111.11 * ± 3.49	23.42 ± 2.11
F2	6.33	274.49 ± 10.09	89.45 * ± 6.49
F3	6.26	258.10 ± 9.86	110.12 * ± 5.26
F4	6.34	253.13 ± 6.99	224.88 ± 9.04
F5	6.35	259.98 ± 6.43	
F6	6.37	175.58 ± 3.08	67.78 * ± 1.38
Bulk 1	6.66	102.22 ± 1.34	
Bulk 2	6.62	68.75 ± 5.82	
Bulk 3	6.66	90.27 ± 3.99	
Bulk 3 & B1 serum	6.53	138.71 ± 2.22	
Bulk 3 & F2 serum	6.59	122.99 ± 2.69	
B1 & bulk 3 serum	6.64	143.99 ± 5.42	
F1 & bulk 3 serum	6.74	47.93 ± 1.08	

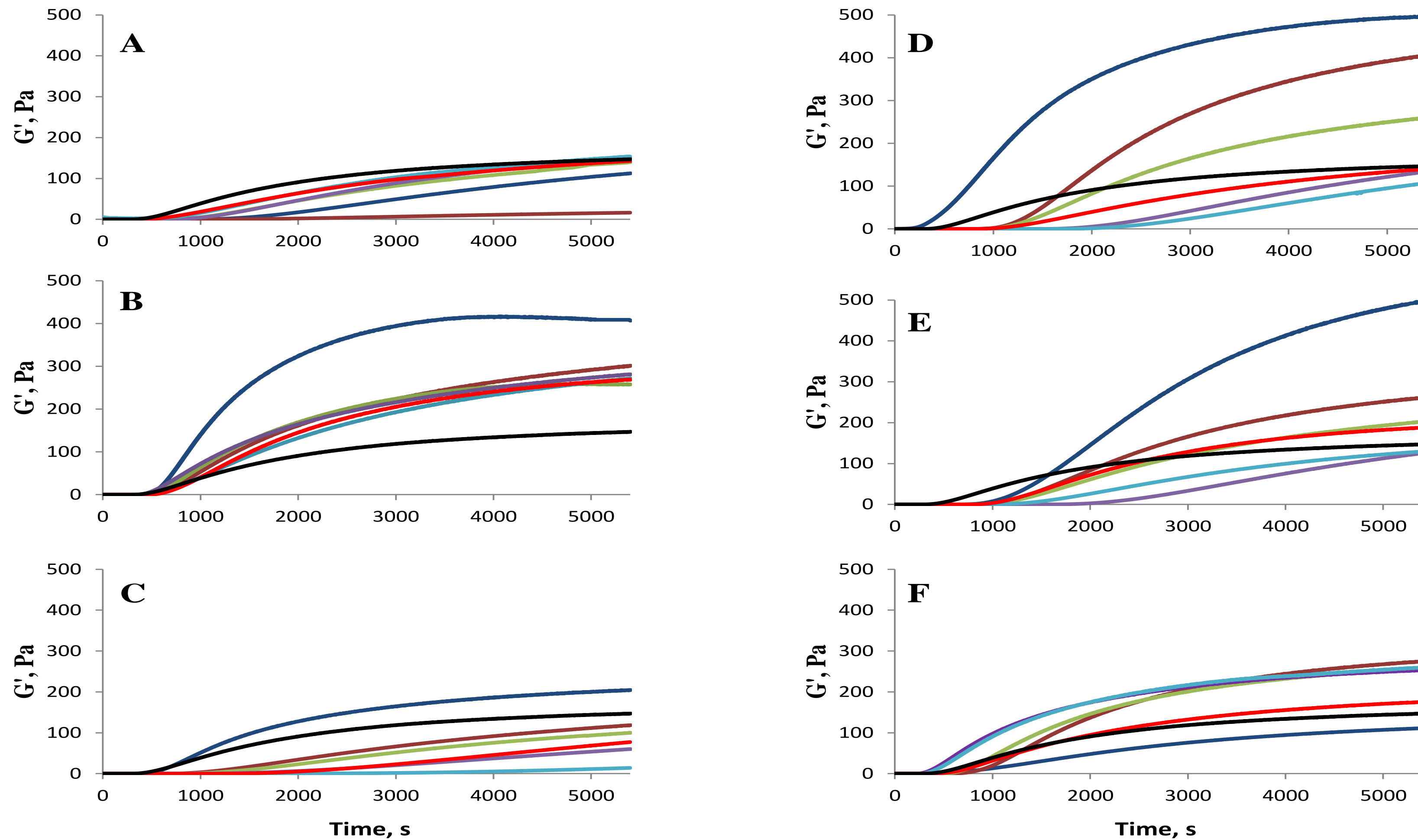


Figure 2.5 Effect of renneting (at 30°C and unadjusted pH) with 10 μL/mL of 1:10 (v/v) diluted Maxiren 180 on the storage modulus, G′, of the first six milkings from cows A to F (— 1st, — 2nd, — 3rd, — 4th, — 5th, — 6th) and bulk mature milk (—). For clarity, the result from a single analysis is shown; the coefficient of variation was < 5% of the reported value for each data point

2.3.10 Acidification of colostrum samples

GDL can be hydrolysed to gluconic acid in aqueous solution, which results in a reduction in the pH of milk, typically to pH 4.6. At this pH, gelation occurs as a result of the aggregation of casein particles. The influence of acidification by GDL on the storage modulus, G', of colostrum samples was measured using small-amplitude oscillatory rheology, and the results are shown in Table 2.12 and Figure 2.6. G' describes the elastic properties of a gel. GDL was added to colostrum samples at concentrations which caused the pH to decrease to 3.9 in 90 min. This pH value was selected because, in certain colostrum samples, casein did not precipitate at pH 4.6, as described in Section 2.3.11. Varying levels of GDL were required to reach pH 3.9 due to the highly variable protein and mineral content, and consequently highly variable buffering capacity, of colostrum samples. The G' value of the gel formed from mature milk was 198.2 Pa. In all cases, the G' value of acid gels formed from colostrum was significantly lower than that from mature milk. No gelation was observed for the sixth milking from cows B and E, the first and third milking from D and the first milkings from cows E and F. The range of G' values for the remaining colostrum samples was 3.7 to 56.7 Pa, but no apparent trends were observed.

The formation and physical properties of acid milk gels have been reviewed by Lucey and Singh (1998). Protein fortification is one of the most important processing parameters which affects the textural properties of acid milk gels. Addition of whey protein concentrate (WPC) to milk has been shown to increase the firmness and viscosity of yogurt (Robinson and Tamine, 1986), but only when the mixture is heat-treated. Lucey and Singh (1998) suggested that cross-linking or bridging by denatured whey proteins within gels made from heated milk was responsible for the increase in G'. Lucey *et al.* (1999) found that addition of native (unheated) whey proteins to milk results in a reduction in G' and suggested that they act as an inert filler in acid milk gels. This may be the case in the current study, where colostrum samples with high levels of native whey proteins (Table 2.1) had poor acid coagulation properties. Colostrum samples with the highest

concentration of whey protein (D1, E1 and F1) did not form a gel on acidification. The studies mentioned previously (Robinson and Tamine, 1986; Lucey *et al.,* 1999) used WPC as the whey protein source. WPC is produced from mature bovine milk and contains predominantly β-Lg and α-La. As already discussed in Section 2.3.3, colostrum has high levels of Ig, LF, and BSA; to the best of our knowledge, no work has been reported on the effect of these proteins on acid-induced gelation of milk.

Table 2.12 Mean initial pH values, amount of glucono-δ-lactone (GDL; % w/v) required to acidify colostrum to pH 3.9, and G' values of the first, third and sixth milking from cows A to F and bulk mature milk sample 1.

Sample	Initial pH	GDL (% w/v)	G'
A1	6.31	3.5	23.5
A3	6.4	3.5	47.1
A6	6.46	3	22.0
B1	6.37	3	38.6
B3	6.32	3	56.7
B6	6.47	3.5	no gel
C1	6.39	3	34.7
C3	6.51	3	34.3
C6	6.5	3	no gel
D1	6.28	3.5	no gel
D3	6.39	3.5	no gel
D6	6.67	3	54.6
E1	6.39	3.5	no gel
E3	6.42	3	36.7
E6	6.44	3.5	32.4
F1	6.35	3.5	no gel
F3	6.26	3	3.7
F6	6.37	3	27.7
Bulk Mature	6.66	2.2	198.2

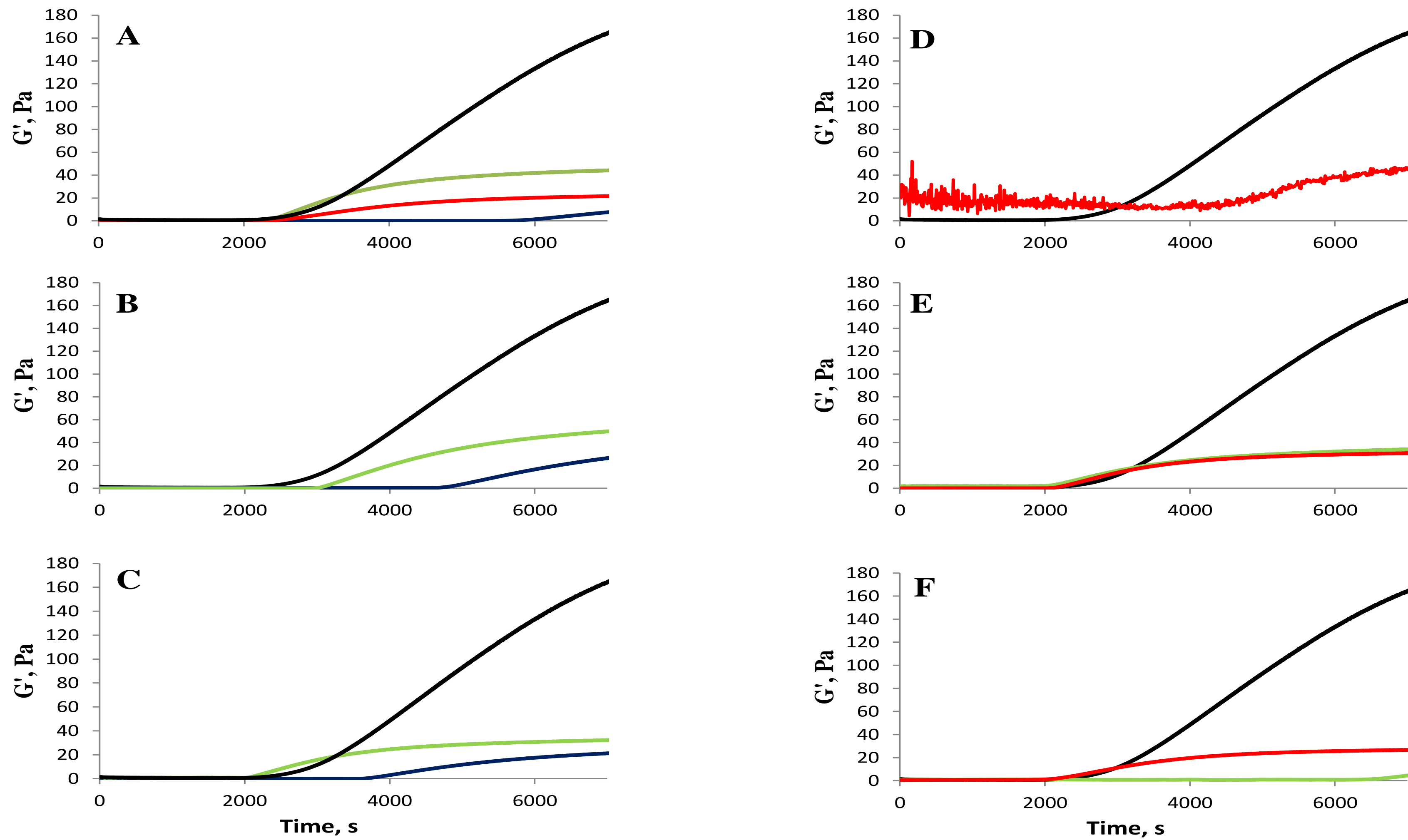


Figure 2.6 Influence of acidification to pH 3.9 by glucono-δ-lactone (GDL) at 30 C on the storage modulus, G', of the first (▬), third (▬) or sixth (▬) milking from cows A to F and bulk mature milk (▬).

2.3.11 Isoelectric precipitation of casein

Table 2.13 presents average values for protein solubility of the first six milkings *post partum* from cows A to F and bulk mature milk samples 1 to 3, after adjustment to pH 4.6. In this case, protein solubility is defined as the percentage of total protein which remained soluble after adjustment to pH 4.6. Colostrum samples were diluted with distilled water to investigate the effect of dilution on the isoelectric precipitation of casein. Results shown include those for undiluted colostrum, and those for a 1:1 and 1:2 dilution of colostrum with distilled water before pH adjustment. Colostrum was also diluted 1:3 and 1:4, but no further change occurred above a 1:2 dilution and so the results are not shown. Mature bovine milk has a pH of approximately 6.7, at which value the casein micelles have a net negative charge. The stability of casein micelles against precipitation is complex; it involves neutralising the electrostatic repulsion of the charged protein molecules by decreasing the pH to the pI of the caseins (pH 4.6). Adjusting the pH of milk to 4.6 neutralises the outer surface of the micelles, followed by irreversible destruction of the micellar structure and precipitation of the caseins (Fox, 2003).

In the current study, it was found that casein did not precipitate at pH 4.6 in 10 out of a total of 36 colostrum samples. Of these ten samples, five were first milkings, three were second milkings, one was third milking, and one was fourth milking. For all cows, with the exception of cow C, casein did not precipitate at pH 4.6 in the first milking. Cows A and B followed a similar trend, i.e., casein did not precipitate at pH 4.6 in the first milking, but did for the subsequent five milkings. A 1:1 dilution of colostrum with distilled water restored acid precipitability in five out of ten samples and a 1:2 dilution with distilled water restored acid precipitability in all cases. In the case of the first milking from cows A and B, a 1:1 dilution with distilled water resulted in casein precipitating at pH 4.6. For cow C, casein precipitated at pH 4.6 for all six milkings. For cow D, casein did not precipitate at pH 4.6 for the first four milkings. In the first and second milking from cow D, a 1:1 dilution with distilled water had no effect on precipitability at pH 4.6, but a 1:2 dilution resulted in casein

precipitating at pH 4.6. For the third and fourth milking from cow D, a 1:1 dilution with distilled water resulted in casein precipitating at pH 4.6. Cows E and F followed a similar trend, i.e., casein did not precipitate at pH 4.6 in the first two milkings, but did in the subsequent four milkings. In the case of the first milking from cows E and F, a 1:2 dilution with distilled water was required to cause precipitation at pH 4.6. For the second milking from cows E and F, a 1:1 dilution with distilled water was sufficient. To the best of our knowledge, non-precipitability of casein in colostrum at pH 4.6 has not been reported previously.

Table 2.13 Mean values (± S.D., n=3) for protein solubility of undiluted, 1:1 and 1:2 dilution of the first six milkings from cows A to F and bulk mature milk samples 1 to 3, after adjustment to pH 4.6. Values without an asterisk (*) were significantly different ($p < 0.05$) from control bulk milk samples.

Sample	No dilution		1:1 dilution		1:2 dilution	
	precipitation at pH 4.6 yes/no	% of total protein soluble at pH 4.6	precipitation at pH 4.6 yes/no	% of total protein soluble at pH 4.6	precipitation at pH 4.6 yes/no	% of total protein soluble at pH 4.6
A1	N	96.1 ± 0.33	y	54.1 ± 0.76	y	56.3 ± 0.23
A2	Y	35.8 ± 0.05	y	39.0 ± 0.17	y	38.6 ± 0.29
A3	Y	21.2 * ± 0.43	y	26.9 ± 1.64	y	26.8 * ± 0.75
A4	Y	20.6 * ± 0.06	y	23.9 * ± 0.78	y	24.3 * ± 0.52
A5	Y	20.6 * ± 0.09	y	22.5 * ± 0.60	y	22.6 * ± 0.89
A6	Y	20.5 * ± 0.70	y	22.5 * ± 0.69	y	22.1 * ± 0.57
B1	N	84.1 ± 0.56	y	31.7 ± 0.15	y	32.3 ± 0.65
B2	Y	15.9 ± 0.62	y	19.0 * ± 0.22	y	21.1 * ± 0.34
B3	Y	17.5 * ± 0.00	y	21.5 * ± 2.17	y	20.9 * ± 0.34
B4	Y	15.4 ± 0.84	y	18.6 * ± 0.21	y	14.5 * ± 0.66
B5	Y	17.4 * ± 1.84	y	20.5 * ± 1.97	y	19.9 * ± 0.50
B6	Y	18.0 * ± 0.82	y	19.9 * ± 0.24	y	21.2 * ± 0.32
C1	Y	50.9 ± 3.06	y	44.6 ± 0.38	y	40.4 ± 2.85
C2	Y	24.9 * ± 1.13	y	27.3 ± 0.24	y	27.5 ± 0.00
C3	Y	23.7 * ± 0.22	y	23.2 * ± 0.99	y	21.8 * ± 0.39
C4	Y	22.2 * ± 0.34	y	22.7 * ± 1.94	y	22.5 * ± 2.61
C5	Y	21.5 * ± 0.84	y	23.2 * ± 0.00	y	25.0 * ± 0.80
C6	Y	20.2 * ± 0.12	y	17.6 * ± 0.57	y	20.2 * ± 0.45
D1	N	89.0 ± 0.23	N	87.8 ± 0.59	Y	80.1 ± 0.93
D2	N	96.3 ± 2.07	N	93.4 ± 2.01	Y	52.3 ± 4.50
D3	N	93.7 ± 0.14	Y	57.3 ± 3.89	Y	55.1 ± 0.95
D4	N	91.4 ± 0.20	Y	36.1 ± 0.17	Y	39.4 ± 0.81
D5	Y	22.7 * ± 1.21	Y	29.8 ± 0.43	Y	29.7 ± 0.66
D6	Y	20.5 * ± 0.19	Y	20.3 * ± 0.46	Y	21.8 * ± 0.00
E1	N	90.7 ± 0.16	N	91.0 ± 0.23	Y	77.4 ± 4.55
E2	N	94.4 ± 0.45	Y	44.1 ± 1.77	Y	44.2 ± 3.12
E3	Y	22.1 * ± 1.30	Y	30.6 ± 0.37	Y	32.1 ± 0.29
E4	Y	22.5 * ± 0.00	Y	21.2 * ± 0.23	Y	26.0 * ± 0.72
E5	Y	17.4 * ± 0.57	Y	23.5 * ± 0.52	Y	16.0 * ± 2.09
E6	Y	18.8 * ± 0.00	Y	19.3 * ± 0.26	Y	15.8 * ± 1.59
F1	N	87.7 ± 0.29	N	90.9 ± 4.17	Y	71.4 ± 0.45
F2	N	89.8 ± 0.43	Y	39.1 ± 0.40	Y	40.9 ± 2.23
F3	Y	32.6 ± 0.74	Y	32.1 ± 0.20	Y	31.7 ± 0.00
F4	Y	21.8 * ± 0.28	Y	24.6 * ± 0.48	Y	14.3 ± 1.13
F5	Y	17.4 * ± 0.21	Y	20.2 * ± 0.28	Y	22.6 * ± 1.30
F6	Y	19.6 * ± 0.13	Y	23.5 * ± 1.51	Y	22.5 * ± 1.87
Bulk 1	Y	18.7 ± 0.16	Y	17.5 ± 0.33	Y	19.6 ± 1.02
Bulk 2	Y	20.3 ± 0.11	Y	19.6 ± 0.35	Y	20.9 ± 0.00
Bulk 3	Y	22.5 ± 0.11	Y	21.9 ± 0.77	Y	21.8 ± 0.54

2.3.11.1 Effect of pH on the precipitation of casein

The pI is the point in a titration curve corresponding to the pH in solution at which the net surface charge, and thus the electrophoretic mobility, of a protein is zero (Righetti, 2004). The effect of varying the pH of acidification of colostrum on the solubility of the caseins was investigated, as shown in Table 2.14 and Figure 2.7. Two colostrum samples in which casein did not precipitate at pH 4.6 were selected; these were the first two milkings from cow F. Samples were adjusted to pH 4.0, 4.2, 4.4, 4.6, 4.8 or 5.0. For the first milking from cow F, the casein did not precipitate in this pH range, as shown in lanes 3-9 (Figure 2.7). However, at pH 4.0 and 4.2, samples became very viscous, which suggests that casein was approaching its pI. For the second milking from cow F, the casein precipitated at pH 4.0, 4.2, and 4.4, which is evident by the absence of casein in lanes 11, 12, and 13, respectively (Figure 2.7). This indicates that there was a shift in the pI of casein in certain colostrum samples. While the isoelectric point of casein in mammalian milk is pH 4.6, generally caseins are insoluble over a pH range (3.5 to 5) and so will precipitate at values above and below the isoelectric point, e.g., the pH range for precipitation of casein in human milk is 4.1 to 4.7 (Khanna *et al.,* 2006). Egito *et al.* (2001) reported that minimal solubility of equine casein is at pH 4.2 instead of pH 4.6, as found for bovine casein. Khaldi and Shields (2011) reported that proteins which display a dramatic shift in pI are those that are highly glycosylated and phosphorylated. Holland *et al*. (2004) identified multiple forms of κ-casein in mature milk, with a pI value ranging from 4.47 to 5.81. High levels of κ-casein, in particular a high proportion of glycosylated κ-casein, in colostrum (Table 2.2) may cause a shift in pI, to a value below pH 4.6. The proportions of κ-casein in colostrum of the first two milkings from cow F, as a percentage of total casein (excluding α_{S2}-casein) were similar, at 20 and 19.7% respectively (Table 2.2), but the first milking contained a higher level of glycosylated κ-casein as a proportion of total κ-casein compared to the second milking (Figure 2.2).

Table 2.14 Mean values (± S.D., n=3) of % protein in samples F1 and F2 soluble at pH values in the range of 4.0 to 5.0

Sample	adjusted pH	precipitate yes/no	soluble protein (as a % of total protein) at adjusted pH
F1	4	no	89.0 ± 0.34
F1	4.2	no	90.1 ± 1.77
F1	4.4	no	90.9 ± 0.30
F1	4.6	no	91.4 ± 0.65
F1	4.8	no	91.7 ± 1.95
F1	5	no	95.1 ± 2.11
F2	4	yes	24.4 ± 0.16
F2	4.2	yes	27.3 ± 0.22
F2	4.4	yes	26.2 ± 0.41
F2	4.6	no	89.9 ± 0.22
F2	4.8	no	96.9 ± 0.69
F2	5	no	97.8 ± 0.75

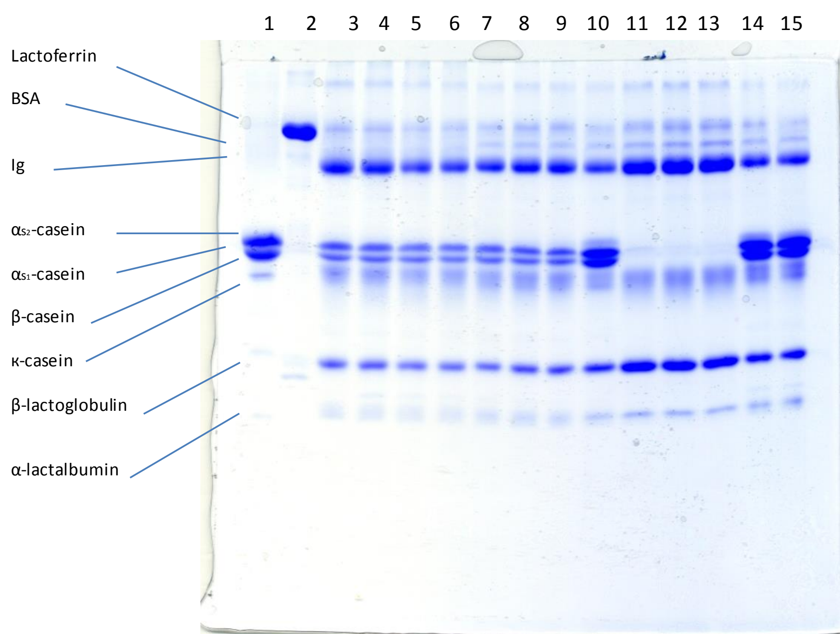


Figure 2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoretograms obtained under reducing conditions; Lane 1, sodium caseinate; Lane 2, Lactoferrin (15 µg); Lanes 3-9, soluble fraction of the first milking from cow F at pH 6.35 (unadjusted), 4.0, 4.2, 4.4, 4.6, 4.8 and 5.0, respectively; Lanes 10-15, soluble fraction of the second milking from cow F at pH 6.33 (unadjusted), 4.0, 4.2, 4.4, 4.6 and 4.8, respectively. Thirty micrograms of protein were loaded for all samples unless otherwise stated.

2.3.11.2 Effect of reversing the serum phase between colostrum and mature milk on the isoelectric precipitation of casein

The effect of the serum phase of colostrum, prepared by ultracentrifugation, on isoelectric precipitation of casein from mature milk was investigated (Figure 2.8). Sedimented casein micelles (100, 000 g x 1 h) of bulk mature milk 1 were dispersed in the serum phase of the first milking from cow E or bulk mature milk 1. In the presence of mature milk serum, casein precipitated at pH 4.6, as shown in Figure 2.8 (lane 6). In the presence of colostral serum, mature milk casein did not precipitate at pH 4.6, as shown in Figure 2.8 (lane 9). This suggests that it was a constituent of the serum phase of colostrum which was causing non-precipitability of casein at pH 4.6. However, as shown in Table 2.7, a significant proportion of casein in colostrum samples was non-sedimentable, and thus would account for a portion of the serum phase.

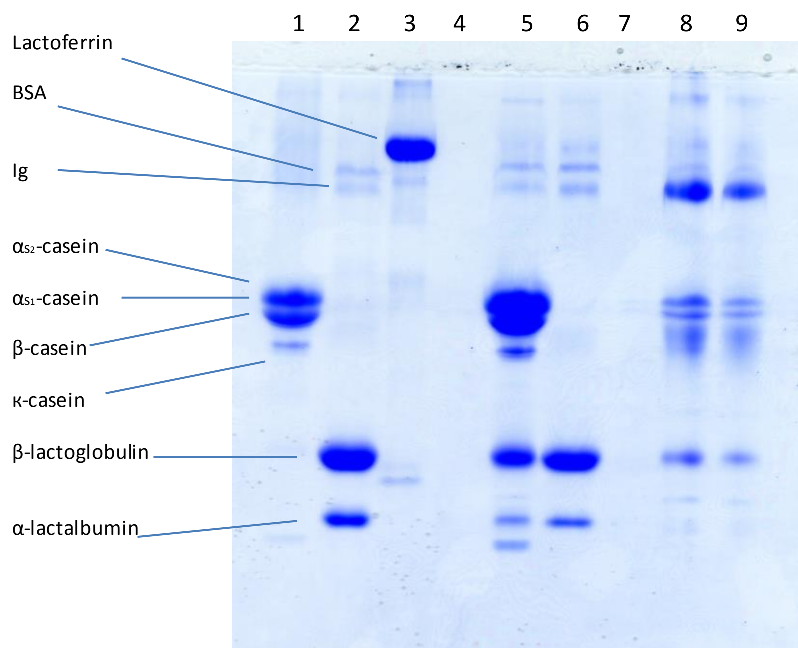


Figure 2.8 Sodium dodecyl sulphate-polyacrylamide gel electrophoretograms obtained under reducing conditions. Lane 1, sodium caseinate (31.5 µg); Lane 2, whey protein isolate (31.5 µg); Lane 3, lactoferrin (15 µg); Lane 5-6, pellet from mature milk reconstituted in serum from mature milk and pH 4.6-soluble fraction thereof, respectively; Lane 8-9, pellet from mature milk reconstituted in serum from colostrum and pH 4.6-soluble fraction thereof, respectively. Samples were not loaded on an equal protein basis.

2.3.11.3 Effect of interchanging the serum minerals between colostrum and mature milk on the isoelectric precipitation of casein

Large variations in the mineral composition of colostrum samples were observed (Table 2.9). The salts in milk play an important role in the stability of the proteins. Of most interest is Ca^{2+}, which can significantly influence the negative charge on casein micelles, and thus reduce the repulsive forces between them (Tsioulpas *et al*., 2007b). Calcium ions are involved in the internal stability of casein micelles as they form linkages between the protein molecules either as CCP or directly bound to caseins (Horne, 1998). The effect of reversing the mineral profile of colostrum and mature milk by equilibrium dialysis was investigated (Table 2.15 and Figure 2.9). Equilibrium dialysis works on the principle of diffusion of solutes across a semi-permeable membrane from an area of high concentration to an area of low concentration. The pH of colostrum and mature milk before dialysis was 6.42 and 6.58, respectively. Following dialysis, the pH of colostrum increased to 6.56, which was close to that of milk, and the pH of mature milk decreased to 6.43, close to that of colostrum. This is a good indication that dialysis was successful in reversing the mineral profiles of colostrum and mature milk; however, it was found that reversal of minerals had no effect on isoelectric precipitability, as shown in Table 2.15 and Figure 2.9. The casein in colostrum remained non-precipitable at pH 4.6 in the presence of the mineral profile of mature milk (lanes 5-8, Figure 2.9) while mature milk casein remained precipitable at pH 4.6 in the presence of the mineral profile of colostrum (lanes 10-13, Figure 2.9).

Table 2.15 Mean values (± S.D., n=3) for total and pH 4.6-soluble protein, in dialysed and undialysed, colostrum and mature milk. Values with different superscript letters are significantly different ($p < 0.05$).

Sample	pH	protein (mg/mL)	
		soluble at natural pH	soluble at pH 4.6
Colostrum	6.42	15.86 [g] ± 0.01	15.28 [f] ± 0.03
Dialysed colostrum	6.56	15.34 [f] ± 0.02	14.65 [e] ± 0.07
Mature milk	6.58	3.39 [c] ± 0.02	0.96 [a] ± 0.04
Dialysed mature milk	6.43	3.66 [d] ± 0.01	1.11 [b] ± 0.03

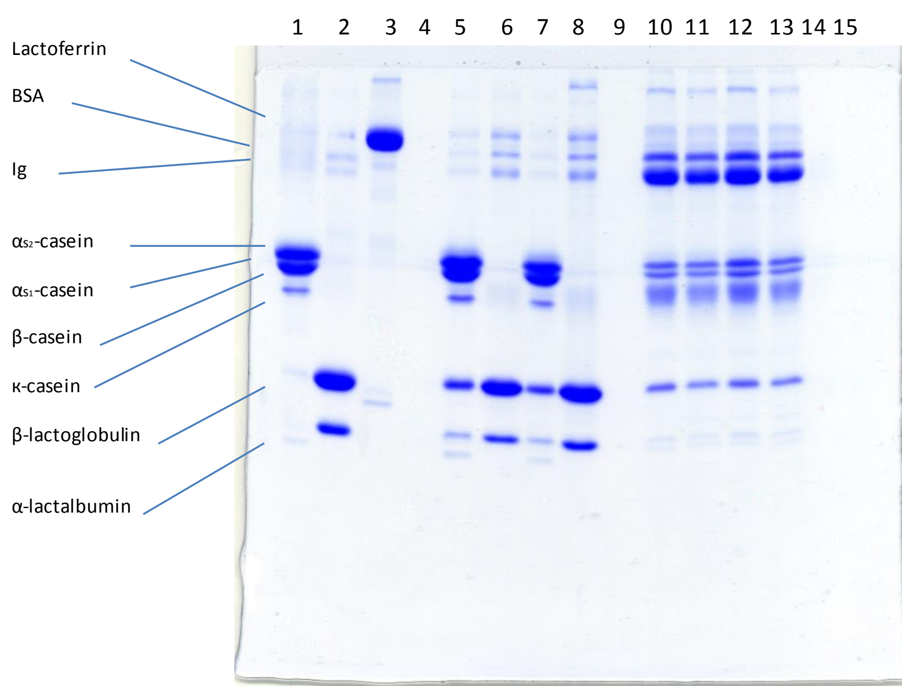


Figure 2.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoretograms obtained under reducing conditions. Lane 1, sodium caseinate (31.5 µg); Lane 2, whey protein isolate (31.5 µg); Lane 3, lactoferrin (15 µg); Lanes 5-8, standard milk, pH 4.6-soluble fraction of standard milk, dialysed standard milk, pH 4.6-soluble fraction of dialysed standard milk, respectively. Lanes 10-13, colostrum, pH 4.6-soluble fraction of colostrum, dialysed colostrum, pH 4.6-soluble fraction of dialysed colostrum, respectively. Thirty micrograms of protein was loaded for all samples unless otherwise stated.

2.3.11.4 Effect of adding lactoferrin to mature milk on the isoelectric precipitation of casein

Lactoferrin (LF) is a non-haem iron-binding protein that is a member of the transferrin family, whose function is to transport iron in the blood (Gonzalez-Chavez *et al.,* 2009). It is an 80 kDa glycoprotein and is strongly cationic with a high pI (~8.9) (Anema and de Kruif, 2012a). Recent studies have shown that lactoferrin is capable of interacting with casein micelles (Anema and de Kruif, 2011; Anema and de Kruif, 2012a,b; Croguennec *et al.,* 2012). At the natural pH of milk, LF carries a net positive charge, while casein micelles are negatively charged (pI 4.6), and therefore it has been speculated that LF interacts with casein micelles through electrostatic interactions (Anema and de Kruif, 2011). In the current study, mature milk samples were found to contain, on average, 0.69 mg/mL LF. This value is higher than the report of Cheng *et al*. (2008), who reported that the LF concentration in mature milk varies in the range 0.032 – 0.49 mg/mL. In the study of Sobczuk-Szul *et al*. (2013), colostrum was reported to contain 2.65 mg/mL LF.

In the current study, LF concentration varied widely, in the range 0.77 – 14.29 mg/mL. LF concentration was very high initially and decreased with time *post partum*. As shown in Table 2.16, all colostrum samples (with the exception of the first milking from cow C) in which casein did not precipitate at pH 4.6, contained greater than 3.43 mg/mL LF. The range of LF concentration in non-precipitable samples was 3.43 to 14.29 mg/mL. To investigate the effect of elevated LF content on the isoelectric precipitability of casein in colostrum, LF was added to mature milk at a concentration ranging from 0 to 25.6 mg/mL. The results in Table 2.17 and Figures 2.10 and 2.11 show that, in all cases, LF had no effect on the isoelectric precipitation of casein. This is evident from the absence of casein in the pH 4.6-soluble supernatants (Figures 2.10 and 2.11); in the pH 4.6-insoluble precipitates, LF was present. The binding of LF to the caseins in casein micelles observed in the current study is in agreement with the studies of Anema and Kruif (2011) and Croguennec *et al*. (2012).

Table 2.16 Approximate concentration of lactoferrin (Lf) (mg/mL) of the first six milkings from cows A to F and bulk mature milk samples 1 to 3, as calculated by densitometirc analysis of SDS-PAGE gels, in relation to isoelectric precipitability at pH 4.6.

Sample	Lf mg/mL	precipitate at pH 4.6 yes/no
A1	7.99	**No**
A2	3.1	Yes
A3	1.32	Yes
A4	1.05	Yes
A5	1.03	Yes
A6	0.87	Yes
B1	3.43	**No**
B2	1.45	Yes
B3	1.44	Yes
B4	1.15	Yes
B5	1.12	Yes
B6	0.77	Yes
C1	3.47	Yes
C2	0.94	Yes
C3	0.74	Yes
C4	0.89	Yes
C5	0.73	Yes
C6	0.68	Yes
D1	13.45	**No**
D2	10.21	**No**
D3	6.48	**No**
D4	3.35	**No**
D5	1.59	Yes
D6	1.02	Yes
E1	12.73	**No**
E2	4.26	**No**
E3	2.46	Yes
E4	1.95	Yes
E5	1.48	Yes
E6	1.42	Yes
F1	14.29	**No**
F2	5.32	**No**
F3	2.82	Yes
F4	1.86	Yes
F5	1.58	Yes
F6	1.17	Yes
Bulk 1	0.77	Yes
Bulk 2	0.67	Yes
Bulk 3	0.64	Yes

Table 2.17 Mean values (± S.D., n=3) for pH 4.6-soluble protein for bulk mature milk enriched with lactoferrin at 0 to 25.6 mg/mL. Values with different superscript letters were significantly different ($p < 0.05$).

Lactoferrin addition (mg/ml)	precipitate at pH 4.6 yes/no	soluble protein at pH 4.6 (% of total protein)
0	yes	16.48 [a] ± 0.11
0.2	yes	16.91 [a] ± 0.43
0.4	yes	16.12 [a] ± 0.11
0.8	yes	16.76 [a] ± 0.05
1.6	yes	18.52 [b] ± 0.49
3.2	yes	19.68 [c] ± 0.70
6.4	yes	22.65 [d] ± 0.22
12.8	yes	28.43 [e] ± 0.09
25.6	yes	32.69 [f] ± 0.46

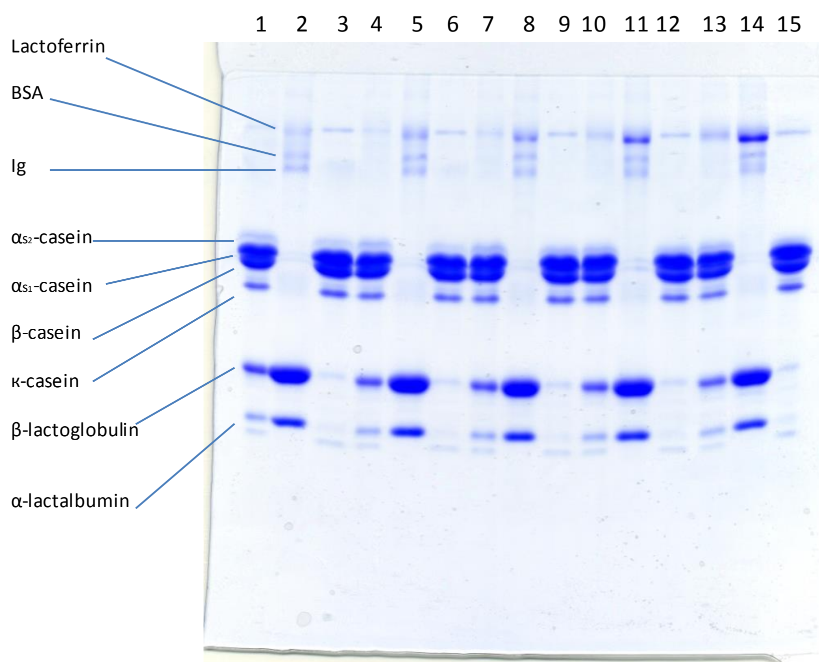


Figure 2.10 Sodium dodecyl sulphate-polyacrylamide gel electrophoretograms obtained under reducing conditions. Crude, pH 4.6-soluble and pH 4.6-insoluble fractions of mature milk with 0 mg/mL (lanes 1-3, respectively), 0.2 mg/mL (lanes 4-6, respectively), 0.4 mg/mL (lanes 7-9, respectively), 0.8 mg/mL (lanes 10-12, respectively) and 1.6 mg/mL (lanes 13-15) added lactoferrin. Thirty micrograms of protein were loaded for crude and pH 4.6-soluble samples, while 31.5 µg protein were loaded for pH 4.6-insoluble samples.

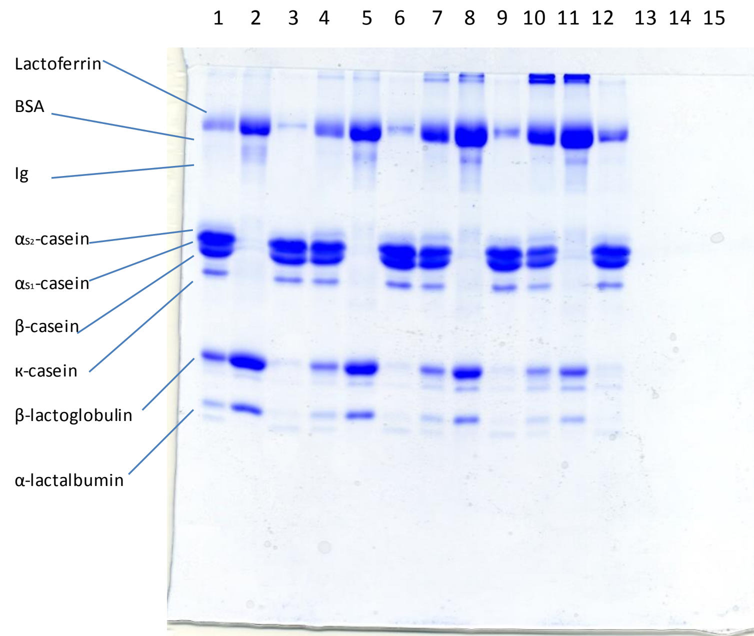


Figure 2.11 Sodium dodecyl sulphate-polyacrylamide gel electrophoretograms obtained under reducing conditions. Crude, pH 4.6-soluble and pH 4.6-insoluble fractions of mature milk with 2 mg/mL (lanes 1-3, respectively), 6.4 mg/mL (lanes 4-6, respectively), 12.8 mg/mL (lanes 7-9, respectively) and 25.6 mg/mL (lanes 10-12, respectively) added lactoferrin. Thirty micrograms of protein were loaded for crude and pH 4.6-soluble samples, while 31.5 µg protein were loaded for pH 4.6-insoluble samples.

2.3.11.5 Effect of adding colostral Ig to mature milk on isoelectric precipitation of casein

The solubility of Ig in colostrum varies according to its ionic strength. Based on this principle, Ig was purified from colostrum by precipitation with ammonium sulphate. A colostrum sample collected immediately after parturition was used because, as previously discussed, the concentration of Ig in colostrum is highest initially. Lanes 3-7 (Figure 2.12) show purified Ig. The first migrating band is IgM, followed by LF, IgG heavy chain, IgG light chain, β-Lg, and α-La. Lanes 8-12 show the soluble material left after ammonium sulphate precipitation. It can be seen from Figure 2.12 that IgG was selectively enriched from the pH 4.6-soluble fraction of skimmed colostrum.

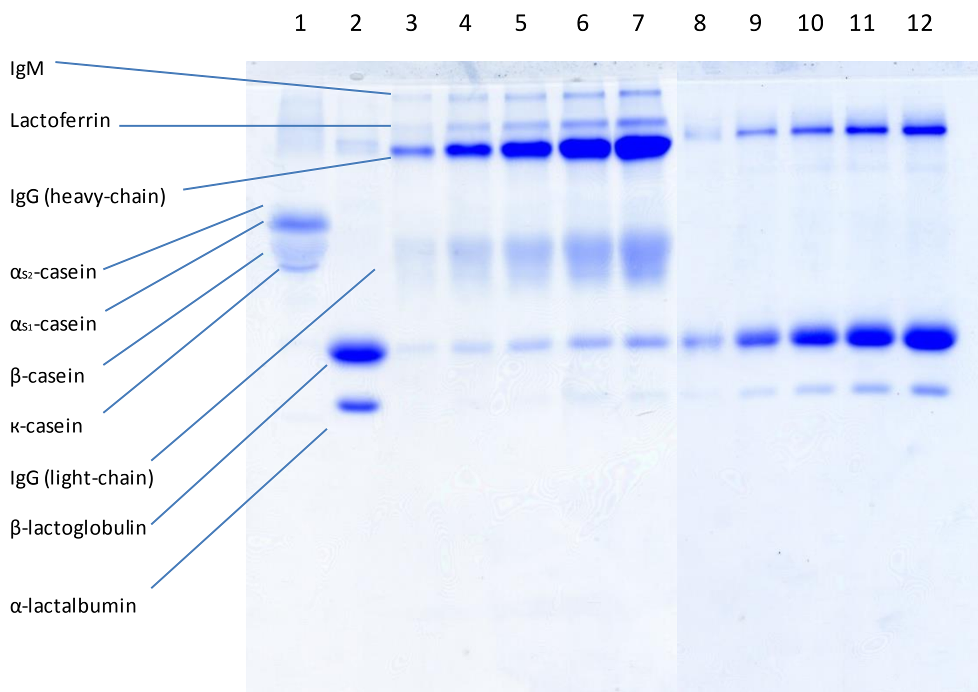


Figure 2.12 Sodium dodecyl sulphate-polyacrylamide gel electrophoretograms obtained under reducing conditions; Lane 1, sodium caseinate (30 μg); Lane 2, whey protein isolate (30 μg); Lane 3-7, Ig purified from colostrum (10 mg/mL) loaded at 1, 2, 3, 4, 5 μl, respectively; Lanes 8-12, supernatant of Ig purified from colostrum (10 mg/mL) loaded at 1, 2, 3, 4, 5 μl, respectively.

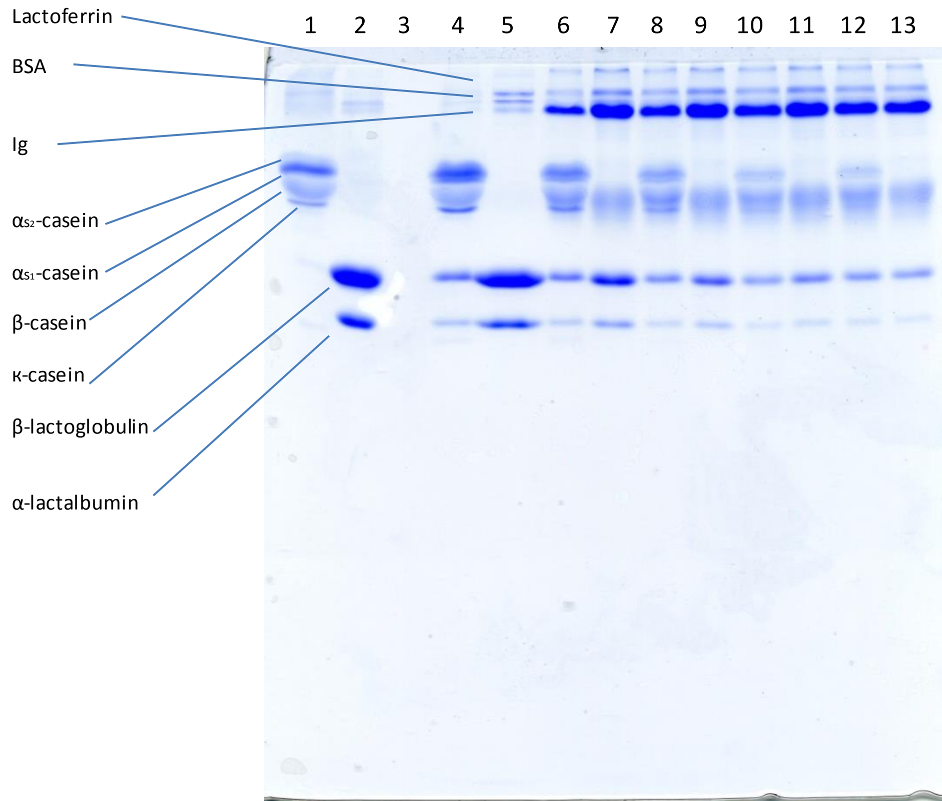


Figure 2.13 Sodium dodecyl sulphate-polyacrylamide gel electrophoretograms obtained under reducing conditions. Lane 1, sodium caseinate; Lane 2, whey protein isolate; Crude and pH 4.6-soluble fraction of mature milk with 0 mg/mL (lanes 4-5, respectively), 25 mg/mL (lanes 6-7, respectively), 50 mg/mL (lanes 8-9, respectively), 100 mg/mL (lanes 10-11, respectively), and 150 mg/mL (lanes 12-13, respectively) added Ig preparation. All samples were loaded on an equal protein basis (30 μg).

As discussed earlier, the principal Ig in colostrum is IgG_1, representing more than 90% of this protein fraction. The effect of elevated levels of IgG on the isoelectric precipitability of casein in mature milk was investigated. Colostrum samples were found to contain highly variable levels of IgG heavy chain, ranging from 1.43 to 41.59 mg/mL (Table 2.3). IgG was purified from colostrum and was added to mature milk at concentrations ranging from 0 to 15% (w/v); elevated levels of IgG had no effect on isoelectric precipitation of casein in this range. This was evident from the absence of casein in pH 4.6-soluble samples, as shown in Figure 2.13.

2.3.12 Multivariate statistical analysis

General linear model analysis of variance (GLM ANOVA) was used to assess the effect of individual cow variability (A to F) and time of milking (1st to 6th milking *post partum*) on the composition and physico-chemical properties of colostrum, i.e., L*, a*,b*, pH, protein concentration, non-sedimentable protein at 4 and 20°C, sedimentable protein at 4 and 20°C, minerals (Na, Mg, P, K, Ca), casein micelle diameter, viscosity at 5 and 20°C, soluble protein at pH 4.6 (undiluted, 1:1 dilution, 1:2 dilution), rennet coagulation time and storage modulus (G') after rennet addition. GLM ANOVA used a regression approach to fit data to specified models. In this study, models were selected which minimised S (standard distance of data values from fitted values, i.e., error of standard deviation) and maximized R^2 (variation in data explained by the results) and adjusted R^2 (variation in data corrected for degrees of freedom in the error) values. Individual evaluation of each variable response made it possible to identify how individuality of each cow and time of milking affected the responses, but, not all variable responses behaved in a similar manner, which meant that GLM ANOVA models had to be adjusted to accommodate the large variability for colostrum samples. For example, examination of scatter-plots of pH values of colostrum revealed that pH generally increased with time *post partum* but, the increase was not consistent across cows, which made it impossible to identify a consistent linear or quadratic effect, therefore, residual *versus* fits and residual *versus* order plots showed that the pH of colostrum from cow D had some 'extreme' values compared with colostrum from other cows (although in pH terms this was only a difference of 0.05 pH units). Scatter-plots for protein concentration showed the same general effect for all samples over time (results not shown), but the protein concentration in the first milking from each cow was highly variable, and so GLM ANOVA had to be adjusted to accommodate this.

For all parameters measured, except concentration of K, there was a statistically significant effect ($p < 0.05$) of individual cow and time of milking, and a significant interaction ($p < 0.05$) between each cow and time of milking. Unusual observations within responses were noted where standardized residuals differed from zero by

more than 2.00, e.g., a* values of the first and second milking of cows E and F were high, as was the viscosity of the first, second and third milking from cow D, measured at both 4 and 20°C.

To simplify the multivariate nature of the data set and find patterns in observations for the samples over time, principal component analysis (PCA) was used. PCA is a statistical procedure that uses orthogonal transformation to convert a set of observations of potentially correlated variables into a set of values of linearly uncorrelated variables called principal components. The first principal component has the largest possible variance, i.e., accounts for as much of the variability in the data as possible, and each succeeding component in turn has the highest variance possible under the constraint that it be orthogonal to, i.e., uncorrelated with, the preceding components. In the current study, scree plots (eigenvalue profile plots in descending order) were examined to judge the relative magnitude of eigenvalues and to visually assess which principal components accounted for most of the variability in the data. Eigenanalysis of the correlation matrix revealed that the first principal component (PC1) accounted for 60% of variation of data and the second component (PC2) accounted for 15%, giving a cumulative value of 75%, indicating that these two components accounted for a large percentage of the variation in data. Thus, most of the structure in the data could be captured with these two underlying dimensions.

The correlations between the original data for each variable and principal component were examined in the Minitab output and the loading plot (Figure 2.14). The first PC was strongly correlated with 13 of the variables measured (L*, protein concentration, non-sedimentable protein at 4 and 20°C, sedimentable protein at 4 and 20°C, Na, Mg, and Ca concentration, casein micelle diameter, and soluble protein at pH 4.6 [undiluted, 1:1 dilution, 1:2 dilution]). All correlations were positive except that for L*, which had a large negative correlation with PC1. Time of milking increased going from right to left on the loading plot. On the PC1 scale, moving left, L* increased with milking number while mineral content and protein concentration decreased. The largest positive correlation was found for protein content. PC1 increased with these 13 variables, indicating that these

criteria were related, e.g., when L* increased, the protein content decreased, as would be expected. pH was also negatively correlated with PC1, although its loading value was considerably lower. PC2 had a large positive correlation with a*, K and P contents, while it was strongly negatively correlated with viscosity at both 4 and 20°C; the lower a PC2 loading, the higher was sample viscosity.

The length of a variable line (in blue) on the loading plot is an indication of the degree of variability, i.e., longer lines have a higher overall variance. The cosine of the angle between lines is an approximate correlation between the variables they represent. The closer the angle is to 90 or 270 degrees, the smaller the correlation. An angle of 0 or 180 degrees reflects a correlation of 1 or -1, respectively. Rennet coagulation times of colostrum showed the least variation throughout the study, as indicated by their having the shortest variance line (Figure 2.14).

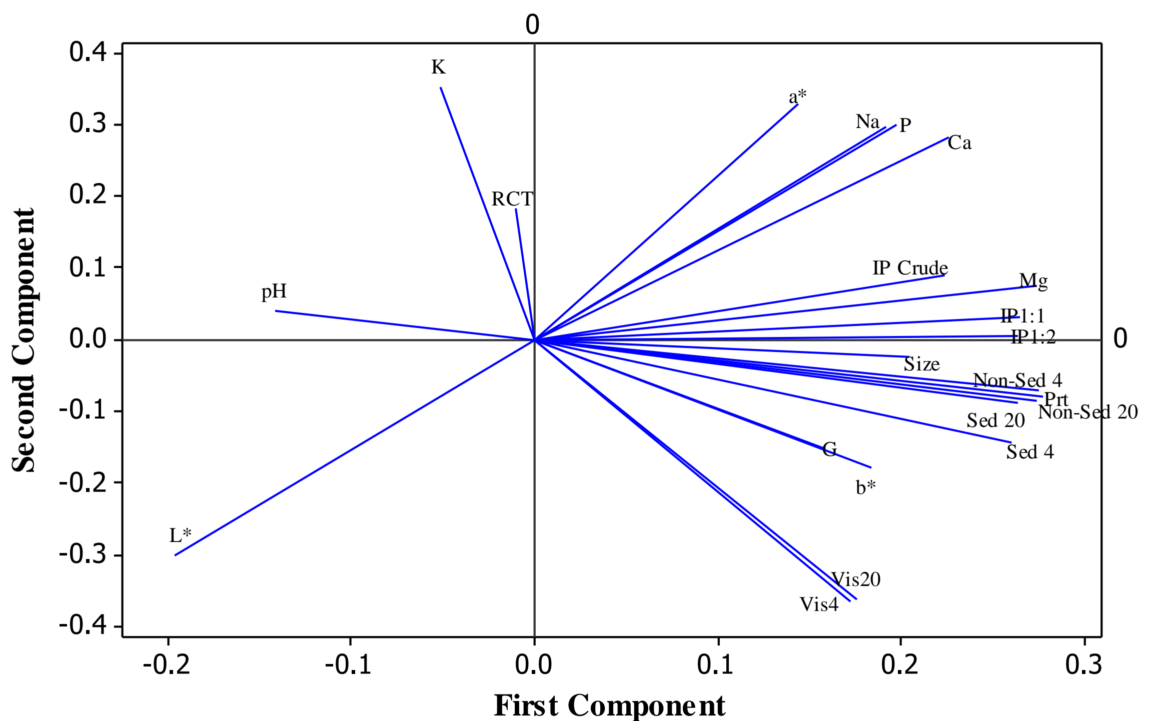


Figure 2.14 Loading plot from PCA showing the relationship between 22 variables of the first six milkings from cows A to F: L*; a*; b*; pH; Prt, protein concentration; Non-Sed 4 and Non-Sed 20, non-sedimentable protein at 4 and 20°C, respectively; Sed 4 and Sed 20, sedimentable protein at 4 and 20°C, respectively; minerals (Na, Mg, P, K, Ca); Size, casein micelle diameter; Vis 5 and Vis 20, viscosity at 5 and 20°C, respectively; IP Crude, IP 1:1 and IP 1:2, soluble protein at pH 4.6 (undiluted, 1:1 dilution, 1:2 dilution), respectively; RCT, rennet coagulation times; and G, storage modulus (G') after rennet addition.

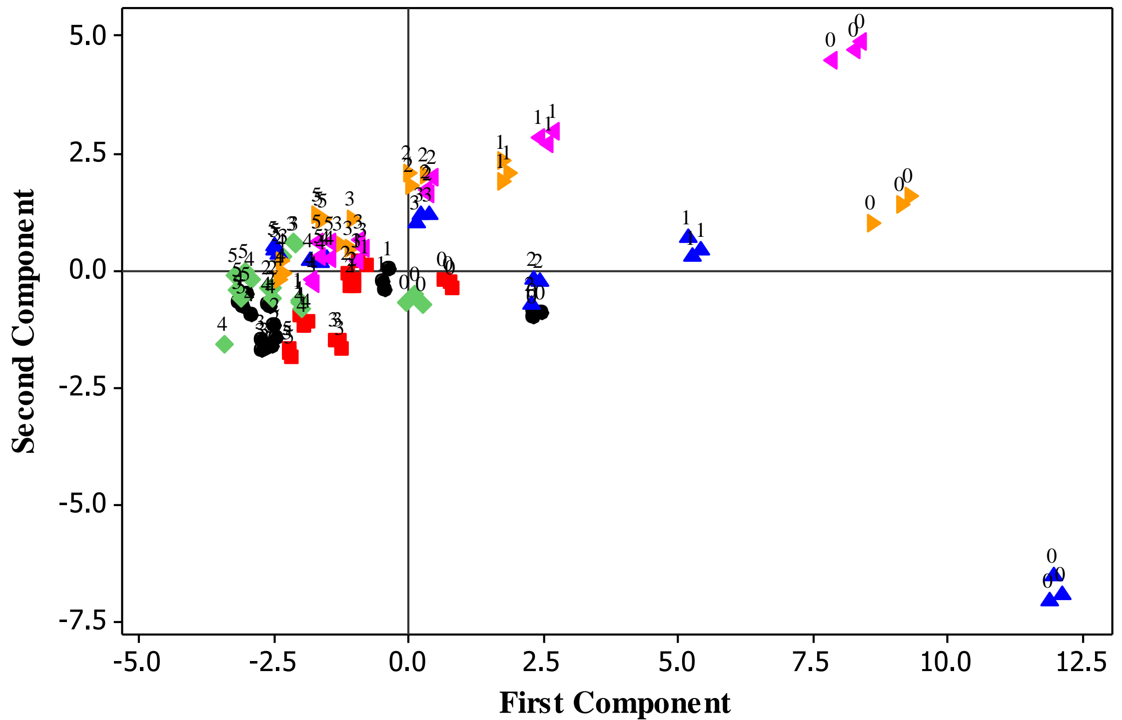


Figure 2.15 Score plot from PCA showing the relationship between individual cows and milking number. 0, 1st milking; 1, 2nd milking; 2, 3rd milking; 3, 4th milking; 4, 5th milking; 5, 6th milking, for cow ●, A; ■, B; ♦, C; ▲, D; ▲, E and ▲, F. Milking number increases from right to left.

While the loading plot from PCA can be used to interpret the relationship between the 22 variables, the score plot (Figure 2.15) is a summary of the relationship among the observations or samples. Grouping by milking number showed how observations generally shifted from right to left, which was strongly influenced by increasing L* values and decreasing mineral and viscosity values. The greatest variation in data occurred at the first milking but, as time progressed, the values tended to converge, with significantly less variation. The first milking of cow D stands alone in the bottom right of the score plot. The overall pattern on the score plot of samples A, B and C is quite similar to one another, as are the patterns for samples E and F. The largest overall change in the measured variables over time occurred for samples D, E and F, as can be seen from the position of the first milking of cows E and F (top right hand corner) and the first milking of cow D (bottom right) and, to a lesser extent, the second milking of cow D.

By comparing the score and loading plots directly in the form of a biplot (Figure 2.16), some relationships between samples and variables could be identified. An overlay of the correlation structure of the variables seen in the loading plot (Figure 2.14) and clustering of observations was revealed. On a biplot, the closer a sample is to each variability line, the more influence that variable has on the sample, e.g., the exceptionally high viscosity of early milkings from cow D, measured at both 4 and 20°C, explains some of the difference between colostrum from cow D with colostrum from other cows. Samples positioned to the right on the biplot were dominated by variables on the right, while those to the left were dominated by variables on the left. Most variables on the biplot were grouped closely together on the right, while the sample observations cluster was on the left, with a few samples grouped on the right. The strong negative correlation of L* values with the first principal component is evident, as is the negative correlation with pH. In general, the biplot shows that colostrum from different cows behaves in a similar trend over time, but some have a wider range of movement around the first and second principal component. The overall pattern was that the composition and physico-chemical properties of colostrum, most notably from cows D, E, and F, were highly variable, in particular at the first, second and third milkings.

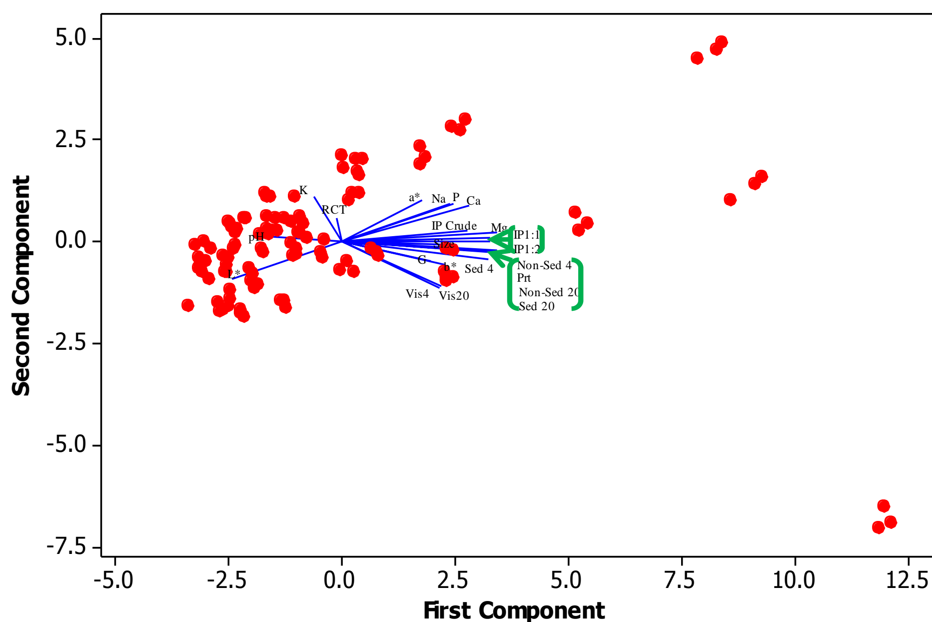


Figure 2.16 Biplot from PCA showing both the loadings and the score for the first and second principal components. Blue lines are the variable lines, while red dots are the observations.

2.4 Conclusion

The composition and physico-chemical properties of colostrum over the first three days *post partum* were highly variable, not only compared to mature milk, but also between individual cows and consecutive milkings. In general, the highest variability was observed between the first and second milking. Most notably, the protein concentration in colostrum was very high initially and decreased with time *post partum*. The level of lactose was low initially and increased with time *post partum*, while early colostral milkings contained significantly higher levels of oligosaccharides than later milkings and mature milk samples. In general, colostrum samples contained elevated levels of Na, Mg, P, K and Ca, which decreased with time *post partum*.

Several authors have examined differences in the composition between colostrum and mature milk, but very little work has been carried out on the physico-chemical properties. The pH of colostrum was very low initially; the exact reason for this was unknown and warrants further investigation. A significant proportion of the casein, in particular in early milkings, was non-sedimentable at 100, 000 g x 1 h, most likely due to the high viscosity of colostrum. The colour of colostrum was highly variable, especially in early milkings, which had a reddish-yellow colour due to the presence of carotenoid pigments and red blood cells. Colostrum had poor heat stability due to a low pH and high whey protein content and early milkings were the most heat-labile. Colostrum had good rennet coagulation properties, i.e., lower RCT and higher G' values than mature milk, but poor acid gelation properties. Casein did not precipitate at pH 4.6 in certain colostrum samples, but did at lower pH values, indicating a shift in pI, possibly due to high levels of glycosylated κ-casein. Further work is required to determine the exact cause of non-precipitability of casein at pH 4.6 in certain colostrum samples.

Results from the current study indicate that, at the sixth milking post partum, milk may still be considered as colostrum and consequently would not be suitable for dairy processing due to its highly variable composition and physico-chemical properties, i.e., high level of minerals, low level of lactose and poor heat stability,

which would lead to problems for many processes, including UHT treatment, sterilisation, and powder production.

2.5 Acknowledgements

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Chapter 3

Characterisation of bovine colostrum and potential anti-inflammatory and insulinotrophic properties of fractions thereof

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Declaration: Fractionation and characterisation of colostrum were performed by Brian McGrath at University College Cork. Mass spectrometry was performed by Michael Kinsella, Aisling Robinson, Paula O'Conor and Brian McGrath at University College Cork, University College Dublin and Teagasc, Moorepark Food Research Centre. Fatty acid analysis was performed by Alan Hennessy at Teagasc, Moorepark Food Research Centre. Cell exposures and cytokine analysis were performed by Kieran Holohan, Maeve McArdle and Sarah Flynn at University College Dublin. All experimental data/results were analysed and the chapter written by Brian McGrath.

Abstract

The aim of this study was to generate and characterise various fractions of bovine colostrum and to test the effect of these fractions on the secretion of pro- and anti-inflammatory cytokines from a macrophage cell line and bone marrow dendritic cells, as well as insulin secretion from a pancreatic beta cell line. Colostrum was fractionated into cream, casein, whey, 30 kDa retentate of whey and 30 kDa permeate of whey. Casein from colostrum had no inflammatory or insulinotrophic properties, while colostrum cream caused a significant reduction in the secretion of TNF-α, a central pro-inflammatory cytokine, without compromising macrophage cell viability. Colostrum cream was further fractionated to produce buttermilk, which was found to significantly reduce the secretion of NF-κB, the key transcription factor that regulates the inflammatory response, as well as significantly reducing the secretion of the pro-inflammatory cytokines TNF-α, IL-6, IL-1β and IL-12. This buttermilk also caused a significant increase in the secretion of the anti-inflammatory cytokine IL-10. Buttermilk from colostrum was analysed by two dimensional gel electrophoresis and mass spectrometry, and major milk fat globule membrane (MFGM) proteins such as xanthine dehydrogenase/oxidase, lactadherin, adipophilin and butyrophilin were identified. Colostrum whey, 30 kDa retentate of whey and 30 kDa permeate of whey were all found to increase significantly insulin secretion when administered to pancreatic beta cells in combination with 16.7 mM glucose, at levels similar to that obtained with the positive control. Cell viability was not affected by the presence of colostrum fractions and increased insulin secretion was not due to the presence of growth factors in colostrum. The 30 kDa permeate was the most potent activator of insulin secretion *in vitro,* and the biological activity was found to be most concentrated in the 3 kDa permeate of this fraction. Based on the findings of this study, colostrum buttermilk and whey may represent novel functional food products with immunomodulatory and insulinotrophic properties.

3.1 Introduction

The World Health Organization (WHO) reports that at least one billion adults are overweight and 300 million are obese and, without intervention, these numbers are expected to rise (WHO, 2010). In Europe, >$1x10^6$ deaths annually are attributable to diseases related to excess bodyweight (WHO, 2006a). Substantial evidence indicates that obesity is associated with a state of chronic low-grade inflammation characterised by inflamed adipose tissue, with increased infiltration of macrophages that produce pro-inflammatory cytokines (McArdle *et al*., 2013). These cytokines directly reduce insulin sensitivity through the insulin-signalling pathway (Peraldi *et al*., 1996) by activation of the mitogen-activated protein kinases and the activation of transcription factors such as NF-ΚB, causing both the down-regulation and decreased activation of insulin-signalling proteins (McArdle *et al*., 2013). The inflammatory trigger in obesity is metabolic and caused by the excess consumption of nutrients. Metabolic signals are produced by metabolic cells (such as adipocytes), which start the inflammatory response and damage metabolic homeostasis (Gregor and Hotamisligil, 2011). Adipose tissue traditionally functions in lipid storage, storing triacylglycerides (TAG) following energy excess, and mobilizing these stores during periods of nutrient deprivation (Gregoire *et al*., 1998). With obesity and insulin resistance there is increased lipolysis; consequently, there is an inappropriate spill-over of triacylglycerol (TAG)-derived free fatty acids (FFAs) (Sethi and Vidal-Puig, 2007) and these FFAs can activate inflammatory pathways and impair insulin signalling. Macrophages are known to be important contributors to inflammation; however, adipocytes also demonstrate significant intrinsic inflammatory properties (Berg and Scherer, 2005). Adipose tissue acts as an endocrine organ, secreting biologically active substances including interleukin (IL)-6, IL-1β, TNF-α, leptin and adiponectin (McArdle *et al*., 2013). Macrophages can be classified based on their cytokine secretion profile; M1 macrophages secrete pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-12, whereas M2 macrophages secrete anti-inflammatory cytokines including IL-10 and IL-1 receptor antagonist (Lumeng *et al*., 2007).

Approaches to modulating the release of pro- and anti-inflammatory cytokines may minimise a pro-inflammatory insulin resistance state. Pharmaceutical strategies that target inflammation may have some potential. Currently, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used of therapeutic agents (McGettiga and Henry, 2013). They reduce pain and inflammation by blocking cyclo-oxygenases (COX) that are needed to produce prostaglandins (Cashman, 1996). However, there are a number of safety concerns, as NSAIDs are known to have serious adverse effects, including gastrointestinal bleeding, peptic ulcer disease, hypertension, edema and renal disease (Dugowson and Gnanashanmugam, 2006).

Recent studies have suggested that the consumption of dairy products is inversely associated with low-grade systemic inflammation (Esmaillzadeh and Azadbakht, 2010; Panagiotakos *et al*., 2010). Milk proteins may represent a novel alternative to pharmaceutical strategies which target inflammation, while they may be less potent than some pharmaceutical anti-inflammatory agents, which may be beneficial for long-term therapy. The current study focussed on proteins from colostrum as it is rich in biologically active constituents, which are also most likely present in mature milk but at significantly reduced levels.

Colostrum is the initial milk secreted by mammals and is typically defined as the first five to six milkings *post partum.* The composition of bovine colostrum is highly variable and differs substantially from that of mature milk (Foley and Otterby, 1978; Madsen *et al*., 2004; Tsioulpas *et al*., 2007). The main factors responsible for variation in colostrum composition are: time *post partum*, individuality of the animal, breed, mastitic infection, and feed (Walstra et al., 2006). Colostrum is a complete source of nutrients such as protein, carbohydrates, fat, vitamins and minerals, but is also rich in biologically active constituents. These include growth factors, immunoglobulins, lactoperoxidase, lysozyme, lactoferrin, cytokines, nucleosides, vitamins, peptides and oligosaccharides, which are all either not present in mature milk or are present at substantially lower concentrations (Wu *et al*., 2011). Hagiwara *et al*. (2000) demonstrated that colostrum contains significantly higher concentrations of the cytokines IL-1β, IL-6, TNF-α, INF-γ and IL-1

than mature milk. The natural function of these bioactive components is to provide passive immunity to the newborn calf, as well as facilitating the growth and immune maturation of the digestive tract (Kelly, 2003).

Bovine colostrum has a wide range of reported biological activities and clinical uses. Products made from bovine colostrum have been marketed for their health benefits for many years and are generally regarded as safe (Davis *et al*., 2007). Boosting the natural concentrations of immune components in colostrum through vaccination of cows offers great potential in the development of hyperimmune colostrum products for prophylactic or therapeutic use in humans (Stelwagen *et al*., 2009). A commercial example of a hyperimmune milk product is the range of Stolle products (Stolle Milk Biologics Inc., Cincinnati, OH) which contain elevated levels of IgG brought about by vaccination of lactating cows with a multivalent killed bacterial antigen. Mitra *et al*. (1995) demonstrated that hyperimmune colostrum from cows vaccinated with human rotavirus was an effective therapeutic in reducing the severity and duration of rotavirus-caused diarrhea in infants. Tzipori *et al*. (1986) reported that bovine colostrum might be of benefit for the treatment of chronic diarrhea among persons with immune deficiency syndromes. Bovine colostrum has also been used for the prevention or treatment of infectious diseases such as cholera, cryptosporidiosis, Helicobacter pylori, rotavirus and shigellosis (Kelly, 2003). Playford *et al*. (1999) and Playford *et al*. (2001) reported that bovine colostrum prevents non-steroidal anti-inflammatory drug (NSAID) induced gut injury.

The objectives of this study were (1) to generate and characterise various fractions of bovine colostrum, (2) to test the effect of these fractions on the secretion of pro- and anti-inflammatory cytokines from a murine macrophage cell line and bone marrow dendritic cells, (3) to test the effect of these fractions on insulin secretion from a pancreatic beta cell line.

3.2 Materials and Methods

3.2.1 Collection of bovine colostrum

Colostrum samples were obtained from the Teagasc, Moorepark, Animal and Grassland Research and Innovation Centre, Fermoy, Co. Cork. Colostrum samples from either the first or second milking *post partum*, within 0 to 24 h of calving, were collected from three individual Holstein Friesian cows. The colostrum samples were mixed and immediately frozen and stored at -20°C. A bulk mature milk sample from cows in mid-lactation was also obtained for purposes of comparison.

3.2.2 Processing of colostrum

Samples were thawed at 4°C overnight. After thawing, the lipid fraction was removed by centrifugation at 3,000 g for 15 min at 4°C (Sorvall RC-5C, DuPont, Wilmington, DE, USA). The skimmed colostrum was filtered through glass wool and Whatman no. 113 filter paper. Colostrum cream and skimmed colostrum were immediately frozen and stored at -20°C.

3.2.3 Preparation of pH 4.6-soluble fractions

Casein and whey were prepared from skimmed colostrum by isoelectric precipitation according to the method of Kuchroo and Fox (1982), as described in Section 2.2.11. Portions of the pH 4.6-soluble and insoluble material were then lyophilised.

The pH 4.6-soluble fraction was further fractionated by ultrafiltration (20°C, regenerated cellulose membrane, 30 kDa nominal molecular weight limit, Merck Millipore Ltd, Cork, Ireland) including a 2X diafiltration step using ultra-pure water (Elga purelab option-Q, Veolia Water Solutions and Technologies, Marlow, UK). The 30 kDa retentate and permeate were then separately lyophilised.

The lyophilised 30 kDa permeate was reconstituted (4%, w/v) in ultra-pure water and left to hydrate overnight at 4°C. The solution was further fractionated by ultrafiltration (20°C, regenerated cellulose membrane, 3 kDa nominal molecular weight limit, Merck Millipore Ltd, Cork, Ireland) including a 2X diafiltration step using ultra-pure water (Elga purelab option-Q, Veolia Water Solutions and Technologies, Marlow, UK). The 3 kDa retentate and permeate were then separately lyophilised.

3.2.4 Preparation of buttermilk

Buttermilk was prepared from colostrum by mechanical agitation of raw cream in the presence of air. Briefly, an equal volume of distilled water was added to cream and heated to 50°C. The solution was cooled and processed into butter at 20°C using an overhead stirrer (Kenwood Food Processor, model no. 8910D, Thorn EMI, Hants, UK) until fat granules appeared; the granules were kneaded at *ca.* 20 rpm to release the buttermilk. The buttermilk was cooled to 4°C and filtered through cheese cloth to remove any residual butter grains. A portion of the filtrate was then lyophilised. Buttermilk was prepared from mature milk in the same manner for comparison purposes.

3.2.5 Protein content

Protein content was determined by the macro-Kjeldahl method (IDF, 1986), as described in Section 2.2.2. In some cases, the Bradford assay was used. Briefly, a standard curve was generated from a 5 mg/mL working solution of bovine serum albumin (BSA). BSA standards and samples to be quantified were prepared in triplicate. Samples were typically diluted 1:50 with distilled H_2O. Diluted (1:5) protein assay concentrated dye reagent (200 µl) was added to 10 µL of the BSA standards and diluted samples in 96 well plates (Greiner Bio-One Ltd., Stonehouse, UK). Following 15 min of incubation at room temperature, the absorbance of each sample was read at 595 nm (Varioskan Flash Multimode Reader, Thermo Scientific,

Waltham, MA, USA). The protein concentration was then determined from the standard curve.

3.2.6 One-dimensional gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Laemmli (1970), as described in Section 2.2.13.

3.2.7 Two-dimensional gel electrophoresis (2-DE)

2-DE was performed on the pH 4.6-soluble fractions of buttermilk and serum from colostrum or mature milk. Lyophilised samples were reconstituted (10% w/v) in distilled water. Samples were adjusted to pH 4.6 by adding 30 µL of 33% acetic acid to 1 mL sample and vortexing the mixture; after 10 min, 30 µL of 3.3 M-Na-acetate was added and the mixture centrifuged for 30 min at 14,000 g at 20°C. After centrifugation, the supernatant was carefully removed and used for further analysis. Isoelectric focusing (IEF) was carried out using a PROTEAN IEF (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The pH 4.6-soluble fraction of each sample was diluted accordingly with a rehydration buffer (8 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM dithiothreitol (DTT), 0.2% Bio-Lyte 3/10 ampholyte, 0.001% Bromophenol Blue) to give a final volume of 125 µL and concentration of 500 µg protein. Samples (125 µl) were loaded on to immobilised pH gradient (IPG) strips (pH 4-7, 7cm, Bio-Rad) which were then passively hydrated for 8 h, followed by active rehydration for 8 h. The IPG strips were then focused at 20°C until 20 kV was reached. After focussing, the strips were immediately used for second-dimension sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out under reducing conditions using a 15% acrylamide gel, in a Criterion© Dodeca Cell unit (Bio-Rad, USA). The focussed IPG strips were equilibrated with 50 mM Tris/HCl,

pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 130 mM DTT and alkylated in the same buffer containing 130 mM iodoacetamide (IAA) instead of DTT. The gels were then run at 200 V for 50 min, stained using colloidal Coomassie blue (Chevalier *et al*. 2004) and images for stained gels were digitised at 300 dpi using a calibrated GS-800 densitometer (Bio-Rad Laboratories).

3.2.8 Reversed-phase ultra-performance liquid chromatography (RP-UPLC)

Colostrum fractions were filtered through 0.22 µm pore filters (Millex, low protein binding durapore, syringe driven PVDF membrane; Millipore Ireland Ltd, Tullagreen, Carrigtwohill, Co Cork, Ireland). The filtrate was analysed at room temperature (21°C) by RP-UPLC using a Waters Acquity UPLC H-Class Core System. The column used was an Acquity UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm). The sample was eluted for 0.37 min with 100% solvent A (0.1% trifluoroacetic acid [sequencing grade; Sigma-Aldrich Ireland Ltd, Arklow, Co Wicklow, Ireland] in ultra-pure water [Elga purelab option-Q, Veolia Water Solutions and Technologies, Marlow, UK]), then with a linear gradient to 50% solvent B (0.1% trichloroacetic acid in acetonitrile [HPLC far UV grade; Labscan Ltd, Dublin, Ireland]) over 6.23 min, maintained at 50% B for 0.68 min, then with a linear gradient to 95% B over 0.46 min, maintained at 95% B for 0.68 min, then with a linear gradient to 100% B over 0.45 min, before returning to the original conditions. Eluate was monitored at 214 nm using a Waters Acquity UPLC TUV Detector (dual wavelength) interfaced with Empower 3 software. The flow rate was maintained at 0.46 mL/min.

3.2.9 Mass Spectrometry

3.2.9.1 In-gel digestion

3.2.9.1.1 Manual excision of Coomassie stained protein spots from gels

Coomassie-stained gels from which spots were to be cut were washed in distilled H_2O before being placed on a clean level surface protected with a plastic gel bag.

Spots of interest were manually excised with a 1.5 mm One Touch Plus Spot Picker (Web Scientific, Crewe, Cheshire, UK) using a new pipette tip for each spot. Gel plugs were placed in individual eppendorfs and covered with 100 µL of distilled H_2O in order to prevent dehydration. The samples were stored at 4°C until required.

3.2.9.1.2 Reduction and protection of cysteine residues

Fifty µL of freshly prepared 10 mM DTT/0.1 M ammonium bicarbonate was added to the gel pieces and the samples were incubated at 56°C for 60 min with agitation at 500 rpm (Eppendorf Thermomixer Compact, Sigmaaldrich, St. Louis, MO, USA). The solution was allowed to cool to room temperature and was removed. Fifty µL of freshly made 50 mM IAA/0.1M ammonium bicarbonate was applied to the gel pieces and incubated at 37°C for 30 min in the dark with agitation at 500 rpm. The IAA solution was removed and replaced with 250 µL of 100 mM ammonium bicarbonate for 15 min at 37°C with agitation at 300 rpm. This was removed and replaced with 250 µL of 20 mM ammonium bicarbonate/ACN 1:1 for 15 min at 37°C with agitation at 300 rpm. The ammonium bicarbonate/ACN solution was then replaced by 100 µL of ACN for 10 min at 37°C with agitation at 300 rpm, followed by removal of the ACN solution.

3.2.9.1.3 In-gel tryptic digestion

The gel pieces were re-suspended in 25 µL of trypsin digestion buffer [8 ng/µL sequencing grade modified trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate]. The samples were incubated overnight at 37°C with agitation at 500 rpm (Eppendorf Thermomixer Compact, Sigmaaldrich, St. Louis, MO, USA).

3.2.9.1.4 Extraction of peptides

The eppendorfs containing the samples were briefly spun down and the supernatant was taken off and placed in a fresh eppendorf. Eighty µL of 30%

ACN/0.2% TFA was added to each sample and incubated at 37°C for 10 min with agitation at 500 rpm (Eppendorf Thermomixer Compact, Sigmaaldrich, St. Louis, MO, USA). The samples were spun down briefly and the supernatants pooled with their respective original tryptic supernatants. Eighty µL of 70% ACN/0.2% TFA was added to each sample and incubated at 37°C for 10 min with agitation at 500 rpm. The samples were spun down briefly and the supernatants combined with their respective samples. The pooled supernatants were then dried in a vacuum centrifuge (Eppendorf Concentrator 5301, Hamburg, Germany) at 45°C.

3.2.9.2 In-solution digestion

In-solution digestion of complex protein mixtures (100 µg) was performed as described in Sections 3.2.9.1.2 to 3.2.9.1.4.

3.2.9.3 LTQ linear ion trap mass spectrometry

Samples were analysed by liquid chromatography mass spectrometry (LC-MS) on an LTQ linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) connected online to a surveyor chromatography system incorporating an autosampler. Dried tryptic samples were re-suspended in 0.1% formic acid and separated on a modular CapaLC system (Finnegan) connected directly to the source of the LTQ. Samples were loaded onto a Biobasic C18 Picofrit™ column (100 mm length, 75 µm ID) at a flow rate of 30 nL/min and eluted from the column by an increasing acetonitrile gradient. The gradient length for a LC separation is dependant upon the complexity of the sample and a 20 min gradient was used for 2-DE spots. The mass spectrometer was operated in positive ion mode with a capillary temperature of 200°C, a capillary voltage of 46 V, a tube lens voltage of 140 V and a potential of 1800 V applied to the frit. Data was collected by the mass spectrometer operating in automatic data dependent switching mode. A zoom scan was performed on the 5 most intense ions to determine charge state prior to MS/MS analysis. MS/MS spectra were sequence database searched using TurboSEQUEST (Xcalibur Software, Thermo Fisher Scientific, Waltham, MA, USA).

The MS/MS spectra were searched against the non-redundant UniProt/SwissProt (release 6.0) database. Methionine oxidation and cysteine carboxyamidomethylation were specified as variable modifications. The precursor-ion mass tolerance was set at 1.5 Da, fragment ion tolerance was set at 1.0 Da and a maximum of 2 missed cleavage sites were allowed.

3.2.9.4 *Quadropole time-of-flight (Q-TOF) mass spectrometry*

Samples were analysed by mass spectrometry using a Waters Acquity G2 Q-TOF LC-MS. This system consisted of a Waters Acquity Nano-Flow LC system coupled to a Quadrupole Time-of-flight mass spectrometer. A sample (1 µL) was injected onto a Waters Acquity UPLC BEH C18 column (1.7 µm particle size, 100 mm x 75 µm) with a flowrate of 300 nL/min. The column temperature was maintained at 30°C while samples were refrigerated at 4°C. The nano-flow system was fitted with a trapping column to aid column loading on to the analytical column using 99.5% solvent A (0.1% formic acid [sequencing grade; Sigma-Aldrich Ireland Ltd, Arklow, Co Wicklow, Ireland] in deionised water [Milli Q System; Millipore Corp]) and 0.5% solvent B (0.1% formic acid in acetonitrile [HPLC far UV grade; Labscan Ltd, Dublin, Ireland]) with a flow rate of 15 µL/min and a sample loading time of 1 min. Once the sample was transferred from the trapping column to the analytical column, it was eluted for 0.1 min with 97% solvent A, then with a linear gradient to 40% solvent B over 29 min, then with a linear gradient to 85% B over 2 min, maintained at 85% B for 2 min, then with a linear gradient to 3% B over 1 min and maintained at 3% B for 15 min.

Mass spectrometry detection was conducted through electrospray ionization using an ms^e centroid experiment in positive mode in the m/z scan range 50-2000 Da. The following MS settings were used in conjunction with the nano-flow ESI source: capillary voltage 3.5 kV, sampling cone 20 V, extraction cone 5 V, source temperature 100 °C, desolvation temperature 150 °C, desolvation gas flow 600 L/h, cone gas flow 50 L/h. The accurate mass of the instrument was initially calibrated through direct infusion of a sodium iodide calibrant solution prior to sample

analysis. In addition, leucine enkephalin (Leuenk) lockmass solution (2 ng/uL) and Glu-fib (1 ng/µL) were infused at 500 nL/min using an auxiliary solvent manager and attached to the reference position of the nano-flow source. This reference mixture was scanned, but not applied, to verify exact mass, as this correction was conducted at the data analysis stage. Masslynx v4.1 software (Waters, Millford, Massachusetts) was used to control the instrument while Proteinlynx Global Server (PLGS) software (Waters, Millford, Massachusetts) was used to assist data analysis and aid in determination of peptides present. Within PLGS, a non-specific or tryptic digest was specified and appropriate intensity thresholds of 80 and 20 were used for low and elevated energy thresholds, respectively. Lockmass correction was also conducted in PLGS rather than during data acquisition. A species specific database for *Bos taurus* was downloaded from the Uniprot website and only reviewed hits were included. The analytical data were searched against this database resulting in a number of peptides identified within the samples.

3.2.9.5 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

Samples were analysed by mass spectrometry with an Axima TOF^2 MALDI-TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). Aliquots (0.5 µL) of matrix solution (α-cyano 4-hydroxy cinnamic acid, 10 mg/mL, in 50 % acetonitrile/0.1 % (v/v) trifluoroacetic acid) were deposited onto the target and left for 5 seconds before being removed. The residual solution was allowed to air-dry and 0.5 µL of the sample solution was deposited onto the pre-coated sample spot. Aliquots (0.5 µL) of matrix solution were added to the deposited sample and allowed to air-dry. The sample was subsequently analysed in positive-ion reflectron mode.

Protein identification was carried out via peptide mass fingerprinting (PMF) using the Mascot search engine (http://www.matrix-science.com). The monoisotopic, positive ion data +/- 0.25 Da was searched using the following parameters: NCBInr database or Swiss Prot, taxonomy mammalian, trypsin digest with one missed

cleavage. Variable modifications including cysteine modified by carbamidomethylation, and methionine modified by oxygen were also checked.

Tandem mass spectrometry (MS/MS) was carried out on peaks from spots that did not score well using PMF. The MS/MS positive ion, averaged data +/- 0.8 Da was again searched using the Mascot engine (http://www.matrix-science.com) and the following parameters: NCBInr database or Swiss Prot, taxonomy mammalian trypsin digest with one missed cleavage or semi trypsin with one missed cleavage, variable modifications including cysteine modified by carbamidomethylation, methionine modified by oxygen and/or acetylated.

3.2.10 Fatty acid analysis

Following extraction and methylation according to International Organization for Standardization (ISO) standards 14156:2001 (ISO, 2001) and 15884:2002 (ISO, 2002), the fatty acid methyl esters (FAME) of cream from colostrum or mature milk were separated using a CP Sil 88 column (100 mm, 30.25 mm i.d., 0.20 mm film thickness) (Chrompack, Middelburg, the Netherlands) and quantified using a gas liquid chromatograph (Varian 3400 GC, Harbor City, CA, USA). The GLC instrument was calibrated using a range of commercial fatty acid methyl ester standards. FAMEs were analysed by gas liquid chromatography (GLC) using the parameters described by Childs *et al*. (2008). The GLC instrument was fitted with a flame ionisation detector (FID) and helium (2.55 bar) was used as the carrier gas. The injector temperature was held isothermally at 225°C for 10 min, and the detector temperature was 250°C. The column oven was held at an initial temperature of 140°C for 8 min, and was then increased at a rate of 8.5°C per min, to reach a final temperature of 200°C, which was held for 50 min. The data were recorded and analysed on a Varian Datastar system (Harbor City, CA, USA).

3.2.11 Cell culture (inflammation)

3.2.11.1 *Cell lines and reagents*

NIH3T3/NF-κB-luc fibroblasts were purchased from Panomics (Affymetric Inc, Santa Clara, California, USA). J774.2 macrophages were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Bone marrow-derived mononuclear (BM-MNC) cells were isolated from the femurs and tibia of C57Bl/6 mice.

3.2.11.2 *Cell culture*

NIH3T3/NF-κB-luc fibroblasts, J774.2 macrophages and viable BM-MNC were enumerated by a Trypan blue assay according to the method of Strober (1997). After enumeration, J774.2 macrophages were re-suspended in Dublecco's Modified Eagle's Medium (DMEM, Lonza Walkersville Inc, MD, USA) supplemented with 10% fetal calf serum (FBS, Gibco®, Invitrogen, Paisley, UK) and 1% penicillin/streptromycin. NIH3T3/NF-κB-luc fibroblast were re-suspended in DMEM supplemented with 10% fetal calf serum, 1% penicillin/streptromycin and 0.1% hygromycin B. BM-MNCs were re-suspended in DMEM medium containing L929 conditioned medium and differentiated for 7 days. On day 7, fully differentiated macrophages (BMDM) were treated.

3.2.11.3 *Preparation of lipopolysaccharide (LPS)*

LPS is a cell wall component of Gram-neagtive bacteria such as *Escherichia coli*. LPS binds to the pattern recognition receptor (PRR) Toll-like receptor–4 (TLR 4) to induce a potent inflammatory response and was used in the current study to induce inflammation in NIH3T3/NF-κB-luc fibroblasts, J774.2 macrophages and bone marrow-derived macrophages (BMDM). *E. coli* (Serotype 0111:B4) derived LPS (Alexis, Enzo Life Sciences, Exeter, UK), at 1 mg/mL, was stored at 4°C and diluted to a working concentration prior to use.

3.2.11.4 *Treatment of macrophages with milk samples*

BMDM or J774.2 macrophages in log-growth phase were seeded on 24-well tissue culture plates (Cell Star®, Greiner Bio-One, Stonehouse, UK) with 1.25×10^5 cells in 500 µL of medium and incubated overnight. Following overnight incubation, the medium was removed and replaced with fresh medium. The cells were incubated with various concentrations of lyophilised colostrum or mature milk samples for 24 h. Following 24 h incubation the medium was removed and the cells were washed gently with phosphate buffered saline (PBS, Gibco®, Invitrogen, Paisley, UK). Fresh medium was added to the cells with/without 10 ng/mL LPS (3 h for TNF-α, IL-6 and IL-1β analysis; 24 h for IL-10 and IL-12 analysis). Following LPS stimulation, the supernatant was collected and stored at -20°C for cytokine analysis.

3.2.11.5 *Cytokine secretion*

The supernatant was thawed and centrifuged at 300 x g for 10 min prior to ELISA to pellet cell debris. The concentrations of TNF-α, IL-6, IL-1β, IL-10 and IL-12 were determined using DuoSet ELISA development systems (R&D Systems Europe, Abdingdon, UK).

3.2.11.6 *NF-κB-luciferase reporter assay*

Conditioned medium was aspirated and the cells were rinsed gently with 200 µL of sterile PBS. After rinsing, 50 µL 1X Reporter Lysis Buffer (Promega Corporation, Madison, WI, USA) was added to each well and the plate was gently agitated on a shaker table for 5 min at room temperature to lyse cells. Cell lysate (10 µL) was transferred to a 96 well fluorescence sterile polystyrene Fluoro Nunc white plate (Thermo Fisher Scientific, Waltham, MA, USA). Luciferase Assay Reagent (50 µL) was added to the cell lysate and luminescence was measured using a luminometer (Synergy HT Multi-Mode Micro plate Reader, BioTek, Winooski, VT, USA).

3.2.12 *Cell culture (insulin secretion)*

3.2.12.1 *Culture of the BRIN-BD11 cell line*

BRIN-BD11 cells were grown and maintained in RPMI 1640 tissue culture medium with 10% (v/v) FBS, 0.1% antibiotics (100 units/mL penicillin and 0.1 mg/mL streptomycin) and 11.1 mmol/L D-glucose at 37^oC in a humidified atmosphere of 5% CO_2 and 95% air using a Thermo HEPA class 100 incubator (Thermo Fisher Scientific, Waltham, MA, USA). The cells were gently washed in 10 mL PBS prior to detachment from the tissue culture flask by adding 5 mL of pre-warmed 0.025% (w/v) trypsin containing 1 mM ETDA, and trypsin was removed before cells began to attach. Once detached, cells were re-suspended with 6 mL of pre-warmed tissue culture medium. To maintain culture, 1 mL of cell suspension was seeded back into a tissue culture flask and supplemented with 50 mL of fresh tissue culture medium. For experimental use, cells aliquots of cell suspension (100 μL) were stained with trypan blue (1:2 dilution) and were counted using a Neubauer haemocytometer (Scientific Supplies Co, UK) and were viewed under a phase contrast microscope (Olympus CKX 41). Once the cell count was determined, cells were seeded to the required plates and were allowed to attach overnight as monolayers.

3.2.12.2 *Acute insulin secretion test*

Acute insulin secretion was induced with a high level of glucose (16.7 mM) plus fractions from colostrum or mature milk which are meant to mimic meal conditions where there is a starvation period followed by high glucose plus protein. BRIN-BD11 cells were seeded at a concentration of 1×10^5 cells/well in 1 mL of medium in 24-well plates for 24 h in RPMI-1640 medium containing 11.1 mM D-glucose. The following day, the tissue culture medium was removed gently and the cells were washed with 1 mL warmed PBS. One mL Kreb's Ringer Bicarbonate buffer (KRB) (pH 7.4) (115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2.6H_2O$, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, 0.1% BSA), supplemented with 1.1 mM D-glucose was then added to each well for a 40 min incubation period at 37°C. At the end of the incubation period, the buffer was removed and the cells were washed with 1 mL

pre-warmed PBS to remove any trace of insulin. The cells were stimulated with 1 mL KRB supplemented with 16.7 mM glucose plus 1 mg/mL of colostrum or mature milk fractions for precisely 20 min at 37°C. The buffer was removed from each well and transferred to eppendorf tubes and samples were centrifuged at 100 x g for 5 mins to remove cell debris. The supernatant was collected from each tube and stored at -20°C for subsequent insulin determination by ELISA. Cells were lysed with RIPA (1x) and protein content was determined as above.

3.2.12.3 *Insulin secretion measurement*

The Mercodia Ultrasensitive Rat Insulin ELISA (Mercodia, Uppsala, Sweeden) was used to determine the concentration of insulin in samples. This is a solid phase two-site enzyme immunoassay, which is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with anti-insulin antibodies bound to microtitration wells and peroxidase-conjugated anti-insulin antibodies. A washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3, 3’, 5, 5’-tetramethylbenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm. The exact concentration of insulin in each sample was extrapolated from a standard curve.

3.2.12.4 *Procedure for insulin secretion measurement*

Standards (50 µl) ranging from 0.02-1.0 µg/L or test samples (colostrum or mature milk fractions) were added to separate anti-insulin wells. Conjugate solution (50 µL), which contains the peroxidase conjugated mouse monoclonal anti-insulin antibody in conjugate buffer, was added to each well and the plate was then placed on a plate shaker at room temperature for 2 h. Following incubation, the conjugate buffer was removed and the wells were washed 6 times with 350 µL ELISA wash buffer. After the final wash the plate was inverted and tapped firmly against absorbent paper. Peroxidase substrate (TMB) (200 µl) was then added and

incubated at room temperature for 30 min. To stop the reaction, 50 µL of stop solution (1 M H_2SO_4) was added and mixed on a plate shaker for 5 seconds. The plate was then read at 450 nm using a Molecular Devices V_{max} kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). Insulin content present in each well was extrapolated from the standard curve.

3.2.12.5 *Cell proliferation assay - WST-1*

The WST-1 assay is a colorimetric assay for the quantification of cell proliferation and cell viability, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. BRIN-BD11 cells (4×10^4) were seeded in a 96-well plate in 250 µL of medium and were left to adhere overnight. Control wells containing medium alone were also included. Following overnight incubation, the medium was removed and the cells were washed with PBS before adding 250 µL medium containing the treatment to be analysed, or with medium alone as a control. After 24 hours incubation, 25 µL WST-1 reagent was added to each well and cells were incubated for a total of 90 min in the incubator, removing the plate for a reading at 30, 60 and 90 min. The cells were shaken for 1 min and bubbles were removed prior to reading, and then read at 450 nm using a Molecular Devices V_{max} kinetic microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA).

3.2.13 *Data analysis*

Analysis of variance (one-way ANOVA) was conducted using Minitab version 16 (Minitab Inc., State College, PA, USA). When differences were significant ($P \leq 0.05$), the means were analysed using Tukey's test.

3.3 Results and Discussion

3.3.1 *Colostrum buttermilk*

Buttermilk is defined as the aqueous phase released during the churning of cream in butter manufacture (Corredig and Dalgleish, 1997). Mature milk buttermilk contains lactose, minerals and skimmed milk proteins in the same proportion as skimmed milk (Corredig *et al*., 1997). It has traditionally been considered a waste product; however, in recent years, it has gained considerable attention due to its specific composition of proteins and polar lipids from the milk fat globule membrane (MFGM). The MFGM is a thin membrane, about 10-20 nm in cross-section, which surrounds the triglyceride core of fat globules in milk (Dewettinck *et al*., 2008). The MFGM consists of 3 distinct layers: a monolayer of lipid droplet-associated proteins and polar lipids, an electron-dense proteinaceous coat and a bilayer membrane of polar lipids and proteins (Keenan and Mather, 2006). The MFGM comprises mainly membrane-specific glycoproteins and polar lipids including phospho- and sphingolipids (Fox and McSweeney, 1998). MFGM can be isolated by a four-step procedure in which the fat globules are separated from whole milk and washed several times with buffers. The membrane is then released from the surface of the globules by physical or chemical means and collected by centrifugation (Mather, 2000). The washing step is carried out to remove lactose, minerals and skim milk protein. In the current study, colostrum cream was not washed before mechanical agitation, which resulted in a large proportion of lactose, minerals and skim milk proteins remaining in the buttermilk. An alternative method for isolating MFGM is freezing and thawing the washed globules (Keenan, 1970). In the current study, buttermilk was produced from colostrum which had been frozen, which, on thawing, may have resulted in the loss of some MFGM material into the serum phase of colostrum. While extensive work has been carried out on MFGM proteins in colostrum and mature milk (Mather *et al*., 2000; Reinhardt and Lippolis, 2006; Dewettinck *et al*., 2008; Vanderghem *et al*., 2010), to the best of our knowledge, very little work has been carried out on the protein

composition and biological activities associated with buttermilk produced from bovine colostrum.

The protein profile of the pH 4.6-soluble fraction of (a) colostrum buttermilk and (b) mature milk buttermilk were analysed using 2-DE (Figure 3.1). Colostrum buttermilk and mature milk buttermilk were adjusted to pH 4.6 according to the method of Kuchroo and Fox (1982) in order to remove pH 4.6-insoluble material and improve the resolution of whey proteins on 2-DE electrophoretograms. The pH 4.6-insoluble fraction of colostrum was shown to have no inflammatory (Figure 3.2) or insulinotrophic properties (as discussed later, Figure 3.12). The pH 4.6-soluble fraction of colostrum buttermilk contained a higher proportion of high molecular weight protein than the pH 4.6-soluble fraction of mature milk buttermilk. This was to be expected because, as already mentioned, the protein profile of colostrum buttermilk is very similar to that of skimmed colostrum. Colostrum has a distinctive whey protein composition, which includes elevated levels of IgG, IgA and IgM (Smolenski *et al*., 2007). Colostrum also has a high concentration of bioactive components, e.g., growth factors, especially insulin-like growth factor-1 (IGF-1), transforming growth factor beta-2 (TGF-β2) and growth hormone (GH) as well as lysozyme and lactoperoxidase (Elfstrand *et al*., 2002). Proteins secreted by the mammary gland such as β-lactoglobulin, α-lactalbumin, lactoferrin, proteinase inhibitors and some enzymes are present in higher concentrations in colostrum than in mature milk (Levieux and Ollier, 1999). Proteins derived from the blood such as albumin, α_2-macroglobulin, and transferrin are also present at higher concentration in colostrum (Levieux and Ollier, 1999). In addition, colostrum contains cytokines and leukocytes, including activated neutrophils, macrophages and lymphocytes. The most abundant proteins associated with the bovine MFGM are MUC1, xanthine dehydrogenase/oxidase, CD36, butyrophilin, adipophilin, periodic acid Schiff 6/7 (PAS 6/7) and fatty-acid-binding protein (Mather, 2000).

To identify some of the proteins which were abundant in colostrum buttermilk compared with mature milk buttermilk, LC-MS was performed on 2-DE excised gel spots (Figure 3.1). Identification of the 13 spots analysed are shown in Table 3.1. It should be noted that 2-DE is problematic when analysing membrane proteins

associated with the MFGM as many are lost due to precipitation at the isoelectric point, and these proteins can be difficult to get into solution prior to iso-electric focussing (Reinhardt and Lippolis, 2006). The predominant proteins identified in the pH 4.6-soluble fraction of colostrum buttermilk by LC-MS of 2-DE gel spots, in decreasing order, were immunoglobulin light chain lambda (IGL), immunoglobulin light chain kappa (IGK), lactadherin, polymeric immunoglobulin receptor (pIgR), serum albumin, lactotransferrin, alpha 1 antiproteinase and zinc alpha 2 glycoprotein.

The immunoglobulin (Ig) proteins in milk protect the gut mucosa against pathogenic microorganisms. In bovine milk, the predominant species of Ig is IgG, in particular IgG_1, but IgA and IgM are also found. Colostrum contains 40-300 times the concentration of IgG than mature milk (Edwards *et al*., 2009). All monomeric immunoglobulins have the same basic molecular structure, being composed of two identical heavy chains and two identical light chains, with a total molecular mass of approximately 160 kDa (Hurley *et al*., 2011). Heavy and light chains are linked together by disulphide bonds, resulting in a Y-shape. The number and location of disulphide bonds is dependent on the class of immunoglobulin (Mix *et al*., 2006). Under reducing conditions used in 2-DE, the disulphide bonds linking together the heavy and light chains are broken. There are two types of light chain, referred to as kappa (κ) and lambda (λ or L). Of the 13 gel spots analysed, IgL was present in 7 spots and IgK was present in 6 spots (Table 3.1). Because light chains of the various Ig species are identical, there is no way of determining from which particular species of Ig these light chains originated.

Lactadherin (formerly known as PAS-6/7) is a glycoprotein and is an abundant peripheral protein associated with the MFGM (Andersen *et al*., 1997). It is secreted by mammary epithelial cells (Butler *et al*., 1980), epididymal epithelial cells (Ensslin *et al*., 1995), vascular cells (Silvestre *et al*., 2005) and macrophages (Hanayama *et al*., 2002). Lactadherin comprises two EGF-like domains and two C-like domains found in blood clotting factors V and VIII (Andersen *et al*., 2000). Lactadherin was found in 6 out of 13 gel spots analysed (Table 3.1). Lactadherin has been linked with intestinal development. Zhou *et al.* (2010) demonstrated that lactadherin increases

production of IL-10, an anti-inflammatory cytokine, when present in culture medium compared with lactadherin-absent culture. Lactadherin-associated glycans may also bind to rotaviruses and protect the neonatal gut from infection (Newberg *et al*., 1998).

Polymeric immunoglobulin receptor (pIgR) is a transmembrane glycoprotein which mediates transport of polymeric immunoglobulins (IgA and to a lesser extent IgM) across mucosal epithelial cells (Kaetzel, 2005), the pathway of which has been well characterised (Mostov *et al*., 2005). The pro-inflammatory cytokines interferon-γ, tumor necrosis factor and interleukin-1, play a key role in upregulation of pIgR expression (Kaetzel, 2005). pIgR was found in 5 out of 13 gel spots analysed (Table 3.1).

Serum albumin was found in 4 out of 13 gel spots analysed (Table 3.1); this is a minor protein in milk which gains entry *via* leakage from blood serum (Walstra *et al*., 2006). It is a large molecule having three globular domains, resulting in an elongated shape, and consists of 9 separate disulphide-bonded loops connected by peptide links of 11-26 residues (Beretta *et al*., 2001). BSA is involved in milk allergy, where it represents one of the most important antigens from whey proteins (Goldman *et al*., 1963).

Alpha 1 antiproteinase (also known as alpha 1 antitrypsin or serpin A1) is a glycoprotein synthesised in the liver and is composed of a single polypeptide chain (M_r, 50 kDa) and three carbohydrate chains (Kurachi *et al*., 1981). It is an important protease inhibitor present in mammalian blood (Kurachi *et al*., 1981), which has also been identified as a low abundance protein in bovine colostrum (Yamada *et al*., 2002). Its major physiological function appears to be the inhibition of neutrophil elastase; however, it also inhibits many other serine proteinases, including trypsin, chymotrypsin, collagenase, thrombin, kallikrein and plasmin (Laurell and Jeppsson, 1975). Alpha 1 antiproteinase was found in 3 out of 13 gel spots analysed (Table 3.1).

Lactotransferrin (also known as lactoferrin) is a monomeric, globular, iron binding glycoprotein with a molecular mass of 80 kDa (Edwards *et al*., 2009). It is present in high concentrations in milk, especially colostrum (Sanchez *et al*., 1988) and was found in 3 out of 13 spots analysed (Table 3.1). Lactoferrin possesses numerous biological functions, including roles in iron metabolism, cell proliferation and differentiation, and antibacterial, antiviral and antiparasitic activity (Adlerova *et al*., 2008). Lactoferrin has been shown to reduce the production of pro-inflammatory cytokines such as tumor necrosis factor or interleukins IL-1β and IL-6 (Haversen *et al*., 2002) and increase the production of the anti-inflammatory interleukin IL-10. Lactoferrin may also prevent the development of inflammation and tissue damage by binding components of bacterial cell walls, such as LPS (Adlerova *et al*., 2008).

Zinc alpha 2 glycoprotein (ZAG) is a 40 kDa single-chain polypeptide (Burgi and Schmid, 1961), the exact function of which is still unknown. ZAG is a multidisciplinary protein and is considered an adipokine due to its high sequence homology with lipid-mobilizing factor and high expression in cancer cachexia. ZAG may also play a role in the immune response as its structural organization and fold is similar to MHC class I antigen-presenting molecule (Hassan *et al*., 2008). ZAG was found in 2 out of 13 spots analysed (Table 3.1).

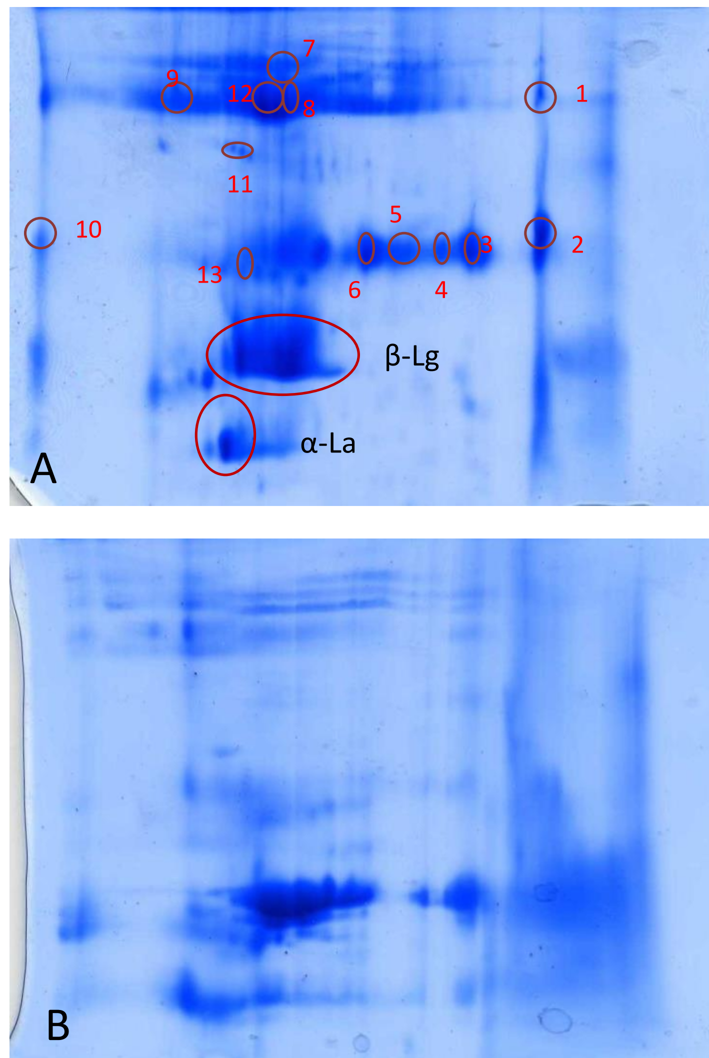


Figure 3.1 Two-dimensional gel electrophoretograms of the pH 4.6-soluble fraction of (a) colostrum buttermilk and (b) mature milk buttermilk, separated under reducing conditions using a 7 cm pH 4-7 pI range for the first dimension and a 12% gradient acrylamide SDS-PAGE gel for the second dimension. Circled and numbered spots were excised and analysed by LC-MS.

Table 3.1. Identification of spots from a 2-DE gel of the pH 4.6-soluble fraction of colostrum buttermilk, by LC-MS using a LTQ linear ion trap

Spot No.	Identified protein	Uniprot Accession Number	Mol. mass (Da)	% Coverage	Peptides	Score
1	Actin, cytoplasmic 1	P60712	42052	45.6	18	506
	Lactotransferrin	P24627	80002	12	7	228
	Fibrinogen beta chain	P02676	53933	19.2	5	181
	Serpin H1	Q2KJH6	46591	16	6	177
	Elongation factor 1-alpha 1	P68103	50451	10.6	3	177
	Alpha-enolase	Q9XSJ4	47639	18.2	4	136
2	Immunoglobulin light chain lambda	A5PK49	24919	43.6	29	493
	Immunoglobulin light chain kappa	Q05B55	26589	12.5	3	124
3	Immunoglobulin light chain lambda	A5PK49	24919	43.6	27	507
	Immunoglobulin light chain kappa	Q05B55	26859	33.3	7	386
4	Immunoglobulin light chain lambda	A5PK49	24919	35.5	21	446
	Immunoglobulin light chain kappa	Q05B55	26589	19.2	4	211
5	Immunoglobulin light chain lambda	A5PK49	24910	59.1	27	608
	Immunoglobulin light chain kappa	Q05B55	26859	45.4	8	274
6	Immunoglobulin light chain lambda	A5PK49	24919	40.2	20	432
	Immunoglobulin light chain kappa	Q05B55	26859	45.4	7	353
7	Polymeric immunoglobulin receptor	P812165	83695	40	43	1660
	Serum albumin	P02679	71244	46.3	27	1427
	Lactotransferrin	P24627	80002	36	23	1305
	Serotransferrin	Q29443	79870	37.1	21	1151
	CD5L protein	A6QNW7	52118	24.6	9	426
	Hemopexin	Q3SZV7	52974	17.2	7	379
8	Lactadherin	Q95114	48520	40.3	15	968
	Polymeric immunoglobulin receptor	P81265	83695	20.2	12	732
	Serum albumin	P02679	71244	21.6	10	540
	α-1-antiproteinase	P34955	46417	20.2	9	395
	Vitamin D-binding protein	Q3MHN5	54904	16.7	4	287

(Continued)

Table 3.1. (*Continued*)

Spot No.	Identified protein	Uniprot Accession Number	Mol. mass (Da)	% Coverage	Peptides	Score
9	Lactadherin	Q95114	48520	39.8	18	822
	Polymeric immunoglobulin receptor	P81265	83695	17	9	617
	Serum albumin	P02679	71244	20.1	9	369
	Lactotransferrin	P24627	80002	10.9	6	272
	Endopin 2C	Q32T06	46858	14.7	5	258
	FGG protein	Q3SZZ9	49763	10.1	3	173
	α-2-HS-glycoprotein	P12763	39193	11.7	3	161
10	Lactadherin	Q95114	48520	27.9	11	635
	Polymeric immunoglobulin receptor	P81265	83695	13.1	7	478
	Immunoglobulin light chain lambda	A5PK49	24910	22.6	8	224
	Immunoglobulin light chain kappa	Q05B55	26589	32.1	4	214
11	Zinc-α-2-glycoprotein	B2BX70	34059	55.2	16	970
	Lactadherin	Q95114	48520	27.4	11	529
	Polymeric immunoglobulin receptor	P81265	83695	12.4	7	442
	Apolipoprotein A-IV	Q32PJ2	42991	23.2	9	422
	Apolipoprotein E	Q03247	36015	28.8	7	409
	Actin, cytoplasmin 1	P60712	42052	30.4	8	319
	Serum albumin	P02679	71244	17	8	312
12	Lactadherin	Q95114	48520	44.7	16	910
	Alpha-1-antiproteinase	P34955	46417	23.8	11	503
	Antithrombin-III	P41361	52827	23.4	7	449
	Serum albumin	P02679	71244	16.5	7	376
	α-2-HS-glycoprotein	P12763	39193	18.4	3	179
	Zinc-α-2-glycoprotein	B2BX70	34059	10.7	2	71
13	Lactadherin	Q95114	48520	32.6	13	652
	Immunoglobulin light chain kappa	Q05B55	24863	35.5	8	398
	Immunoglobulin light chain lambda	A5PK49	24910	33.2	7	370
	β-lactoglobulin	PO2754	20269	26.4	9	330
	α-1-antiproteinase	P34955	46417	10.6	4	226
	Actin, cytoplasmin 1	P60712	42052	18.9	5	201
	κ-casein	PO2668	21370	13.7	10	147

Unfractionated colostrum buttermilk was analysed by LC-MS using a G2 Q-TOF (Table 3.2). As already mentioned, buttermilk contains the same proportion of proteins as skimmed milk. Therefore, it is not surprising that the caseins (α_{S1}-, α_{S2}-, β- and κ-casein) were identified, along with whey proteins such as β-Lg and α-La (Table 3.2). Several proteins which were identified from 2-DE gel spots of the pH 4.6-soluble fraction of colostrum buttermilk by LC-MS using a linear ion trap (Table 3.1) were also identified using this approach. These include lactadherin, polymeric immunoglobulin receptor, serum albumin and lactotransferrin, and will not be discussed further. Major MFGM proteins which were identified include lactadherin, xanthine oxidoreductase, butyrophilin and adipophilin.

Xanthine oxidoreductase (XOR) is the second most abundant protein in the MFGM, after butyrophilin, comprising approximately 20% of globule-associated protein (Fox and Kelly, 2006). XOR is a molybdenum-containing enzyme which comprises a homodimer of M_r approximately 300,000 Da (Mather, 1999). XOR exists in two forms, xanthine oxidase XO (EC 1.1.3.22) and xanthine dehydrogenase XDH (EC 1.1.1.204). The most important role of XOR in milk is in the secretion of milk fat globules from mammary secretory cells; it is also involved in lipid oxidation, atherosclerosis, production of H_2O_2, purine catabolism and bactericidal activity (Fox and Kelly, 2006). XOR has also been linked with the inflammatory process because expression of the XOR gene, protein and enzyme activity are induced by cytokines and steroids (Pfeffer *et al*., 1994; Page *et al*., 1998). Pfeffer *et al*. (1994) and Page *et al*. (1998) demonstrated that XOR may produce reactive oxygen species, which either cause tissue damage and exacerbate inflammation or induce expression of genes that modulate tissue healing.

Butyrophilin is a type 1 transmembrane glycoprotein with a cytoplasmic C-terminal tail and is concentrated in the apical plasma membranes of mammary epithelial cells (Jack and Mather, 1990). It is the most abundant protein associated with the MFGM and constitutes more than 40% by weight of the total protein (Mather and Jack, 1993). The exact function of butyrophilin is unclear; however, it, along with XOR, is thought to be involved in milk fat globule secretion by linking milk secretory granules to the plasma membrane for secretion by interacting with adipophilin at

the milk secretory granule surface, facilitating the envelopment of the granule with plasma membrane during milk fat droplet formation (Robenek *et al*., 2006).

Adipophilin (also known as perilipin-2) is enriched in the MFGM and has a Mr of 52 kDa (Nielsen *et al*., 1999). Adipophilin is relatively insoluble in SDS sample buffers (Mather, 2000) which may explain why it was not detected by LC-MS identification of 2-DE gel spots (Table 3.1). It has been shown that adipophilin, butyrophilin and xanthine oxidoreductase are present in constant proportions in high-salt-insoluble MFGM material and therefore share physicochemical characteristics (Heid *et al*., 1996). Jiang and Serrero (1992) proposed that interactions between butyrophilin, xanthine oxidoreductase and adipophilin mediate formation of the protein coat of the MFGM. Physiological roles of adipophilin in cytoplasmic lipid droplet accumulation and triglyceride metabolism have been established in adipose and hepatic tissues (Chang *et al*., 2006). Recent studies have shown that adipophilin might be involved in the inflammatory processes in macrophages (Sarov-Blat *et al*., 2007). Chen *et al*. (2010) showed that adipophilin augmented the secretion of the pro-inflammatory cytokines TNF-α, MCP-1 and IL-6 in macrophages.

Glycosylation-dependant cell adhesion molecule 1 (GlyCAM1) (also known as lactophorin/proteose peptone component 3) is a mucin-like endothelial glycoprotein that mediates leukocyte-endothelial cell adhesion by presenting carbohydrate ligands to the lectin domain of L selectin (Imai *et al*., 1992). In milk, a high molecular weight aggregate of several mucin like glycoproteins that is associated with the MFGM has been shown to have antiviral activity against rotavirus (Yolken *et al*., 1992). A similar high molecular weight mucin aggregate in milk was shown to inhibit the replication of respiratory syncytial virus (Laegreid *et al*., 1986).

Clusterin (also known as apolipoprotein J) is a multifunctional protein implicated in several immunological and pathological conditions (Sonn *et al*., 2010). Clusterin is a glycoprotein which is expressed in a wide variety of tissues and has been shown to play a role in the regulation of inflammation and immunity (Trougakos and Gonos, 2006). Jeong *et al*. (2012) showed that clusterin helps prevent the onset of

inflammation by maintaining fluid-epithelial interface homeostasis. Sonn *et al*. (2010) presented clusterin as a novel regulator of NK cells via providing co-stimulation required for cell proliferation and IFN-γ secretion which would have a positive effect in chronic inflammatory and autoimmune conditions.

Angiogenesis is a key process in the pathogenesis of inflammatory arthritis. Angiogenin is a heparin-binding 14.1 kDa polypeptide and ribonucleic enzyme (Soncin *et al*., 1997; Shapiro *et al*., 1986) and is one of the most active angiogenic factor in experimental models *in vivo* (Liote *et al*., 2003). Kourtroubakis *et al*. (2004) found that angiogenin may mediate angiogenesis and vascular permeability in the mucosa of patients with inflammatory bowel disease, while Liote *et al*. (2003) suggested that angiogenin may mediate local inflammation in arthritis via effects on angiogenesis and leukocyte regulation.

Table 3.2 Identification of proteins from colostrum buttermilk by LC-MS using a G2 Q-TOF

Identified Protein	Uniprot Accession Number	Mol. mass (Da)	% Coverage	Peptides	PLGS Score
α_{S1}-casein	P02662	24513	67.76	31	44615
κ-casein	P02668	21255	50.53	16	21349
β-lactoglobulin	P02754	19870	73.03	25	18422
β-casein	P02666	25091	24.55	20	15599
Lactadherin	Q95114	47380	54.33	36	14554
α-lactalbumin	P00711	16235	45.77	10	11554
α_{S2}-casein	P02663	26002	42.34	35	10823
Butyrophilin subfamily 1 member A1	P18892	59238	39.92	21	3768
Glycosylation dependent cell adhesion molecule 1	P80195	17141	43.14	7	3019
Polymeric immunoglobulin receptor	P81265	82383	43.59	30	2880
Serum albumin	P02769	69248	56.84	33	2488
Polyubiquitin C	P0CH28	77522	32.61	18	1903
β-2-microglobulin	P01888	13668	10.17	2	989
Apolipoprotein E	Q03247	35957	37.97	9	779
Lactotransferrin	P24627	78006	44.63	29	767
Xanthine dehydrogenase oxidase	P80457	146696	30.03	34	622
Platelet glycoprotein 4	P26201	52906	13.98	6	454
Perilipin 2	Q9TUM6	49337	34.00	11	293
Ribonuclease pancreatic	P61823	16450	27.33	2	271
Serotransferrin	Q29443	77702	33.24	18	240
Angiogenin 1	P10152	16958	30.41	4	220
Junctional sarcoplasmic reticulum protein 1	Q2YDF7	36931	28.78	10	211
Golgi SNAP receptor complex member 1	Q2TBU3	28523	25.60	5	167
Monocyte differentiation antigen CD14	Q95122	39641	12.60	4	163
UPF0740 protein C1orf192 homolog	Q3SZT6	21171	31.63	7	155
Post GPI attachment to proteins factor 3	A7YWP2	36032	23.20	4	148
Clusterin	P17697	51081	20.50	9	123

3.3.2 *Fatty acid profile*

Milk fat consists mainly (approximately 96%) of triglycerides, while other milk lipids include diacylglycerol (about 2% of the lipid fraction), cholesterol (less than 0.5%), phospholipids (about 1%) and free fatty acids (about 0.1%) (Jensen and Newburg, 1995). In addition, there are trace amounts of ether lipids, hydrocarbons, fat-soluble vitamins, flavour compounds and compounds introduced by the feed (Parodi, 2004). Fatty acids in milk are derived from two sources, the feed and

microbial activity in the rumen of the cow (Parodi, 2004). Bovine milk fat contains approximately 400 different fatty acids, making it one of the most complicated naturally occurring fats. The fatty acid profile of colostrum cream, colostrum buttermilk, mature milk cream and mature milk buttermilk were analysed by GC-MS (Table 3.3). The most abundant fatty acids across all samples were palmitic acid, oleic acid, myristic acid and stearic acid, as reported by Kaylegian and Lindsay (1995).

In general, the fatty acid content of mature milk cream and mature milk buttermilk were in line with values reported by Jensen (2002). The fatty acid content of colostrum cream and colostrum buttermilk differed in comparison to mature milk cream and mature milk buttermilk. Cream and buttermilk from colostrum contained lower levels of caproic acid, caprylic acid, pelargonic acid, capric acid, lauric acid, myristic acid, myristoleic acid, pentadecanoic acid and oleic acid, and higher levels of valeric acid, palmitic acid, palmitoleic acid, margaric acid, stearic acid, linoleic acid and arachidonic acid than mature milk cream and buttermilk (Table 3.3). Laakso *et al*. (1996) found that colostrum triglycerides contained less oleic acid and more palmitic acid than mature milk fat, which is in agreement with the current study; however, the same author found that colostrum triglycerides contained less stearic acid and more myristic acid than mature milk fat, which does not agree with results from the current study.

It has been shown that increased concentrations of fatty acids in blood plasma are associated with obesity and metabolic syndrome (Reaven *et al*., 1988), and saturated long-chain fatty acids can activate inflammatory and innate immune responses in the body (Kennedy *et al*., 2009). In particular, the saturated fatty acids palmitic acid, lauric acid and myristic acid have been shown to activate inflammatory genes in adipocytes (Laine *et al*., 2007; Han *et al*., 2010). Excess consumption of nutrients causes expansion of white adipose tissue and an increase in the size and number of adipocytes, leading to adipocyte dysfunction (Lago *et al*., 2007). These swollen adipocytes secrete pro-inflammatory agents that promote systemic inflammation (Bays *et al.,* 2008). Also, these lipid-engorged adipocytes will eventually die and release their contents, resulting in the recruitment of

neutrophils and macrophages (Weisberg *et al*., 2003). Therefore, excess consumption of fat is associated with obesity-mediated macrophage recruitment to white adipose tissue, resulting in chronic, low-grade inflammation (Kennedy *et al*., 2009).

Table 3.3 Fatty acid composition of colostrum cream, colostrum buttermilk, mature milk cream and mature milk buttermilk, measured by GLC and expressed as percent by weight of total fatty acids (results shown are based on a single analysis).

Fatty Acid	Lipid Number	Colostrum Cream % wt	Colostrum Buttermilk % wt	Mature Milk Cream % wt	Mature Milk Buttermilk % wt
Butyric acid	C4:0	1.07	0.81	0.99	0.91
Valeric Acid	C5:0	0.17	0.1	0.08	0
Caproic Acid	C6:0	1.08	1.1	1.8	1.57
Enanthoic Acid	C7:0	0.09	0	0	0.12
Caprylic Acid	C8:0	0.72	0.65	1.29	1.37
Pelargonic Acid	C9:0	0	0	0.05	0.05
Capric Acid	C10:0	1.61	1.52	3.46	3.56
	C10:1	0	0	0.27	0.26
	C11:0	0	0	0	0.06
Lauric Acid	C12:0	2.16	2.13	4.23	4.33
	C12:1	0	0	0.06	0.09
	C13:0	0	0	0.13	0.15
	Iso C14:0	0	0	0.13	0.15
Myristic Acid	C14:0	10.43	10.5	12.24	12.6
Myristoleic Acid	C14:1	0.93	1.03	1.92	2.02
Pentadecanoic Acid	C15:0	1	0.94	1.37	1.4
	Iso C16:0	0.15	0.17	0.27	0.26
Palmitic Acid	C16:0	35.49	36.26	26.98	27.79
Palmitoleic Acid	C16:1	2.77	2.87	2.52	2.25
Margaric Acid	C17:0	1.17	1.13	0.84	0.73
	C17:1	0.45	0.53	0.38	0.25
Stearic Acid	C18:0	11.33	11.62	10.43	10.23
Oleic Acid	C18:1	23.83	23.4	24.71	24.17
Linoleic Acid	C18:2	1.34	1.35	0.87	0.85
	CLA cis-9 trans-11	0	0	1.37	1.05
	CLA cis-10 cis-12	0	0	0	0
	C18:3 n-6	0.91	0.94	0.7	0.32
	C18:3 n-3	0.87	0.84	1.01	0.99
Arachidic Acid	C20:0	0.19	0.18	0.2	0.22
	C20:1	0	0	0.08	0
	C20:2	0	0	0	0
	C20:3 n-6	0.09	0.07	0	0
	C20:3 n-3	0.06	0.08	0.07	0.06
Arachidonic Acid	C20:4	0.14	0.12	0.05	0.05
	C20:5	0	0.08	0	0
Behenic Acid	C22:0	0	0	0	0
Eruric Acid	C22:1	0	0	0	0
	C22:2	0.16	0.15	0.14	0.14
	C22:4	0.07	0	0.05	0
	C22:5	0.33	0.33	0.16	0.13
	C22:6	0	0	0	0
Lignoceric Acid	C24:0	0.1	0.07	0.06	0
Nervonic Acid	C24:1	0	0	0	0
	Others	1.27	1.05	1.1	1.85
	Total	98.73	98.95	98.9	98.15

3.3.3 Effect of colostrum fractions on LPS-induced TNF-α secretion

Tumor necrosis factor (TNF) is among the most studied and central pro-inflammatory cytokines (Gillett *et al*., 2010). TNF is a key regulator of the inflammatory response whose ligands are known to play a central role in a variety of inflammatory conditions, e.g., rheumatoid arthiritis, inflammatory bowel disease, multiple sclerosis, diabetes and asthma (Croft *et al*., 2012). Inhibition of TNF by colostrum fractions could potentially lead to suppression of autoimmune and inflammatory diseases. TNF-α and its receptors TNFR1/TNFR2 are the major members of a gene superfamily which consists of approximately 50 membrane-bound and soluble proteins (Dhama *et al*., 2013). TNF-α is predominantly produced by activated macrophages and T-lymphocytes and is known to have numerous biological properties relating to inflammation, proliferation, differentiation and cancer growth (Patil *et al*., 2011).

In the current study, murine macrophage cells (J774.2) were isolated, cultured and incubated with 5 mg/mL of colostrum cream, casein (pH 4.6-insoluble), whey (pH 4.6-soluble), 30 kDa retentate of whey or 30 kDa permeate of whey for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h. These samples were tested for their effect on LPS-induced TNF-α secretion by J774.2 cells (Figure 3.2). LPS is a key inflammatory component of Gram-negative bacteria, which induces a distinctive pattern of cytokine release that regulates inflammation (Singh and Jiang, 2003). In the current study, LPS was used to initiate an inflammatory response which resulted in increased secretion of TNF-α from J774.2 cells. It can be seen from Figure 3.2 that colostrum fractions had an effect on TNF-α secretion from J774.2 cells, compared to the positive control; colostrum cream caused the greatest reduction in the secretion of TNF-α from J774.2 cells, followed by the 30 kDa retentate of whey and unfractionated whey. The colostrum casein had no effect on the secretion of TNF-α from J774.2 cells, while the 30 kDa permeate of whey increased the secretion of TNF-α. Based on these findings, it was decided to focus on colostrum cream as it was the most potent inhibitor of LPS-induced TNF-α secretion from J774.2 cells.

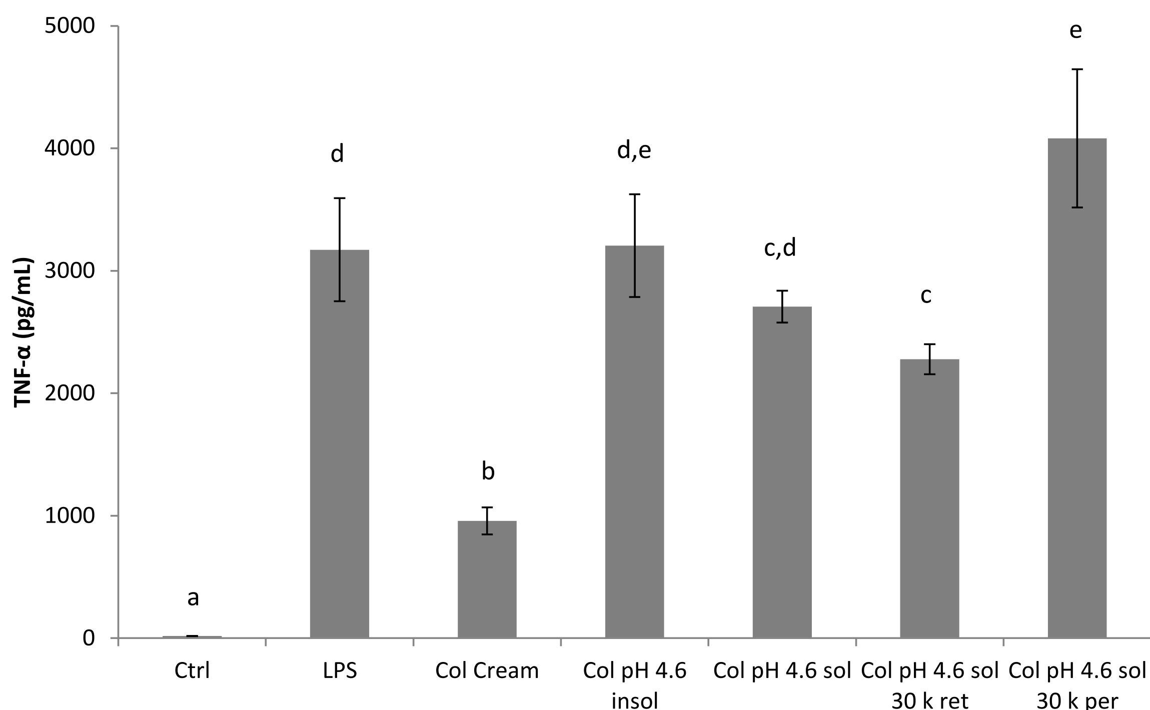


Figure 3.2 Effect of colostrum fractions on LPS-induced TNF-α secretion by J774.2 macrophages. Exponentially growing cells were incubated with 5 mg/mL of colostrum fractions for 24 h and stimulated with 10 ng/mL of LPS for 3 h. The supernatant was removed and the concentration of cytokine quantified using ELISA. Data shown represent the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

J774.2 cells were isolated, cultured and incubated with various concentrations of colostrum cream ranging from 0.01 to 5 mg/mL for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h. The dose-response effect of colostrum cream on LPS-induced TNF-α secretion by J774.2 cells is shown in Figure 3.3. It can be seen that colostrum cream, at concentrations greater than 0.1 mg/mL, significantly reduced TNF-α secretion in a dose-dependent manner.

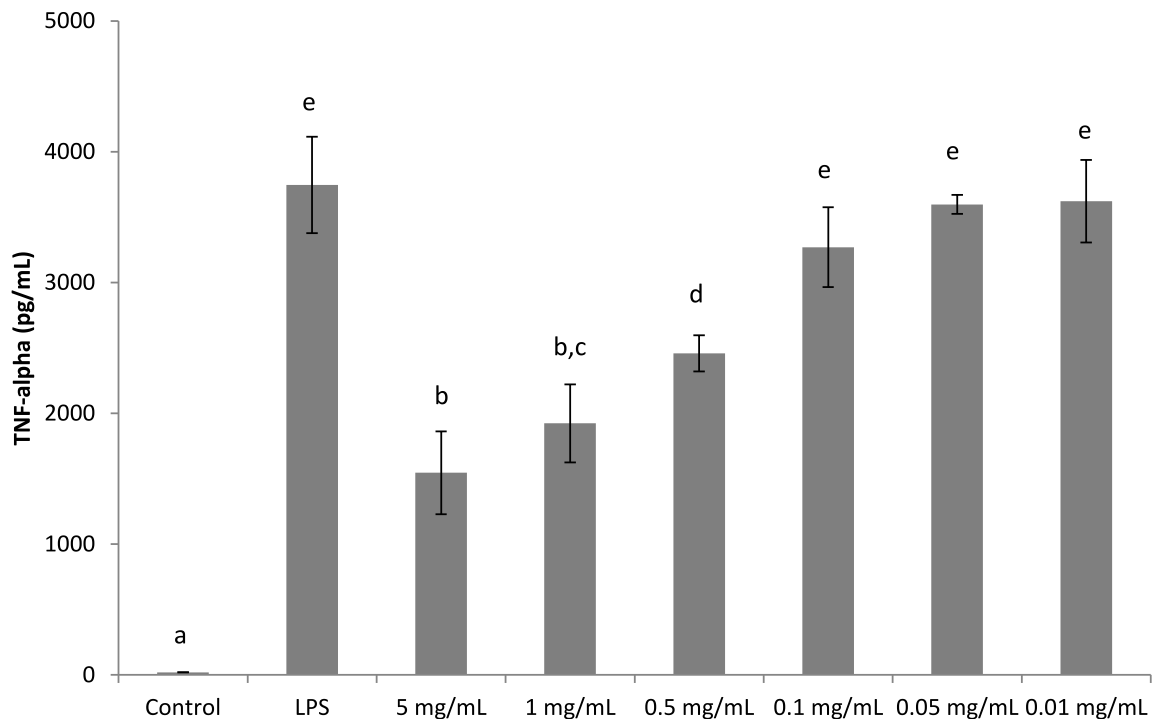


Figure 3.3 Effect of colostrum cream on LPS-induced TNF-α secretion by J774.2 macrophages. Exponentially growing cells were incubated with various concentrations (mg/mL) of colostrum cream for 24 h and stimulated with 10 ng/mL of LPS for 3 h. The supernatant was removed and cytokine secretion levels were quantified using ELISA. Data shown represent the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

3.3.4 Effect of colostrum cream on macrophage cell viability

Necrosis occurs as a result of harmful insults such as physical damage, hypoxia, hyperthermia, starvation, complement attack and chemical injury and elicits an inflammatory reaction in adjacent viable tissue in response to the released cell debris (Haanen and Vermes, 1995). To assess the possibility that colostrum cream may cause necrosis, its effect on macrophage viability was examined (Figure 4). J774.2 cells were isolated, cultured and incubated with various concentrations of colostrum cream ranging from 0.01 to 5 mg/mL for 24 h. The effect of colostrum cream on J774.2 macrophage cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) cleavage assay. It was found

that colostrum cream had no significant effect on J774.2 macrophage cell viability when tested in the range 0.01 to 5 mg/mL (Figure 3.4).

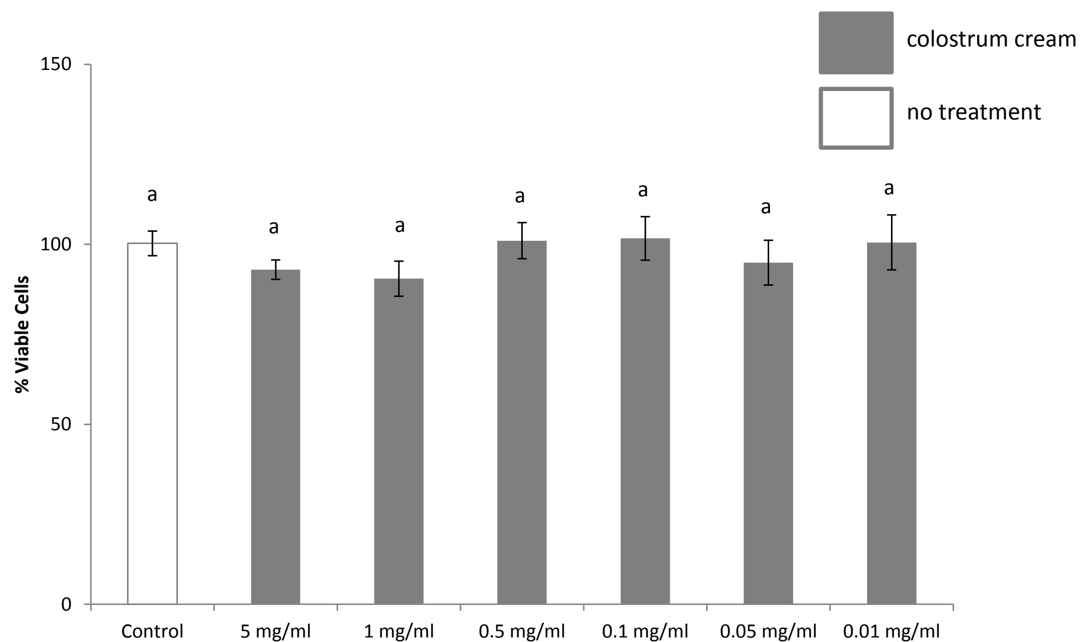


Figure 3.4 Effect of colostrum cream on J774.2 macrophage cell viability. Exponentially growing cells were incubated with various concentrations (mg/mL) of colostrum cream for 24 h. Cell toxicity was determined by an MTT assay. The values expressed are normalized to complete (100%) viability of untreated cells. Data shown represent the mean (± SD, n=3) of three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

3.3.5 Effect of buttermilk from colostrum and mature milk on LPS-induced TNF-α secretion

Colostrum cream was fractionated into butter and buttermilk, as described in Section 3.2.4, in an attempt to identify the biologically active component with inflammatory potential, i.e., reduced TNF-α secretion. Colostrum butter and colostrum buttermilk were both found to have inflammatory potential compared to mature milk butter and mature milk buttermilk prepared in the same way (results not shown). Prior to cell treatment, reconstituted powders were filtered through 0.45 µm filters, thereby removing any lipid material in colloidal suspension. For this reason, it was decided to proceed only with the buttermilk fraction as any

biologically active components present in the soluble fraction of butter would also be present in buttermilk.

J774.2 macrophages were isolated, cultured and incubated with various concentrations of buttermilk from colostrum or mature milk ranging from 0.05 to 5 mg/mL for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h. The dose-response effect of buttermilk from colostrum or mature milk on LPS-induced TNF-α secretion by J774.2 macrophages is shown in Figure 3.5. Colostrum buttermilk caused a significant reduction in the secretion of TNF-α from J774.2 macrophages, in a dose-dependent manner, at concentrations greater than 0.05 mg/mL. Mature milk buttermilk also caused a significant reduction in the secretion of TNF-α at concentrations greater than 0.5 mg/mL, but, in all cases, the reduction was not as pronounced as that for colostrum buttermilk when tested at equal concentrations. This was most evident at higher concentrations of buttermilk from colostrum and mature milk.

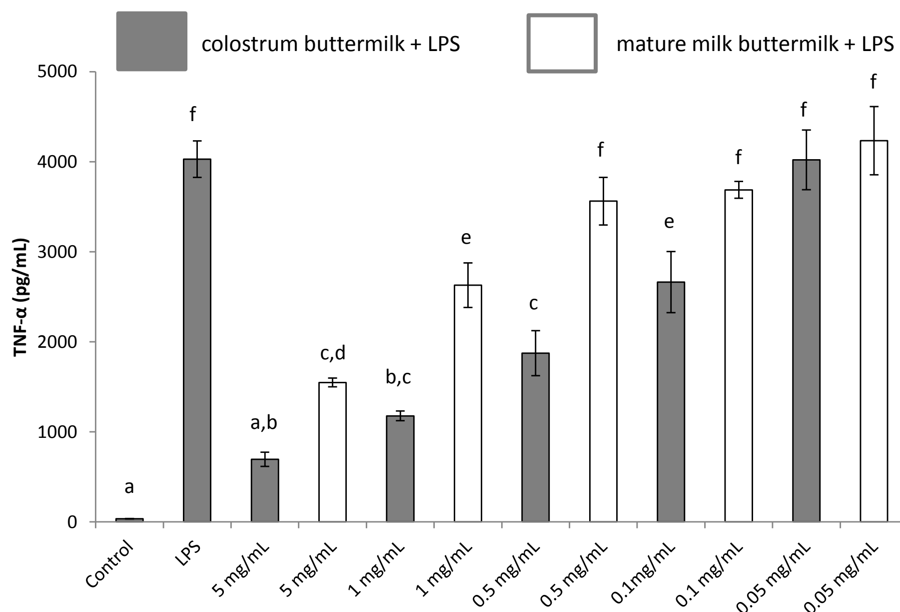


Figure 3.5 Dose response effect of buttermilk from colostrum or mature milk on LPS-induced TNF-α secretion by J774.2 macrophages. Exponentially growing cells were incubated with various concentrations (mg/mL) of buttermilk from colostrum or mature milk for 24 h and stimulated with 10 ng/mL of LPS for 3 h. The supernatant was removed and cytokine secretion levels were quantified using ELISA. Data shown represent the mean (± SD, n=3) of three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

3.3.6 Effect of buttermilk from colostrum or mature milk on LPS-induced NF-κB luciferase activity

The transcription factor nuclear factor-kappa B (NF-κB) is an important intracellular regulator of many inflammatory responses as it controls the expression of many genes involved in the inflammatory process (Ciesielski *et al*., 2002), and is therefore a target for anti-inflammatory treatment. Other transcription factors, such as activator protein 1 (AP-1) and the nuclear factor of interleukin-6, are also involved in the regulation of inflammatory and immune genes (Stein and Baldwin, 1993). The activation of NF-κB causes an increase in the expression of many genes whose products mediate inflammatory and immune responses. These include pro-inflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors and adhesion molecules that play a key role in the initial recruitment of leukocytes to the initial site of inflammation (Barnes and Karin, 1997). The pro-inflammatory cytokines interleukin-1β and TNF-α both activate and are activated by NF-κB, creating a positive regulatory loop which has the potential to amplify and perpetuate a local inflammatory response (Barnes and Karin, 1997).

NIH3T3/NF-κB-luc fibroblasts were isolated, cultured and incubated with various concentrations of buttermilk from colostrum or mature milk ranging from 0.05 to 5 mg/mL for 24 h before inflammation was induced with 50 ng/mL LPS for 6 h. Figure 3.6 shows the dose response effect of buttermilk from colostrum or mature milk on LPS-induced NF-κB luciferase activity. Colostrum buttermilk caused a significant reduction in NF-κB luciferase activity, in a dose-dependant manner, at concentrations greater than 0.05 mg/mL. Mature milk buttermilk also caused a slight reduction in NF-κB luciferase activity; however, the effect was not dose-dependent. In all cases, the reduction in NF-κB luciferase activity associated with colostrum buttermilk was greater than with mature milk buttermilk when tested at equal concentrations (Figure 3.6). This was most evident at higher concentrations of buttermilk from colostrum and mature milk.

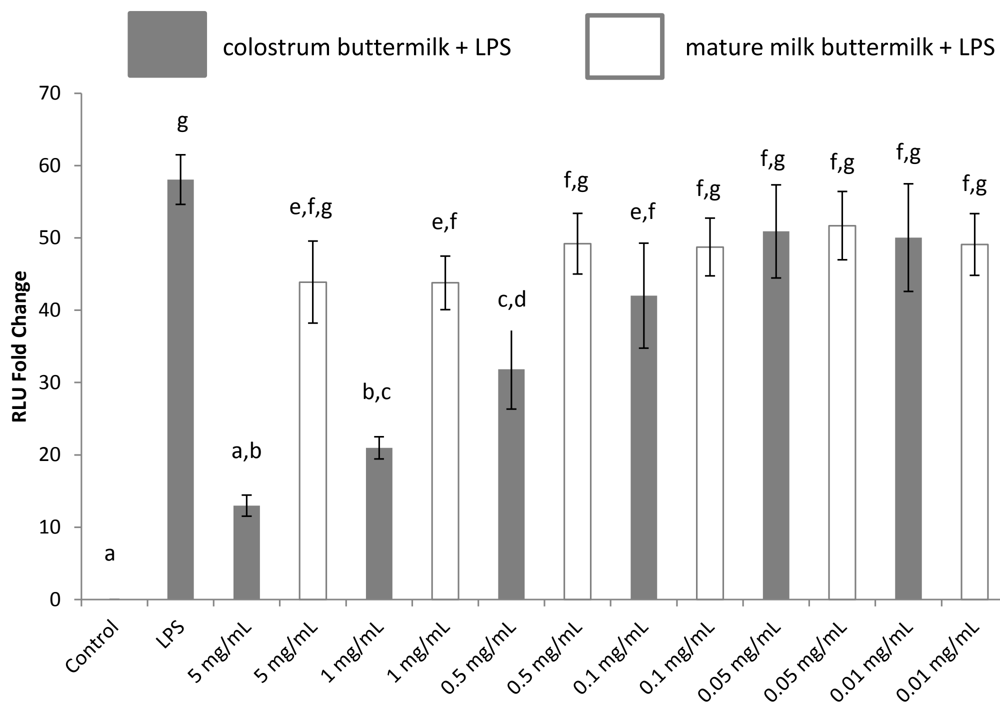


Figure 3.6 Dose response effect of buttermilk from colostrum or mature milk on LPS-induced NF-κB luciferase after 24 h pre-treatment. Exponentially growing cells were incubated with various concentrations (mg/mL) of buttermilk from colostrum or mature milk for 24 h and stimulated with 50 ng/mL of LPS for 6 h. Cell extracts were prepared for assessment of luciferase activity. Luciferase activity is expressed as the fold change between untreated cells relative to LPS-treated cells. Data shown represent the mean (± SD, n=3) of three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

3.3.7 Effect of buttermilk from colostrum or mature milk on the secretion of the pro-inflammatory cytokines IL-6 and IL-1β

Interleukin (IL)-6 is a pro-inflammatory cytokine that is produced at the site of inflammation and plays a key role in the acute phase response (Gregor and Hotamisligil, 2011). IL-6 dictates the transition from acute to chronic inflammation by changing the nature of leukocyte infiltration from polymorphonuclear neutrophils to monocyte/macrophages (Gabay, 2006). Furthermore, circulating IL-6 stimulates the hypothalamic-pituitary-adrenal axis, which is associated with central obesity, hypertension, and insulin resistance (Berg and Scherer, 2005).

IL-1β is a pro-inflammatory cytokine which mediates its actions through the IL-1 receptor 1 (IL-1R1) and potentially induces the production of inflammatory cytokines including IL-6 (Mc Ardle *et al*., 2013). IL-1β is not secreted *in vivo* in a biologically active state, i.e., activation is dependent on cleavage of pro-IL-1β by capase-1 (Dinarello *et al*., 2010). Increased concentrations of circulating IL-1β are associated with a greater risk of type 2 diabetes (Spranger *et al*., 2003).

In the current study, J774.2 macrophages were isolated, cultured and incubated with 500 µg/mL of buttermilk from colostrum or mature milk for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h and 25 µM ATP for 30 min. The effect of buttermilk from colostrum or mature milk on IL-6 and IL-1β secretion by J774.2 macrophages is shown in Figures 3.7a and 3.7b, respectively. Buttermilk from colostrum was found to decrease significantly the secretion of IL-6 and IL-1β from J774.2 macrophages. Buttermilk from mature milk caused a slight decrease in the secretion of IL-6 and IL-1β from J774.2 macrophages; however, the effect was not as pronounced as with colostrum buttermilk (Figure 3.7a and 3.7b). Buttermilk from colostrum or mature milk were also tested in the absence of LPS to investigate whether these samples alone induced IL-6 and IL-1β secretion. In the absence of LPS, it was found that colostrum buttermilk did induce secretion of IL-6, while no effect was observed in the presence of mature milk buttermilk (Figure 3.7a). Similarly, in the absence of LPS, buttermilk from colostrum and mature milk had no effect on the secretion of IL-1β (Figure 3.7b).

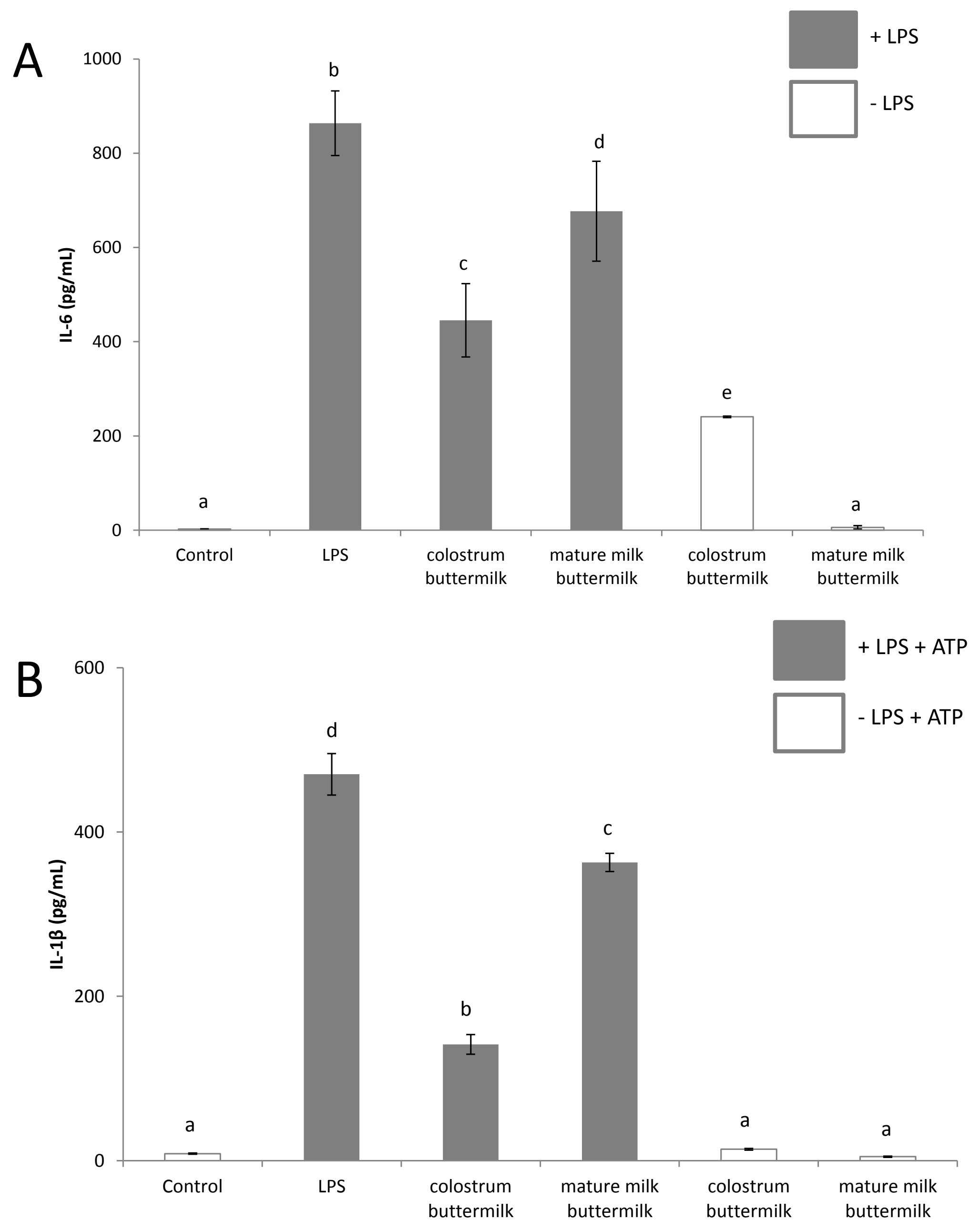


Figure 3.7 Effect of buttermilk from colostrum or mature milk on (a) IL-6 and (b) IL-1β secretion by J774.2 macrophages after 24 h pre-treatment. Exponentially growing cells were incubated with 500 µg/mL of buttermilk from colostrum or mature milk for 24 h and stimulated with 10 ng/mL of LPS for 3 h and 25 µM ATP for 30 min. The supernatant was removed and the concentration of cytokine quantified by ELISA. Data shown represent the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

3.3.8 Effect of buttermilk from colostrum or mature milk on the secretion of the pro-inflammatory cytokine IL-12

IL-12 is a pro-inflammatory cytokine that is produced mainly by dendritic cells (Hamza *et al*., 2010). IL-12 is a multifunctional cytokine that acts as a key regulator of cell-mediated immune responses by inducing T helper 1 differentiation (Del Vecchio *et al*., 2007). In the current study, BMDC were isolated and cultured for 7 d and incubated with buttermilk from colostrum or mature milk for 24 h before inflammation was induced with 10 ng/mL LPS for a further 24 h. The effect of buttermilk from colostrum or mature milk on IL-12 secretion from BMDC is shown in Figure 3.8. Colostrum buttermilk significantly reduced the secretion of IL-12 from BMDC. Mature milk buttermilk also caused a reduction in the secretion of IL-12; however, the effect was not as pronounced as with colostrum buttermilk.

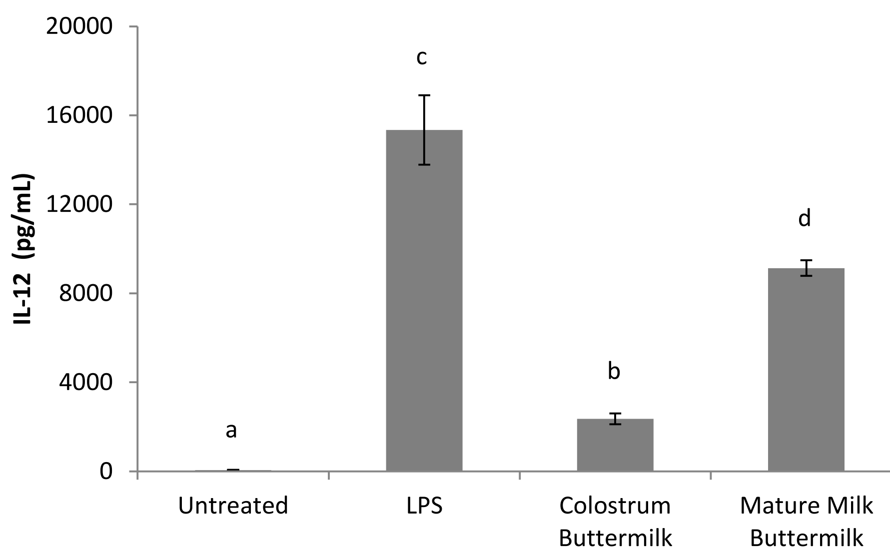


Figure 3.8 Effect of buttermilk from colostrum or mature milk on IL-12 secretion from BMDC. BMDC from male WT C57Bl/6 mice were cultured for 7 d then seeded at a density of 1 x 10^6 cells/mL and treated for 24 h with buttermilk from colostrum or mature milk (1 mg/mL) or H_2O vehicle control and then stimulated with LPS (10 ng/mL) for 24 h. Supernatants were collected and IL-12 concentrations were measured by ELISA. Data shown represent the mean (± SEM, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

3.3.9 Effect of buttermilk from colostrum or mature milk on the secretion of the anti-inflammatory cytokine IL-10

IL-10 is an anti-inflammatory cytokine which is produced by numerous immune cells including monocytes, macrophages and dendritic cells (Mc Ardle *et al*., 2013). IL-10 was first recognized for its ability to inhibit activation and effector function of T cells, monocytes and macrophages (Moore *et al*., 2001). The main function of IL-10 appears to be in limiting and terminating inflammatory responses (Villalta *et al*., 2011). In the current study, J774.2 macrophages were isolated, cultured and incubated with 500 µg/mL of buttermilk from colostrum or mature milk for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h and 25 µM ATP for 30 min. The effect of buttermilk from colostrum or mature milk on IL-10 secretion by J774.2 macrophages is shown in Figure 3.9. Colostrum buttermilk significantly increased the secretion of IL-10 from J774.2 macrophages while mature milk buttermilk caused no significant change compared to the LPS-positive control. Buttermilk from colostrum or mature milk were also tested in the absence of LPS to investigate whether these samples alone induced IL-10 secretion. In the absence of LPS, it was found that colostrum buttermilk did induce secretion of IL-10, as shown in Figure 3.9, while no effect was observed in the presence of mature milk buttermilk.

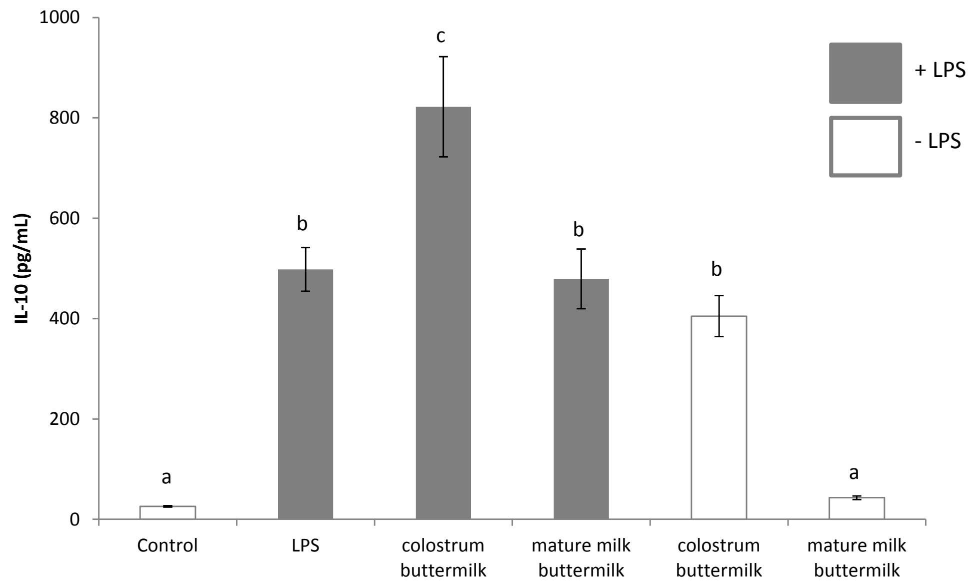


Figure 3.9 Effect of colostrum buttermilk and mature milk buttermilk on IL-10 secretion by J774.2 macrophages after 24 h pre-treatment. Exponentially growing cells were incubated with 500 µg/mL of colostrum buttermilk and mature milk buttermilk for 24 h and stimulated with 10 ng/mL of LPS for 24 h. The supernatant was removed and the concentration of cytokine quantified by ELISA. Data shown represent the mean (± SEM, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

3.3.10 Characterisation of the serum phase of colostrum

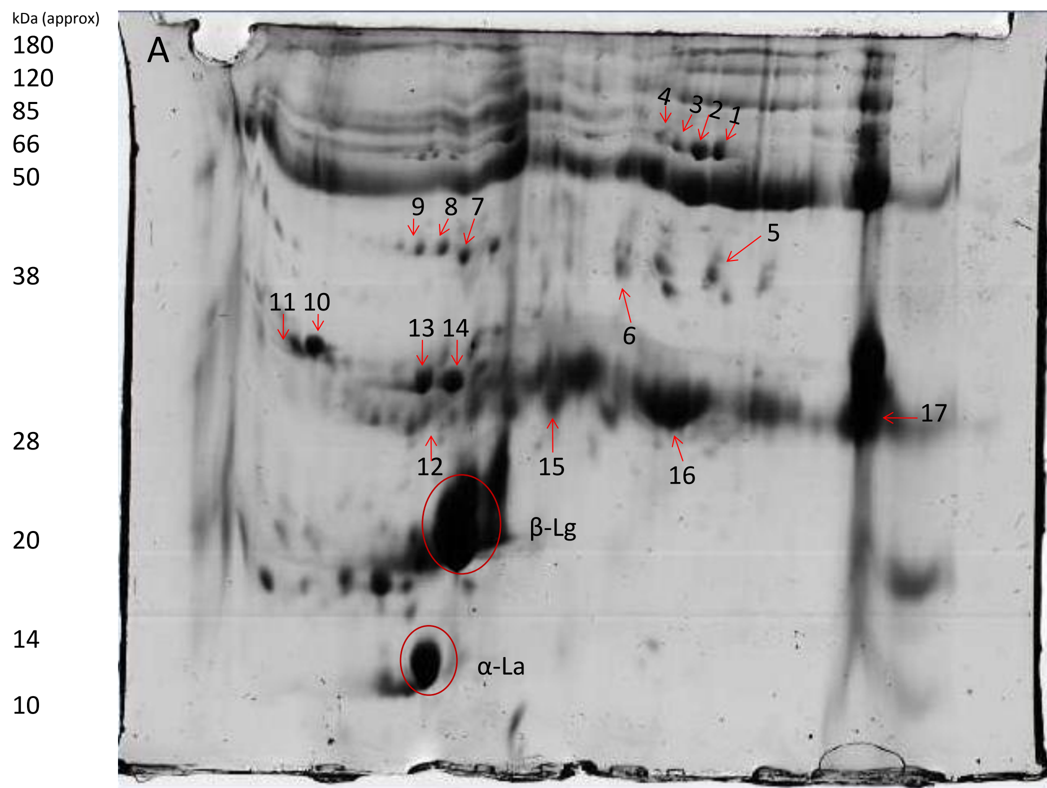
The protein profile of the pH 4.6-soluble fraction of (a) colostrum and (b) mature milk were analysed by 2-DE (Figure 3.10). Skimmed colostrum and mature milk were adjusted to pH 4.6 according to the method of Kuchroo and Fox (1982) in order to remove pH 4.6-insoluble material and improve the resolution of whey proteins on 2-DE electrophoretograms. The whey fraction of mature milk contains more than 200 different proteins (Sgarbieri, 2004); however, it is dominated by a small number of so-called major proteins, which collectively constitute over 80% of whey protein (Tremblay *et al*., 2003). Of these, β-Lg and α-La are the most abundant, as shown in Figure 3.10. As previously discussed (Section 3.3.1), colostrum has a distinct whey protein composition which is quite different from that of mature milk. The protein composition of colostrum is dominated by the presence of immunoglobulins, in particular IgG, whose natural function is the transfer of passive immunity from the cow to the calf (Elizondo-Salazar and Heinrichs, 2008).

The pH 4.6-soluble fraction of colostrum contained significantly more high molecular weight material in the range 30 – 180 kDa compared with the pH 4.6-soluble fraction of mature milk (Figure 3.10). To identify some of the proteins which were abundant in the pH 4.6-soluble fraction of colostrum compared with the pH 4.6-soluble fraction of mature milk, LC-MS was performed on 2-DE excised gel spots. Identification of the 17 spots analysed are shown in Table 3.4. Despite adjustment of skimmed colostrum to pH 4.6 in order to remove caseins by isoelectric precipitation, α_{S1}-casein was identified in 2 spots, β-casein was identified in 3 spots, and κ-casein was identified in 3 spots.

Spots 7-9 were identified as zinc-alpha-2-glycoprotein (ZAG), which was also identified in the pH 4.6-soluble colostrum buttermilk (Table 3.1) and discussed briefly in Section 3.3.1. ZAG is a multidisciplinary protein involved in many important functions in the human body, including fertilization and lipid mobilization (Hassan, *et al*., 2008). ZAG has previously been reported in bovine milk (Fong *et al*., 2008; Golinelli *et al*., 2011). Bao *et al.* (2005) reported that human adipocytes

express and secrete ZAG, with ZAG expression being regulated particularly through TNF-α and the PPARγ nuclear receptor. TNF-α is known to have *in vivo* effects on lipid metabolism and adipose tissue (Beutler and Cerami, 1988). TNF-α has been implicated as a causative factor in obesity-associated insulin resistance and the pathogenesis of type 2 diabetes (Moller, 2000). Similarly, Barroso *et al*. (1999) reported that PPARγ is associated with severe insulin resistance and diabetes mellitus.

Spot 6 was identified as alpha 1 acid glycoprotein (AGP). Milk-AGP is made up of at least two isoforms, a low MW group (44kDa) that is produced in the mammary gland, and a higher MW group (55-77 kDa) that is produced by somatic cells (Ceciliani *et al*., 2007). Ceciliani et *al.* (2005) reported that bovine colostrum contains 162 (+/- 63.7) μg/mL and 114.5 (+/- 67.8) μg/mL AGP during the first 12h and 24h *post partum*, but, AGP was not detectable in mature milk. AGP is a lipocalin (Ceciliani *et al*., 2005) with immunomodulatory and anti-inflammatory properties, and plays an important role in the regulation of local inflammatory reactions (Tilg *et al*., 1993). AGP has been shown to reduce the activation state of neutrophils and increase the expression of anti-inflammatory cytokines by macrophages (Hochepied *et al*., 2003). Spots 1-4 were identified as serum albumin, which was also identified in the pH 4.6-soluble fraction of buttermilk from colostrum (Table 3.1) and discussed briefly in Section 3.3.1.



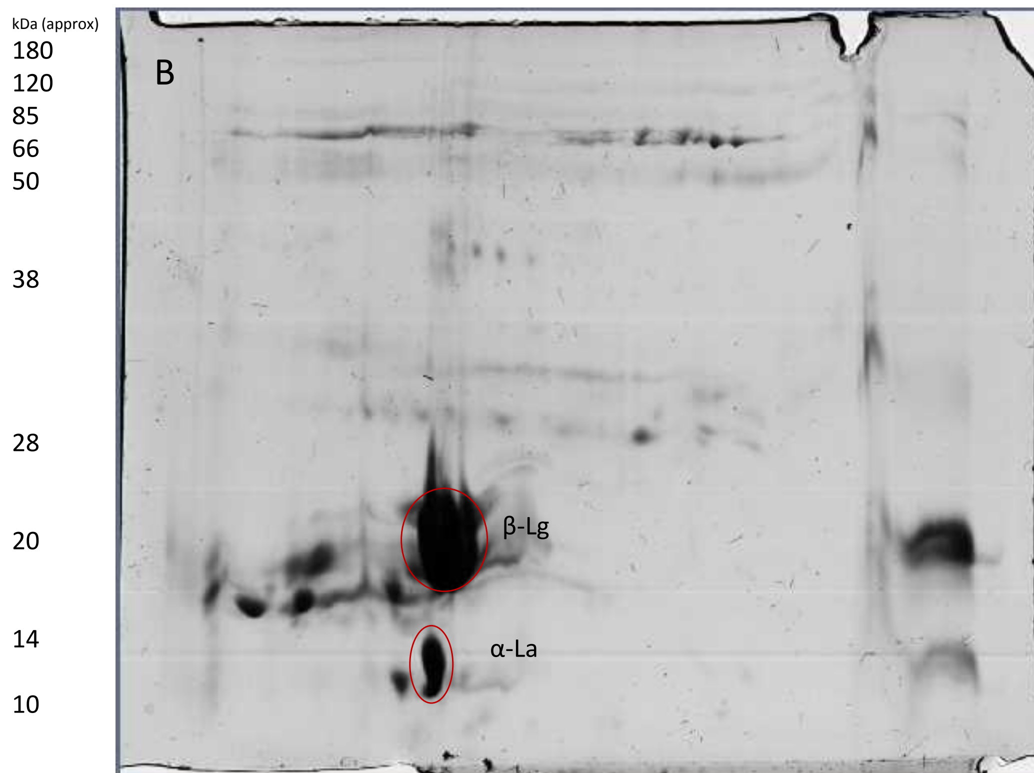


Figure 3.10 Two-dimensional gel electrophoretograms of the pH 4.6-soluble fraction of (a) colostrum and (b) mature milk separated under reducing conditions using a 7 cm pH 4-7 pI range for the first dimension and a 12% gradient acrylamide SDS-PAGE gel for the second dimension. Circled and numbered spots were excised and analysed by LC-MS.

Table 3.4 Identification of spots from the 2-D gel of pH 4.6-soluble colostrum by peptide mass fingerprinting using MALDI-TOF

Spot No.	Identified Protein	Uniprot Accession Number	Mol. mass (Da)	Peptides	Score
1	Serum albumin	PO2769	69248	8	84
2	Serum albumin	PO2769	69248	10	95
3	Serum albumin	PO2769	69248	9	68
4	Serum albumin	PO2769	69248	7	73
5	β-casein	PO2666	25091	4	52
6	α-1-acid glycoprotein	Q5GN72	23168	6	78
7	Zinc-α-2-glycoprotein	B2BX70	33830	11	79
8	Zinc-α-2-glycoprotein	B2BX70	33830	17	112
9	Zinc-α-2-glycoprotein	B2BX70	33830	12	156
10	α_{S1}-casein	B5B3R8	24513	8	65
11	α_{S1}-casein	B5B3R8	24513	5	46
12	κ-casein	PO2668	21256	4	55
13	β-casein	PO2666	25091	5	50
14	β-casein	PO2666	25091	4	46
15	κ-casein	PO2668	21256	3	59
16	κ-casein	PO2668	21256	6	71
17	β-lactoglobulin	PO2754	19870	6	78

The pH 4.6-soluble fraction of colostrum, and subsequent fractions, were analysed by RP-UPLC, as shown in Figure 3.11 a-e. β-Lg and α-la were the predomainant proteins identified in the pH 4.6-soluble fraction of colostrum and 30 kDa retentate, but, the concentration of each was greatly reduced in the 30 kDa permeate. The 30 kDa permeate was further fractionated by ultrafiltration and the products analysed. Compared to the 30 kDa permeate, β-Lg and α-La were concentrated slightly in the 3 kDa retentate; but, both proteins were virtually absent from the 3 kDa permeate. Approximately 9 peaks (excluding the injection peak) were observed within the first min of each chromatogram in Figure 3.11 a-e. Material eluting in this region is most likely hydrophilic peptides or individual amino acids.

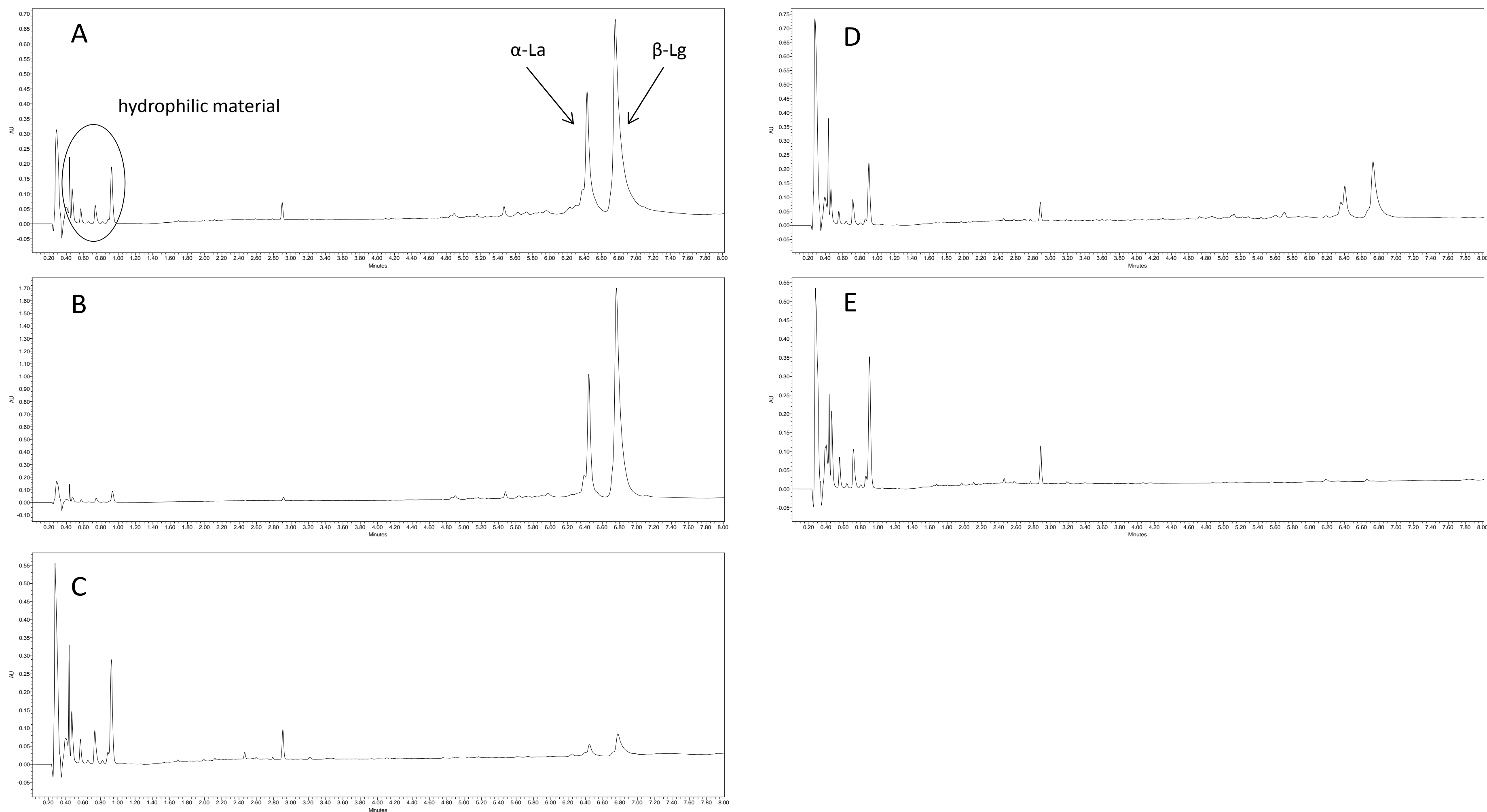


Figure 3.11 Reversed-phase UPLC chromatograms of (a) pH 4.6-soluble colostrum; (b) pH 4.6-soluble colostrum 30 kDa retentate; (c) pH 4.6-soluble colostrum 30 kDa permeate; (d) 3 kDa retentate of pH 4.6-soluble colostrum 30 kDa permeate; (e) 3 kDa permeate of pH 4.6-soluble colostrum 30 kDa permeate

The pH 4.6-soluble fraction of skimmed colostrum and subsequent 30 kDa UF retentate and permeate, were analysed by LC-MS using a LTQ linear ion trap (Table 3.5). The most abundant proteins identified, based on the greatest number of peptide matches, in the pH 4.6-soluble fraction of colostrum were α_{S1}-, α_{S2}- and β-casein. Even though colostrum was adjusted to pH 4.6 in order to remove casein by isoelectric precipitation, it is not unusual for some casein to remain. However, it is unusual that the caseins represented the most abundant proteins in the pH 4.6-soluble colostrum. This may be explained by Section 2.3.11 of this thesis where it was found that casein did not precipitate at pH 4.6 in certain colostrum samples. The most abundant proteins in the pH 4.6-soluble 30 kDa retentate were α_{S1}-casein, β-Lg and β-casein while the most abundant proteins in the pH 4.6-soluble 30 kDa permeate were α_{S1}-casein, β-Lg and α_{S2}-casein. κ-Casein was present in a relatively high abundance in all three samples. α-La was identified in the pH 4.6-soluble colostrum and pH 4.6-soluble 30 kDa retentate but not in the pH 4.6-soluble 30 kDa permeate. Serum albumin, which was identified in colostrum buttermilk and discussed briefly in Section 3.3.1, was only identified in the pH 4.6-soluble colostrum. Polymeric immunoglobulin receptor (pIgR), which was identified in colostrum buttermilk and discussed briefly in Section 3.3.1, was found in the pH 4.6-soluble colostrum and 30 kDa retentate of pH 4.6-soluble colostrum but not in the 30 kDa permeate of the pH 4.6-soluble fraction.

Lactoferrin (also known as lactotransferrin), which was identified in colostrum buttermilk and discussed briefly in Section 3.3.1, was found in the pH 4.6-soluble fraction of colostrum and pH 4.6-soluble 30 kDa retentate but not in the pH 4.6-soluble 30 kDa permeate. This was to be expected as lactoferrin is an 80 kDa glycoprotein which would not pass through a 30 kDa UF membrane. Lactoferrin is known to down-regulate pro-inflammatory cytokine production in cell lines by acting via NF-κB (Haversen *et al*., 2002), which leads to a decrease in the secretion of TNF-α and IL-6 in mice (Kruzel *et al*., 1998). In an insulin-resistance state, pro-inflammatory cytokines activate several kinases (Gual *et al*., 2005) which have been shown to inhibit insulin action by promoting phosphorylation of serine residues of the insulin-signalling pathway (Schenk *et al*., 2008).

Lipoprotein lipase (LPL) was identified in the pH 4.6-soluble fraction of colostrum only. LPL is a multifunctional enzyme which is produced by many tissues, including adipose tissue, cardiac and skeletal muscle, islets and macrophages (Wang and Eckel, 2009). The main function of LPL is the hydrolysis or core triglycerides and very low-density lipoproteins (Eckel, 1989). Plasma LPL activity has been shown to be reduced in insulin-resistance subjects without diabetes (Knudsen *et al*., 1995) and, overall, plasma LPL activity is inversely correlated with insulin resistance in individuals with type 2 diabetes (Erikkson *et al.*, 2003).

Table 3.5. Identification of proteins from the ph 4.6-soluble fraction of colostrum, pH 4.6-soluble 30 kDa retentate and pH 4.6-soluble 30 kDa permeate, by LC-MS using a LTQ linear ion trap

			pH 4.6-soluble colostrum			pH 4.6-soluble 30 kDa retentate			pH 4.6-soluble 30 kDa permeate		
Identified protein	Uniprot Accession Number	Mol. mass (Da)	% Coverage	Peptides	Score	% Coverage	Peptides	Score	% Coverage	Peptides	Score
α_{S1}-casein	PO2662	24529	41.6	36	464	31.3	22	461	35	15	263
α_{S2}-casein	PO2663	26019	20.7	8	310	15.3	3	172	18.9	4	131
β-casein	PO2666	25107	54	7	303	33.5	3	156	3.6	1	66
β-lactoglobulin	PO2754	19883	33.7	6	198	21.3	6	252	32.6	6	235
Lactotransferrin	P24627	78056	5.2	3	115	1.8	1	46	N/A	0	0
NEK4 protein	A6QQ15	79280	0.7	2	32	N/A	0	0	N/A	0	0
ACSL4 protein	A7E3D9	72659	1.1	2	46	N/A	0	0	N/A	0	0
Serum albumin	PO2769	69293	4.4	2	130	N/A	0	0	N/A	0	0
κ-casein	PO2668	21269	23.7	2	132	28.9	3	119	28.9	3	105
Lipoprotein lipase	P11511	53378	6.1	2	73	N/A	0	0	N/A	0	0
Polymeric immunoglobulin receptor	P81265	52435	4.4	2	113	5.9	2	51	N/A	0	0
TWF2 protein	A2VDX0	39564	1.4	1	31	N/A	0	0	N/A	0	0
Heart fatty acid-binding protein	P10790	14779	9.8	1	68	9.8	1	45	N/A	0	0
UPF0488 protein C8orf33 homolog	Q2K1D8	20972	4.3	1	30	N/A	0	0	N/A	0	0
Protein kinase C gamma type	PO5128	77156	0.9	1	44	N/A	0	0	N/A	0	0
α-lactalbumin	P00711	16247	9.9	1	42	9.9	1	55	N/A	0	0
Enterotoxin-binding glycoprotein PP20K	Q9TRB9	2266	70	1	66	70	1	54	70	1	62
RecQ-mediated genome instability protein 1	A41F98	70051	1.4	1	37	N/A	0	0	N/A	0	0
Retinoid isomerohydrolase	Q28175	60944	0.9	1	28	N/A	0	0	N/A	0	0
RUVBL1 protein	A7MBG8	50154	N/A	0	0	3.3	1	32	N/A	0	0
Glycosylation-dependent cell adhesion molecule 1	P80195	17152	N/A	0	0	7.2	1	62	N/A	0	0
Glutamine synthetase	P15103	42031	N/A	0	0	2.9	1	30	N/A	0	0
Inter-alpha-trypsin inhibitor heavy chain H1	Q0VCM5	101237	N/A	0	0	N/A	0	0	1.1	1	47
PAX-interacting protein 1	A0JNA8	109167	N/A	0	0	1.3	1	49	N/A	0	0
Protein-glutamine gamma-glutamyltransferase 2	P51176	77112	N/A	0	0	3.8	1	29	N/A	0	0

N/A = not applicable

3.3.11 In vitro *insulinotrophic effects of colostrum fractions*

There are two classifications of diabetes; type 1 diabetes is an autoimmune condition which is characterised by the destruction of pancreatic β-cells, resulting in reduced or complete loss of insulin secretion, while type 2 diabetes is characterised by insulin resistance in peripheral tissues (adipose tissue, liver and skeletal muscle) which, in some cases, may develop into deficiencies of insulin secretion and β-cell dysfunction (Schrezenmeir and Jagla, 2000). The WHO predicts that the incidence of type 2 diabetes will increase to 366 million by 2030 (WHO 2006b). The consumption of milk proteins has been associated with a reduction in the risk of type 2 diabetes (Choi *et al*., 2005; Tremblay and Gilbert, 2009) by regulating postprandial glycaemia and insulin secretion in normoglycemic and type 2 diabetic individuals (Frid *et al*., 2005). The objective of this study was to assess the potential of colostrum in beneficially modulating insulin secretion by examining their possible insulinotrophic properties on pancreatic β-cells *in vitro*. BRIN-BD11 is a hybrid cell line formed by the electrofusion of a primary culture of NEDH rat pancreatic islets with RINm5F (a cell line derived from a NEDH rat insulinoma). The cells grow as monolayers with an epitheliod morphology and, when confluent, they take on a pavemental pattern characteristic of epithelial cells. Figure 3.12 shows the effect of colostrum fractions on insulin secretion when administered to the pancreatic β-cells in combination with 16.7 mM glucose; 10 mM alanine in combination with 16.7 mM glucose was used as a positive control.

The colostrum lipid and casein fractions had no effect on insulin secretion; however, the pH 4.6-soluble, pH 4.6-soluble 30 kDa retentate and pH 4.6-soluble 30 kDa permeate of colostrum were found to significantly increase insulin secretion in pancreatic β-cells, at levels similar to that obtained with the positive control, in the order pH 4.6-soluble 30 kDa permeate > pH 4.6-soluble > pH 4.6-soluble 30 kDa retentate. However, colostrum fractions were tested on an equal solids and not equal protein basis. As can be seen from Table 3.6, the protein content of these colostrum fractions varied widely. Assuming that the bioactive component was protein-based, it would be fair to assume that, if these samples were tested on an

equal protein basis, insulin secretion from pancreatic β-cells in response to the pH 4.6-soluble 30 kDa permeate would be greatly amplified. These colostrum fractions were naturally high in lactose. To rule out the possibility of lactose causing an insulinotrophic response, lactose monohydrate was also tested, however, it was found to have no effect on insulin secretion (results not shown).

Table 3.6 Protein concentration of the pH 4.6-soluble fractions of colostrum, as determined by the Bradford protein assay

Colostrum fraction	% Protein
pH 4.6-soluble	42.6
pH 4.6 soluble 30 kDa retentate	70.2
pH 4.6-soluble 30 kDa permeate	8.9
pH 4.6-soluble 30 kDa permeate 3 kDa retentate	15.4
pH 4.6-soluble 30 kDa permeate 3 kDa permeate	5.4

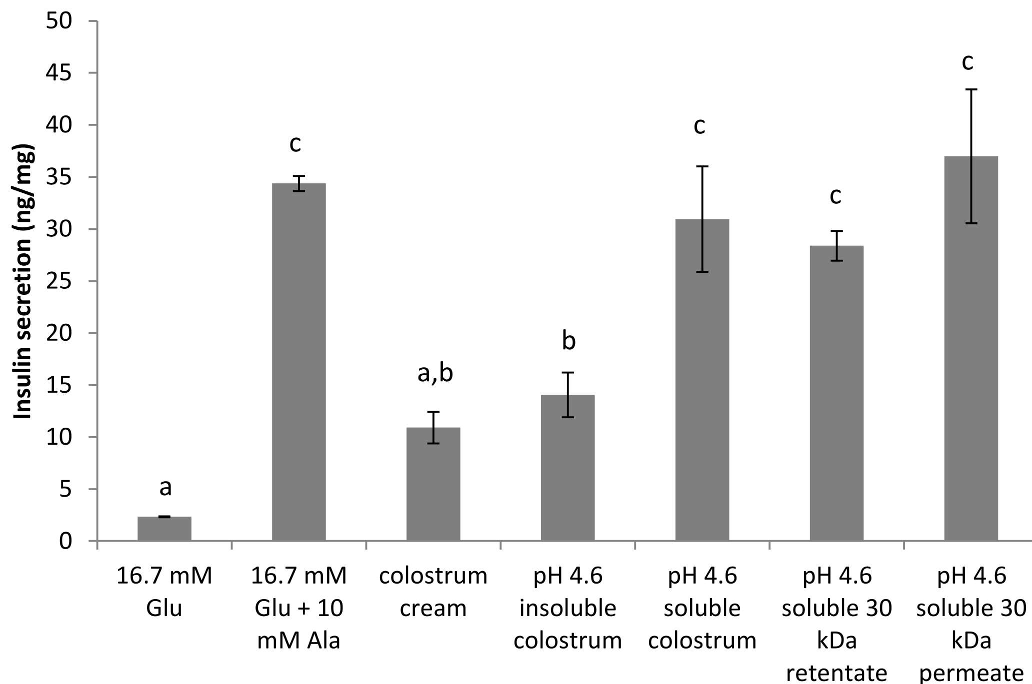


Figure 3.12 Acute Insulin secretion in BRIN-BD11 cells. BRIN-BD11 cells ($1.5x10^5$) were treated with basal levels of glucose with 1.1 mM Glucose Krebs for 40 min, followed by treatment with high glucose (16.7 mM krebs) plus colostrum fractions which stimulated insulin secretion. Data shown represent the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

The addition of colostrum fractions to BRIN-BD11 cells did not affect cell viability within the concentration range tested (results not shown). Therefore, BRIN-BD11 cells were treated with the 30 kDa permeate of the pH 4.6-soluble fraction of colostrum at concentrations ranging from 0.1 to 5 mg/mL. The addition of the 30 kDa permeate of the pH 4.6-soluble fraction of colostrum led to a concentration-dependant increase in the concentration of insulin secreted (Figure 3.13).

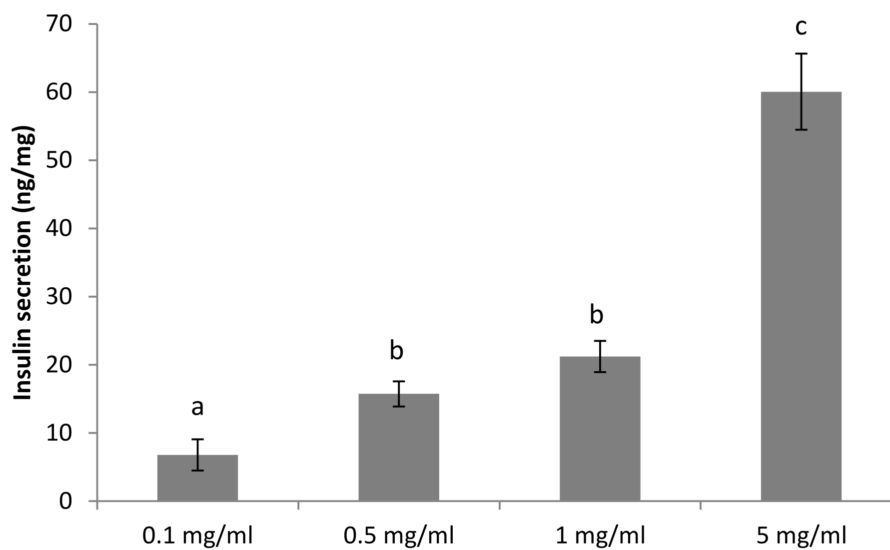


Figure 3.13 Concentration-dependent insulin secretion from BRIN-BD11 cells. BRIN-BD11 cells ($1.5x10^5$) were treated with basal levels of glucose with 1.1 mM glucose Krebs for 40 min, followed by treatment with high glucose (16.7 mM Krebs) plus pH 4.6-soluble colostrum 30 kDa permeate at concentrations of 0.1, 0.5, 1 and 10 mg/mL which stimulated a dose response insulin secretion. Data shown represent the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

Colostrum is rich in growth factors, especially insulin-like growth factor-1 (IGF-1), transforming growth factor beta-2 (TGF-β2) and growth hormone (GH) (Elfstrand *et al*., 2002). Acutely, a high dose of IGF-1 results in hypoglycaemia (Guler *et al*., 1987), whereas prolonged IGF-1 therapy has been shown to ameliorate hyperglycaemia in type 1 and type 2 diabetes mellitus, as well as in severe insulin-resistant states (Dunger *et al*., 1995). A cell proliferation assay was carried out in order to determine if growth factors present in colostrum may be causing an insulinotrophic response. However, as can be seen from Figure 3.14, no differences were observed between the control and colostrum samples, ruling out growth factors as a possible cause of insulin secretion.

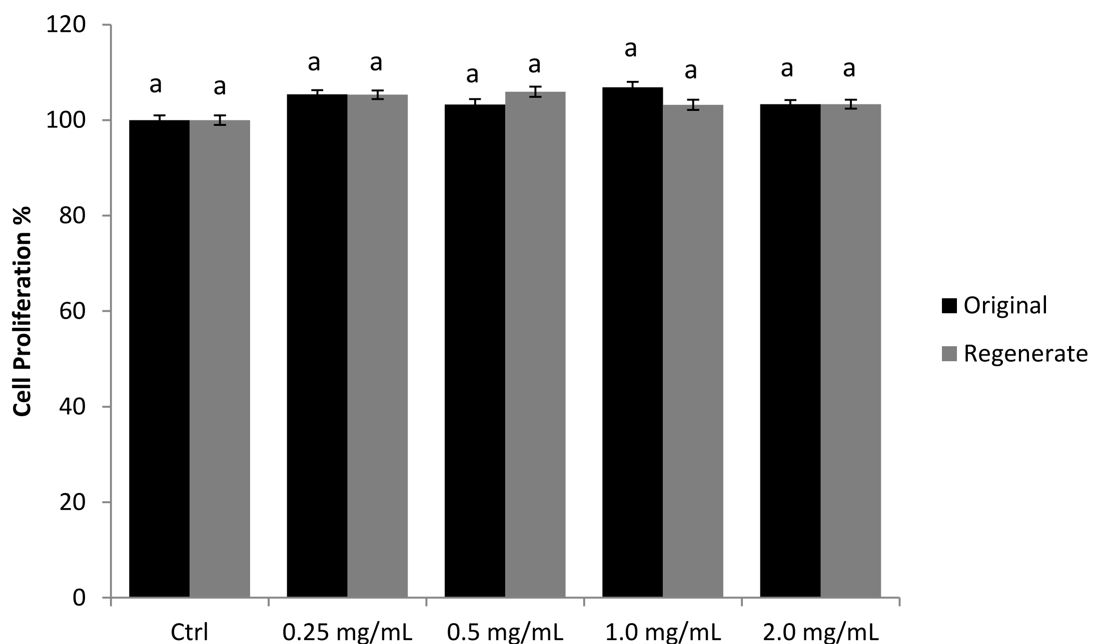


Figure 3.14 Measurement of mitochondrial function reflecting cell proliferation following treatment with colostrum fractions as determined by the WST-1 assay. BRIN-BD11 cells ($4x10^4$) were incubated for 24 h in RPMI-1640 medium supplemented with the original pH 4.6-soluble colostrum 30 kDa permeate or a regenerated sample at various concentrations ranging from 0 to 2.0 mg/mL. Cell viability was determined by the WST-1 assay. Results are relative to control of RPMI-1640 medium with no treatment (100%) and expressed at t = 90 min. Data shown represent the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

Based on the results shown in Figure 3.13 and Table 3.6, it was decided to further fractionate the pH 4.6-soluble 30 kDa permeate of colostrum by ultrafiltration to produce a 3 kDa retentate and permeate. This step was undertaken in order to remove any intact protein material from the pH 4.6-soluble 30 kDa permeate. Essentially what was left in the 3 kDa permeate was peptide material, lactose and ash. The 3 kDa permeate caused a significant increase in insulin secretion from pancreatic β-cells, while no effect was observed with the 3 kDa retentate.

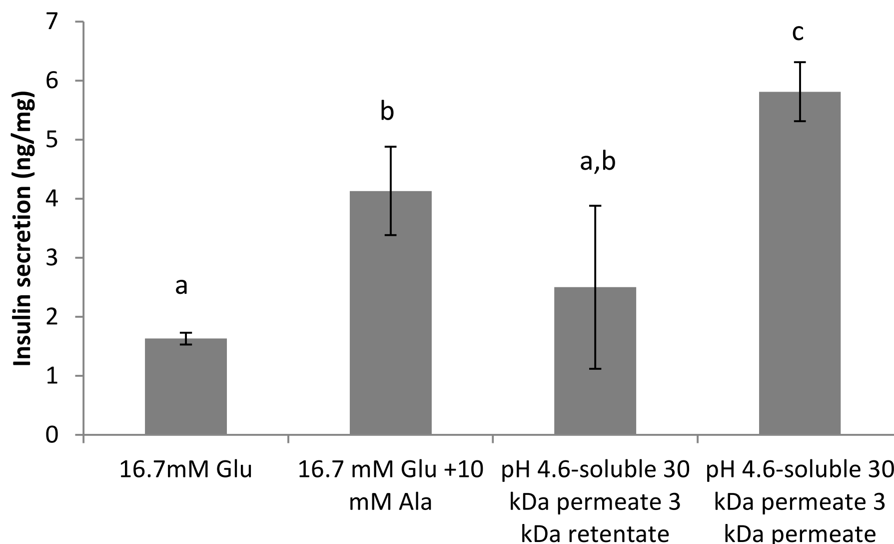


Figure 3.15 Acute insulin secretion from BRIN-BD11 cells. BRIN-BD11 cells ($1.5x10^5$) were treated with basal levels of glucose with 1.1mM Glucose Krebs for 40 min, followed by treatment with high glucose (16.7mM Krebs) plus pH 4.6-soluble colostrum 30 kDa permeate 3 kDa retentate or permeate which stimulated insulin secretion. Data shown represent the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

3.4 Conclusion

The current study focussed on the characterisation of bovine colostrum and the potential anti-inflammatory and insulinotrophic properties of fractions thereof. The protein profile of the serum phase of colostrum and colostrum buttermilk were analysed by two-dimensional electrophoresis and mass spectrometry. Marked differences were observed between the protein profile of colostrum and mature milk. GC-MS revealed variations between the fatty acid profiles of colostrum and mature milk.

Based on the results described in the current study, cream and, in particular, buttermilk from colostrum, have the potential to modulate immune function, i.e., significantly reduce LPS-induced secretion of the transcription factor NF-κB and pro-inflammatory cytokines TNF-α, IL-6, and IL-1β from J774.2 cells, and IL-12 from BMDCs, as well as increasing LPS-induced secretion of the anti-inflammatory cytokine IL-10 from J774.2 cells. While several proteins associated with the MFGM which were identified in colostrum buttermilk in the current study have reported biological activities relating to inflammation and immune function, further work is needed to pin-point the specific biological activity of each protein and their precise mode of action, in addition to any possible synergistic effects.

It should be noted that buttermilk from colostrum is a complex fluid which, in addition to a very heterogeneous group of proteins, also contains carbohydrates, lipids and minor constituents such as vitamins and minerals. The effect of fatty acids on inflammation was discussed briefly in Section 3.3.3. One aspect which was not investigated in the current study was the effect of the carbohydrate fraction of colostrum on inflammation. Compared to mature milk, colostrum contains much higher concentrations of oligosaccharides and other glycoconjugates (Nakamura *et al*., 2003; Urashima *et al*., 2009) which are known to exhibit a range of immunological effects, e.g., inhibit immune cell recruitment and adhesion in endothelial cells (Bode *et al*., 2004) and stimulate the production of cytokines in blood-borne immune cells (Eiwegger *et al*., 2004).

The serum phase of colostrum caused a significant increase in insulin secretion when administered to pancreatic β-cells cells in combination with 16.7 mM glucose. Fractionation by ultrafiltration revealed that bioactivity was most concentrated in the 3 kDa permeate.

The consumption of dairy products has been negatively correlated with type 1 diabetes (Scott, 1991); however, Mensink (2006) reported a protective relationship between the consumption of low-fat dairy products and type 2 diabetes. In particular, the consumption of milk proteins and milk-derived peptides has been associated with a reduction in the risk of type 2 diabetes (Choi *et al*., 2005; Tremblay and Gilbert, 2009) by regulating postprandial glycaemia and insulin secretion in normoglycemic and type 2 diabetic individuals (Frid *et al*., 2005). Glucose is the primary stimulus for insulin secretion (Bryan and Aguilar-Bryan, 1997). Essential amino acids are the primary regulators of the protein-mediated insulin response and are heterogeneous in their insulinotropic potency (Floyd *et al*., 1996). L-Glutamine, L-leucine and L-arginine are known to regulate insulin secretion acutely and chronically from pancreatic β cells *in vitro* and *in vivo* (Bratusch-Marrain *et al*., 1980). It is possible that the insulinotrophic properties of the serum phase of colostrum is due to the presence of free amino acids. In addition to glucose and amino acids, fatty acids are also known to stimulate insulin secretion (Grill and Qvigstad, 2000). The presence of free fatty acids in the serum phase of colostrum is another possible explanation for its insulinotrophic properties.

Based on the results obtained in this study, colostrum may represent a novel treatment for inflammatory- and immune-related disorders; however, the current study focused on *in vitro* experimental approaches. Assuming the bioactivity of colostrum was protein-derived, it is likely that, during digestion, enzymatic modification of proteins will alter their biological activities. For this reason, further work is needed to investigate whether the *in vitro* bioactive effects can be replicated *in vivo*. This work would help in the formulation of a food product which maintains *in vivo* bioactivity.

3.5 Acknowledgements

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Chapter 4

Characterisation of the protein component of milk from cows with lipopolysaccharide-induced mastitis and its potential immunomodulatory properties

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Declaration: LPS-induced mastitic milk samples were supplied by Olga Wellnitz of the University of Bern, Switzerland, who also measured the SCC. Fractionation and characterisation of milk samples were performed by Brian McGrath at University College Cork. Cell exposures and cytokine analysis were performed by Stacey Kelly at University College Dublin. All experimental data/results were analysed and the chapter written by Brian McGrath.

Abstract

Mastitis is an inflammation of the mammary gland and is characterised by physical, chemical and bacteriological changes in the milk. Gram-negative *Escherichia coli* is among the most common bacteria known to cause mastitis. Lipopolysaccharide (LPS) is an endotoxin and is located in the outer membrane of all Gram-negative bacteria. The objective of this study was to investigate the early proteomic changes in the milk of 8 cows in response to infusion with LPS at quarter level in a model mastitic system. In addition, the immunological potential of a peptide fraction of this LPS-induced mastitic milk was investigated. One udder quarter of each cow was challenged with 200 µg LPS. From these quarters, milk samples were obtained after 8 h, while milk samples from respective control quarters were obtained prior to LPS infusion. An increase in the SCC of milk from infused quarters was observed for all 8 cows. One- and two-dimensional SDS-polyacrylamide gel electrophoretograms revealed marked differences in the protein profiles of milk samples from LPS-challenged quarters compared with milk samples from control quarters, i.e., milk samples from LPS-challenged quarters showed hydrolysis of the caseins due to indigenous proteolytic activity. Similarly, reversed phase-ultra performance liquid chromatography showed an increase in the quantity of pH 4.6-soluble peptide material in milk from LPS-challenged quarters compared with milk from control quarters. In addition, these samples were found to contain increased concentrations of serum proteins, most notably serum albumin and immunoglobulins, due to increased permeability of the blood-milk barrier in response to an elevated SCC. Subsequently, the pH 4.6-soluble protein content of milk from LPS challenged quarters was found to be greater than that of the corresponding control milk samples, but to varying degrees across all 8 cows. A pH 4.6-soluble fraction of bulked milk from LPS challenged quarters of all 8 cows was prepared and was found to cause a substantial induction in the secretion of the anti-inflammatory cytokine, IL-10, from a murine macrophage cell line, while no effects on the secretion of the pro-inflammatory cytokine, TNF-α, were observed. Based on the results obtained in this study, milk from cows with experimentally induced mastitis may represent a functional food product/ingredient with immune-stimulating properties.

4.1 Introduction

Bovine mastitis is defined as inflammation of mammary gland parenchyma and most commonly results from a bacterial infection (Seegers *et al*., 2003, Sharma *et al*., 2011). Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* are among the most prevalent bacteria known to cause mastitis (Ibeagha-Awemu *et al*., 2010). Mastitis is one of the greatest influences on milk composition (Kitchen, 1981; Barbano *et al*., 2006; Forsback *et al*., 2010, 2011) and has a negative effect on the physico-chemical properties of milk (Cunha *et al*., 2008). Clinical mastitis is a costly disease amongst dairy farmers due to a reduction in milk volume and quality, increased culling rates, and the need to discard milk (Oliver and Mitchell, 1984; Smith *et al*., 1985; Oliver, 1988; DeGraves and Fetrow, 1993; Todhunter *et al*., 1995). Mastitis has also been shown to have harmful effects on the reproductive efficiency of dairy cows (Moore *et al*., 1991; Ahmadzadeh *et al*., 2009). Mastitis is estimated to cost the New Zealand and US dairy industries €180 million and €1.3 billion per annum, respectively (Geary *et al*., 2012).

Somatic cells are indicators of both resistance and susceptibility of cows to mastitis; somatic cell count (SCC) is a predictor of intramammary infection. Somatic cells found in healthy milk are primarily leukocytes, which include macrophages, lymphocytes and polymorphonuclear neutrophils (PMN) (Sharma *et al.,* 2011). The SCC in milk from a healthy mammary gland is usually lower than 1×10^5 cells/mL, while bacterial infection causes it to increase to above 1×10^6 cells/mL (Bytyqi *et al*., 2010). Somatic cells contain lysosomes that release active proteolytic enzymes, i.e., elastase, collagenase and cathepsins (Kelly and McSweeney, 2003). Several authors have correlated increased proteolysis in milk with an elevated SCC (de Rham and Andrews, 1982; Andrews, 1983; Le Roux *et al*., 1995; Moussaoui *et al*., 2004; Hinz *et al*., 2012), due mainly to increased levels of proteases (Larsen *et al*., 2006). Previous studies have shown that cathepsin D activity (O'Driscoll *et al*., 1999) and cysteine protease activity were elevated with increasing SCC in milk (Suzuki & Katoh, 1990; O'Driscoll *et al*., 1999). Similarly, previous results have shown activation of the plasmin system during experimentally induced *E. coli* mastitis

infections in cows (Grieve and Kitchen, 1985; Saeman *et al*., 1988; Moussaoui *et al*., 2002; Hinz *et al*., 2012).

As already mentioned, *E. coli* is the most prevalent Gram-negative bacterium which causes mastitis in cows and is commonly used experimentally to induce mastitis (Andrews, 1983; Hirvonen *et al*., 1999; Moussaoui *et al*., 2002; Sladek *et al*., 2002; Bannerman *et al*., 2004; Moussaoui *et al*., 2004; Schmitz *et al*., 2004; Sohn *et al*., 2007; Hinz *et al*., 2012). Infections caused by *E. coli* are usually associated with a faster and more dramatic immune response than that due to Gram-positive bacteria such as *S. aureus* (Petzl *et al*., 2008; Bannerman, 2009) or *Streptococcus uberis* (Rambeaud *et al*., 2003; Genini *et al*., 2011). The ability of *E. coli* to grow in mammary secretions and to liberate lipopolysaccharide (LPS) is of crucial importance in the pathogenesis of mastitis (Mehrzad *et al*., 2008). LPS is a part of the outer membrane in the cell wall of Gram-negative bacteria (Rietschel *et al*., 1994) and is a potent inducer of inflammation and the acute phase response (Berczi, 1998).

In humans, low grade inflammation has been linked with a range of metabolic conditions such as obesity, insulin resistance, metabolic syndrome and coronary heart disease (Rosa *et al.,* 2012). Bovine milk is known to contain a number of biologically active peptides. These peptides are not active within the parent protein but can be produced from precursor milk proteins in the following ways: (a) enzymatic hydrolysis by digestive enzymes (Korhonen and Pihlanto, 2003), (b) fermentation of milk with proteolytic starter cultures (Fugslang *et al.,* 2003), and/or (c) proteolysis by enzymes derived from microorganisms or plants (Yamamoto *et al.,* 1994). Immunomodulatory peptides are those which can enhance immune function *in vivo* through stimulation or suppression of the immune system (Gauthier *et al.*, 2006). They work by stimulating the proliferation of lymphocytes, the phagocytic activities of macrophages (Nagpal *et al.,* 2011), antibody synthesis and cytokine expression (Udenigwe and Aluko, 2012).

The objectives of the current study were to investigate the early proteomic changes in the milk of 8 cows in response to infusion with LPS at quarter level. In addition,

the effects of a pH 4.6-soluble fraction of the experimentally induced mastitic milk on the secretion of the anti-inflammatory cytokine, interleukin (IL)-10, and the pro-inflammatory cytokine, tumor necrosis factor (TNF)-α, from a murine macrophage cell line, were investigated.

4.2 Materials and Methods

4.2.1 Experimental animals

Eight dairy cows (Holstein Friesian [n=6] and Swiss Fleckvieh [n=2]), referred from hereon in as cows A to H, were selected based on their milk having a SCC < 130,000 cells/mL in all four quarters (morning foremilk samples) for at least 5 days before the experiment. Parities ranged from 1 to 5 and none of the udders showed clinical signs of mastitis. The cows were housed in tie stall barns during experiments. SCC measurements were performed with a DeLaval cell counter (DeLaval International AB, Tumba, Sweden).

4.2.2 Experimental procedures

After the morning milking, one quarter of each cow was challenged with 200 µg of *E. coli* LPS (serotype O26:B6; Sigma-Aldrich), diluted in 10 mL of saline solution, that caused mastitis (Yang *et al*., 2008). Another quarter from each cow was used as a control quarter and treated with 10 mL of saline solution. Treated quarters were selected randomly (front or rear, left or right) and equally distributed. Milk samples were taken by hand from control quarters at 0 h, prior to injection, and from LPS-challenged quarters at 8 h post challenge.

4.2.3 *Determination of protein content*

Protein content of milk samples was determined by the Bradford assay, as described in Section 3.2.5.

4.2.4 One-dimensional gel electrophoresis (1-DE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Laemmli (1970), as described in Section 2.2.13.

4.2.5 Two-dimensional gel electrophoresis (2-DE)

2-DE was performed on the pH 4.6-soluble skimmed milk samples, as described in Section 3.2.7, with slight modifications. Immediately after 2-DE, gels were immersed in an initial fixing solution (50% methanol, 10% acetic acid) in clean glass dishes overnight on a microplate shaker (VWR, Dublin, Ireland). Following this, the gels were fixed, pre-treated, stained and developed using the reagents provided by the Pierce Silver Stain Kit (Thermo Fisher Scientific, Waltham, MA, USA). Once the developing was stopped the gels were washed thoroughly in distilled H_2O before being scanned. Images of gels were captured using the GS-800 calibrated densitometer (Bio-Rad) with quantity one software (Bio-Rad).

4.2.6 Reversed phase-ultra performance liquid chromatography (RP-UPLC)

Samples were adjusted to pH 4.6 as described in Section 3.2.7. RP-UPLC was performed on the pH 4.6-soluble skimmed milk samples, as described in Section 3.2.8 with slight modifications. The sample was eluted for 0.37 min with 100% solvent A (0.1% trifluoroacetic acid [sequencing grade; Sigma-Aldrich Ireland Ltd, Arklow, Co Wicklow, Ireland] in deionised water [Milli Q System; Millipore Corp]), then with a linear gradient to 50% solvent B (0.1% trifluoroacetic acid in acetonitrile [HPLC far UV grade; Labscan Ltd, Dublin, Ireland]) over 6.23 min, maintained at 50% B for 2.68 min, then with a linear gradient to 95% B over 0.46 min, before returning to the starting conditions.

4.2.7 Preparation of samples for bioassay testing

The pH 4.6-soluble fraction of milk from LPS challenged quarters and control quarters was prepared by isoelectric precipitation of casein according to the method of Kuchroo and Fox (1982), as described in Section 2.2.11. A portion of the pH 4.6-soluble material was then lyophilised.

4.2.8 Cell Culture

The effect of the pH 4.6-soluble fraction of milk from control quarters and LPS-challenged quarters on the secretion of IL-10 and TNF-α by J774.2 macrophages was determined as described in Section 3.2.11.

4.2.9 *Data analysis*

Analysis of variance (one-way ANOVA) was conducted using Minitab version 16 (Minitab Inc., State College, PA, USA). When differences were significant ($P \leq 0.05$), the means were analysed using Tukey's test.

4.3 Results and Discussion

4.3.1 Somatic cell count of milk samples

The SCC of milk samples collected from control quarters (prior to injection) and from LPS challenged quarters (8 h post-challenge) are shown in Table 4.1. In general, the SCC of milk samples taken from control quarters were in line with reported values for healthy animals, i.e., Eberhart *et al*. (1979) estimated that 50% of uninfected cows had SCC under 100,000 cells/mL and 80% were under 200,000 cells/mL. The SCC of milk from cow B, taken from a control quarter, was 222,000 cells/mL, which was slightly higher than expected for a healthy animal. However, this result was not necessarily indicative of a bacterial infection. Even though the most important factor influencing the SCC of milk is mammary gland infection, several other factors have been shown to influence the number of somatic cells, i.e., SCC increases with stage of lactation (Dohoo and Meek, 1982), increasing age of the animal (Beckley and Johnson, 1966; Blackburn, 1966), advanced parities (Skrzypek *et al*., 2004), elevated stress levels (Smith *et al*., 1985) and diurnal variation (White and Rattray, 1965). With the exception of cow B, SCC of milk from control quarters ranged from 22,000 to 125,000 cells/mL, with an average value of 56,000 cells/mL. Laevens *et al*. (1997) reported a mean SCC of 49,000 cells/mL for 44 uninfected cows in their first to third lactation.

Large increases in SCC were observed in the milk collected from LPS challenged quarters from all 8 cows, 8 h after injection. Values ranged from 2.6×10^6 to 5.1×10^6 cells/mL, with an average value of 3.9×10^6 cells/mL. These values are consistent with the studies of Wellnitz *et al*. (2011) and Hinz *et al*. (2012). This increase in SCC was a result of stimulation of the innate immune system within the mammary gland of the cow in response to lipid A, which is the toxic and immunomodulating component of LPS that is recognized by the host (Rietschel *et al*., 1987). One of the initial components of the inflammatory response is the influx of polymorphonuclear neutrophils (PMN) into the mammary tissue (Paape *et al*., 1979; Harmon and Heald, 1982; Nickerson and Pankey, 1984; Craven and Williams,

1985), resulting in an increase in the SCC of milk. The level of PMN in somatic cells in mastitic milk has been estimated at over 90% (Miller and Paape, 1985; Harmon, 1994). The European Union Directives (92/46 CEE and 94/71 CEE) set a SCC limit of 400, 000 cells/mL for raw milk, while some researchers consider a normal SCC of milk to be as high as 500, 000 cells/mL.

Table 4.1 Somatic cell count (SCC) of milk from the control quarters and lipopolysaccharide (LPS) challenged quarters of cows

Cow	SCC (x 1000/mL)	
	pre challenge	8 h post challenge
A	22	2942
B	222	3656
C	87	2620
D	43	3296
E	28	4739
F	125	4181
G	46	5058
H	43	4805

4.3.2 Characterisation of the protein component of milk samples

The protein profile of milk samples collected from control quarters (prior to injection) and from LPS-challenged quarters (8 h post challenge) were studied using SDS-PAGE (Figures 4.1 to 4.3). Electrophoretograms revealed marked differences in the protein profiles of milk samples from control quarters and LPS challenged quarters. Milk from control quarters showed little signs of proteolysis (Figure 4.1, lane 6, 8, 10, 12; Figure 4.2, lane 6, 8, 10, 12) and were composed predominantly of casein, i.e., α_{S1}-, α_{S2}-, β-, κ-casein, and the major whey proteins, i.e., β-lactoglobulin (β-lg) and α-lactalbumin (α-la). A substantial increase in overall proteolysis was observed in milk samples from LPS-challenged quarters. Electrophoretograms showed hydrolysis of the caseins, as evident by a decrease in band intensity of α_{S1}-, α_{S2}-, and β-casein.

In an attempt to increase band intensity and improve resolution of the proteolysis products on SDS-PAGE electrophoretograms in the size range 10-15 kDa, samples were purposely overloaded (Figure 4.3). Besides α-la and β-lg, up to 7 additional bands were present in milk samples from LPS challenged quarters in this region, most of which were not present in milk samples from control quarters. This was most likely due to an increase in the level of indigenous proteases in milk, which has been associated with an elevated SCC (de Rham and Andrews, 1982; Andrews, 1983; Le Roux *et al*., 1995; Moussaoui *et al*., 2004; Hinz *et al*., 2012). Plasmin is the main proteolytic enzyme in normal milk (Grufferty and Fox, 1988; Bastian *et al*., 1991; Bastian and Brown, 1996) and is part of a complex system consisting of plasmin, its zymogen (plasminogen), plasminogen activators, plasminogen inhibitors, and inhibitors of plasminogen activators (Vassalli *et al*., 1991). Previous studies have shown that, during mastitis, the plasminogen activator activity in milk is increased, leading to increased plasmin activity (Heegaard *et al*., 1994; White *et al*., 1995; Zhao and Lacasse, 2008). β-Casein is the milk protein most susceptible to plasmin action, followed by α_{S2}- and α_{S1}-casein, while κ-casein appears to be quite resistant (Fox and McSweeney, 1998). This is consistent with results from the current study, in which α_{S1}-, α_{S2}- and β-casein were readily hydrolysed following LPS infusion, while, κ-casein was not hydrolysed to the same extent. Several authors have previously reported that hydrolysis of the caseins in milk from cows suffering with mastitis was not exclusively due to plasmin (Saeman *et al*., 1988; Le Roux *et al*., 1995; Somers *et al*., 2003; Wedholm *et al*., 2008; Larsen *et al*., 2010). Other proteases in milk which may have contributed to proteolysis include cysteine and aspartate proteases (Suzuki and Katoh, 1990; Bach-Larsen *et al*., 1993), both of which have been reported to be positively correlated with proteolytic activity and SCC (O'Driscoll *et al.*, 1999; Somers *et al*., 2003; Larsen *et al*., 2006).

In contrast to the increase in the level of hydrolysis of the caseins in the milk of cows following LPS infusion, β-lg appeared quite resistant to proteolysis. In some cases, α-la was also resistant to breakdown, but, not to the same extent as β-lg. α-La is usually resistant to tryptic hydrolysis (Bertrand-Harb *et al*., 2002), while native β-lg is resistant to acidic environments and to proteolytic enzymes (Chobert *et al*.,

1995; Dalgalarrondo *et al.,* 1995; Wit, 1998). This may explain the observed resistance of these proteins to enzymatic hydrolysis.

In addition to an increased level of hydrolysis in milk from LPS-challenged quarters, elevated levels of high molecular weight proteins were also observed (Figure 4.1, lane 7, 9, 11, 13; Figure 4.2, lane 7, 9, 11, 13). This was a result of leakage of blood components into milk due to increased permeability of the blood-milk mammary epithelial barrier in response to LPS (Burton and Erskine, 2003; Hogarth *et al*., 2004). The most notable increase was the level of serum albumin in milk from LPS challenged quarters compared with milk from control quarters. This was not surprising as serum albumin is the most common protein found in blood serum, i.e., the normal serum protein level is 6 to 8 mg/mL, of which albumin constituents 3.5 to 5 mg/mL (Busher, 1990). This is in agreement with results from the studies of Hogart *et al*. (2004) and Boehmer *et al*. (2008) who both reported increases in the concentration of bovine serum albumin in mastitic bovine whey. The milk from cow F appeared to have the highest concentration of serum albumin following LPS infusion, followed by the milk from cow H. With the exception of the milk from cow B, the milk from all other cows showed a notable increase in the concentration of serum albumin following LPS infusion. Immunoglobulin G is the major immunoglobulin in the milk of healthy cattle (Butler, 1983), of which the subclass IgG_1 represents more than 90 % (Elfstrand *et al*., 2002). However, in mastitic milk, IgG_2 becomes the predominant immunoglobulin (Caffin and Poutrel, 1988) as it is the main opsonin supporting neutrophil phagocytosis and, therefore, plays a role in fighting mastitic pathogens (Burton and Erskine, 2003). In the current study, the concentration of Ig in milk followed the same trend as that of serum albumin, i.e., the milk from cow F had the highest concentration of Ig following LPS infusion, followed by the milk from cow H, while the milk from all other cows, with the exception of milk from cow B, showed a notable increase in the concentration of Ig following LPS infusion.

Three additional bands, ranging in size from approximately 70 to 250 kDa, were present at various concentrations in milk samples from LPS challenged quarters. Of these, one band was estimated to have an MW of approximately 76 kDa. It is likely

that this band represents either serotransferrin and/or lactoferrin, both of whose concentration has been shown to be elevated in milk during mastitis (Kawai *et al.,* 1999; Hogarth *et al*., 2004). It is not clear which proteins the remaining two bands represented; however, proteins which have previously been identified within this size range in mastitic milk samples include polymeric immunoglobulin receptor, complement C3 precursor (Boehmer *et al*., 2008) and α_2-macroglobulin (Perez *et al*., 1989).

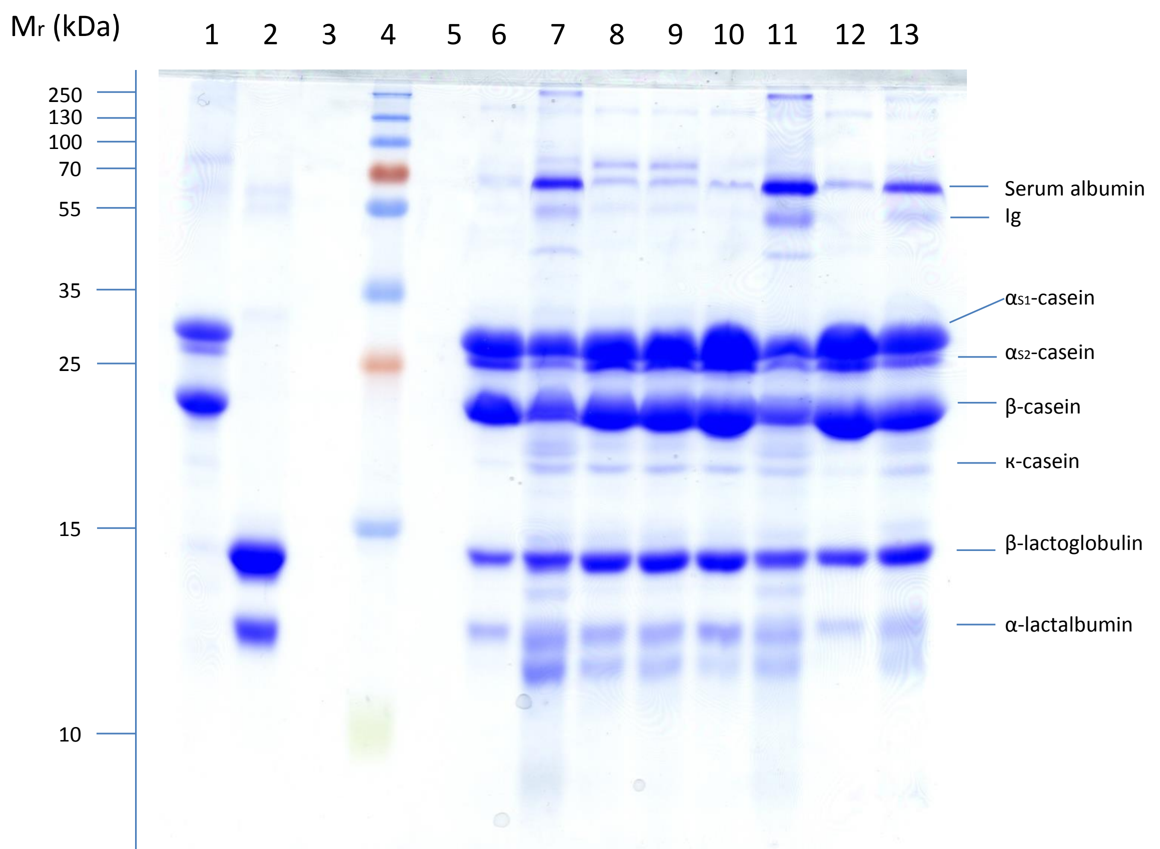

Figure 4.1 SDS-Polyacrylamide gel electrophoretograms of milk from the control quarters and LPS challenged quarters of trial cows. Lane 1, sodium caseinate; Lane 2, whey protein isolate; Lane 4, molecular mass marker; Lane 6-13, A pre challenge, A post challenge, B pre challenge, B post challenge, C pre challenge, C post challenge, D pre challenge and D post challenge, respectively.

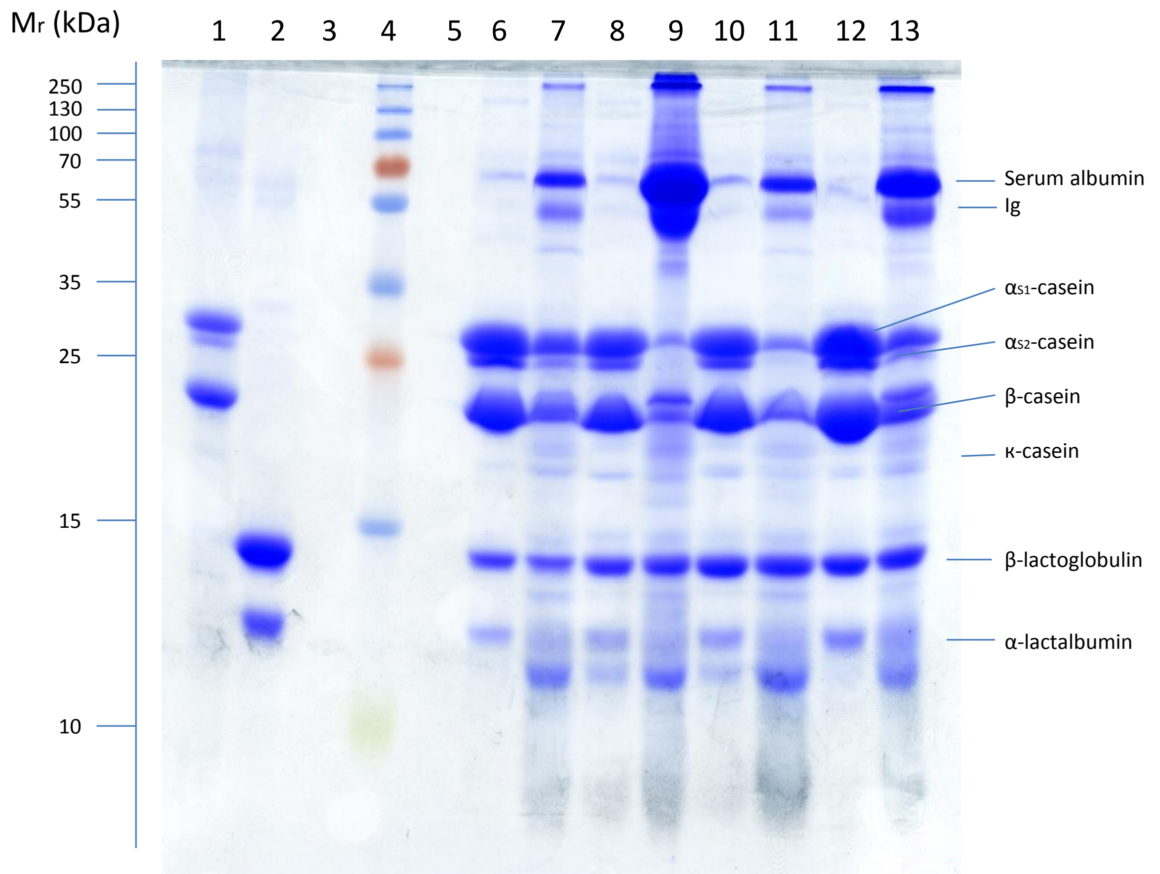

Figure 4.2 SDS-Polyacrylamide gel electrophoretograms of milk from the control quarters and LPS challenged quarters of trial cows. Lane 1, sodium caseinate; Lane 2, whey protein isolate; Lane 4, molecular mass marker; Lane 6-13, E pre challenge, E post challenge, F pre challenge, F post challenge, G pre challenge, G post challenge, H pre challenge and H post challenge, respectively.

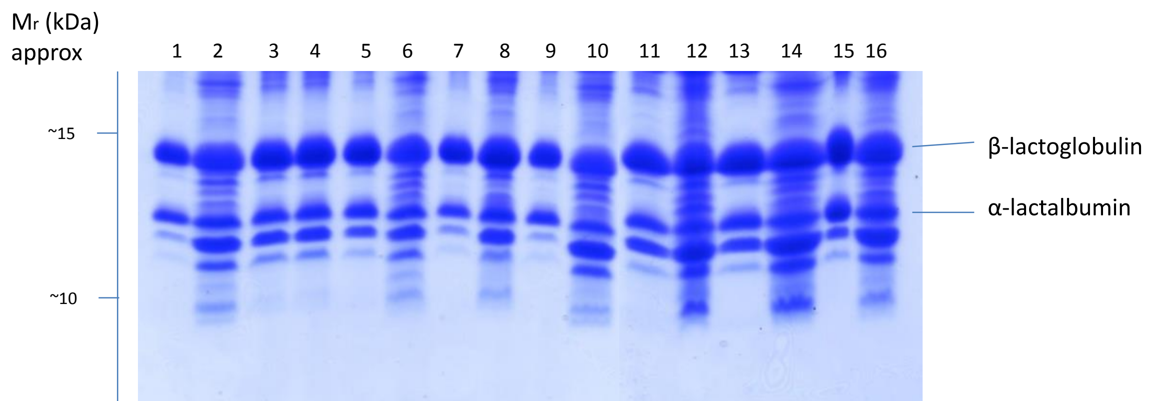

Figure 4.3 Section of an SDS-polyacrylamide gel electrophoretograms of milk from the control quarters and LPS challenged quarters of trial cows. Lane 1-16, A pre challenge, A post challenge, B pre challenge, B post challenge, C pre challenge, C post challenge, D pre challenge, D post challenge, E pre challenge, E post challenge, F pre challenge, F post challenge, G pre challenge, G post challenge, H pre challenge and H post challenge, respectively.

The proteins in bovine milk can be separated into two groups based on their solubility at pH 4.6. Caseins are insoluble at pH 4.6 and constitute approximately 80% of the proteins in mature milk, while whey proteins are soluble at pH 4.6 and account for the remaining 20% of milk protein (Fox and McSweeney, 1998). As already mentioned, proteolysis increases in bovine milk at elevated SCC, as was seen in the current study (Figures 4.1 to 4.3). This increase in proteolysis, mainly of the caseins, led to an increase in the quantity of pH 4.6-soluble protein (Figure 4.4). An increase in the quantity of pH 4.6-soluble protein in milk from LPS-challenged quarters compared with milk from control quarters was seen for all 8 cows. The average pH 4.6-soluble protein content of milk from control quarters ranged from 0.19% to 0.6%, with an average value of 0.45%. These values were slightly below average for the whey protein content of mature bovine milk, with a reported average value of 0.6% (Fox and McSweeney, 1998). The average pH 4.6-soluble protein content of milk from LPS challenged quarters ranged from 0.58% to 3.56%, with an average value of 1.42%. This increase in pH 4.6-soluble protein in milk from LPS challenged quarters compared with milk from control quarters was due to a combination of increased proteolysis of the caseins, the products of which are mostly soluble at pH 4.6, and increased permeability of the blood milk barrier, which resulted in an influx of high molecular weight protein into the milk. The largest increase in the pH 4.6-soluble protein content of milk following LPS infusion was for cow F, in which case an increase of 2.96% was observed, i.e., the pH 4.6-soluble protein content increased from 0.6% in milk from the control quarter to 3.56% in milk from the LPS challenged quarter. The lowest increase in the pH 4.6-soluble protein of milk following LPS infusion was for cow B, where an increase of 0.07% was observed. The quantity of pH 4.6-soluble protein in the milk of all 8 cows following LPS infusion was of the order; cow F > cow H > cow G > cow E >cow C > cow D > cow A > cow B. This is in agreement with the observed band intensities of the whey proteins in Figures 4.1 and 4.2. No correlation between the pH 4.6-soluble protein content and SCC was observed.

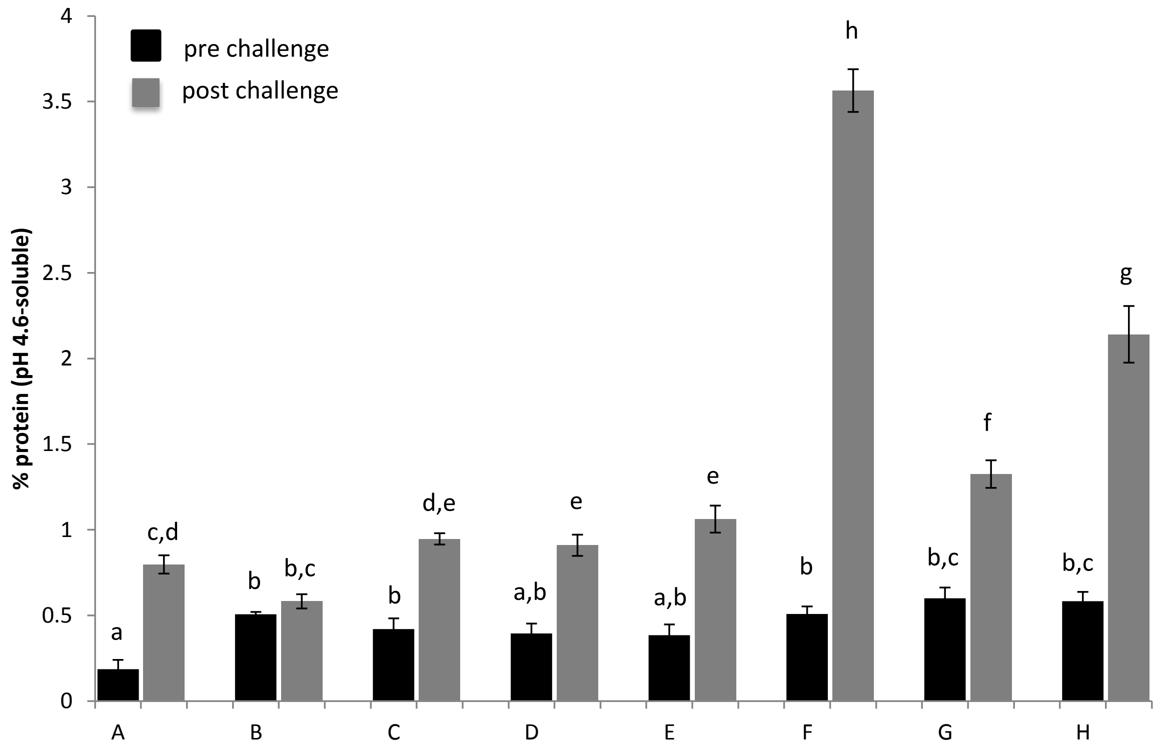

Figure 4.4 Mean values (± S.D., n=3) for protein content of the pH 4.6-soluble fractions of milk from the control quarters and LPS challenged quarters of cows A to H. Values with different superscript letters were significantly different ($p < 0.05$).

The pH 4.6-soluble fraction of milk samples from control quarters and LPS-challenged quarters were studied by RP-UPLC (Figure 4.5 and 4.6). An increased level of proteolysis was observed in milk samples from LPS-challenged quarters compared with control quarters for all 8 cows, with the exception of cow B, as evident by an increase in the overall peak area on RP-UPLC chromatograms (Figure 4.5 and 4.6). This is consistent with SDS-PAGE electrophoretograms (Figures 4.1 to 4.3) and pH 4.6-soluble protein values (Figure 4.4). The amount of peptide material present in milk samples from control quarters varied throughout the 8 cows, i.e., greater amounts of peptide material were present in the pH 4.6-soluble fraction of milk from control quarters of cows B, C, F, G and H than in the corresponding samples from cows A, D and E. This was not surprising as bovine milk is known to contain naturally occurring indigenous peptide material, e.g., proteose-peptones represent about 10% of whey proteins and are constituted by 38 components

(Buccioni *et al*., 2013), several of which are hydrolysis products arising from the action of plasmin on β-casein, in addition to glycoproteins and traces of other proteins (Andrew, 1978; Innocente *et al*., 1998). Intact α-la and β-lg eluted at 6.4 min and 6.8 min on chromatographic profiles, respectively, and were present in relatively constant amounts and proportions in milk samples from control quarters. The levels of β-lg and α-la in milk samples from LPS infused quarters from cows A, B, D and H were virtually identical to those found in milk samples from the corresponding control quarters, but, milk samples from LPS-challenged quarters from cows C, E and F contained lower levels of β-lg and α-la than milk from control quarters.

In general, the peptide profiles of milk samples from LPS-challenged quarters were quite similar for all 8 cows. The majority of peptide material was hydrophobic, eluting between 2.5 and 6 min. On average, approximately 35 peaks were observed within this range, in milk from LPS challenged quarters. Relative to the overall peak area, excluding β-lg and α-la, milk from the LPS challenged quarter of cow G contained the greatest amount of peptide material, while milk from cow B contained the least. It is clear from the RP-UPLC chromatogram shown in Figure 4.5 that milk from the control quarter of cow B had already been subjected to proteolysis prior to LPS infusion, i.e., milk from the LPS challenged quarter of cow B had an identical pH 4.6-soluble profile to milk from the control quarter, indicating that the LPS challenge did not cause an increase in proteolysis. This is in agreement with SDS-PAGE (Figures 4.1 to 4.3) and pH 4.6-soluble protein values (Figure 4.4). It should also be noted that the SCC of milk from the control quarter of cow B prior to LPS infusion was 222 x 10^3 cells/mL, which was, albeit still quite low, significantly higher than milk from control quarters of the remaining 7 cows.

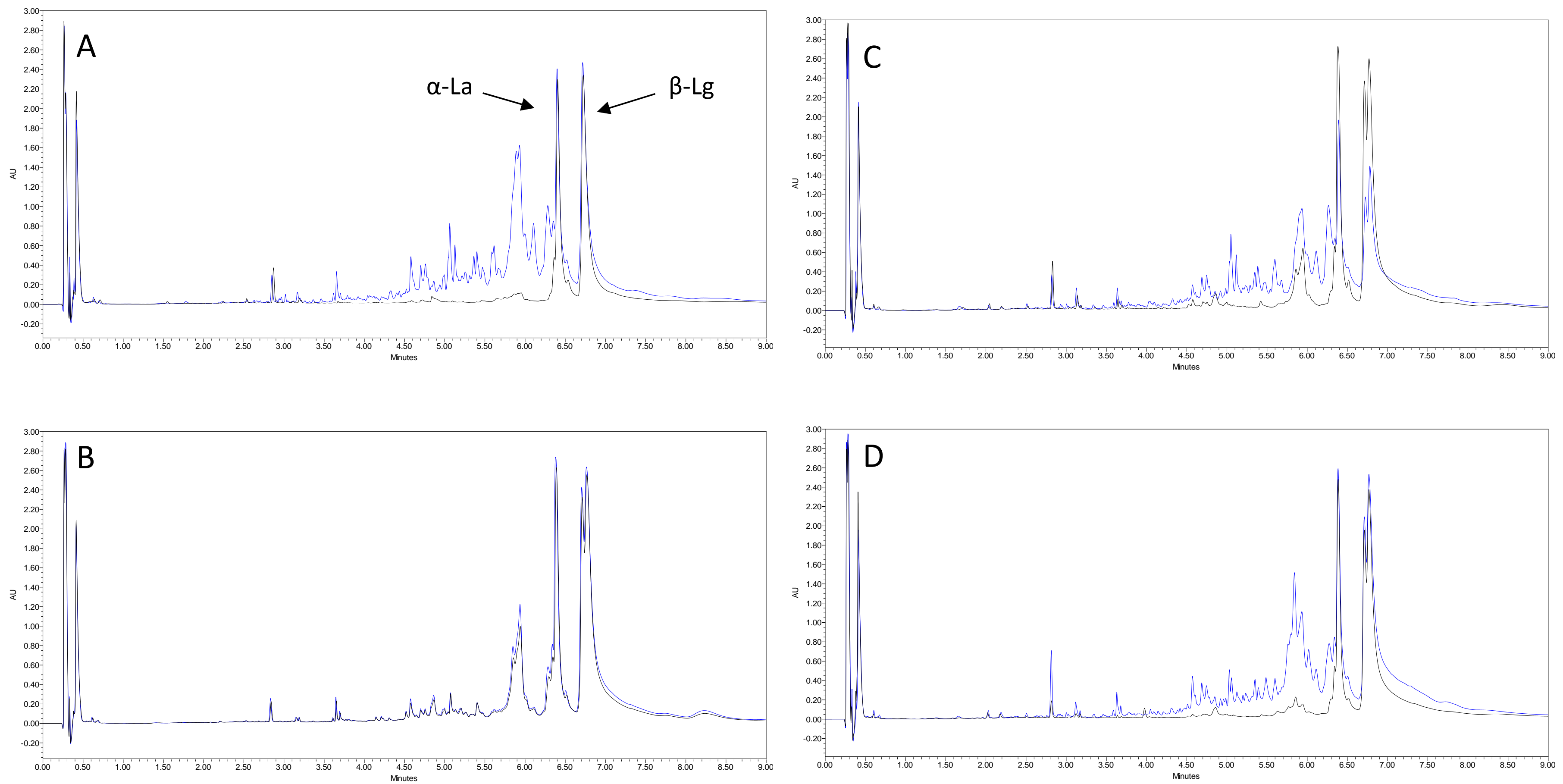

Figure 4.5 RP-UPLC chromatograms of the pH 4.6-soluble fraction of milk from the control quarters (▬) or LPS-challenged quarters (▬) of cows A, B, C and D.

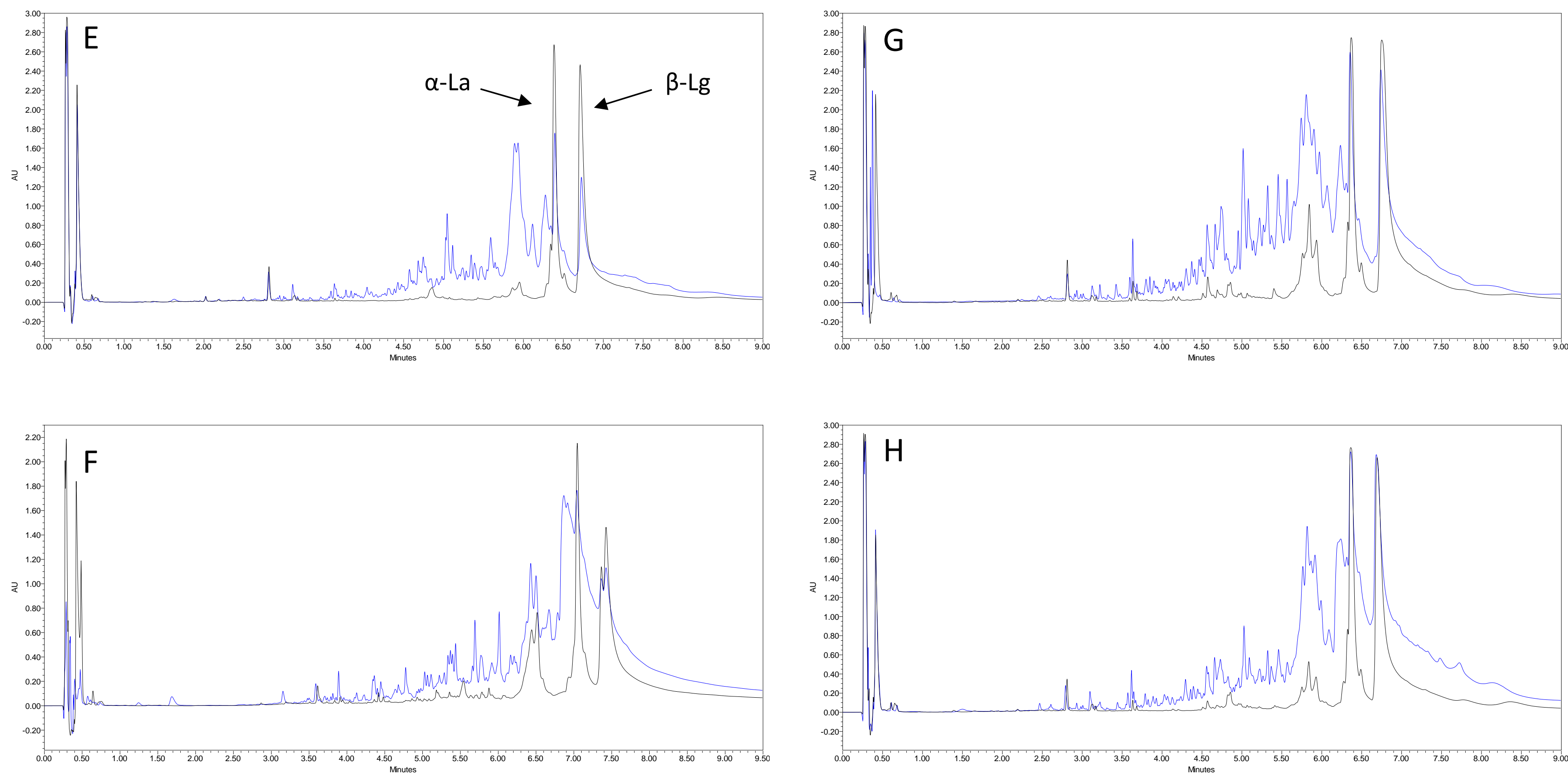

Figure 4.6 RP-UPLC chromatograms of the pH 4.6-soluble fraction of milk from the control quarters (▬) or LPS-challenged quarters (▬) of cows E, F, G and H.

The protein profile of milk samples collected from control quarters (prior to injection) and from LPS challenged quarters (8 h post-challenge) were further analysed by 2-DE. Representative gels of milk samples from control quarters and LPS challenged quarters were chosen to illustrate the protein profiles generated in the current study (Figure 4.7 a to f and Figure 4.8 g to l). Samples were adjusted to pH 4.6 prior to analysis in order to remove any casein. The resulting protein maps demonstrated that infusion with LPS caused a profound change in the protein profiles of milk samples from LPS-challenged quarters compared with milk from control quarters. In general, β-lg and α-la were the predominant whey proteins present in the milk from control quarters; but, some high molecular weight proteins were also present, i.e., most likely immunoglobulins, serum albumin and lactoferrin. Infusion with LPS at quarter level resulted in an increase in the secretion of high molecular weight proteins in milk. These results were consistent with those of Hinz *et al.* (2012) who reported a marked increase in the concentration of serum albumin and serotransferrin in milk from infused quarters. As mentioned earlier, this was due to increased permeability of the blood milk barrier in response to an elevated SCC, which resulted in an influx of serum proteins into milk. Based on observed molecular weights, the high molecular weight proteins of the LPS challenged milk on 2-DE gels (Figure 4.7 b, d, f; Figure 4.8 h, j, l) were most likely serum albumin, immunoglobulins, lactoferrin and serotransferrin. These results were consistent with those obtained by SDS-PAGE (Figures 4.1 and 4.2). It should be noted that 2-DE gels were silver stained, mainly because of its high sensitivity, i.e., it is able to detect less than 1 ng protein (Weis *et al*., 2009). However, despite the improved sensitivity of silver staining (silver staining is reported to be 100-fold more sensitive than Coomassie Brilliant Blue (Switzer *et al*., 1979), the results obtained were not quantitative.

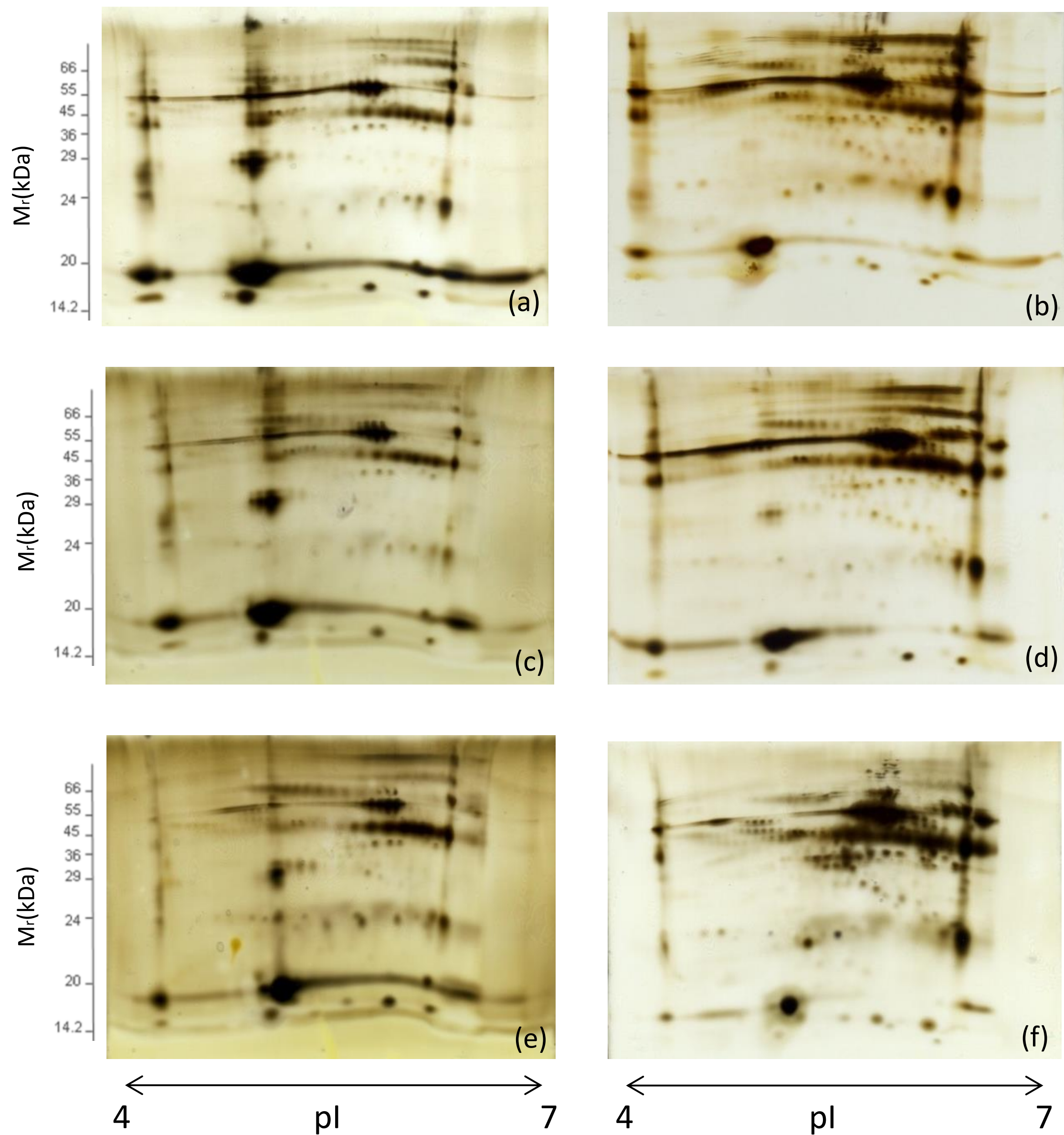

Figure 4.7 Two-dimensional gel electrophoretograms of the pH 4.6-soluble fraction of milk from control and LPS-challenged quarters of cows; (a) A pre challenge, (b) A post challenge, (c) B pre challenge, (d) B post challenge, (e) C pre challenge, (f) C post challenge. The gels show molecular weights (Mr) and isoelectric points (pI) in the range 4-7.

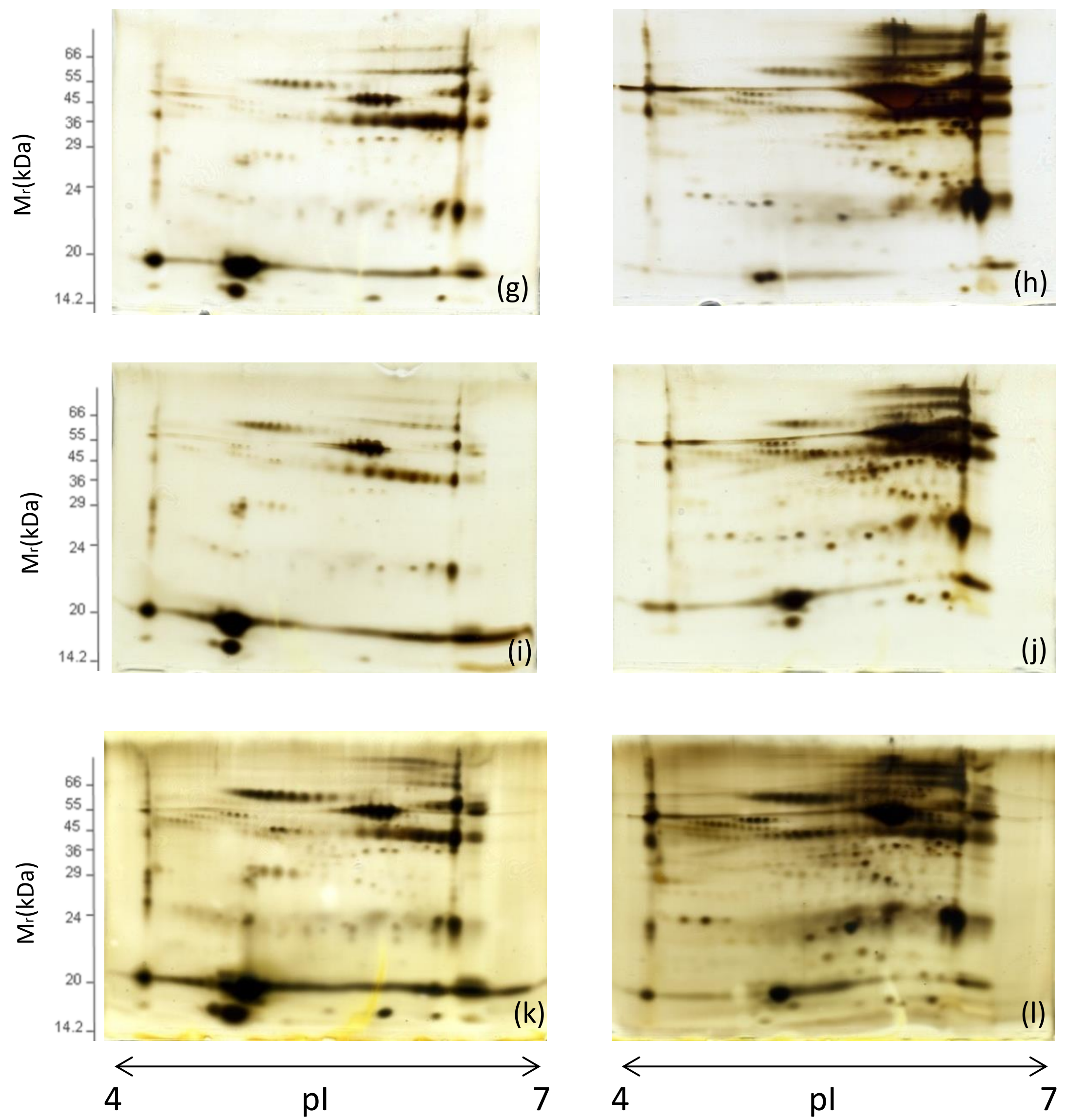

Figure 4.8 Two-dimensional gel electrophoretograms of the pH 4.6-soluble fraction of milk from control and LPS-challenged quarters of cows; (g) D pre challenge, (h) D post challenge, (i) E pre challenge, (j) E post challenge, (k) F pre challenge, (l) F post challenge. The gels show molecular weights (Mr) and isoelectric points (pI) in the range 4-7.

4.3.3 Effect of milk samples on LPS-induced IL-10 secretion

Interleukin (IL)-10 is an important anti-inflammatory cytokine which is produced by many cell populations, i.e., monocytes, macrophages and dendritic cells (McArdle *et al*., 2013). Its main biological function appears to be the limitation and termination of inflammatory responses (Villalta *et al*., 2011) and the regulation of differentiation and proliferation of several immune cells such as T cells, B cells, natural killer cells, antigen presenting cells, mast cells, and granulocytes (Asadullah *et al*., 2003). In the current study, murine macrophage cells (J774.2) were isolated, cultured and pre-treated with 1 mg/mL of the pH 4.6-soluble fraction of milk from LPS-challenged quarters and control quarters for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h. Samples were also tested for their effect on IL-10 secretion from J774.2 cells in the absence of LPS. As discussed earlier, LPS is a key inflammatory component of Gram-negative bacteria, which induces a distinctive pattern of cytokine release that regulates inflammation (Singh and Jiang, 2003). LPS was used in the current study to initiate an inflammatory response which resulted in increased secretion of IL-10 from J774.2 cells (Figure 4.9 a). The effect of the pH 4.6-soluble fraction of milk from LPS-challenged quarters and control quarters on IL-10 secretion by J774.2 macrophages is shown in Figures 4.9 a and 4.9 b.

The pH 4.6-soluble fraction of milk from LPS-challenged quarters was found to cause a substantial induction in the secretion of IL-10. This IL-10 induction was seen in the absence of LPS (Figure 4.9a) and, when LPS was present, IL-10 secretion was amplified to a level which was greater than the effect seen with LPS alone (Figure 4.9b). No effect on IL-10 secretion was observed with the pH 4.6-soluble fraction of milk from control quarters. As shown earlier, milk from LPS-challenged quarters and control quarters differed in both their protein and peptide profiles. Milk from LPS-challenged quarters contained substantially more peptide material and high molecular weight proteins compared to milk from control quarters. Therefore, it is likely that the induction of IL-10 from J774.2 cells seen in the current study was as a result of one or more of these up-regulated components.

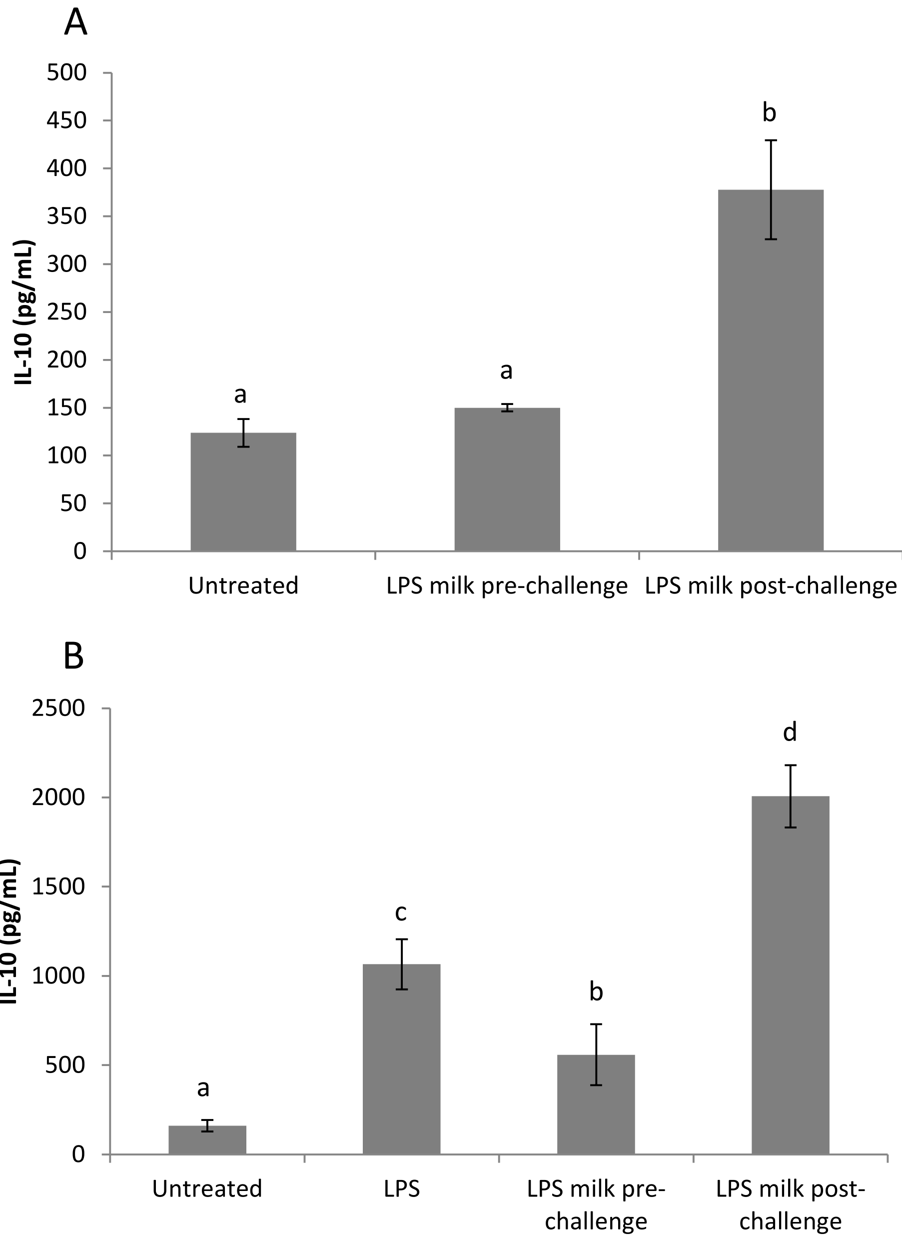

Figure 4.9 Effect of the pH 4.6-soluble fraction of milk from control quarters and LPS-challenged quarters on IL-10 secretion by J774.2 macrophages. Exponentially growing cells were incubated with 1 mg/mL of the lyophilised pH 4.6-soluble fraction of milk from LPS challenged quarters or control quarters for 24 h (A), followed by stimulation with 10 ng/mL LPS for 3 h (B). The supernatant was removed and the concentration of IL-10 quantified by ELISA. Data shown represents the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

4.3.4 Effect of milk samples on LPS-induced TNF-α secretion

Tumor necrosis factor (TNF) is among the most studied and central pro-inflammatory cytokines (Gillett *et al*., 2010). TNF is a key regulator of the inflammatory response whose ligands are known to play a central role in a variety of inflammatory conditions, i.e., rheumatoid arthiritis, inflammatory bowel disease, multiple sclerosis, diabetes and asthma (Croft *et al*., 2012). TNF-α is produced predominantly by activated macrophages and T-lymphocytes and is known to have numerous biological properties relating to inflammation, proliferation, differentiation and cancer growth (Patil *et al*., 2011). In the current study, J774.2 macrophages were isolated, cultured and incubated with 1 mg/mL of the pH 4.6-soluble fraction of milk from LPS-challenged quarters and control quarters for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h. Samples were also tested for their effect on TNF-α secretion from J774.2 cells in the absence of LPS. The effect of the pH 4.6-soluble fraction of milk from LPS-challenged quarters and control quarters on TNF-α secretion by J774.2 macrophages are shown in Figures 4.10 a and 4.10 b. The pH 4.6-soluble fraction of milk from LPS-challenged quarters did not reduce or induce TNF-α secretion in the presence or absence of LPS. In the absence of LPS, the pH 4.6-soluble fraction of milk from control quarters caused a substantial induction of TNF-α, albeit at a much lower level than in response to LPS alone. This most likely represented the J774.2 cellular response to potentially antigenic material.

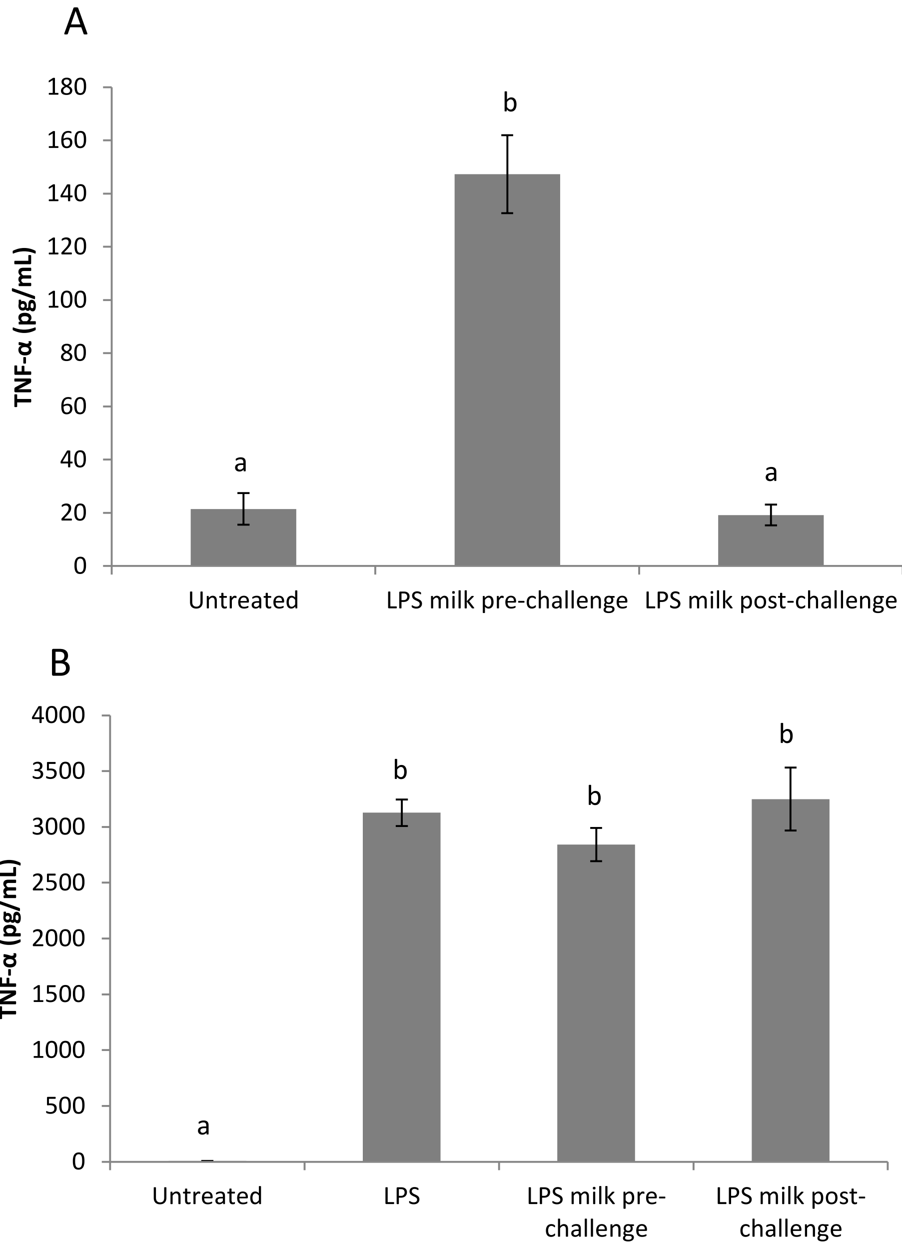

Figure 4.10 Effect of pre and post LPS-challenged milk samples on TNF-α secretion by J774.2 macrophages. Exponentially growing cells were incubated with 1 mg/mL of the lyophilised pH 4.6-soluble fraction of milk from LPS challenged quarters and control quarters for 24 h (A), followed by stimulation with 10 ng/mL LPS for 3 h (B). The supernatant was removed and the concentration of TNF-α quantified by ELISA. Data shown represents the mean (± SD, n=3) of data from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

4.4 Conclusion

Intramammary challenge with the endotoxin LPS, from *E. coli*, induced an immune response *in vivo*, the results of which were seen in the milk of challenged cows, i.e., increased SCC, level of hydrolysis, and concentration of serum proteins. These changes were due to an increased permeability of the blood-milk barrier in response to an elevated SCC.

A pH 4.6-soluble fraction of bulked milk from LPS challenged quarters and control quarters was prepared in order to examine the immunological potential of these samples. The pH 4.6-soluble fraction of milk from LPS challenged quarters was found to cause a substantial induction in the secretion of the anti-inflammatory cytokine, IL-10, from a murine macrophage cell line, both in the presence and absence of *in vitro* LPS stimulation, while, the corresponding control sample had no effect. Based on the results of the current study, it was speculated that this bioactivity was in response to either the abundant serum proteins and/or the proteolysis products which were up-regulated in milk from LPS challenged quarters. The pH 4.6-soluble fraction of milk from LPS challenged quarters was found to have no effect on the secretion of the pro-inflammatory cytokine, TNF-α; however, the corresponding control sample caused a substantial induction of TNF-α in the absence of *in vitro* LPS stimulation. It is thought that this was a cellular response to potentially antigenic material.

The results obtained in the current study represent a useful contribution in understanding the immunological response of cows to LPS. In particular, correlations between an elevated milk SCC and alterations in the milk proteome were further elucidated. Considerable variation in response to LPS was observed between individual cows and further investigation is required in this area. It is not known as to whether this milk could be produced on a large scale due to animal welfare concerns; however, experimentally induced mastitic milk may represent a functional food product/ingredient with immune stimulating properties.

4.5 Acknowledgements

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4.6 References

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Chapter 5

Heat-induced hydrolysis of sodium caseinate and potential production of biologically active peptides

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Declaration: Generation, fractionation and characterisation of heat-hydrolysed sodium caseinate was performed by Brian McGrath at University College Cork. Mass spectrometry was performed by Michael Kinsella and Brian McGrath at University College Cork. Cell exposures and cytokine analysis were performed by Stacey Kelly at University College Dublin. All experimental data/results were analysed and the chapter written by Brian McGrath.

Abstract

Milk is a very heat-stable system; while the whey proteins are easily denatured, caseins are remarkably heat-stable and can be heated at 100°C at the natural pH of milk for up to 24 h. Despite this, some changes occur on heating under extreme conditions, e.g., dephosphorylation, cleavage of peptide bonds and intermolecular cross-linking. The aim of this study was to investigate heat-induced hydrolysis of sodium caseinate during heating for up to 120 min at pH 7.0 and 130°C, from the dual viewpoints of protein breakdown and peptide formation. The formation of 2% trichloroacetic acid-soluble peptides was studied using spectrofluorimetry and liquid chromatography-mass spectrometry (LC-MS) while sodium dodecyl sulphate (SDS)- and urea-polyacrylamide gel electrophoresis (PAGE) were used to study the hydrolysis of the caseins. LC-MS and spectrofluorimetry showed an increase in 2% trichloroacetic acid-soluble peptides over time, confirming that proteolysis had occurred. Similarly, SDS- and urea-PAGE electrophoretograms showed a decrease in band intensity for the main caseins over time. In total, 1023 peptides were identified and characterised. Eleven of the most abundant peptides were studied, and their production and breakdown modelled as a function of time. Of the eleven major peptides, seven came from α_{S1}-casein (six from the C-terminal region, one from the N-terminal region), one from β-casein (from its C-terminal region) and three from κ-casein (two from the C-terminal region, one from the N-terminal region). Peptide bonds containing proline, serine, asparagine and/or aspartic acid were preferentially hydrolysed during heating. Subsequently, a peptide fraction produced by heat-induced hydrolysis of sodium caseinate was tested for its effect on lipopolysaccharide (LPS)-induced cytokine secretion from a murine macrophage cell line and was found to cause a significant increase in the secretion of the anti-inflammatory cytokine interleukin-10. The production of peptides in this manner may represent a novel alternative to microbial or enzymatic hydrolysis for the generation of biologically active peptides.

5.1 Introduction

Heat treatments of milk and dairy products are commonly used in the dairy industry, ranging from thermization (65°C for 15 s) to sterilization (120°C for 10-20 min) or ultra-high temperature (UHT) treatment (typically 135-145°C for several seconds). The effect of heat on the milk protein system is an important consideration as it can affect both the functional properties and nutritional quality of milk. The heat stability of milk has been reviewed by several authors (Davies & White, 1966; Robertson & Dixon, 1969; Fox & Morrissey, 1977; Fox & Hearn, 1978; O'Connell & Fox, 2003; Singh, 2004) and may be defined as the ability of milk to withstand high processing temperatures without visible coagulation or gelation (Singh, 2004).

Milk is a complex biological fluid containing a very heterogeneous mix of proteins, carbohydrates, lipids, vitamins and minerals, in soluble, colloidal or emulsified states (Fox and McSweeney, 1998). Despite this, the milk protein system is very heat-stable and can withstand heating at 140°C for 15-20 min at pH 6.7 (Fox, 1982; Singh and Creamer, 1992; McCrae and Muir, 1995). The whey proteins in milk are typical globular proteins with well defined secondary and tertiary structures and therefore are susceptible to denaturation. They retain their conformation only within limited temperature ranges and exposure to extremes of temperatures results in denaturation and aggregation of these proteins (Anema, 2009). The order of heat stability of the whey proteins, as measured by loss in solubility, is: α-lactalbumin (α-la) > β-lactoglobulin (β-lg) > blood serum albumin (BSA) > immunoglobulins (Ig). Heating β-Lg at neutral pH to 70°C causes dimers to dissociate into monomers, and a thiol group and hydrophobic residues become exposed. This may lead to heat-induced gelation as aggregates are formed *via* intermolecular thiol-disulphide exchange, thiol-thiol oxidation and noncovalent interactions (Hoffmann and van Mill, 1997; McSwiney *et al.,* 1994; Mulvhill and Kinsella, 1987). Similarly, α-la can be denatured at temperatures as low as 63°C (Jelen and Rattray, 1995).

On the other hand, casein is a remarkably heat-stable protein system; sodium caseinate can be heated at pH 6.7 at 140°C for at least 40 min before coagulation occurs (Fox and Hoynes, 1975). This high heat stability is due largely, in part, to its relative lack of secondary and tertiary structures. However, under certain conditions of temperature and pH, the colloidal stability of the casein micelles may be lost, as evident by visible flocculation, gelation or protein separation (Fox, 1982). High heat treatment can lead to an increase in casein micelle size (Mohammad and Fox, 1987; McMahon, 1996), intermolecular cross-linking (Singh, 1995) and dephosphorylation of the caseins, subsequently disrupting the native micelle structure, which is largely held together by calcium phosphate links (Dalgleish *et al.,* 1987; Singh, 2004). Calcium phosphate present in the serum phase of milk becomes less soluble during heat treatment (Holt, 1995) and it is thought that formation of insoluble calcium phosphate and its deposition on casein micelles is largely responsible for the instability of casein micelles in UHT-treated milks (Wahlgren *et al.,* 1990; Dalgleish, 1992). Guo *et al*. (1999), who studied the effect of heat on the functional properties of sodium caseinate, found that, with heating, solubility increased, viscosity decreased, as did its foaming and emulsifying capacity, while the foam stability increased.

Severe heat treatment of milk has also been shown to cause proteolysis, but little detailed study has been carried out in this area. White and Davies (1966) showed that 10-20% of the total protein nitrogen in milk is converted to non-protein nitrogen (NPN) when heated at 135°C for 60 min. Similarly, Saidi and Wrathesen (1993) found an 18% increase in the NPN content of milk following typical sterilization conditions (120°C for 20 min). Hustinx *et al.* (1997) found a linear increase in pH 4.6- and 2% trichloroacetic acid-soluble peptides on heating a sodium caseinate solution at 140°C and pH 7.0 for 100 min; fifteen of the pH 4.6-soluble peptides were isolated and identified. Gaucheron *et al.* (1999) related an increase in the NPN content of casein micelle suspensions in a salt solution, in milk ultrafiltrate and in milk during sterilization (120°C for 10, 20 or 30 min) to an increase in proteolysis and subsequently characterised ten peptides. In a later study, Gaucheron *et al.* (2001) studied the effect of pH on the proteolysis of sodium

caseinate induced by heating at 120°C and observed an increase in soluble nitrogen content with increasing pH (6.0, 7.0, 8.0, 9.0); eighteen low molar mass peptides found in the soluble nitrogen fraction were subsequently characterised. Van Boekel (1999) studied the kinetics of deamidation, dephosphorylation and protein breakdown in heated sodium caseinate solutions; the extent of deamidation was related to the extent of amide present in asparagine, while protein breakdown and dephosphorylation increased with increasing heating temperature and time.

Low-grade inflammation has been linked with a range of metabolic conditions such as obesity, insulin resistance, metabolic syndrome and coronary heart disease (Rosa *et al.,* 2012). Bovine milk is known to contain a number of biologically active peptides (Korhonen, 2009). These peptides are not active within the parent protein but can be produced from precursor milk proteins in the following ways: (a) enzymatic hydrolysis by digestive enzymes (Korhonen and Pihlanto, 2003), (b) fermentation of milk with proteolytic starter cultures (Fugslang *et al.,* 2003), and/or (c) proteolysis by enzymes derived from microorganisms or plants (Yamamoto *et al.,* 1994). Immunomodulatory peptides are those which can enhance immune function *in vivo* through stimulation or suppression of the immune system (Gauthier *et al.*, 2006). They function by stimulating the proliferation of lymphocytes, the phagocytic activities of macrophages (Nagpal *et al.,* 2011), antibody synthesis and cytokine expression (Udenigwe and Aluko, 2012).

The aim of this study was to identify some of the peptides produced during heat treatment of sodium caseinate at 130°C over a 120 min period, with the aim of identifying the most heat-labile bonds in the casein system. In addition, heat-hydrolysed sodium caseinate was tested for its effect on the secretion of pro- and anti-inflammatory cytokines from a murine macrophage cell line, with the view of developing a functional food product which targets inflammation in humans. Sodium caseinate was chosen in order to avoid numerous other changes that occur on heating the more complex milk system.

5.2 Materials and Methods

5.2.1 Sodium caseinate preparation

Sodium caseinate (Kerry Group, Listowel, Ireland) was made up in distilled water (2.5%, w/v) and was allowed to hydrate overnight at 4°C. Following hydration, the solution was adjusted to pH 7.0 using 1 N NaOH and dialysed for three 24 hour periods against fifty volumes of distilled water using a 3.5 kDa dialysis membrane (Medicell International, London, UK). Following dialysis, the pH was re-checked and re-adjusted, if necessary, to pH 7.0 using 1 N NaOH.

5.2.2 Heat treatment

Aliquots of sodium caseinate (3 mL) were placed in glass tubes (length, 150 mm; diameter, 15 mm; wall thickness, 2 mm), sealed with silicone rubber stoppers. Tubes were heated in an oscillating oil bath at 130°C for up to 120 min. A 2 min equilibration time was also allowed. Following heating, the tubes were immediately cooled in ice water at 4°C.

5.2.3 Preparation of the pH 4.6-soluble fraction

Unhydrolysed casein was removed from heat-treated sodium caseinate samples by isoelectric precipitation according to the method of Kuchroo and Fox (1982), as described in Section 2.2.11. A portion of the pH 4.6-soluble and insoluble material were then lyophilised.

5.2.4 Preparation of the 2% trichloroacetic acid (TCA) soluble fraction

The 2% TCA-soluble fraction of sodium caseinate was prepared by adding 166 µL 12% TCA to 833 µL sodium caseinate solution and vortexing the mixture; after 10

min, the mixture was centrifuged for 30 min at 18,000 g at 20°C. After centrifugation, the supernatant was carefully removed and used for further analysis.

5.2.5 Spectrofluorimetric measurement

The relative fluorescence intensity of the 2% TCA-soluble fractions of sodium caseinate heated at 130°C for 0 to 120 min were measured at 20°C using a luminescence spectrometer (Varioskan Flash, Thermo Scientific, Waltham, MA, USA) at excitation and emission wavelengths of 280 and 340 nm, respectively. Slit widths were 5 nm. Under these conditions of excitation and emission, fluorescence measurement was used to evaluate the release of peptides containing tyrosine and/or tryptophan.

5.2.6 Reverse phase-ultra performance liquid chromatography

RP-UPLC was performed on the 2% TCA-soluble fractions, as described in Section 3.2.8 with slight modifications. The sample was eluted for 0.5 min with 100% solvent A (0.1% formic acid [sequencing grade; Sigma-Aldrich Ireland Ltd, Arklow, Co Wicklow, Ireland] in deionised water [Milli Q System; Millipore Corp]), then with a linear gradient to 30% solvent B (0.1% formic acid in acetonitrile [HPLC far UV grade; Labscan Ltd, Dublin, Ireland]) over 9.5 min, then with a linear gradient to 50% B over 2 min, then with a linear gradient to 85% B over 0.5 min, maintained at 85% B for 0.5 min and finally with 0% B over 1.1 min.

5.2.7 Urea–polyacrylamide gel electrophoresis

Electrophoresis in polyacrylamide gels (12.5% T, 4% C, pH 8.9) was performed using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Andrews (1983) using a 12%, w/v, acrylamide

separating gel and a 4%, w/v, stacking gel. Solutions of heated sodium caseinate (2.5%, w/v, total solids) were prepared in 0.124 M Tris-HCl buffer containing 49%, w/v, urea, 1.4%, v/v, 2-mercaptoethanol and 0.012%, w/w, bromophenol blue. Gels were stained directly by the method of Blakeley and Boezi (1977) with Coomasie Brilliant Blue G250 for visualization of proteins.

5.2.8 SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Laemmli (1970), as described in Section 2.2.13. In some cases, gels were silver stained using the Pierce® Silver Stain Kit (Thermo Scientific, Waltham, MA, USA), as described in Section 4.2.5.

5.2.9 Mass Spectrometry

5.2.9.1 Identification of 2% TCA-soluble peptides by Quadropole Time-of-flight Mass Spectrometry

TCA-soluble peptides were identified using a Waters Acquity G2 Q-TOF LC-MS. This system consisted of a Waters Acquity UPLC system coupled to a Quadrupole Time-of-flight mass spectrometer. A sample (6.9 µL) was injected onto a Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm) with a flowrate of 0.46 mL/min. The column temperature was maintained at ambient temperature (25°C) while samples were refrigerated at 4°C. Sample was eluted for 0.37 min with 100% solvent A (0.1% formic acid [sequencing grade; Sigma-Aldrich Ireland Ltd, Arklow, Co Wicklow, Ireland] in deionised water [Milli Q System; Millipore Corp]), then with a linear gradient to 20% solvent B (0.1% formic acid in acetonitrile [HPLC far UV grade; Labscan Ltd, Dublin, Ireland]) over 2.13 min, then with a linear gradient to 50% B over 7.5 min, then with a linear gradient to 95% B over 0.1 min, maintained at 95% B for 1 min, then with a linear gradient to 0% B over 0.1 min before

returning to starting conditions. UV detection was carried using an Acquity PDA detector where a scan of 200-450 nm was conducted in parallel to measuring aborbance at 214 nm.

Mass spectrometry detection was conducted through electrospray ionization using an ms^e centroid experiment in positive mode in the m/z scan range 50-2000 Da. The following MS settings were used: capillary voltage 3 kV, sampling cone 40 V, extraction cone 4 V, source temperature 120 °C, desolvation temperature 450 °C, desolvation gas flow 1000 L/h, cone gas flow 50 L/h. The accurate mass of the instrument was initially calibrated through direct infusion of a sodium iodide calibrant solution prior to sample analysis. In addition, leucine enkephalin (Leuenk) lockmass solution (2 ng/uL) and Glu-fib (1 ng/µL) were infused at 5 µL/min in parallel to the mobile phase flow, scanned and automatically corrected to verify exact mass. Masslynx v4.1 software (Waters, Millford, Massachusetts) was used to control the instrument while Proteinlynx Global Server (PLGS) software (Waters, Millford, Massachusetts) was used to assist data analysis and aid in determination of peptides present. Within PLGS, a non-specific digest was specified and appropriate intensity thresholds of 80 and 20 were used for low and elevated energy thresholds, respectively. Lockmass correction was also conducted in PLGS rather than during data acquisition. A species specific database for *Bos taurus* was downloaded from the Uniprot website and only reviewed hits were included. The analytical data were searched against this database resulting in a number of peptides identified within the samples.

5.2.9.2 Digestion of protein bands from SDS-PAGE electrophoretograms and identification of peptides by Quadropole Time-of-flight Mass Spectrometry

Protein bands from SDS-PAGE electrophoretograms were digested, as described in Section 3.2.9.1. Tryptic peptides were identified using a Waters Acquity G2 Q-TOF LC-MS, as described in Section 3.2.9.4.

5.2.10 Cell Culture

The effect of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate on the secretion of IL-10 and TNF-α by J774.2 macrophages was determined as described in Section 3.2.11.

5.2.11 Data analysis

Analysis of variance (one-way ANOVA) was conducted using Minitab version 16 (Minitab Inc., State College, PA, USA). When differences were significant ($P \leq 0.05$), the means were analysed using Tukey's test.

5.3 Results and Discussion

Preliminary experiments were performed on sodium caseinate at pH 1.0, 3.0, 5.0 and 7.0 to investigate the effect of heating temperature on hydrolysis at these pH values. Heat treatment at 140°C resulted in extensive hydrolysis of the caseins (α_{S1}-, α_{S2}-, β- and κ-casein) over 30 min, as measured by urea-PAGE, SDS-PAGE and RP-HPLC (results not shown). The degree of protein degradation and peptide formation increased with decreasing pH. One of the aims of this study was to identify the most heat-labile bonds in the casein system. For this reason, a temperature of 130°C and pH value of 7.0 were used for all further heat treatment studies, as a heating temperature of 140°C and pH values of 1.0, 3.0 and 5.0 proved too severe to monitor the early stages of heat-induced hydrolysis of the caseins.

5.3.1 *Fluorescence intensity of 2% TCA-soluble peptides*

Heat-induced hydrolysis of sodium caseinate at 130°C and pH 7.0 led to a linear increase in the fluorescence intensities, as a function of time, of the 2% TCA-soluble fractions during the first 40 min of heating (Figure 5.1). The fluorescence intensity continued to increase until 120 min, but, the increase was not linear, i.e., the rate of increase in relative fluorescence intensity slowed after 40 min of heating.

5.3.2 *SDS-PAGE of heat-treated sodium caseinate*

SDS-PAGE was used to study heat-induced hydrolysis of sodium caseinate at 130°C and pH 7.0 over a period of 120 min. SDS-PAGE was carried out under reducing (Figure 5.2) and non-reducing (Figure 5.3) conditions to study any disulphide protein interactions which may have taken place during heating. The SDS-PAGE electrophoretograms in Figure 5.2 showed a decrease in band intensity for the main caseins with heating over time, indicating that hydrolysis had occurred. After 60 min of heating, α_{S1}- and α_{S2}-casein were no longer visible, while low levels of β- and κ-casein remained. After 120 min of heating, a blank lane was observed, indicating extensive hydrolysis with no intact protein or large polypeptide material remaining.

Based on the results shown in Figure 5.2, it appears that α_{S1}- and α_{S2}-casein are the most heat-labile of the caseins, followed by β- and κ-casein. As expected, increased protein breakdown was accompanied by an increase in the appearance of peptide material in the size range 6.5 - 20 kDa. These results are in agreement with the study of Guo *et al.* (1999), who showed that several peptides were formed after heating sodium caseinate (120 and 132°C for 60 min), due to heat-induced hydrolysis. Four distinct protein bands were present on the SDS-PAGE electrophoretogram (Figure 5.2) in the undialysed sodium caseinate in the size range 50-200 kDa. These bands gradually disappeared on heating and were no longer visible after 6 min. Some aggregation of protein was observed after 40 and 60 min of heating, as evidenced by material remaining in the slots of the SDS-PAGE electrophoretograms (Figure 5.2). These aggregates were not present after 120 min, suggesting that they were both formed and broken down during heating.

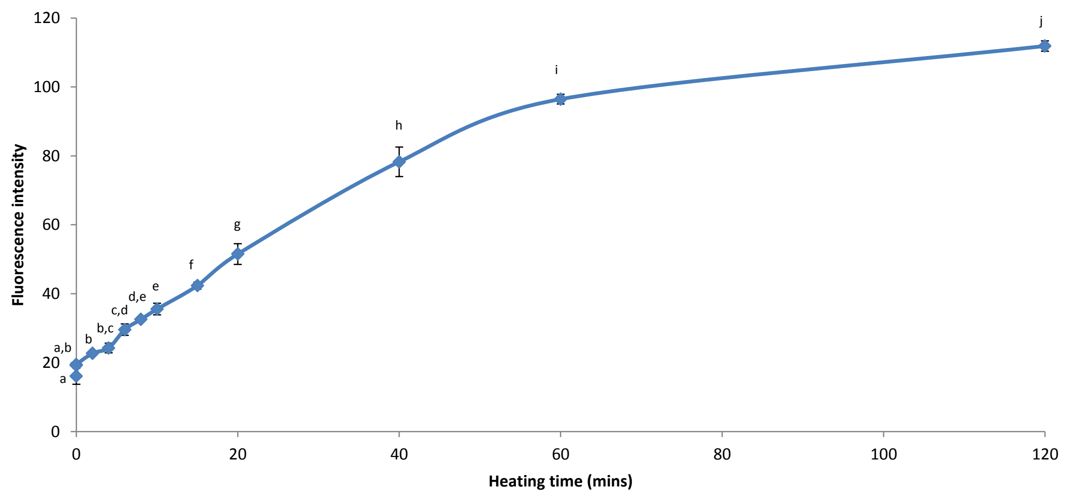


Figure 5.1 Mean values (± S.D., n=3) of fluorescence intensity of the 2% TCA-soluble fractions of sodium caseinate heated at 130°C for 0 to 120 min. The relative fluorescence intensity was measured at 20°C using excitation and emission wavelengths of 280 and 340 nm, respectively. Values with different superscript letters were significantly different ($p < 0.05$).

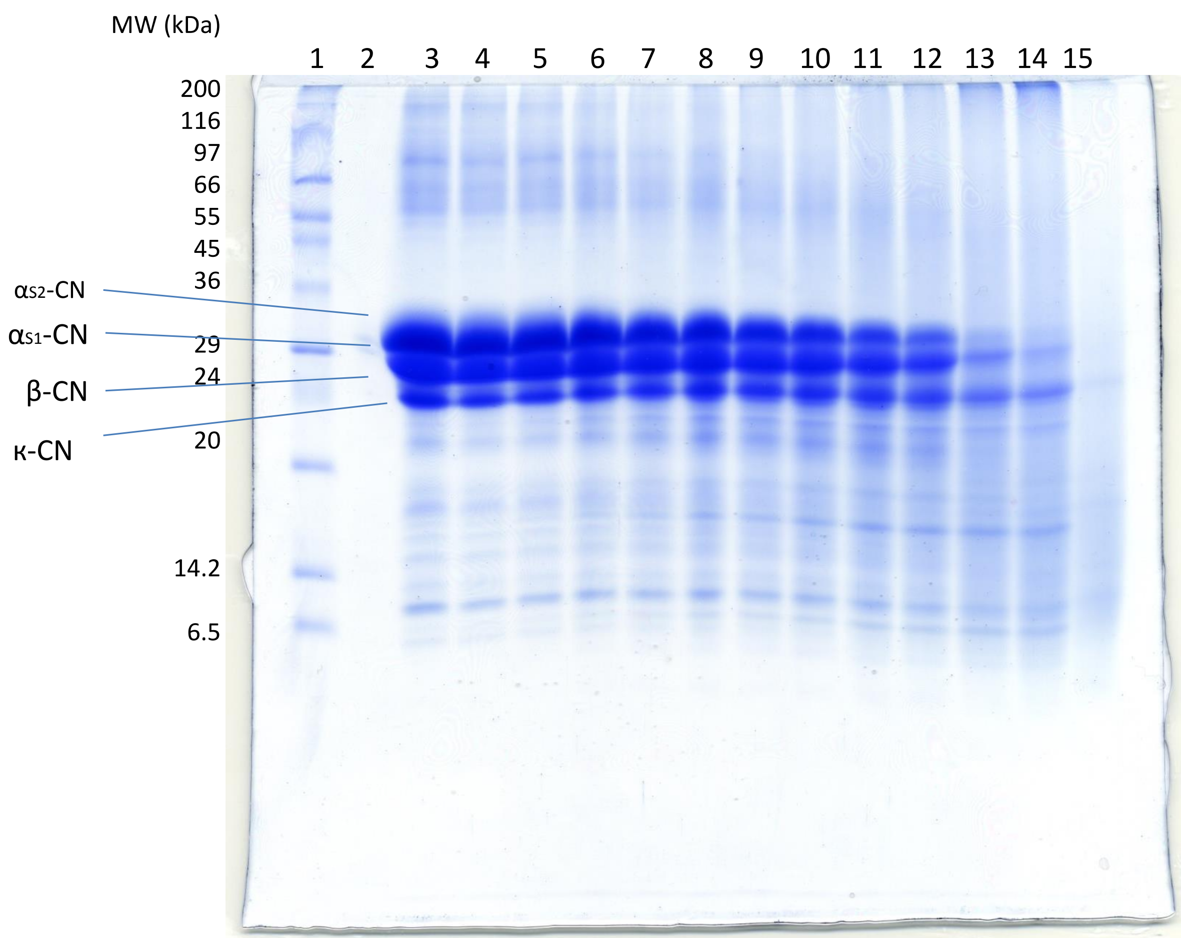


Figure 5.2 SDS-PAGE electrophoretograms (Coomassie stain) of sodium caseinate heated at 130°C for 0 to 120 min, separated under reducing conditions. Lane 1, molecular weight standard; lane 3, undialysed sodium caseinate; lane 4, unheated dialysed sodium caseinate; lane 5, heated (2 min come up time) dialysed sodium caseinate; lanes 6 – 15, dialysed sodium caseinate heated for a further 2, 4, 6, 8, 10, 15, 20, 40, 60 and 120 min, respectively.

The SDS-PAGE electrophoretograms in Figure 5.3 show sodium caseinate heated at 130°C and pH 7.0 over a period of 120 min, separated under non-reducing conditions. The protein profile of the heat-hydrolysed sodium caseinate in Figures 5.2 and 5.3 within the < 30 kDa range were quite similar. However, the protein profiles within the > 30 kDa range differ slightly, i.e., the 4 distinct protein bands present in Figure 5.2 in the undialysed sodium caseinate in the size range 50-200 kDa were also present in Figure 5.3, but at greater intensities. As shown in Figure 5.2, these bands gradually disappeared on heating; however, in Figure 5.3, they were present for longer. These bands were most likely κ-casein, which, on heating,

is known to aggregate due to the presence of free sulfhydryl groups, which allow the formation of intramolecular disulphide bonds (Groves *et al.,* 1998). It should be noted that disulphide bonds are naturally present within the κ-casein structure. Another possibility is that these bands were complexes formed between κ-casein and heat-denatured β-lactoglobulin *via* disulphide bonds (Cho *et al.,* 2002), as the sodium caseinate used in the current study was found to contain a low level of β-lactoglobulin (results not shown). It is important to note that these bands were present in the unhydrolysed sodium caseinate, suggesting that they were not formed during heat-induced hydrolysis. However, it is possible that they were formed during the manufacture of the sodium caseinate, e.g., as a result of exposure to extremes of pH during localised addition of alkali. Irrespective of this, it is clear from Figure 5.3 that heating sodium caseinate at 130°C and pH 7 for 120 min was not a causative factor in protein aggregation but, instead, resulted in the breakdown of any aggregates which were already present.

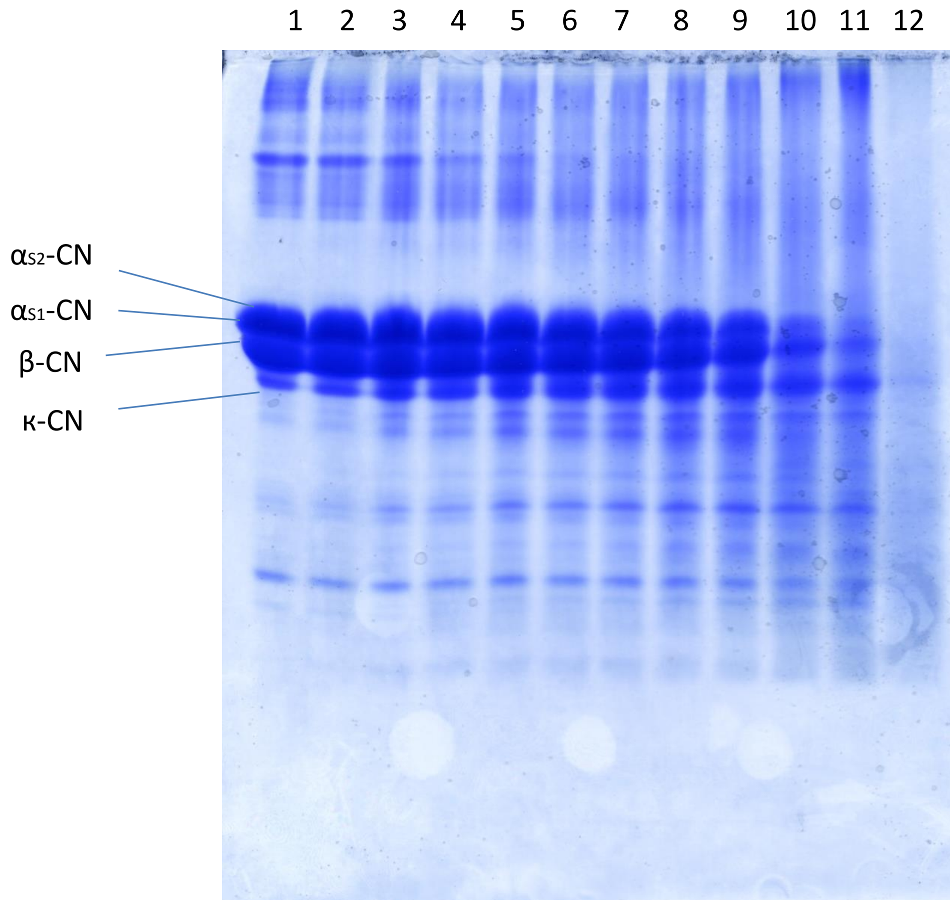


Figure 5.3 SDS-PAGE electrophoretograms (Coomassie stain) of sodium caseinate heated at 130°C for 0 to 120 min, separated under non-reducing conditions. Lane 1, unheated sodium caseinate; lane 2, heated (2 min come up time) sodium caseinate; lanes 3 - 12, sodium caseinate heated for a further 2, 4, 6, 8, 10, 15, 20, 40, 60 and 120 min, respectively.

The most common method for in-gel protein detection is staining with Coomassie blue; however, this method sometimes lacks sensitivity. The most sensitive colorimetric method for detecting proteins is silver staining; this technique involves silver ions interacting with and binding to certain functional groups of proteins, which in turn are reduced to metallic silver, resulting in a brown-black colour. The SDS-PAGE electrophoretograms of Figure 5.4 show the pH 4.6-soluble fraction of sodium caseinate, heated at 130°C and pH 7.0 for up to 120 min, separated under reducing conditions in the approximate size range 6 – 30 kDa and stained using the

silver stain method. The dominant band across all lanes, with the exception of lane 15 (120 min), was at approximately 27 kDa. This is the region where the main caseins would be expected; however, these samples had been adjusted to pH 4.6 and so it is unlikely that any intact casein would remain. This band was present at a relatively constant level over the first 20 min (lanes 3-12), after which its intensity was reduced, i.e., 40 and 60 min (lanes 13 and 14). By 120 min (lane 15), only a faint band was evident. Eleven additional bands were present on the SDS-PAGE electrophoretograms in Figure 5.4. Of these, only one band (A) was consistently present at all heating times. Of the remaining bands, four were present in the unheated sodium caseinate and subsequently broken down during heating (B, C, D, H), while six were not present initially and were produced on heating (J, K, E, F, G).

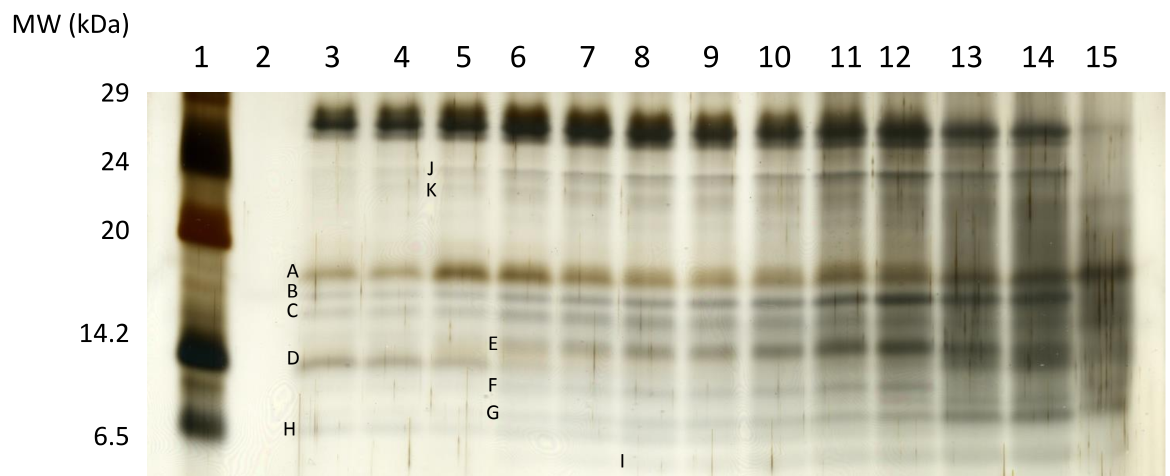


Figure 5.4 Section of an SDS-PAGE electrophoretograms (silver stained) of the pH 4.6-soluble fraction of sodium caseinate heated at 130°C and pH 7.0 for up to 120 min, separated under reducing conditions. Lane 1, molecular weight standard; lane 3, undialysed sodium caseinate; lane 4, unheated dialysed sodium caseinate; lane 5, heated (2 min come up time) dialysed sodium caseinate; lanes 6 – 15, dialysed sodium caseinate heated for a further 2, 4, 6, 8, 10, 15, 20, 40, 60 and 120 min, respectively.

5.3.3 *Urea-PAGE of heat-treated sodium caseinate*

Samples of heat-hydrolysed sodium caseinate (130°C, pH 7.0) were analysed by urea-PAGE (Figure 5.5). The electrophoretograms were rather streaky, which is usually indicative of intermolecular covalent bonds (Hustinx *et al*., 1997). This is supported by the SDS-PAGE electrophoretograms (Figures 5.2 and 5.3) which showed some protein aggregation occurring in sodium caseinate after 40 and 60 min of heating. Hydrolysis was observed in the urea-PAGE electrophoretograms, as evident by a decrease in the band intensity of α_{S1}-, α_{S2}-, β-, and κ-casein over time. After a period of 120 min, no intact α_{S1}-, α_{S2}- or β-casein remained.

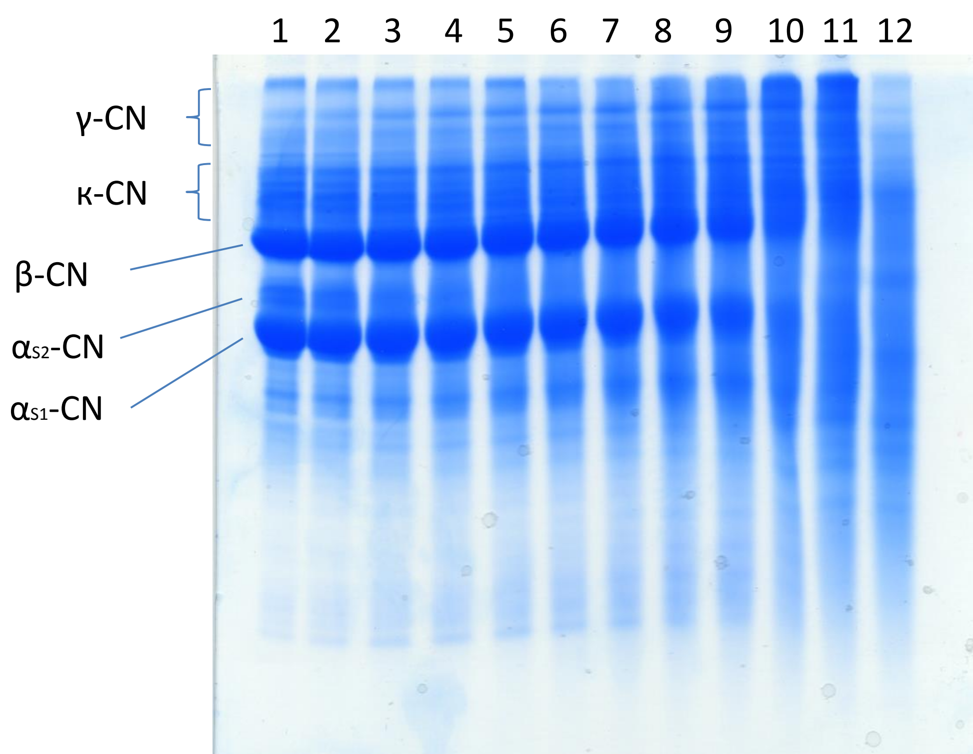
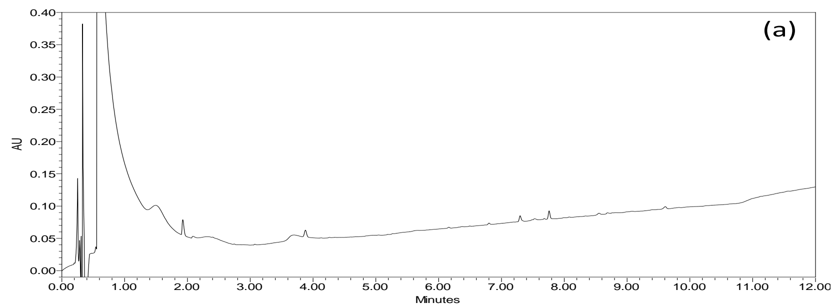
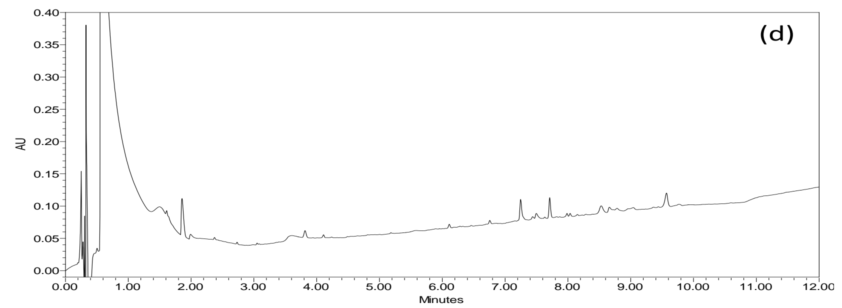


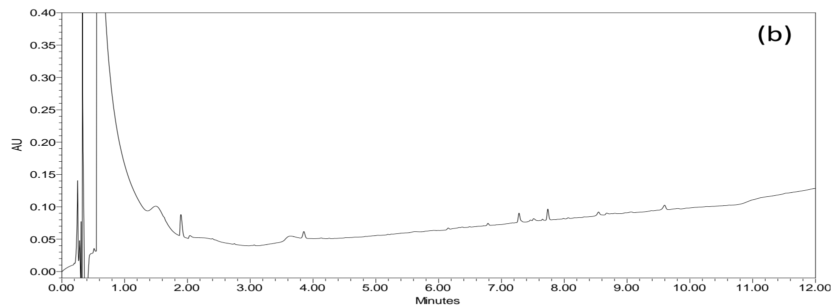
Figure 5.5 Urea-PAGE electrophoretograms of sodium caseinate heated at 130°C for 0 to 120 min. Lane 1, unheated sodium caseinate; lane 2, heated (2 min come up time) sodium caseinate; lanes 3-12, sodium caseinate heated for a further 2, 4, 6, 8, 10, 15, 20, 40, 60 and 120 min, respectively.

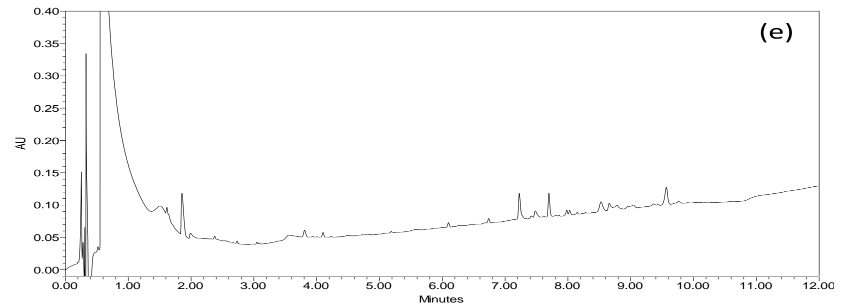
5.3.4 *RP-UPLC of 2% TCA-soluble fractions*

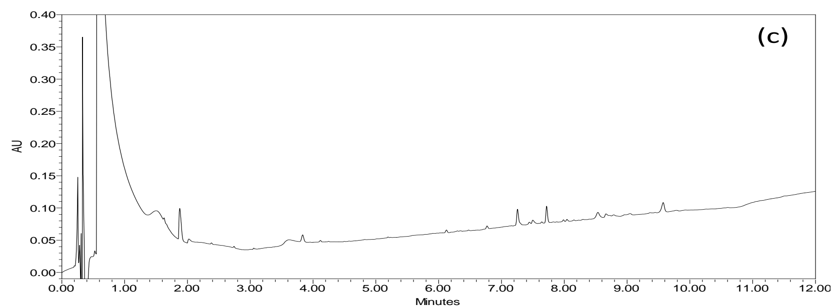
The 2% TCA-soluble fractions of sodium caseinate heated at 130°C, pH 7.0, over 120 min were analysed by RP-UPLC (Figures 5.6 and 5.7). RP-UPLC showed an increase in 2% TCA-soluble peptides on heating, confirming that hydrolysis had occurred. Six minor peaks were visible in the unhydrolysed sodium caseinate, i.e., eluting at 2.0, 3.9, 7.4, 7.8, 8.6 and 9.6 min, indicating some peptide material was naturally present in the unhydrolysed sodium caseinate. This peptide material was most likely not due to the action of indigenous proteolytic enzymes in milk, as any peptide material produced in this manner would have been removed during the production of sodium caseinate, in which several washing steps were carried out. As previously mentioned, this indigenous peptide material may have been formed during the manufacture of sodium caseinate. The majority of peptides produced on heating were hydrophobic, i.e., late eluting; however, a substantial minority of peptide material eluted in the hydrophilic range. The reproducibility of heat-induced hydrolysis of sodium caseinate was investigated by repeating the experiment and RP-UPLC analysis (results not shown); identical chromatographic profiles were obtained, indicating that heat-induced hydrolysis of sodium caseinate was selective, i.e., that protein breakdown and peptide formation did not occur at random.











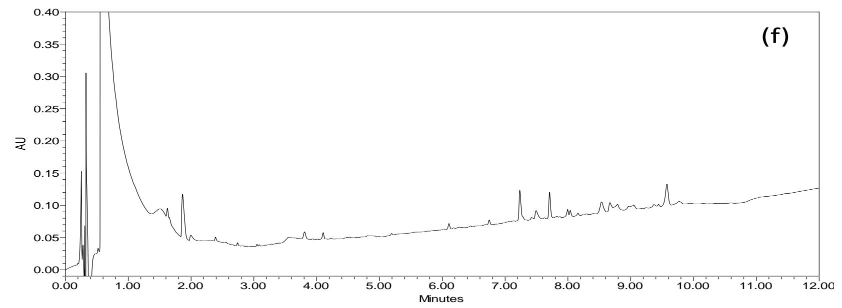
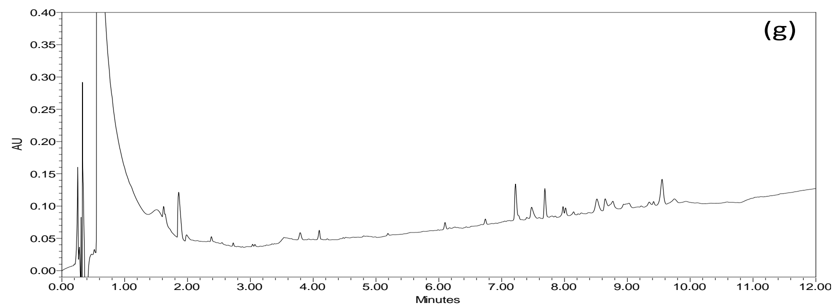
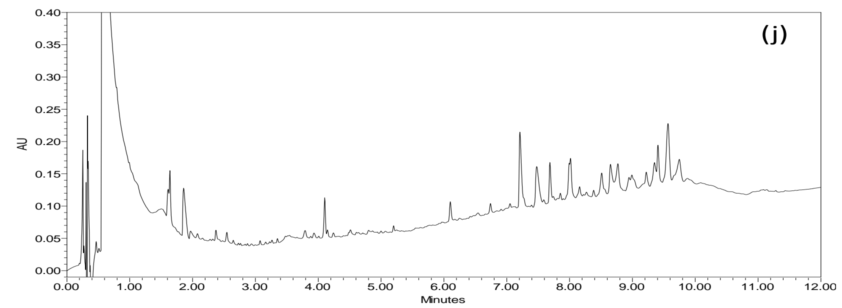
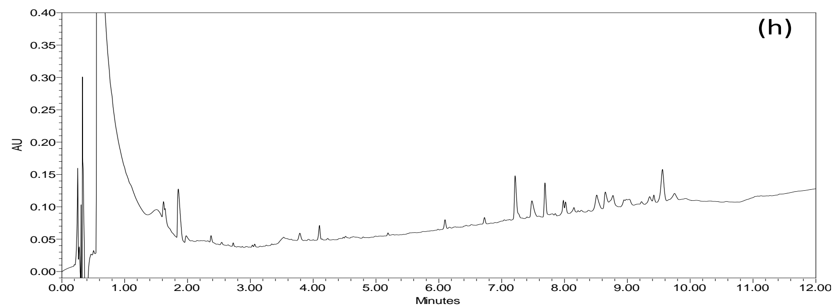
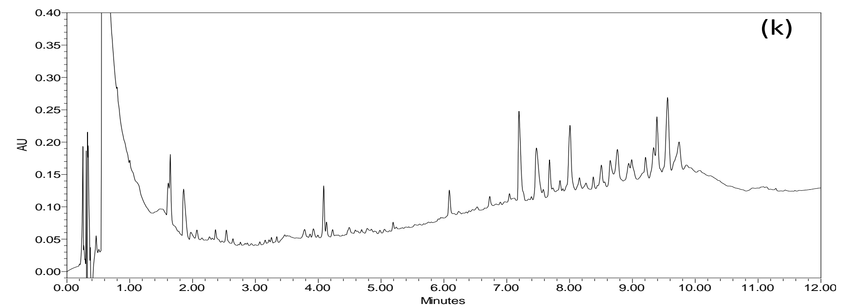


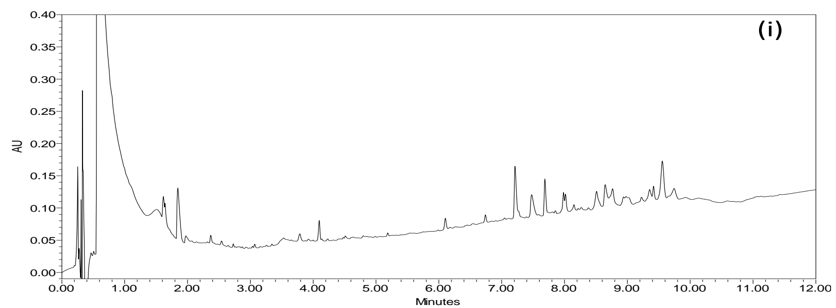
Figure 5.6 RP-UPLC chromatograms of the 2% TCA-soluble fractions of sodium caseinate (2.5% w/v), pH 7.0, heated at 130°C for 0-8 min; (a) 0 min, (b) 2 min come up time, (c) to (f) a further 2, 4, 6, and 8 min, respectively.











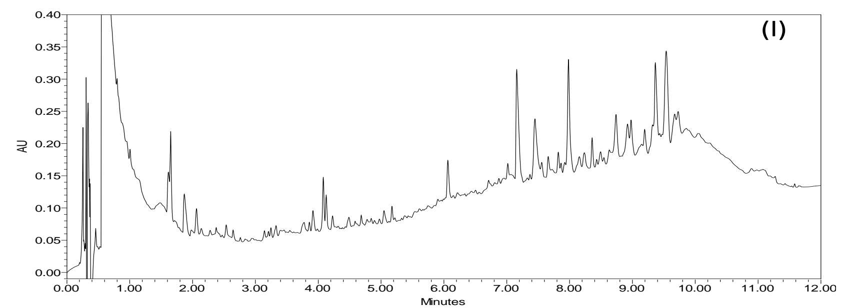


Figure 5.7 RP-UPLC chromatograms of the 2% TCA-soluble fractions of sodium caseinate (2.5% w/v), pH 7.0, heated at 130°C for 10-120 min; (g) to (l) 10, 15, 20, 40, 60 and 120 min (not including 2 min come up time), respectively.

5.3.5 *LC-MS identification of peptides*

The 2% TCA-soluble peptides were separated by RP-UPLC and detected by mass spectrometry (Figures 5.8 to 5.11). The chromatographic eluent was by-passed from the mass spectrometer during the first two minutes after samples injection, in order to prevent disturbance in mass spectrometry analysis due to the presence of residual TCA, lactose and minerals. In total, 1023 peptides were identified and characterised. Of these peptides, 326 originated from α_{S1}-casein (32%) (Figure 5.8), 125 from α_{S2}-casein (12%) (Figure 5.9), 353 from β-casein (35%) (Figure 5.10) and 219 from κ-casein (21%) (Figure 5.11).

The 326 peptides identified from α_{S1}-casein were evenly spread throughout its entire sequence. Peptides ranged in size from 3 (α_{S1}-casein [f197-199]) to 45 (α_{S1}-casein [f1-45]) amino acid residues in length. It is highly probable that dipeptides and individual amino acids were also produced during heat-induced hydrolysis of sodium caseinate, but were not retained on the RP-UPLC column. Based on the peptides identified in the current study, the only bonds within α_{S1}-casein which were not hydrolysed during heating at 130°C over 120 min were Glu(39)-Leu(40), Ile(44)-Gly(45), Ser(48)-Thr(49), Lys(83)-Glu(84), Glu(84)-Asp(85), Tyr(94)-Leu(95), Gln(97)-Leu(98), Arg(100)-Leu(101) and Leu(101)-Lys(102). It is interesting to note that leucine was present in five of these peptide bonds, glutamic acid was present in three and lysine in two.

A peptide bond was designated a major cleavage site based on at least 6 peptides having a common N- and/or C-terminus. The following peptide bonds were accordingly identified as major cleavage sites within α_{S1}-casein; Pro(2)-Lys(3), His(4)-Pro(5), Leu(21)-Arg(22), Asn(38)-Glu(39), Asp(56)-Ile(57), Val(167)-Pro(168), Asp(175)-Ala(176), Pro(177)-Ser(178), Asp(181)-Ile(182), Asn(184)-Pro(185), Gly(187)-Ser(188), Asn(190)-Ser(191) and Leu(198)-Trp(199). Proline was present in five of these major cleavage sites, while asparagine, aspartic acid or serine were each present in three. Of the 326 α_{S1}-casein derived peptides, only one had a reported biological activity, i.e., α_{S1}-casein (f194-199); this hexapeptide has been shown to have antihypertensive (Maruyama and Susuki, 1982; Maruyama *et al.,*

1987; Meisel and Schlimme, 1994; Pihlanto-Leppala *et al.,* 1998) and immunomodulatory properties (Parker *et al.,* 1984; Migilore-Samour and Jolles, 1988).

Similarly, the 125 peptides identified from α_{S2}-casein were evenly spread throughout its entire sequence. Based on the peptides identified in the current study, 47 bonds within α_{S2}-casein were not hydrolysed during heating. Lysine and threonine were present in thirteen of these peptide bonds, glutamine was present in nine and leucine in eight. No major cleavage sites were identified in α_{S2}-casein. Relative to the number of peptide bonds hydrolysed during heating, α_{S2}-casein appeared to be the most heat-resistant of the caseins. None of the peptides identified from α_{S2}-casein were biologically active peptides from data reported in the literature (Silva and Malcata, 2005).

As with α_{S1}- and α_{S2}-casein, the peptides identified from β-casein were evenly spread throughout its entire sequence. Only five peptide bonds were not hydrolysed during heating, i.e., Arg(25)-Ile(26), Pro(112)-Lys(113), Leu(125)-Thr(126), Pro(179)-Tyr(180) and Val(201)-Arg(202). Thirteen major cleavage sites were identified in β-casein, i.e., Val(8)-Pro(9), Asn(27)-Lys(28), Asp(43)-Glu(44), Gln(54)-Thr(55), Val(59)-Tyr(60), Pro(63)-Gly(64), Asn(68)-Ser(69), Pro(76)-Leu(77), Gln(79)-Thr(80), Thr(80)-Pro(81), Val(95)-Ser(96), Ser(96)-Lys(97) and His(148)-Gln(149). Proline was present in four of these major cleavage sites, while threonine, valine and serine were each present in three. The abundance of proline and serine at the major cleavage sites of β-casein is consistent with the major cleavage sites of α_{S1}-casein. Compared to the total number of peptide bonds hydrolysed, β-casein-derived peptides were the most numerous, compared to the other caseins, accounting for 35% of the total peptides identified. Of the 353 β-casein-derived peptides, two had reported biological activites, i.e., β-casein (f58-72), which has reported angiotensin I-converting enzyme (ACE) inhibitory properties (Smacchi and Gobbetti, 1998), and β-casein (f193-199), which has been shown to have immunomdoulatory (Minkiewicz *et al.,* 2000) and antimicrobial properties (Sandre *et al.,* 2001).

The pattern of hydrolysis of κ-casein differed from that of α_{S1}-, α_{S2}- and β-casein, i.e., the majority of peptides originated from its C-terminal region. It is interesting to note that, during cheese-making, the first bond in κ-casein which is cleaved by chymosin, and indeed other aspartic proteases, is Phe(105)-Met(106), which releases a hydrophilic peptide from its C-terminal region, termed the caseinomacropeptide, into the serum phase (Horne and Banks, 2004). As can be seen from Figure 5.10, the vast majority of κ-casein-derived peptides produced on heating originated from this region. In total, 26 bonds within κ-casein were not hydrolysed during heating, of which nine contained tyrosine, six contained proline and five contained lysine. Seven major cleavage sites were identified within κ-casein, i.e., Ile(119)-Pro(120), Thr(121)-Ile(122), Asp(148)-Ser(149), Pro(150)-Glu(151), Gln(163)-Val(164), Thr(165)-Ser(166) and Ala(168)-Val(169). Proline, isoleucine, threonine, serine and valine were each present in two major cleavage sites. As with α_{S1}- and β-casein, proline and serine were abundant in the major cleavage sites of κ-casein. None of the peptides identified from κ-casein were biologically active peptides from data reported in the literature (Silva and Malcata, 2005).

α_{S1}-casein

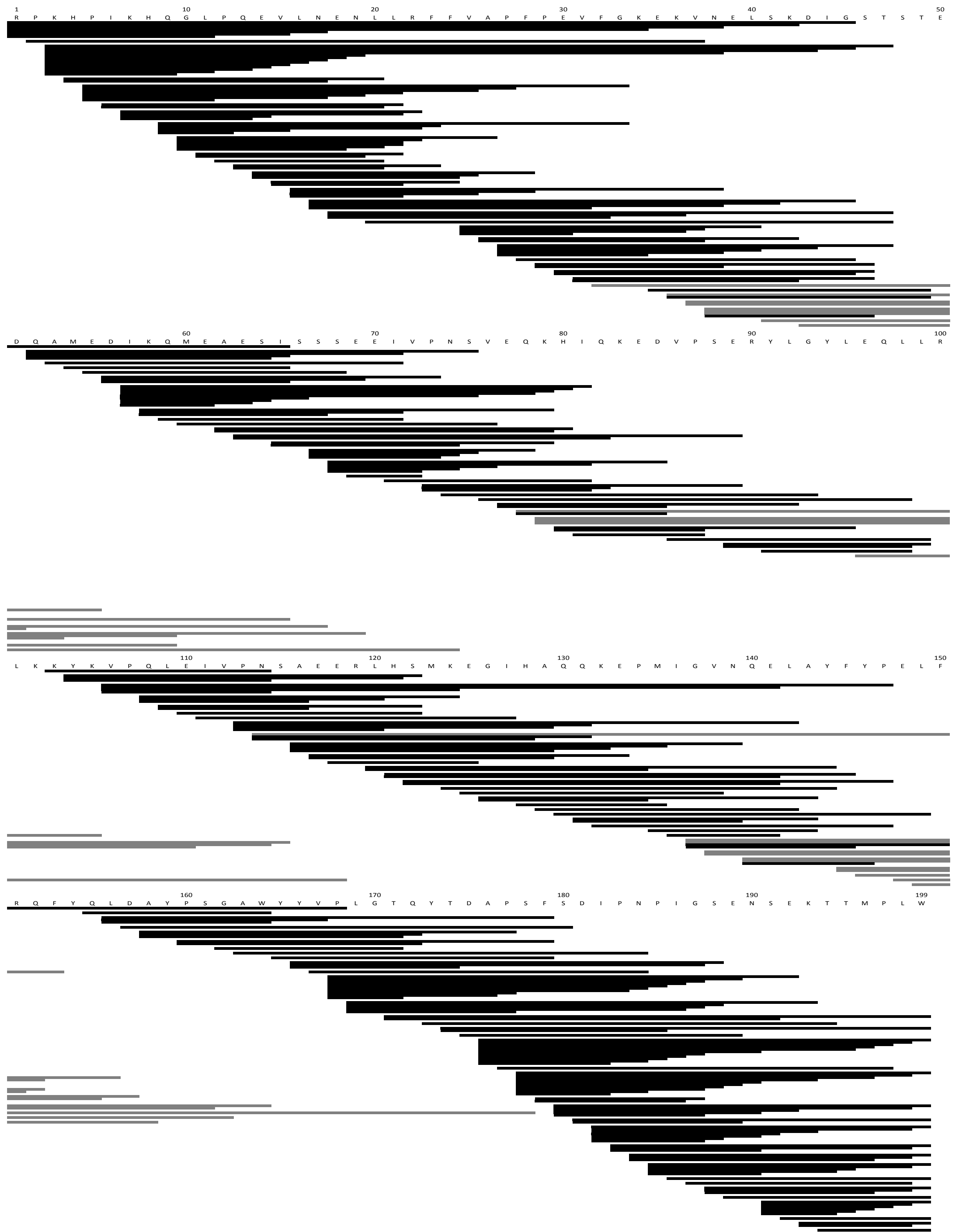


Figure 5.8 α_{S1}-casein-derived peptides released from sodium caseinate during heating at 130°C, pH 7.0, over 120 min. Peptides were identified using quadropole time-of- flight mass spectrometry (n=3).

α_{S2}-casein

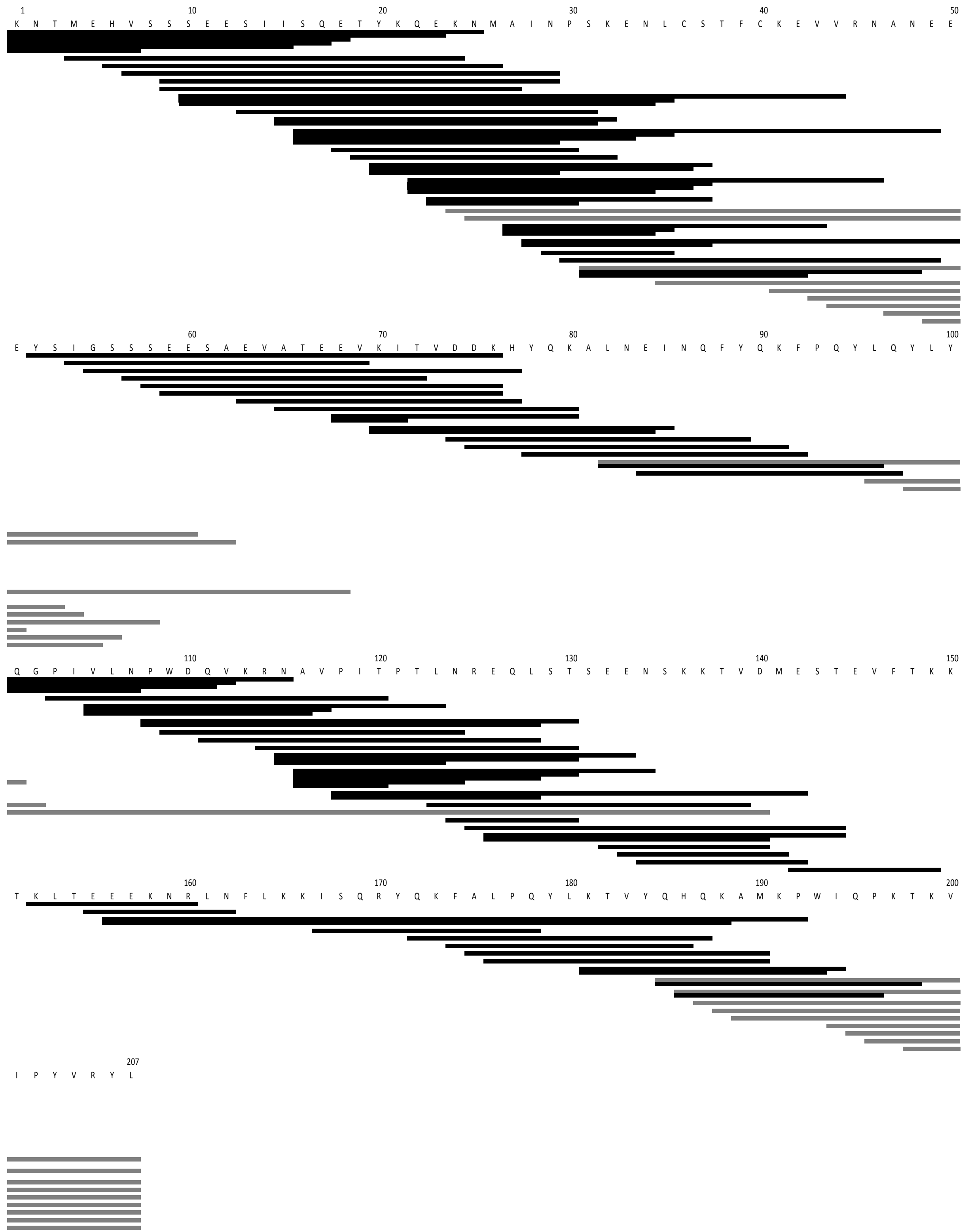


Figure 5.9 α_{S2}-casein-derived peptides released from sodium caseinate during heating at 130°C, pH 7.0, over 120 min. Peptides were identified using quadropole time-of- flight mass spectrometry (n=3).

β-casein

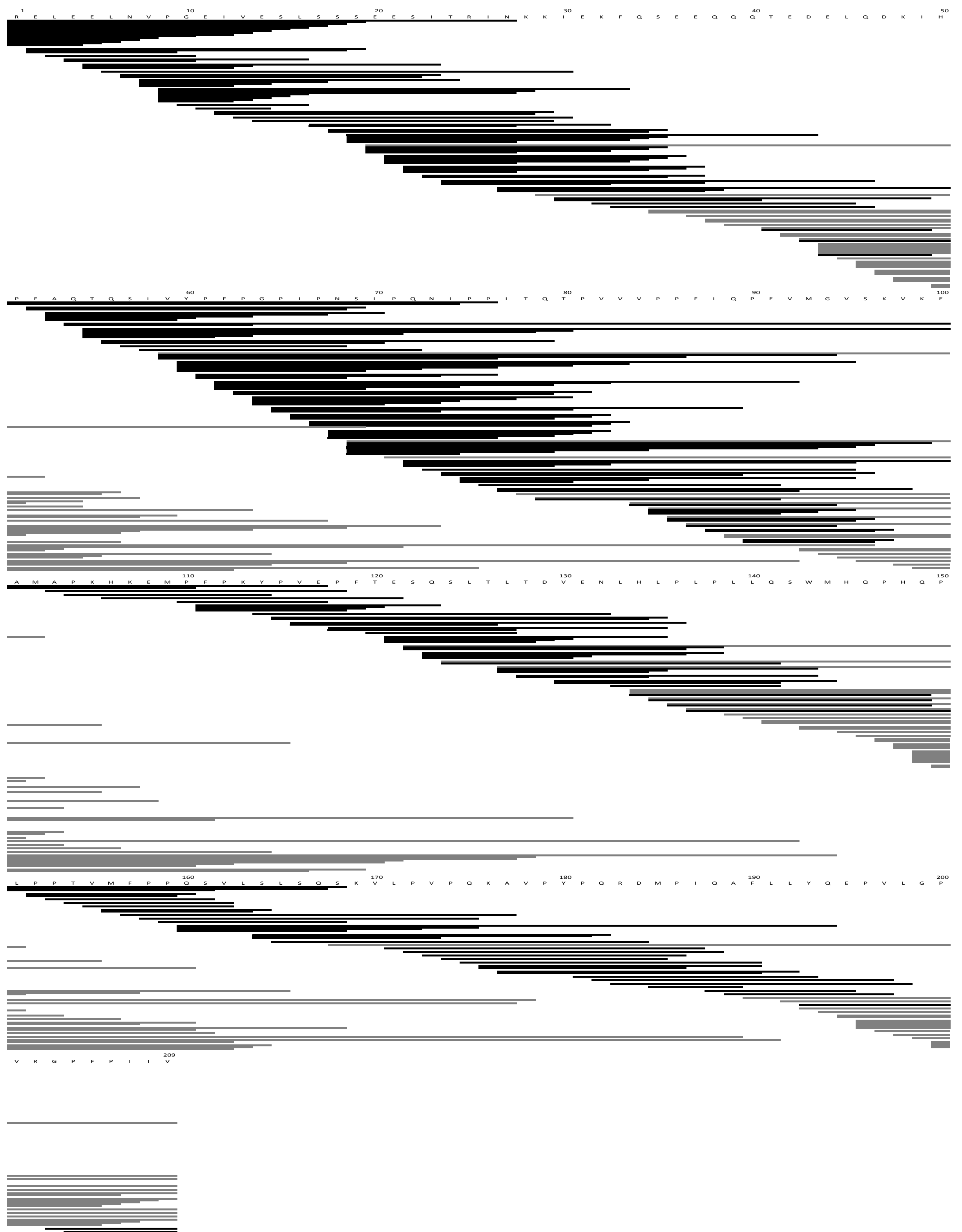


Figure 5.10 β-casein-derived peptides released from sodium caseinate during heating at 130°C, pH 7.0, over 120 min. Peptides were identified using quadropole time-of- flight mass spectrometry (n=3).

κ-casein

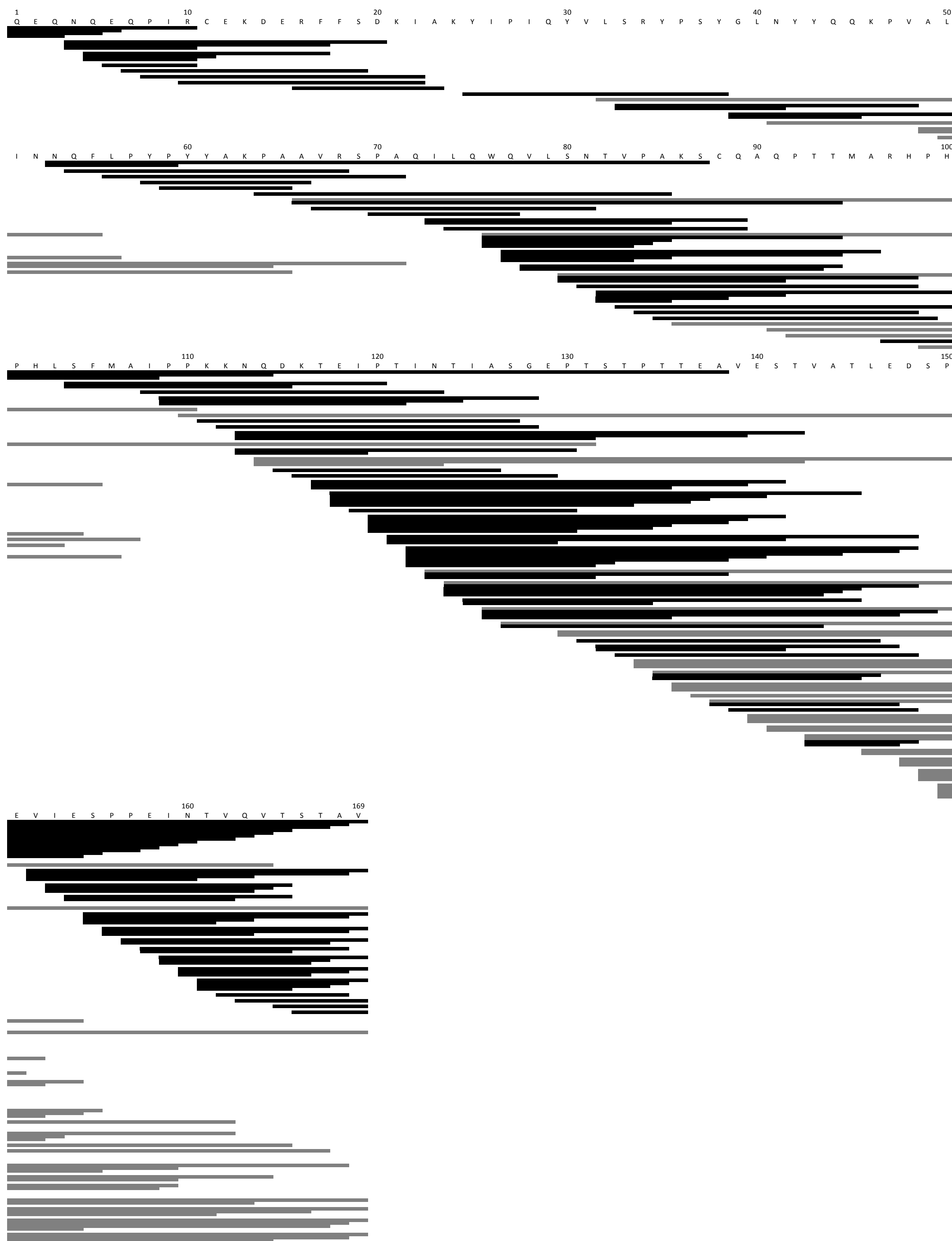


Figure 5.11 κ-casein-derived peptides released from sodium caseinate during heating at 130°C, pH 7.0, over 120 min. Peptides were identified using quadropole time-of- flight mass spectrometry (n=3).

A profile of all the 2% TCA-soluble peptides produced during heat-induced hydrolysis of sodium caseinate that were detected by LC-MS are described in Figures 5.8 to 5.11. This analysis was qualitative and the vast majority of peptides described were present at trace levels. When the relative intensity of each peptide was taken into account, only 11 out of the 1023 peptides identified were present at a significant level. The production and breakdown of the eleven most abundant peptides produced during heating were plotted as a function of time (Figures 5.12, 5.14 & 5.15). Of these eleven peptides, seven came from α_{S1}-casein (Figure 5.12), of which six originated from the C-terminal region and one originated from the N-terminal region. Based on the sequences of these seven peptides, a sequential order of hydrolysis is proposed (Figure 5.13).

It is likely that the first α_{S1}-casein-derived peptide, produced from the C-terminal region on heating, was α_{S1}-casein (f176-199). Production of this peptide required cleavage of α_{S1}-casein at Asp(175)-Ala(176). Of all the α_{S1}-casein-derived peptides shown in Figure 5.12, this peptide had the largest RP-UPLC peak area at each time point throughout the 120 min heating period, suggesting that it was present in the greatest abundance. Subsequently, α_{S1}-casein (f176-199) was cleaved at Pro(177)-Ser(178), Phe(179)-Ser(180), Asp(181)-Ile(182), Asn(184)-Pro(185) and Asn(190)-Ser(191)/Leu(198)-Trp(199) to produce α_{S1}-casein (f178-199), (f180-199), (f182-199), (f185-199) and (f191-198). Four of these peptides were also identified by Gaucheron *et al.* (1999, 2001), i.e., α_{S1}-CN (f176-199), (f178-199), (f182-199) and (f185-199). It should be noted that α_{S1}-CN (f176-199), (f182-199), (f185-199) and (f191-198) were all present in the 2% TCA-soluble fraction of the unheated sodium caseinate, suggesting that these peptides were produced during manufacture. Dialysis of the unheated sodium caseinate prior to heating resulted in the removal of α_{S1}-CN (f182-199) and (f191-198). α_{S1}-CN (f176-199) and (f185-199) remained in the unheated sodium caseinate following dialysis; however, the relative intensity of these peptides was substantially reduced. α_{S1}-CN (f182-199) and (f191-198) reappeared during the two min come up heating time. α_{S1}-CN (f178-199) was detected after two min of heating at 130°C, while α_{S1}-CN (f180-199) was detected after four min. The first α_{S1}-casein-derived peptide, produced from the N-terminal

region, on heating, was α_{S1}-casein (9-22). Production of this peptide required cleavage of α_{S1}-casein at His(8)-Gln(9)/Arg(22)-Phe(23). This peptide was not present in unheated sodium caseinate, but appeared during the two min come-up heating time. Of the nine peptide bonds hydrolysed during the production of the seven major α_{S1}-casein-derived peptides, serine was present in three, while proline, asparagine, phenylalanine and aspartic acid were each present in two. The presence of these amino acids, with the exception of phenylalanine, is in agreement with the qualitative results discussed earlier. The presence of asparagine and aspartic acid at heat-labile bonds is in agreement with the studies of Schultz (1967), Hustinx *et al.* (1997) and Gaucheron *et al.* (1999), who suggested that bonds containing these residues are more labile than other peptide bonds during heat treatment. Given that over 60% of the most abundant peptides produced in sodium caseinate during heating originated from α_{S1}-casein, it is fair to assume that α_{S1}-casein was the most heat-labile of the caseins.

Proteins and peptides are susceptible to a variety of chemical modifications which can affect their structure and biological function. Heat, in particular, has a large effect on proteins. Two α_{S1}-casein-derived peptides were identified as being modified, of which there were two types. The first corresponded to the deamidation of asparagine and glutamine of α_{S1}-casein (f9-22) after heating for 6 min. Deamidation is a spontaneous non-enzymatic reaction (Geiger and Clarke, 1987) which is commonly associated with protein degradation (Vlasak *et al.,* 2009). Deamidation of asparagine occurs more readily than that of glutamine because the amide group of asparagine is closer to adjacent amino residues which may act as catalysts (Wright, 1991). Storage of milk and milk products at elevated temperatures has been reported by several authors to promote chemical changes such as deamidation (Holland *et al.,* 2011, 2012; Le *et al.,* 2012). Van Boekel (1999) reported that the extent of heat-induced deamidation of caseinate corresponds to the level of amide present in asparagine. The second chemical modification, involving α_{S1}-casein (f191-198), was a dehydration reaction which occurred after heating for 2 min. In this case, a water molecule was removed from either Thr(194) or Thr(195) during heating.

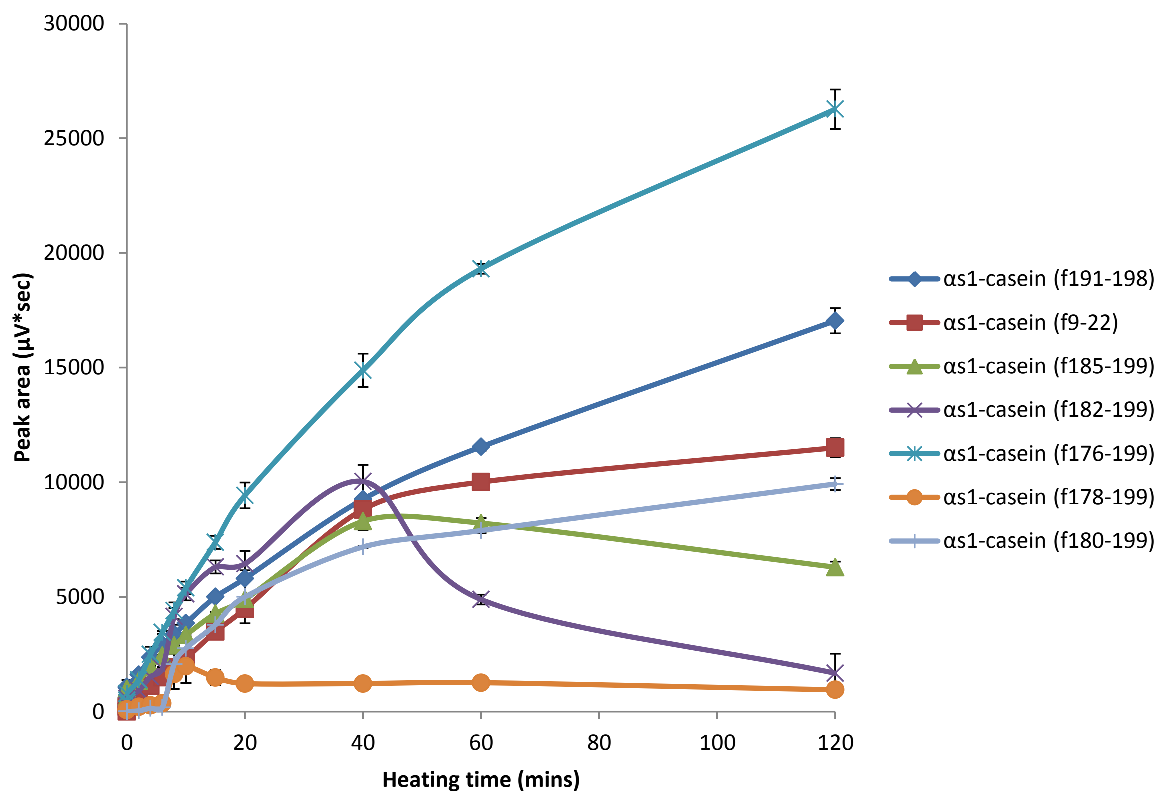


Figure 5.12 Formation of 2% TCA-soluble peptides derived from α_{S1}-casein during heat treatment of sodium caseinate (2.5%, w/v, pH 7.0) at 130°C for 0-120 min. Peptides were identified using quadropole time-of- flight mass spectrometry (n=3).

Figure 5.13 Proposed sequence of heat-induced hydrolysis of α_{S1}-casein. Cleavage sites are indicated by a red line.

Of the eleven major peptides, one came from the C-terminal region of β-casein, i.e., β-casein (f196-209). Production of this peptide required cleavage of Glu(195)-Pro(196). This peptide was not present in the unheated sodium caseinate, but appeared during the two min come-up heating time. Figure 5.14 shows the production of this peptide as a function of time over 120 min. As with asparagine and aspartic acid, bonds containing glutamic acid are more labile than other peptide bonds during heat treatments (Schultz, 1967; Hustinx *et al.,* 1997; Gaucheron *et al.,* 1999). This peptide has not previously been reported as a result of heat treatment in dairy products. No modifications of β-casein (f196-209) were observed during the heating period.

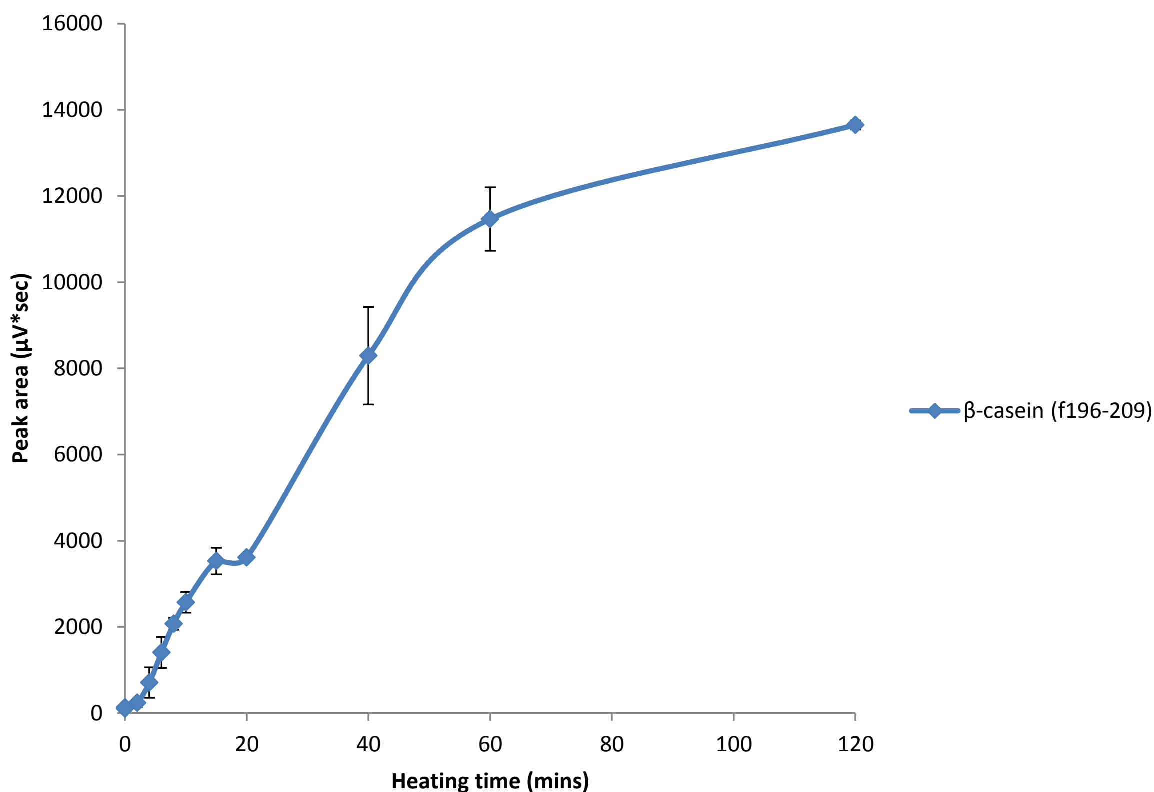


Figure 5.14 Formation of a 2% TCA-soluble peptides derived from β-casein, during heat treatment of sodium caseinate (2.5%, w/v, pH 7.0) at 130°C for 0-120 min. Peptides were identified using quadropole time-of- flight mass spectrometry (n=3).

Three of the 11 major peptides identified in sodium caseinate during heating at 130°C over 120 min originated from κ-casein. Based on the sequences of these peptides, a sequential order of hydrolysis is proposed (Figure 5.16). Two of these peptides were from the C-terminal region, i.e., κ-casein (149-169) and (f161-169). Production of these peptides required cleavage of Asp(148)-Ser(149) and Asn(160)-Thr(161), respectively. The presence of asparagine and aspartic acid at these cleavage sites is consistent with previous results. As already mentioned, these types of bonds are more labile than other peptide bonds during heat treatment. Neither of these peptides were present in the unheated sodium caseinate, but appeared after two min of heating. The third κ-casein derived peptide originated from the N-

terminal region, i.e., κ-casein (f1-10), and required cleavage of Arg(10)-Cys(11). Similarly, this peptide was not present in the unheated sodium caseinate, but appeared after four min of heating. These three peptides have previously been reported during sterilization of milk (Gaucheron *et al.,* 1999) and heat-induced proteolysis of casein molecules (Gaucheron *et al.,* 2001).

In addition to the two chemical modifications which occurred in the 11 most abundant peptides produced during heat-induced hydrolysis of sodium caseinate, several other modifications were observed in the less abundant peptides discussed earlier. These include methylation, glycosylation, oxidation of methionine residues, hydroxylation, carboxylation of glutamic acid residues and formylation (results not shown).

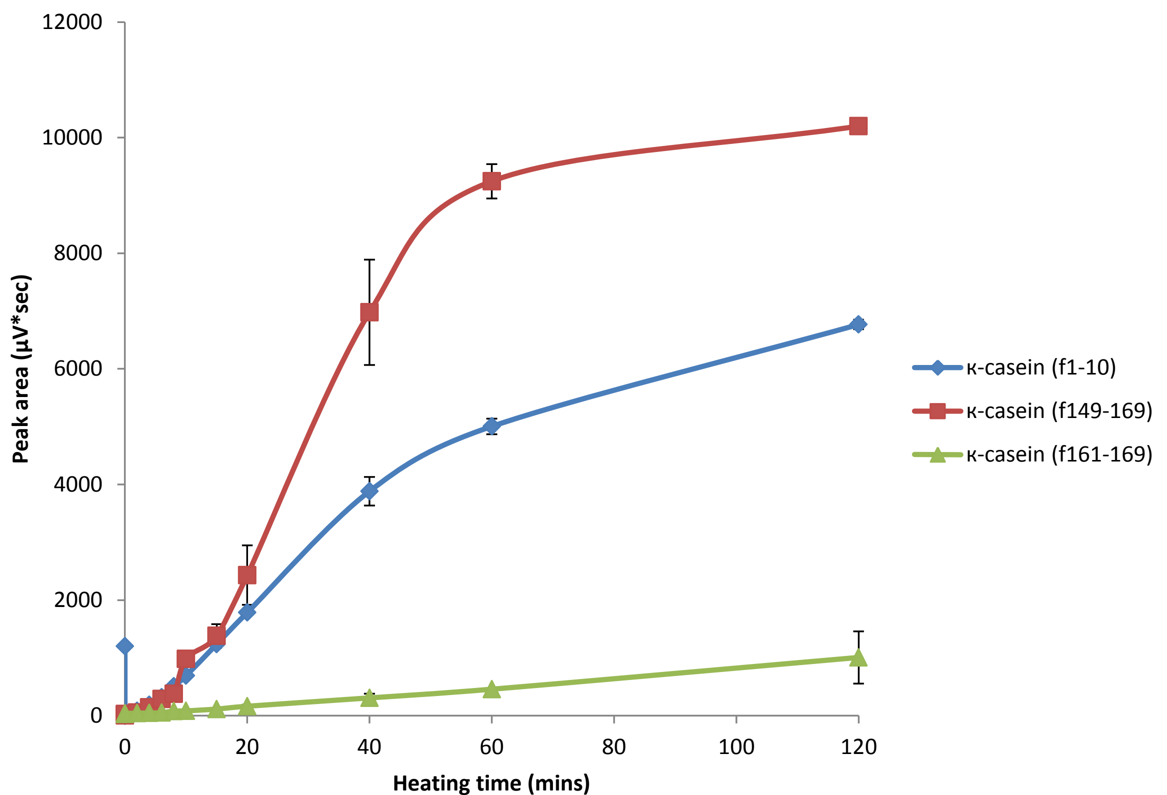


Figure 5.15 Formation of a 2% TCA-soluble peptides derived from κ-casein, during heat treatment of sodium caseinate (2.5%, w/v, pH 7.0) at 130°C for 0-120 min. Peptides were identified using quadropole time-of- flight mass spectrometry (n=3).

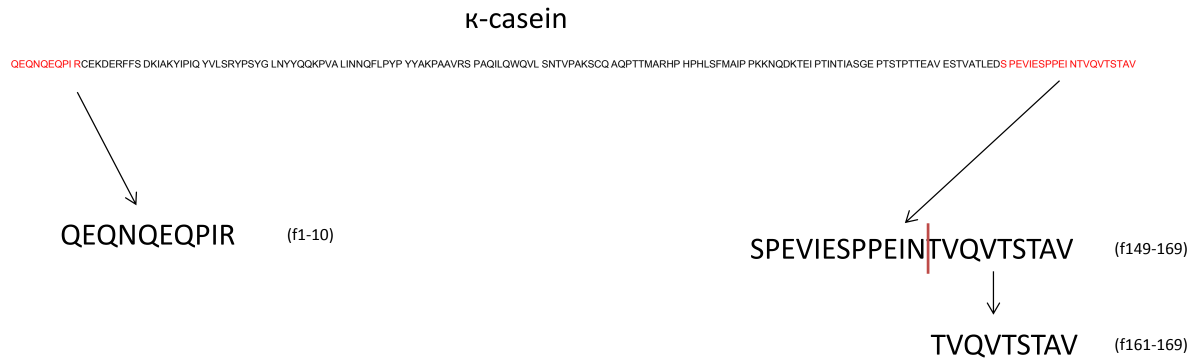


Figure 5.16 Predicted sequence of heat-induced hydrolysis of κ-casein. Cleavage sites are indicated by a red line.

The solubility of peptides in 2% TCA depends on size and hydrophobicity (Yvon *et al.,* 1989). It is likely that peptides of high molar masses were produced during heat-induced hydrolysis of sodium caseinate, but were insoluble in 2% TCA. In an attempt to identify some of these high molar mass peptides, bands were excised from SDS-PAGE electrophoretograms (Figure 5.17) and mass spectrometry was performed. Identification of the five bands analysed are shown in Table 5.1. Of the polypeptides identified, α_{S1}-casein products were present in four of five bands, while α_{S2}-, β- and κ-casein products were present in five, two and four bands, respectively. Individual bands contained multiple protein products due to the nature of 1-D electrophoresis, i.e., proteins/polypeptides were not separated based on their relative isoelectric points, but rather according to their molecular weights. As shown earlier, heat-induced hydrolysis of sodium caseinate resulted in extensive hydrolysis of α_{S1}-, α_{S2}-, β- and κ-caseins. Therefore, it was not surprising that each band contained products from several proteins.

It is interesting to note that α_{S2}-casein products were present in all 5 bands analysed. This is surprising as none of the 11 major peptides discussed earlier originated from α_{S2}-casein. The observed molecular masses of each polypeptide were lower than the theoretical value. This was to be expected as the polypeptides present in each band were hydrolysis products of their respective parent proteins.

The protein coverage of each polypeptide was only a rough estimate of the actual sequence, as it is possible that not all of the peptides produced during tryptic digestion were detected by mass spectrometry.

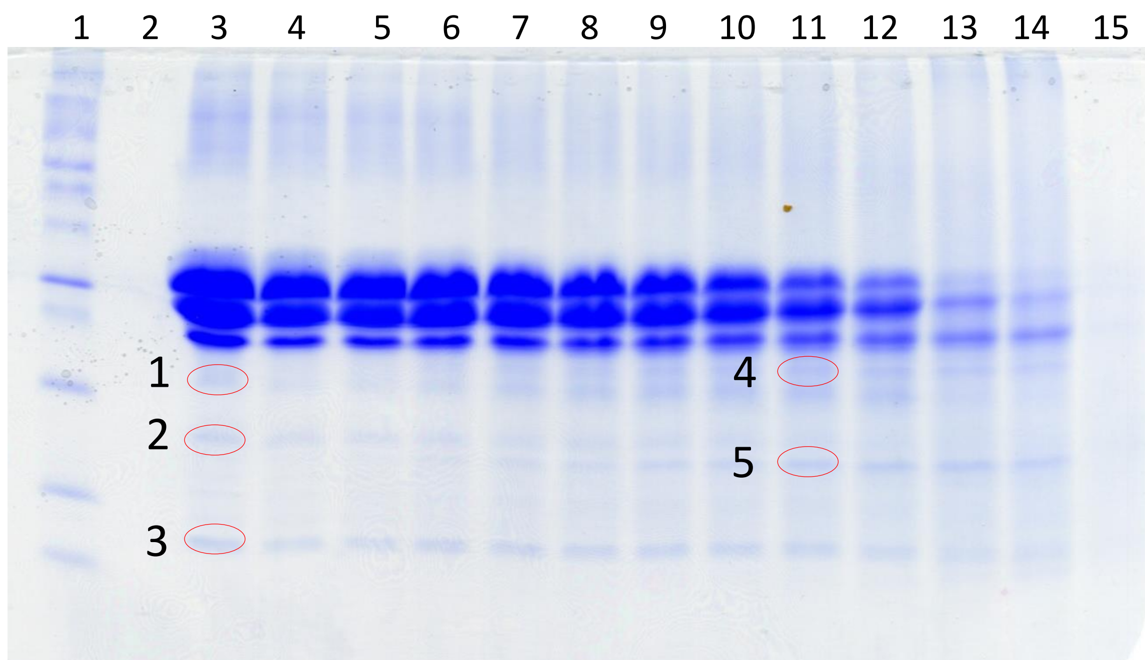


Figure 5.17 SDS-PAGE electrophoretograms of sodium caseinate heated at 130°C and pH 7.0 for up to 120 min. Lane 1, molecular weight standard; lane 3, undialysed sodium caseinate; lane 4, unheated dialysed sodium caseinate; lane 5, heated dialysed sodium caseinate; lanes 6 – 15, dialysed sodium caseinate heated for 2, 4, 6, 8, 10, 15, 20, 40, 60 and 120 min, respectively. Excised bands that were analysed by LC-MS are numbered 1 to 5.

Table 5.1 Identification of bands from the SDS-PAGE electrophoretogram (Figure 16), by LC-MS

Band no.	Identified parent protein	Ref. Uniprot	Mol. mass (kDa) theor/obs	No. of peptides	% Cov.	Peptides found	Protein Cov.
1	α_{S2}-casein	PO2663	26.02/19.73	8	14.86	115-125; 118-125; 161-165; 162-165; 163-165; 174-181; 189-197; 191-197	115-197
	κ-casein	PO2668	21.27/19.73	4	14.74	25-34; 27-34; 69-83; 69-86	25-86
	α_{S1}-casein	P02662	24.53/19.73	4	13.08	23-34; 91-100; 194-198; 194-199	23-199
2	α_{S1}-casein	P02662	24.53/18.13	3	15.42	8-22; 23-34; 194-199	8-199
	κ-casein	PO2668	21.27/18.13	2	14.74	25-34; 69-86	25-86
	β-casein	P02666	25.11/18.13	3	14.73	170-176; 177-183; 184-202	170-202
	α_{S2}-casein	PO2663	26.02/18.13	2	12.61	115-125; 174-181; 189-197	115-197
3	α_{S1}-casein	P02662	24.53/8.44	8	56.07	8-22; 23-34; 91-100; 104-119; 106-119; 133-151; 152-193; 194-199	8-199
	α_{S2}-casein	PO2663	26.02/8.44	6	18.47	115-125; 118-125; 138-150; 174-181; 189-197; 190-197	115-197
	β-casein	P02666	25.11/8.44	8	17.86	170-176; 171-176; 177-183; 181-183; 184-202; 203-207; 203-208; 203-209	170-209
	κ-casein	PO2668	21.27/8.44	3	17.74	25-34; 69-83; 69-86	25-86
4	α_{S2}-casein	P02663	26.02/20.27	15	25.7	81-91; 114-120; 114-125; 115-125; 118-125; 121-125; 138-149; 161-165; 162-165; 163-165; 174-181; 177-181; 189-197; 190-197; 191-197	81-197
	α_{S1}-casein	P02662	24.53/20.27	2	10.3	23-34; 91-100	23-100
	κ-casein	P02668	21.27/20.27	1	5.3	25-34	25-34
5	α_{S2}-casein	P02663	26.02/16.4	5	18	115-125; 138-149; 174-181; 189-197; 190-197	115-197

Protein reference (Ref.) correspond to the UniProt accession number; % cov. refers to sequence coverage. Theoretical molecular mass of proteins are as according to the amino acid and without consideration of degradation or modifications. Observed molecular mass are as observed with the position of the corresponding spots on the 1-DE gels

Biologically active peptides are most commonly produced by enzymatic hydrolysis of whole proteins (Kilara and Panyam, 2003; Korhonen and Pihlanto, 2003) using gastrointestinal enzymes, such as pepsin and trypsin, and enzymes from bacterial and fungal sources (Fitzgerald *et al.,* 2004). In addition, several indigenous proteases have been identified in the milk of various mammalian species (Kelly and Fox, 2006), originating from the animal's blood, somatic cells, and the apical membrane and cytoplasm of secretory cells (Fox and Kelly, 2006). Cleavage sites leading to the eleven most abundant peptides produced during heat-induced hydrolysis of sodium caseinate were compared with the cleavage specificity of elastase, plasmin, cathepsin G, cathepsin B, cathepsin D, chymosin and trypsin on α_{S1}-, β- and κ-casein (Upadhyay *et al.,* 2004) (Table 5.2). Production of these eleven peptides required cleavage of thirteen peptide bonds, of which three could also be cleaved by one or more of the enzymes listed above i.e., Arg(22)-Phe(23) of α_{S1}-casein is cleaved by plasmin and trypsin, Phe(179)-Ser(180) of α_{S1}-casein is cleaved by cathepsin G, elastase and chymosin, and Arg(10)-Cis(11) of κ-casein is cleaved by trypsin. Production of peptides in this manner, i.e. heat-induced hydrolysis, may represent a novel alternative to traditional methods commonly used in the food industry.

Table 5.2 Idenitfication and characterisation by LC-MS of the eleven most abundant peptides produced during heat-induced hydrolysis of sodium caseinate. Also shown is whether these peptide bonds are cleaved by chymosin, trypsin, or the indigenous milk enzymes cathepsin G, cathepsin D, elastase and plasmin (yes/no)

Peptide produced on heating	N-terminal cleavage site	C-terminal cleavage site	Cathepsin G	Cathepsin B	Elastase	Plasmin	Cathepsin D	Chymosin	Trypsin
α_{S1}-CN 191-198	Asn190-Ser191	Leu198-Trp199	N;N	N;N	N;N	N;N	N;N	N;N	N;N
α_{S1}-CN 9-22	His8-Gln9	Arg22-Phe23	N;N	N;N	N;N	N;**Y**	N;N	N;N	N;**Y**
α_{S1}-CN 185-199	Asn184-Pro185		N	N	N	N	N	N	N
α_{S1}-CN 182-199	Asp181-Ile182		N	N	N	N	N	N	N
α_{S1}-CN 176-199	Asp175-Ala176		N	N	N	N	N	N	N
α_{S1}-CN 178-199	Pro177-Ser178		N	N	N	N	N	N	N
α_{S1}-CN 180-199	Phe179-Ser180		**Y**	N	**Y**	N	N	**Y**	N
β-CN 196-209	Glu195-Pro196		N	N	N	N	N	N	N
κ-CN 1-10		Arg10-Cis11	N/A	N/A	N/A	N/A	N/A	N/A	**Y**
κ-CN 149-169	Asp148-SerP149		N/A	N/A	N/A	N/A	N/A	N/A	N
κ-CN 161-169	Asn160-Thr161		N/A	N/A	N/A	N/A	N/A	N/A	N

N;N (N-terminal cleavage site;C-terminal cleavage site)
N/A Information not available

5.3.6 *Effect of heat-treated sodium caseinate on IL-10 secretion from J774.2 macrophages*

Interleukin (IL)-10 is an anti-inflammatory cytokine which is produced by numerous immune cells including monocytes, macrophages and dendritic cells (Mc Ardle *et al.,* 2013). IL-10 was first recognized for its ability to inhibit activation and effector function of T cells, monocytes and macrophages (Moore *et al*., 2001). The main function of IL-10 appears to be in limiting and terminating inflammatory responses (Villalta *et al*., 2011). In the current study, murine macrophage cells (J774.2) were isolated, cultured and pre-treated with 1 mg/mL of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate and unhydrolysed sodium caseinate for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h. LPS is a key inflammatory component of Gram-negative bacteria, which induces a distinctive pattern of cytokine release that regulates inflammation (Singh and Jiang, 2003). In the current study, LPS was used to initiate an inflammatory response which resulted in increased secretion of IL-10 from J774.2 cells.

The effect of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate and unhydrolysed sodium caseinate on IL-10 secretion by J774.2 macrophages is shown in Figure 5.18. The pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate caused a substantial increase the secretion of IL-10 compared to the LPS-positive control. Unhydrolysed sodium caseinate was used as a negative control, as it contained predominantly intact protein with relatively little peptide material. Unhydrolysed sodium caseinate caused a reduction in the secretion of IL-10 compared to the LPS positive control.

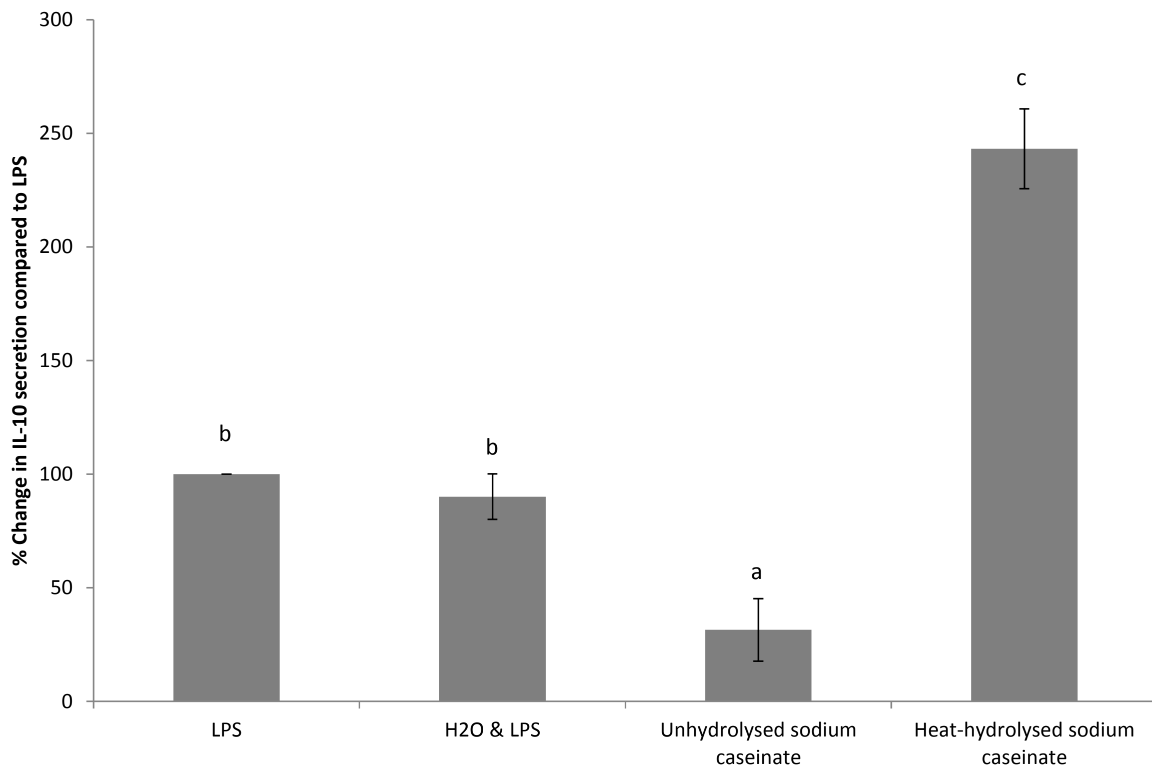


Figure 5.18 Effect of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate (130°C, pH 7.0 for 120 min) and unhydrolysed sodium caseinate on LPS-induced IL-10 secretion by J774.2 macrophages after 48 h pre-treatment. Exponentially growing cells were pre-treated with 1 mg/mL of unhydrolysed and heat-hydrolysed sodium caseinate for 24 h and stimulated with 10 ng/mL LPS for 3 h. The supernatant was removed and the concentration of cytokine quantified by ELISA. Data shown represent the mean (± SD) of measurements from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

J774.2 macrophages were isolated, cultured and pre-treated with various concentrations of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate and unhydrolysed sodium caseinate ranging from 0.001 to 1 mg/mL for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h. The dose-response effect of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate and unhydrolysed sodium caseinate on LPS-induced IL-10 secretion by J774.2 macrophages is shown in Figure 5.19. The pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate caused a substantial increase in the secretion of IL-10 compared to the LPS-positive

control at a concentration of 1 mg/mL, at a level similar to that shown in Figure 5.18; however, no effect was observed in the concentration range 0.001 – 0.1 mg/mL. Unhydrolysed sodium caseinate, which again served as a negative control, had no effect on IL-10 secretion in the concentration range tested.

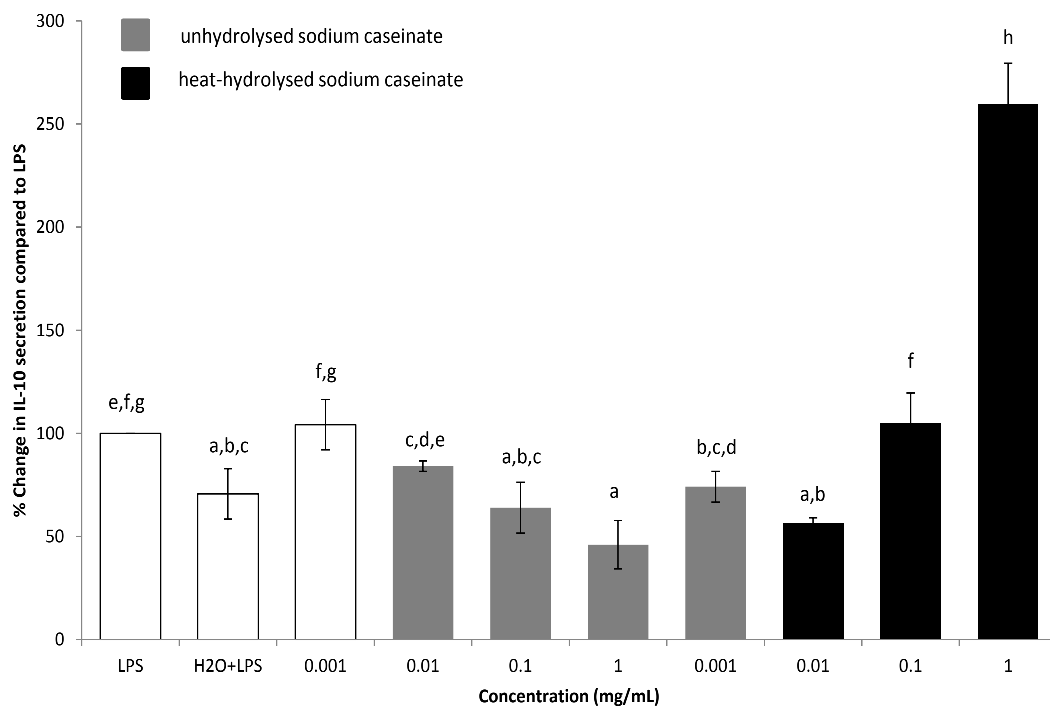


Figure 5.19 Dose-response effect of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate (130°C, pH 7.0 for 120 min) or unhydrolysed sodium caseinate on LPS-induced IL-10 secretion by J774.2 macrophages after 48 h pre-treatment. Exponentially growing cells were pre-treated with various concentrations (mg/mL) of unhydrolysed and heat-hydrolysed sodium caseinate for 24 h and stimulated with 10ng/mL LPS for 3 h. The supernatant was removed and the concentration of cytokine quantified by ELISA. Data shown represent the mean (± SD) of measurements from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

5.3.7 *Effect of heat-treated sodium caseinate on TNF secretion from J774.2 macrophages*

Tumor necrosis factor (TNF) is among the most studied and central pro-inflammatory cytokines (Gillett *et al*., 2010). TNF is a key regulator of the inflammatory response whose ligands are known to play a central role in a variety of inflammatory conditions, e.g., rheumatoid arthiritis and inflammatory bowel disease (Croft *et al*., 2012). TNF-α is produced predominantly by activated macrophages and T-lymphocytes and is known to have numerous biological properties relating to inflammation, proliferation, differentiation and cancer growth (Patil *et al*., 2011). In the current study, J774.2 macrophages were isolated, cultured and pre-treated with 1 mg/mL of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate and unhydrolysed sodium caseinate for 24 h before inflammation was induced with 10 ng/mL LPS for 3 h. The effect of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate and unhydrolysed sodium caseinate on LPS-induced TNF-α secretion is shown in Figure 5.20. The pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate was found to have no effect on TNF-α secretion compared to the LPS-positive control. Dexamethasone is a synthetic member of the glucocorticoid class of steroid drugs that has anti-inflammatory and immunosuppressant properties (Chae *et al.,* 2000). Dexamethasone has been shown to have an inhibitory effect on TNF-α expression (Zilberfarb *et al.,* 2001), as was seen in the current study (Figure 5.20). Unhydrolysed sodium caseinate caused a reduction in the secretion of LPS-induced TNF-α, at a similar level to that of dexamethasone.

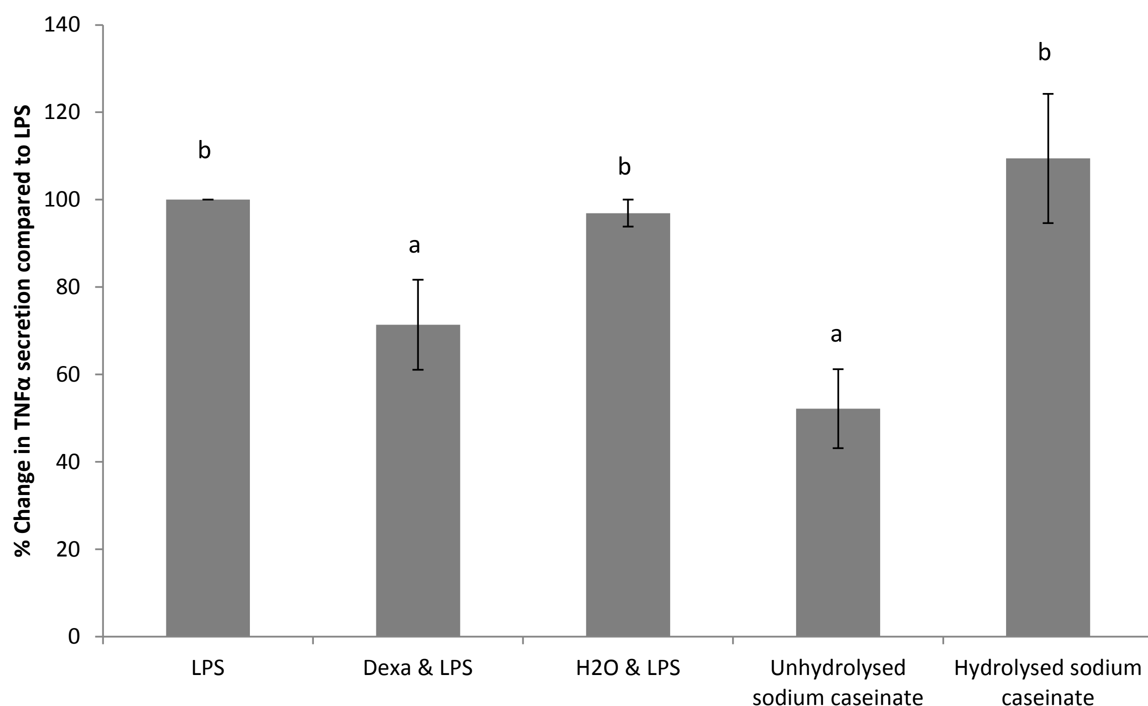


Figure 5.20 Effect of the pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate (130°C, pH 7.0 for 120 min) and unhydrolysed sodium caseinate on LPS-induced TNF-α secretion by J774.2 macrophages. Exponentially growing cells were pre-treated with 1 mg/mL of unhydrolysed and heat-hydrolysed sodium caseinate for 24 h and stimulated with 10 ng/mL LPS for 3 h. The supernatant was removed and the concentration of cytokine quantified by ELISA. Data shown represent the mean (± SEM, n=3) of measurements from three independent experiments. Values with different superscript letters were significantly different ($p < 0.05$).

5.4 Conclusion

Heat-induced hydrolysis of sodium caseinate at 130°C and pH 7.0 resulted in extensive hydrolysis of the caseins over a period of 120 min. Of the caseins, α_{S1}-casein was most susceptible to heat-induced hydrolysis, while κ-casein appeared to be the most resistant. In total, 1023 peptides were identified and characterised in the 2% TCA-soluble fraction of heat-hydrolysed sodium caseinate, of which 326 originated from α_{S1}-casein, 125 from α_{S2}-casein, 353 from β-casein and 219 from κ-casein; however, these peptides were not quantified, and so, were not representative of total proteolysis. For α_{S1}-, α_{S2}- and β-caseins, peptides were evenly dispersed throughout the entire sequence of each protein while, for κ-casein, peptides predominantly originated from the C-terminal region. Hydrolysis appeared to be specific, i.e., certain bonds within the caseins were preferentially hydrolysed, leading to the production of eleven predominant peptides. Of these eleven peptides, seven originated from α_{S1}-casein, one from β-casein and three from κ-casein. Proline, serine, asparagine and aspartic acid were consistently present in peptide bonds which were hydrolysed during the production of these eleven abundant peptides. Of the peptide bonds within the caseins which were not hydrolysed during heating, leucine and lysine were consistently present, suggesting that these amino acids were resistant to heat-induced hydrolysis.

Results from the current study suggest that heat may represent an alternative to enzymatic or microbial hydrolysis of the caseins. Further work is needed to investigate the effect of varying the time, temperature and pH on the production of peptides. Of the peptides identified, 3 have previously been reported to have biological activities relating to the treatment of high blood pressure, modulation of the immune system and inhibition of microbial growth, i.e., α_{S1}-casein (f194-199), β-casein (f58-72) and (f193-199). In the current study, a pH 4.6-soluble fraction of heat-hydrolysed sodium caseinate was found to cause a significant increase in the secretion of the anti-inflammatory cytokine IL-10 and may represent a potential candidate as an ingredient in a functional food product with immunomodulatory properties.

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Chapter 6

Proteomic characterisation of sodium caseinate hydrolysed by chymosin and its potential effect on satiety

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Declaration: Generation, optimisation, fractionation and characterisation of sodium caseinate hydrolysates were performed by Brian McGrath at University College Cork. Cell exposures and GLP-1 analysis were performed by Christine Bruen, Fiona O'Halloran and Triona McCarthy, at Teagasc, Moorepark Food Research Centre. The mouse feed intake trial was performed by Christine Bruen and Harriet Schellekens at University College Cork. Free amino acid analysis was performed by Paula O Conor at Teagasc, Moorepark Food Research Centre. Mass spectrometry was performed by Michael Kinsella and Brian McGrath at University College Cork. All experimental data/results were analysed and the chapter written by Brian McGrath.

Abstract

Bioactive peptides are protein fragments which have a positive impact on body function and conditions and may ultimately influence health; however, most of these biological activities are not active within the parent protein. The aim of this study was to generate a milk-derived bioactive peptide by enzymatic hydrolysis of sodium caseinate which targets gastric satiety signals, in particular glucagon-like peptide-1 (GLP-1). It was found that sodium caseinate (30 mg/mL), adjusted to pH 5.6, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 10, 40 or 120 min, caused an increase in the secretion of GLP-1 from STC-1 cells compared with unhydrolysed sodium caseinate. The hydrolysate produced in 40 min was identified as the most potent activator of GLP-1 *in vitro* and was found to cause a significant reduction in feed intake in mice when injected intraperitoneally, compared with control mice. The hydrolysis protocol was optimised in several ways in order to make it more suitable for commercial production, i.e., solids level increased from 3 to 10%, enzyme:substrate ratio reduced 20-fold (which required an increase in the reaction time from 40 to 220 min), and pH increased from 5.6 to 6.5. The hydrolysate was also fractionated by isoelectric precipitation of casein and ultrafiltration of the soluble fraction in an attempt to identify the bioactive component. Bioactivity was in the order: 3 kDa permeate of a pH 4.6-soluble extract > pH 4.6-soluble > crude hydrolysate > unhydrolysed sodium caseinate > 3 kDa retentate of a pH 4.6-soluble extract > pH 4.6-insoluble; the 3 kDa permeate of a pH 4.6-soluble extract resulted in the greatest secretion of GLP-1. Urea-polyacrylamide gel electrophoresis (PAGE) was used to study protein breakdown and the main polypeptides produced were α_{S1}-casein (f24-199 and f102-199) and β-casein (f1-189/192 and f1-163/165); however, these polypeptides had no effect on GLP-1 secretion. LC-MS was used to study peptide formation and the main peptides produced were β-casein (f193-209) and α_{S1}-casein (f1-23 and f3-23); however, these peptides also did not cause an increase in GLP-1 secretion. While the bioactive component was thus not identified, combined *in vitro* and *in vivo* results from the current study suggest that this hydrolysate could be used in the formulation of a functional food product aimed at targeting obesity and its associated complications.

6.1 Introduction

Obesity and its associated complications present significant public health problems, as obese people have a higher incidence of type two diabetes and cardiovascular disease (Huda *et al.,* 2006). Current International Obesity Taskforce (IOTF) estimates suggest that at least 1.1 billion people are overweight, and 312 million of these are obese (body mass index [BMI] ≥ 30) (James *et al.*, 2004). Peptide hormones released from the gut are important factors in controlling appetite and satiety; these include ghrelin, peptide YY (PYY), pancreatic polypeptide (PP), oxyntomodulin (Oxm), cholecystokinin (CCK), and glucagon-like peptide-1 (GLP-1). With the exception of ghrelin, these hormones act to increase satiety and decrease food intake (Druce *et al.,* 2004). Gut-derived satiety hormones reduce food intake in several ways, such as by reduction of gut motility, delaying gastric emptying, activation of mechanoreceptors, and acting directly on neurons in hypothalamic and brainstem areas (Chaudri *et al.,* 2006).

Milk proteins play an important role in the diet from a nutritional point of view; the protein fraction of milk contains many valuable components and biologically active substances. Bioactive peptides can be defined as specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health (Kitts and Weiler, 2003). These bioactive peptides are inactive within the primary sequence of the native protein (Meisel, 1998); however, they can be liberated in several ways (Korhonen, 2009), i.e., gastrointestinal digestion of milk (Boutrou *et al.*, 2013), fermentation of milk with proteolytic starter cultures (Takano, 2002; Fitzgerald and Murray, 2006) or hydrolysis by proteolytic enzymes (Fitzgerald and Meisel, 2003; Korhonen and Pihlanto, 2003a; Meisel and Fitzgerald, 2003). The most commonly used method of producing bioactive peptides is through enzymatic hydrolysis of milk proteins. Pancreatic enzymes, in particular trypsin, are most commonly used to generate bioactive peptides; however, other enzymes and different combinations of proteinases – including alcalase, chymotrypsin, pancreatin and pepsin as well as enzymes from bacterial and fungal sources – have also been utilised (Clare and Swaisgood, 2000; Korhonen and Pihlanto, 2003b, 2006). Dairy starter cultures, in particular lactic acid bacteria, the proteolytic system

of which is well characterised, are also commonly used to generate bioactive peptides (Phelan *et al.*, 2009).

A wide range of biological activities have been reported with milk-derived bioactive peptides, and can be divided into several categories based on their physiological effect on the body; antihypertensive (Hayes *et al.*, 2006), antithrombotic (Zimecki and Kruzel, 2007), opioid (Clare and Swaisgood, 2000), immunomodulatory (Politis and Chronopoulou, 2008), antimicrobial (Severin and Wenshui, 2005) and cytomodulatory (Parodi, 2007). Recently, they have received much attention as potential functional foods aimed at diet-related chronic diseases, such as obesity (Aoyama *et al.*, 2000), type two diabetes (Liu *et al.*, 2006) and cardiovascular disease (Fitzgerald *et al.*, 2004). Several authors have reported a positive correlation between dairy product consumption and maintenance of healthy body weight (Barr *et al.*, 2000; Phillips *et al.*, 2003; Ranganathan *et al.*, 2005). This is achieved by suppression of short-term food intake, increased subjective satiety and stimulation of the mechanisms known to signal satiation and satiety (Aziz and Andersen, 2007).

Chymosin (EC 3.4.23.4) is an aspartyl proteinase which is secreted in the stomach of young mammals. It is closely related to pepsin A (EC 3.4.23.1), which predominates in the stomach of adult mammals. Chymosin is produced intracellularly as preprochymosin. Preprochymosin is shortened by 16 amino acids during secretion and appears in the stomach as prochymosin, which, in turn, is activated to chymosin by cleavage of an additional 42 amino acids (Kumar *et al.,* 2010). The molecular weight of chymosin is within the range of 23-49 kDa (Kumar *et al.,* 2010); the exact value differs slightly between mammalian species. Chymosin has an acidic pH optimum (Fox, 1969) and its optimum temperature varies between 30 and 40°C (Berridge, 1952). The natural function of chymosin is to increase digestive efficiency by coagulating milk in the stomach of the neonate (McSweeney *et al.,* 1993). It does so by hydrolysing the Phe(105)-Met(106) bond of κ-casein; the removal of the C-terminus of κ-casein causes exposure of the hydrophobic core of casein micelles, leading to aggregation, gel formation and phase separation of the milk into curds and whey (Jensen *et al.,* 2013). Within the food industry, chymosin is the principal

protease used in cheese-making. This is because it has a highly specific milk-clotting activity relative to its proteolytic activity (Hsieh and Pan, 2012). Traditionally, an enzyme extract of calf abomasal tissue was used to coagulate milk in cheese production. However, these extracts often did not meet the necessary requirements for quality and quantity. To meet industrial demand, genetic engineering is increasingly used, whereby chymosin is produced in its exact natural form from bacteria (*E. coli*), yeast (*Kluveryomyces lactis*), and moulds (*Aspergillus niger*) as microbial hosts (Kumar *et al.,* 2010).

The objectives of this study were: (1) to generate a milk protein hydrolysate using chymosin that targets gastric satiety signals, for the development of a functional food product; (2) to optimise and scale up the hydrolysis protocol for commercial production as a food ingredient; and (3) to fractionate and characterise the hydrolysate, with the aim of identifying the bioactive component.

6.2 Materials and Methods

6.2.1 Generation of a sodium caseinate hydrolysate

Sodium caseinate (3% w/v) (Kerry Group, Tralee, Ireland) was made up in distilled water and the solution was allowed to hydrate overnight at 4°C. Following hydration, the solution was adjusted to pH 5.6 using 2N HCl and brought to 37°C in a waterbath (Techne TE-10A, Bibby Scientific, Stone, Staffordshire, UK). One mL chymosin solution (180 IMCU/mL) (Maxiren 180, DSM Food Specialities, Carbon Group, Ringaskiddy, Ireland) was added per gram of sodium caseinate. The solution was maintained at 37°C whilst being stirred using an overhead stirrer (Heidolph R2R-2021, Heidolph Instruments, Schwabach, Germany). Aliquots were taken at 10, 40 and 120 min; at each time point, the enzyme was inactivated by heating in a waterbath at 80° for 10 min. Samples were then lyophilised and stored in a dessicator at -20°C. A negative control was prepared using the same protocol as above, except heat inactivated chymosin was used. Chymosin was inactivated by heating in a waterbath at 80°C for 10 min.

6.2.2 Optimisation of hydrolysis conditions

6.2.2.1 Total solids

The total solids level of the sodium caseinate solution was increased from 3% to 10% (w/v) in order to increase the yield of bioactive material.

6.2.2.2 *pH adjustment*

In some cases, 0.7% (w/v) glucono delta-lactone (GDL) was used instead of HCl to reduce the pH of the sodium caseinate solution (100 mg/mL) to pH 5.6 prior to hydrolysis. In solution, GDL is progressively hydrolysed to gluconic acid, which causes a reduction in pH. Prior to hydrolysis, pH was adjusted to values in the range

5.6 to 7, i.e., 5.6, 6, 6.5, ~6.7 (natural pH of sodium caseinate, no pH adjustment) and 7, with HCl/GDL/NaOH.

6.2.2.3 Enzyme:substrate ratio

For the purpose of this study, the enzyme:substrate ratio will refer to the ratio of chymosin solution:sodium caseinate. The original enzyme:substrate ratio used was 1 mL chymosin (180 IMCU/mL) (Maxiren 180) per gram of sodium caseinate. This ratio was reduced 20-fold so that the enzyme:substrate ratio was 0.05 mL chymosin (9 IMCU) per gram of sodium caseinate. The enzyme:substrate ratio was reduced in order to reduce the cost of the enzyme preparation and also to minimise the addition of exogenous material originating from the enzyme preparation.

6.2.3 *Fractionation of sodium caseinate hydrolysate*

Unhydrolysed sodium caseinate and large polypeptides (pH 4.6-insoluble material) were removed by isoelectric precipitation according to the method of Kuchroo and Fox (1982), as described in Section 2.2.11. The pH 4.6-soluble material was further fractionated by ultrafiltration (20°C, regenerated cellulose membrane, 1 or 3 kDa nominal molecular weight limit; Merck Millipore Ltd, Cork, Ireland). All samples were then lyophilised and stored in a dessicator at -20°C.

pH 4.6-soluble material (6.5% w/v) was redissolved in distilled water and allowed to hydrate overnight at 4°C. Following hydration, the solution was adjusted to pH 6.7 using 1 N NaOH and brought to 50°C in a waterbath. The solution was further fractionated by ultrafiltration. Briefly, the pH 4.6-soluble material was filtered through a 0.2 µm membrane (Vivaflow 200, Sartorius Stedim, Dublin, Ireland) fitted with a variable speed peristaltic pump (Masterflex LS economy drive, Fisher Scientific, Dublin, Ireland). The 0.2 µm retentate was collected while the permeate was filtered through a 10 kDa membrane (Vivaflow 200, Sartorius Stedim, Dublin, Ireland). The 10 kDa retentate was collected while the permeate was filtered

through a 5 kDa membrane (Vivaflow 200, Sartorius Stedim, Dublin, Ireland). The 5 kDa retentate was collected while the permeate was filtered through a 3 kDa membrane (Vivaflow 200, Sartorius Stedim, Dublin, Ireland). The 3 kDa retentate was collected while the permeate was filtered through a 1 kDa membrane (Tangenx Prostream, Novasep Process, France). Both the 1 kDa retentate and permeate were collected. A 5X diafiltration step was carried out using ultra pure water (Elga purelab option-Q; Veolia Water Solutions and Technologies, Marlow, United Kingdom). All samples were lyophilised and stored in a dessicator at -20°C.

6.2.4 One-dimensional gel electrophoresis

One dimensional urea-polyacrylamide gel electrophoresis (PAGE) was used to study proteolysis of the caseins. Electrophoresis in polyacrylamide gels (12.5% T, 4% C, pH 8.9) was performed using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) according to the method of Andrews (1983), as described in Section 5.2.7.

6.2.5 Two-dimensional gel electrophoresis (2-DE)

Two-DE was performed on the sodium caseinate samples hydrolysed for 0, 10, 40 and 120 min, as described in Section 3.2.7.

6.2.6 *Protein content*

Protein content was determined by the macro-Kjeldahl method (IDF, 1986), as described in Section 2.2.2. In some cases, the Bradford assay was used, as described in Section 3.2.5.

6.2.7 Reverse phase-ultra performance liquid chromatography (RP-UPLC)

Samples were adjusted to 2% TCA, as described in Section 5.2.4. RP-UPLC was performed on the 2% TCA-soluble fractions, as described in Section 3.2.8 with slight modifications. The sample was eluted for 0.37 min with 100% solvent A (0.1% trifluoroacetic acid acid [sequencing grade; Sigma-Aldrich Ireland Ltd, Arklow, Co Wicklow, Ireland] in deionised water [Milli Q System; Millipore Corp]), then with a linear gradient to 50% solvent B (0.1% trifluoroacetic acid in acetonitrile [HPLC far UV grade; Labscan Ltd, Dublin, Ireland]) over 6.23 min, maintained at 50% B for 0.46 min, then with a linear gradient to 95% B over 0.68 min, before returning to the original starting conditions.

6.2.8 Free amino acid analysis

Samples were deproteinised by making up to a final concentration of 12% trichloroacetic acid (TCA), i.e., equal volumes of sample and 24% TCA solution were mixed, and allowed to stand for 10 min before being centrifuged at 14,400 g (Microcentaur, MSE, UK) for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2, to give approximately 250 nmol of each amino acid residue. Samples were then diluted 1 in 2 with the internal standard, norleucine, to give a final concentration of 125 nmol/mL. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Welwyn Garden City, Herts, UK) fitted with a Jeol Na^+ high performance cation-exchange column.

6.2.9 Identification of peptides by Quadropole Time-of- flight Mass Spectrometry

Peptides were identified using a Waters Acquity G2 Q-TOF LC-MS, as described in Section 5.2.9.1.

6.2.10 Synthesis of peptides

Select peptides were synthesised using Microwave-assisted Solid Phase Peptide Synthesis (MW-SPPS) performed on a Liberty^{TM} CEM microwave peptide synthesiser. α_{S1}-Casein (f1-23) and (f3-23) were synthesised on an Fmoc-L-Phe Wang resin and β-casein (f190-209) and (f193-209) were synthesised on an Fmoc-L-Val Wang resin (Matrix Innovation, Quebec, Canada). Synthetic peptides were purified using RP-HPLC on a Vydac (10 u, 300A) column (Vydac, California, USA) and peptides eluted using a 20-40% acetonitrile 0.1% TFA gradient over 40 min. The flow rate was 2.5 mL/min and eluent monitored at 214 nm. Fractions containing the desired molecular mass were identified using MALDI TOF Mass Spectrometry (Shimadzu Biotech, Manchester UK) and were pooled and lyophilised on a Genevac HT 4X (Genevac Ltd. Ipswitch, UK) lyophiliser.

6.2.11 Preparation of samples for cell exposure assays

Lyophilised samples (0.5 g powder) were dissolved in 10 mL of sterile distilled water to give a 50 mg/mL stock solution, with stirring. The pH of the solution was adjusted to pH 7.4 using 1 M NaOH and the solution was then hydrated overnight at 4°C. Following hydration, the solution was centrifuged at 500 g for 5 min at room temperature and the supernatant was filter-sterilised using a 0.45 µm sterile filter (Sarstedt, Ireland). The stock solution was diluited in KREBS buffer (Sigma Aldrich, Ireland) containing 1% bovine serum albumin (BSA) (Sigma Aldrich, Ireland) to a 10 mg/mL working solution for cellular bioassays. All test samples were tested at 10 mg/mL, unless otherwise stated.

6.2.12 Cell culture

The STC-1 cell line (ATCC code: SD5482) was purchased from the American Tissue Culture Collection (LGC Standards, Teddington, UK). Cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 4.5 g/L glucose and L-

glutamine (Sigma Aldrich, Dublin, Ireland). The medium was supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were passaged upon reaching confluency, and all cells used in these studies were between passages 15-25.

6.2.13 Measurement of cellular viability

STC-1 cells were seeded into 96-well plates at a density of $2x10^4$ cells/mL and incubated overnight at 37°C in the presence of 5% CO_2. The medium was aspirated off and cells were washed with 50 µL KREBS/1% BSA per well. Cells were pre-incubated for 1 h in 100 µL of Krebs-Hanseleit buffer (KREBS)/1% bovine serum albumin (BSA). Following the pre-incubation step, 100 µL KREBS/1% BSA (as a control) or 100 µL test sample (10 mg/mL) was added to the appropriate wells. Then, 20 µL of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Medical Supply Company Ltd, Fingal, Dublin, Ireland) was added to each well and the plate was incubated for 4 h at 37°C in the presence of 5% CO_2. Absorbance was then measured at 490 nm using a Biotek Synergy HT microtitre plate-reader (Mason Technology, Dublin, Ireland). MTS is converted to formazon by dehydrogenase enzymes in metabolically active cells; therefore, the number of living cells in culture is directly proportional to the quantity of formazon product, as measured by absorbance at 490 nm.

6.2.14 Cell exposures to measure GLP-1 peptide and harvest total RNA

STC-1 cells were seeded into 6-well plates at a density of 1.5×10^6 cells/well and incubated at 37°C in the presence of 5% CO_2 for 18 h prior to test sample addition. The medium was aspirated off and the cell monolayers in each well were washed with 1 mL of KREBS/1% BSA. Cells were pre-incubated for 1 h in 500 µL of KREBS/1% BSA, which was then aspirated off and replaced with 1 mL of test sample at the concentration specified, and plates were incubated for 4 h at 37°C, in the presence of 5% CO_2. Test samples were analysed in duplicate on separate days. KREBS/1%

BSA added to cells alone acted as the basal control. Following the 4 h incubation period, 10 μL of 10 X Halt Protease and Phosphatase Inhibitor (Thermo Fisher scientific, Waltham, MA, USA) was added to each well to inactivate endogenous DPPIV activity. Cellular supernatants were collected by aspiration and stored at -80°C prior to GLP-1 analysis. Cell monolayers were washed with 1 mL per well of Hanks Balanced Salt Solution (HBSS) (Sigma Aldrich, Dublin, Ireland) and then lysed using Bioline Lysis Buffer (MyBio, Kilkenny, Ireland). Total RNA was extracted using the Bioline ISOLATE RNA Mini Kit (MyBio, Kilkenny, Ireland), according to the manufacturer's instructions.

Cellular supernatant levels of total GLP-1 were assayed using MSD® GLP-1 metabolic assay (Cat No: K150JVC-2, Meso Scale Discovery, Gaithersburg, MD, USA), according to the manufacturer's instructions. GLP-1 concentrations (pM) in the samples were quantified by interpolating the intensity of emitted light from standard curves generated in the same assays. Each sample well was assayed in duplicate and plates were read using a Sector Imager 2400 (Meso Scale Discovery, Gaithersburg, MD, USA).

6.2.15 *Data analysis*

Analysis of variance (one-way ANOVA) was conducted using Minitab version 16 (Minitab Inc., State College, PA, USA). When differences were significant ($P \leq 0.05$), the means were analysed using Tukey's test.

6.3 Results and Discussion

6.3.1 *Hydrolysis of individual caseins*

The current study focussed on the generation and proteomic characterisation of a hydrolysate of sodium caseinate produced by chymosin and its potential effect on satiety. Sodium caseinate was selected in order to avoid changes which occur when chymosin is added to milk, i.e., clotting. However, before hydrolysis of sodium caseinate was investigated, it was decided to examine hydrolysis of individual caseins by chymosin. The urea-PAGE electrophoretograms in Figure 6.1 shows α_{S1}-casein, adjusted to pH 5.6, hydrolysed by chymosin (180 IMCU per gram of α_{S1}-casein) at 37°C over a period of 60 min. In 0.1 M phosphate buffer, at pH 6.5, chymosin cleaves α_{S1}-casein at Phe(23)-Phe(24), Phe(28)-Pro(29), Leu(40)-Ser(41), Leu(149)-Phe(150), Phe(153)-Tyr(154), Leu(156)-Asp(157), Tyr(159)-Pro(160), Trp(164)-Tyr(165) (McSweeney *et al.*, 1993). Additionally, in 5% NaCl, at pH 5.2, chymosin also cleaves α_{S1}-casein at Leu(11)-Pro(12), Phe(32)-Gly(33), Leu(101)-Lys(102), Leu(142)-Ala(143) and Phe(179)-Ser(180). Figure 6.1 shows extensive hydrolysis of α_{S1}-casein, as indicated by the decreased intensity of the α_{S1}-casein band, which was virtually absent on the urea-PAGE electrophoretogram after a period of 60 min. The primary site of chymosin action on α_{S1}-casein is Phe(23)-Phe(24) (Carlos and Ribadeau-Dumas, 1985). Initially, α_{S1}-casein (f24-199) was the predominant peptide produced; however, this peptide was further hydrolysed and became less abundant with time. α_{S1}-Casein (f102-199) was also identified by urea-PAGE (Figure 6.1).

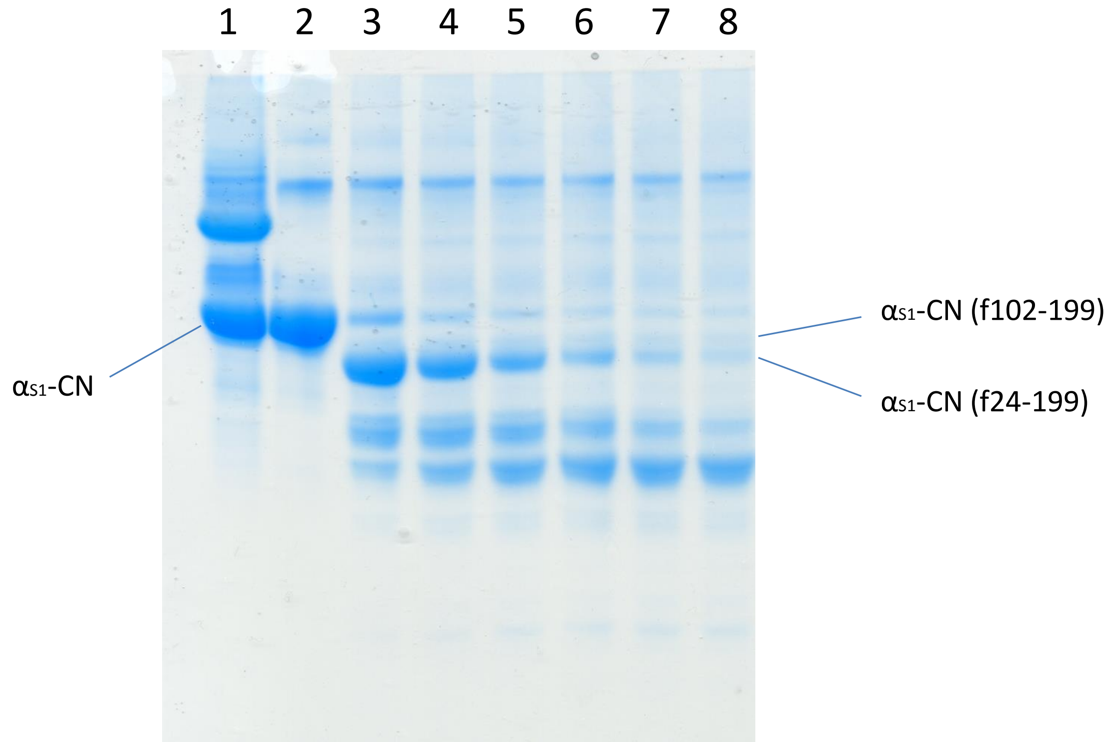

Figure 6.1 Urea-polyacrylamide gel electrophoretograms of sodium caseinate (lane 1) and bovine α_{S1}-CN (10 mg/mL), adjusted to pH 5.6 and hydrolysed by chymosin (180 IMCU per gram of α_{S1}-CN) at 37°C for 0, 10, 20, 30, 40, 50 and 60 min (lanes 2-8).

The urea-PAGE electrophoretograms in Figure 6.2 shows β-casein, adjusted to pH 5.6 and hydrolysed by chymosin (180 IMCU per gram of β-casein) at 37°C over a period of 60 min. A similar trend was seen when compared with the hydrolysis of α_{S1}-casein by chymosin (Figure 6.1), i.e., β-casein was extensively hydrolysed and virtually absent from the urea-PAGE electrophoretograms after a period of 60 min. In 0.05 M Na acetate buffer, pH 5.4, chymosin cleaves β-casein at Leu(192)-Tyr(193), Ala(189)-Phe(190), Leu(165)-Ser(166), Gln(167)-Ser(168), Leu(163)-Ser(164), Leu(139)-Leu(140) and Leu(127)-Thr(128) (Visser and Slangen, 1977). The predominant peptides produced in Figure 6.2 were β-casein (f1-189/192) and β-casein (f1-163/165), both of which increased in concentration as a function of time, as shown by an increase in band intensity.

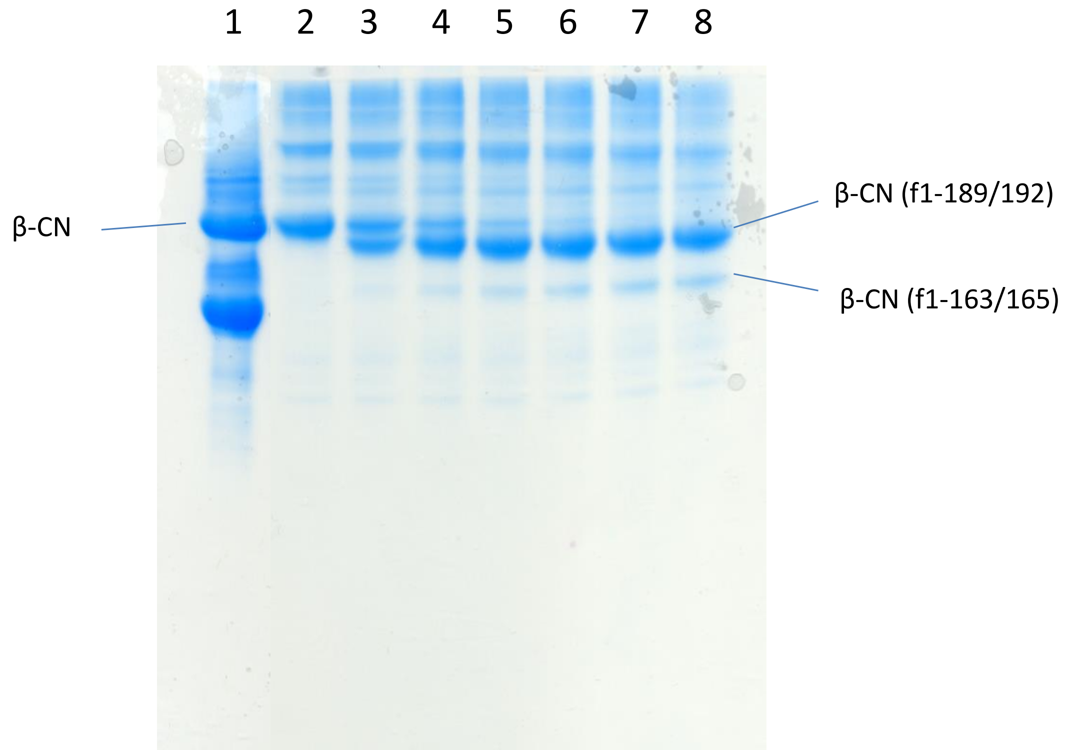

Figure 6.2 Urea-polyacrylamide gel electrophoretograms of sodium caseinate (lane 1) and bovine β-CN (10 mg/mL), adjusted to pH 5.6 and hydrolysed by chymosin (180 IMCU per gram of β-CN) at 37°C for 0, 10, 20, 30, 40, 50 and 60 min (lanes 2-8).

6.3.2 *Hydrolysis of sodium caseinate*

The urea-PAGE electrophoretograms in Figure 6.3 shows sodium caseinate (30 mg/mL), adjusted to pH 5.6 and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C over a period of 120 min. The pattern of hydrolysis is comparable with that shown in Figures 6.1 and 6.2, i.e., both α_{S1}- and β-casein were extensively hydrolysed; however, this was to a lesser extent than previously seen, as slightly more intact protein remained at the 60 min point in Figure 6.3. This was most likely due to a dilution in the ratio of chymosin:α_{S1}/β-casein within sodium caseinate compared to the individual caseins. For this reason, the hydrolysis time was increased from 60 to 120 min in an attempt to ensure that the hydrolysis reaction reached a terminal point. As before, β-casein (f1-189/192) and α_{S1}-casein (f24-199) were identified in the urea-PAGE electrophoretogram (Figure 6.3); however, the rate of production and degradation of α_{S1}-casein (f24-199) does not

match that shown in Figure 6.1, i.e., in Figure 6.1, α_{S1}-casein (f24-199) was the predominant peptide at the 10 min point and subsequently degraded until 60 min, at which point it was virtually absent whereas, in Figure 6.3, the intensity of α_{S1}-casein (f24-199) increased with time, showing no signs of subsequent breakdown. This may have been due to differences in the ionic strength of the sodium caseinate and α_{S1}-casein solutions, which has been shown to influence the hydrolysis of α_{S1}-casein by chymosin (Mulvhill and Fox, 1979). A negative control was prepared under the same conditions used to generate the hydrolysates with the exception that chymosin was heat-inactivated before use (80°C for 10 min); no hydrolysis was seen in the negative control sample, indicating that the enzyme was sufficiently denatured (results not shown).

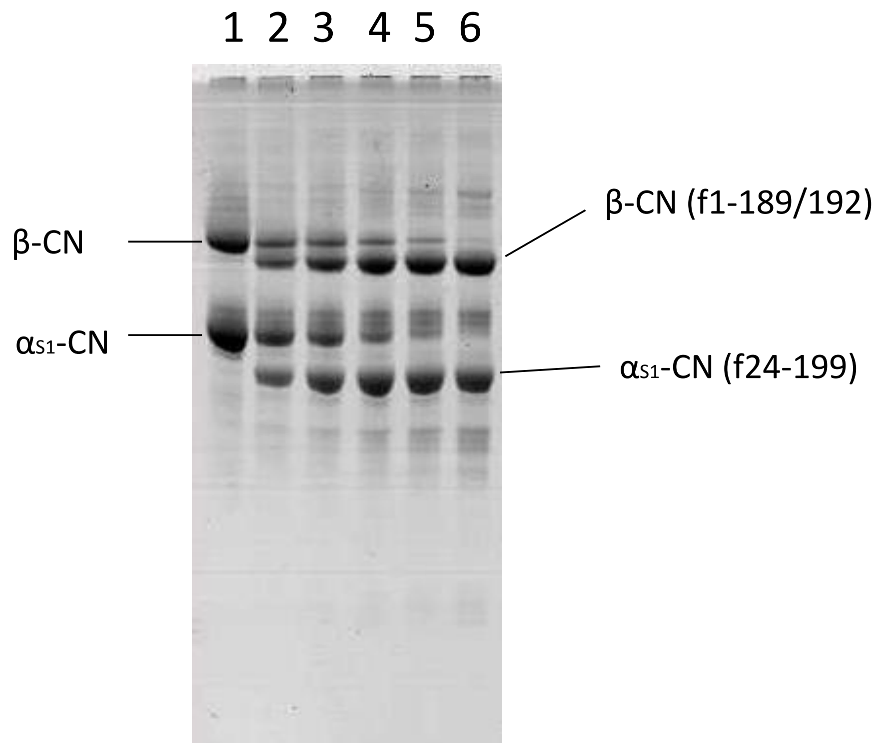

Figure 6.3 Urea-polyacrylamide gel electrophoretograms of sodium caseinate (30 mg/mL), adjusted to pH 5.6 and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 0, 10, 20, 40, 60 and 120 min (lanes 1-6).

The two-dimensional gel electrophoretograms in Figure 6.4 show sodium caseinate (30 mg/mL), adjusted to pH 5.6 and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for (a) 0, (b) 10, (c) 40 and (d) 120 min. Varying degrees of hydrolysis were clearly evident, as shown by changes in the proportion of intact protein and peptide material across the 2D gels. Unhydrolysed sodium caseinate contained mostly intact protein, with small amounts of peptide material, which may have been produced during manufacture. Subsequent 2D gels clearly indicate extensive hydrolysis of α_{S1} and β-casein, and to a lesser extent κ-casein, as shown by decreased band intensity and increased abundance of low molecular weight peptide material in the range 10-20 kDa.

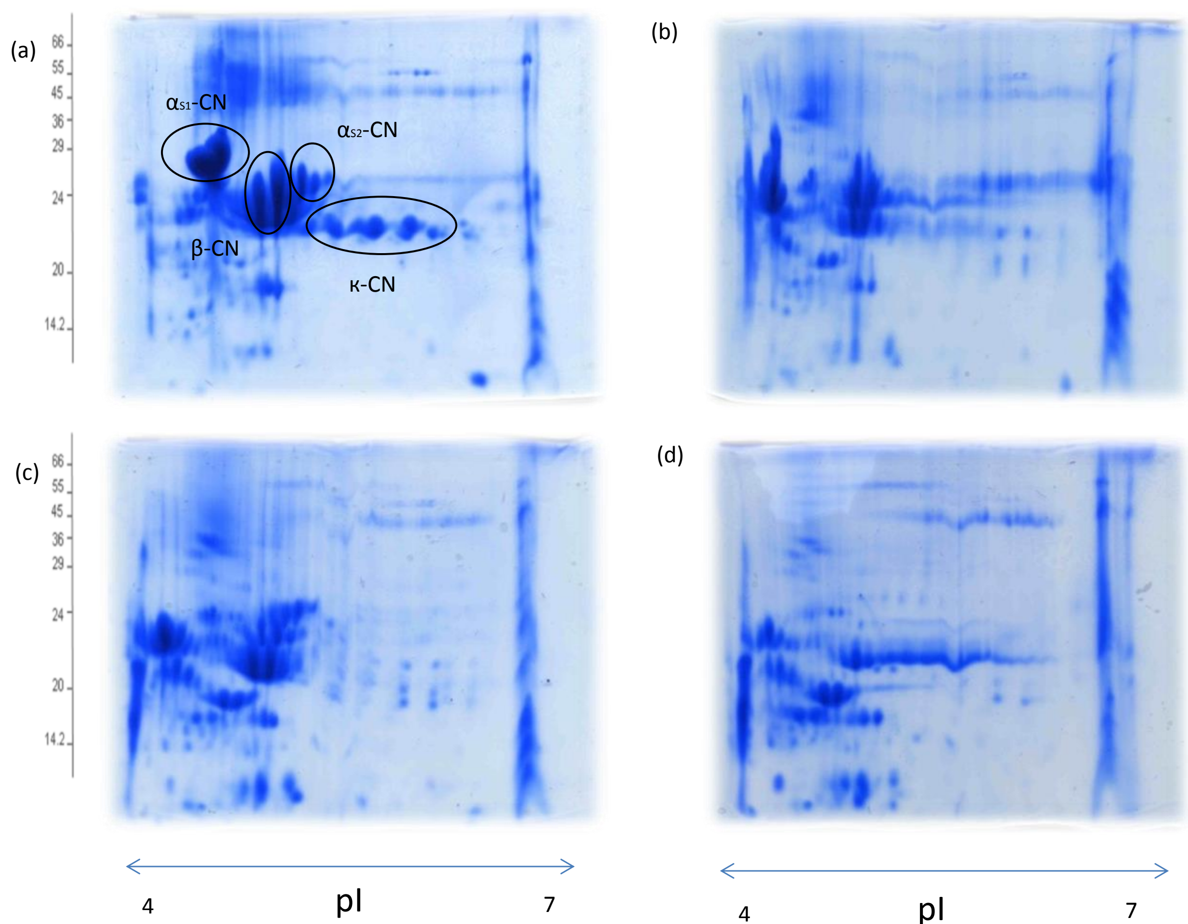

Figure 6.4 Two-dimensional gel electrophoretograms of sodium caseinate (30 mg/mL), adjusted to pH 5.6, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for (a) 0, (b) 10, (c) 40 and (d) 120 min, separated under reducing conditions using a 7 cm pH 4-7 pI range for the first dimension and a 12% gradient acrylamide SDS-PAGE gel for the second dimension.

The RP-UPLC chromatograms in Figure 6.5 show sodium caseinate (30 mg/mL), adjusted to pH 5.6, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) for (a) 0, (b) 10, (c) 40 and (d) 120 min. The RP-UPLC chromatogram of unhydrolysed sodium caseinate was dominated by two large co-eluting peaks, probably representing intact casein, while very little peptide material was found. Subsequent RP-UPLC chromatograms clearly indicated extensive hydrolysis, as shown by a decrease in peak area of intact casein (6.5 to 7.5 min) and increase in the abundance of peptides (4 to 6.5 min). As expected, sodium caseinate hydrolysed by chymosin for 120 min was the most extensively hydrolysed sample, as shown by the greatest abundance of peptide peaks, followed by the 40 min and 10 min samples.

6.3.2.1 Effect of sodium caseinate hydrolysates on GLP-1 secretion

Glucagon-like peptide (GLP-1) is a gut-derived incretin hormone and has multiple physiological effects (Aaboe *et al.,* 2008). In animal studies, GLP-1 stimulates β-cell proliferation and neogenesis (Zhou *et al.,* 1999) and inhibits β-cell apoptosis (Farilla *et al.,* 2003). In humans, GLP-1 enhances satiety and reduces food intake (Turton *et al.*, 1996), stimulates insulin secretion and inhibits glucagon (Orskov *et al.,* 1988) and gastrointestinal secretions and motility (Wettergren *et al.,* 1993; Nauck *et al.,* 1997). Therefore, identification of a sodium caseinate hydrolysate which increases the secretion of GLP-1 would have great potential as a functional food ingredient for weight management. In the current study, the effect of exposure of STC-1 cells to sodium caseinate (30 mg/mL), adjusted to pH 5.6, hydrolysed by chymosin (180 IMCU per gram sodium caseinate) at 37°C for 0, 10, 40 and 120 min, on GLP-1 peptide levels was investigated (Figure 6.6). Samples were tested at 1, 5 and 10 mg/mL. Sodium caseinate hydrolysates had no effect on STC-1 cell viability over 4 hours compared to cells exposed to KREBS buffer only (results not shown). Levels of GLP-1 secreted from STC-1 cells following a 4 hour exposure to hydrolysed sodium caseinate samples were higher compared to levels in response to the unhydrolysed sodium caseinate control. With the exception of the 40 min time point, GLP-1 was not secreted in a dose-dependent manner. At concentrations of 5 and 10 mg/mL, the 40 min time point resulted in the greatest secretion of GLP-1.

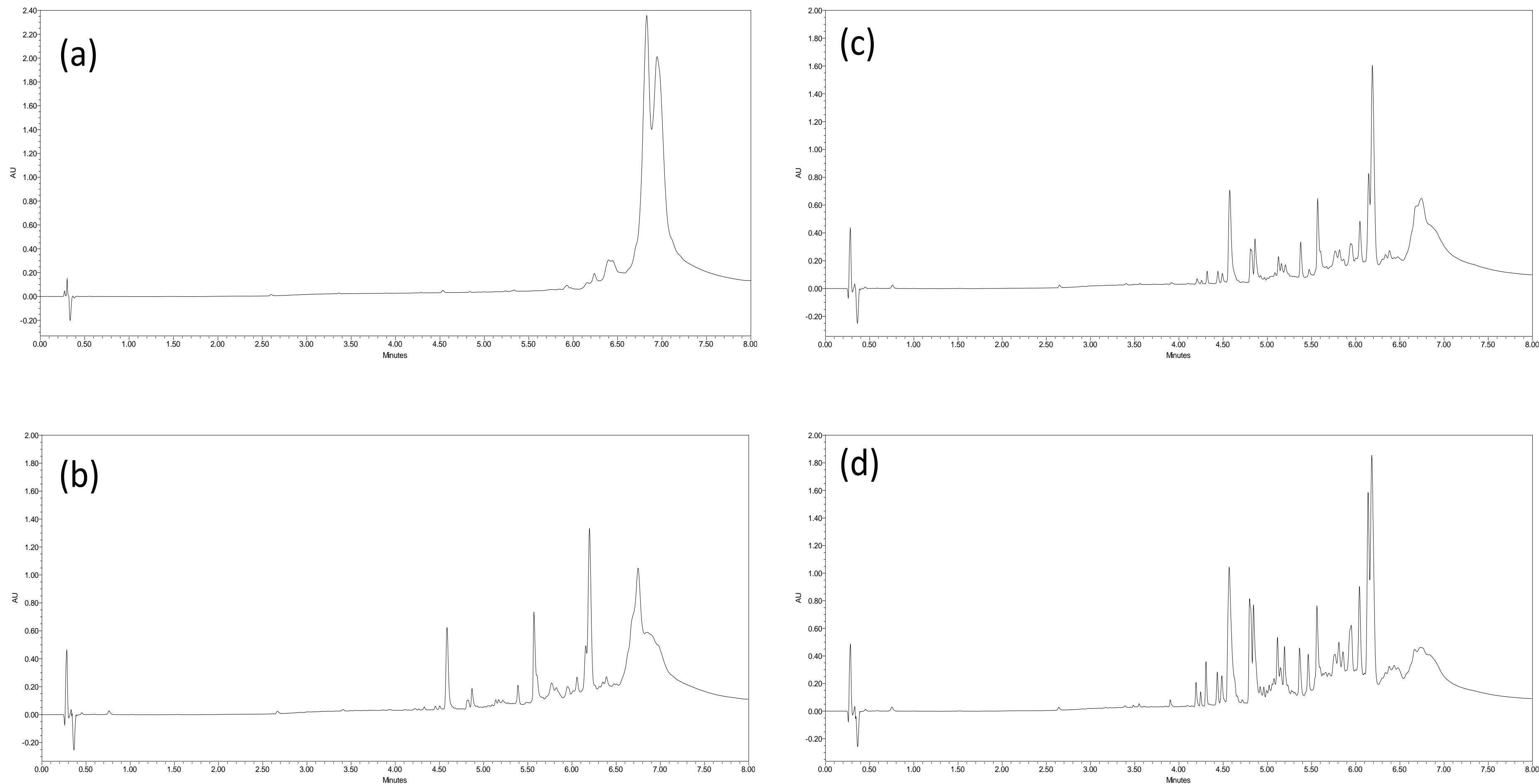

Figure 6.5 Reversed-phase UPLC profiles of (a) unhydrolysed sodium caseinate and sodium caseinate (30 mg/mL), adjusted to pH 5.6 and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for (b) 10, (c) 40 and (d) 120 min.

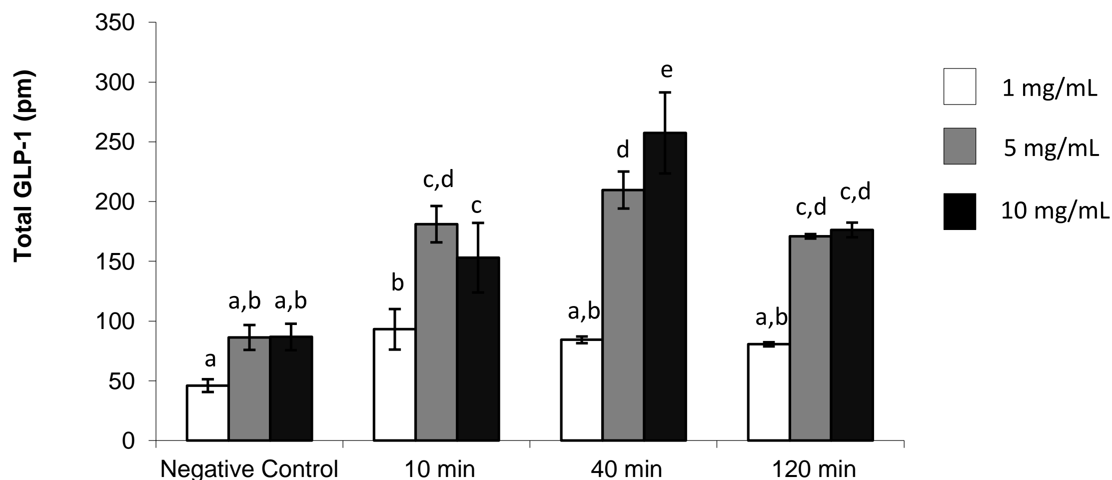

Figure 6.6 The effect of exposure of STC-1 cells to sodium caseinate (30 mg/mL), adjusted to pH 5.6, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 0, 10, 40 and 120 min, on GLP-1 peptide levels at 1, 5 and 10 mg/mL (lyophilised powder), as measured by meso scale discovery (MSD). Values with different superscript letters were significantly different ($p < 0.05$).

6.3.3 Optimisation of hydrolysis conditions

The hydrolysis protocol described thus far was then modified to make it more suitable for large-scale commercial production. The total solids level of dissolved sodium caseinate was increased from 3 to 10%. While the enzyme:substrate ratio remained the same, the amount of enzyme added was increased accordingly. Also, in an attempt to minimise the ash content of the hydrolysate, in particular the low molecular weight fractions produced by ultrafiltration, the chemical used for pH adjustment was changed from hydrochloric acid to glucono-delta lactone (GDL). In solution, GDL is progressively hydrolysed to gluconic acid, which causes a reduction in pH. The amount of GDL required to decrease the pH of a 10% sodium caseinate solution to pH 5.6 was shown experimentally to be 0.7%.

The urea-PAGE electrophoretograms in Figure 6.7 show sodium caseinate (100 mg/mL), adjusted to pH 5.6 (0.7% GDL), hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) over a period of 120 min. The aim here was to optimise hydrolysis conditions while maintaining the same degree of protein breakdown and peptide formation. The pattern of hydrolysis is comparable with that seen in

Figures 6.1, 6.2 and 6.3, i.e., α_{S1}- and β-casein were progressively hydrolysed over 120 min, at which point virtually no intact α_{S1}- or β-casein remained. The predominant peptides produced were β-casein (f1-189/192 and 1-163/165) and α_{S1}-casein (f102-199 and 24-199). Increased sample loading in the urea-PAGE electrophoretograms in Figure 6.7 compared with Figure 6.3 led to better resolution of electrophoretic bands in the lower half the electrophoretogram. Approximately 8 additional bands were observed; however, these bands were not identified. As before, a negative control was prepared under the same conditions used to generate the hydrolysates, with the exception that chymosin was heat-inactivated before use; no hydrolysis was seen in the negative control sample, indicating again that the enzyme was sufficiently denatured (results not shown).

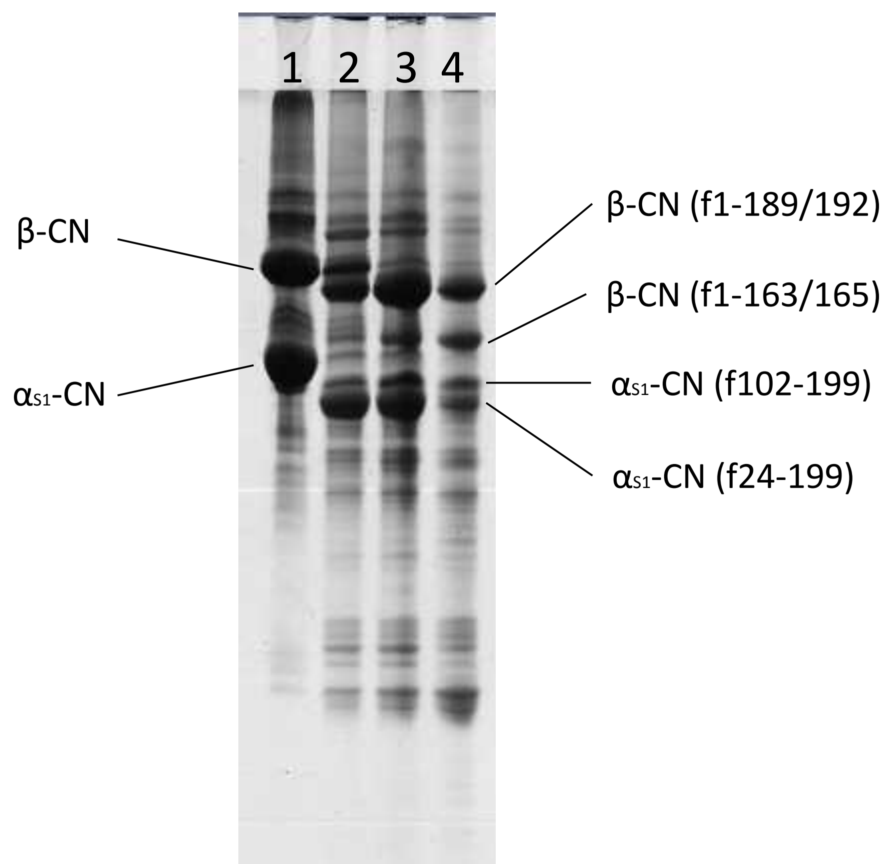

Figure 6.7 Urea-polyacrylamide gel electrophoretograms of sodium caseinate (100 mg/mL), adjusted to pH 5.6 (0.7% GDL) and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 0, 10, 40 and 120 min (lanes 1-4).

6.3.3.1 Effect of optimised sodium caseinate hydrolysates on GLP-1 secretion

Sodium caseinate hydrolysates produced under thus optimised conditions, i.e., sodium caseinate (100 mg/mL), adjusted to pH 5.6 (0.7% GDL) and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 0, 10, 40 and 120 min, were added to STC-1 cells at 1, 5 and 10 mg/mL to measure their effect on GLP-1 secretion (Figure 6.8). In addition to maintaining the same degree of protein breakdown and peptide formation during optimisation of hydrolysis conditions, it was vital to ensure the effect of these hydrolysates on GLP-1 secretion was reproducible. In general, results were similar to those shown in Figure 6.6, i.e., levels of GLP-1 secreted from STC-1 cells following a four-hour exposure to hydrolysed sodium caseinate samples were higher compared to levels in response to the unhydrolysed sodium caseinate sample, except at a concentration of 1 mg/mL. The effect of the 10, 40 and 120 min time points on GLP-1 levels were dose-dependent. As with the original hydrolysis conditions, the 40 min time point caused the greatest secretion of GLP-1.

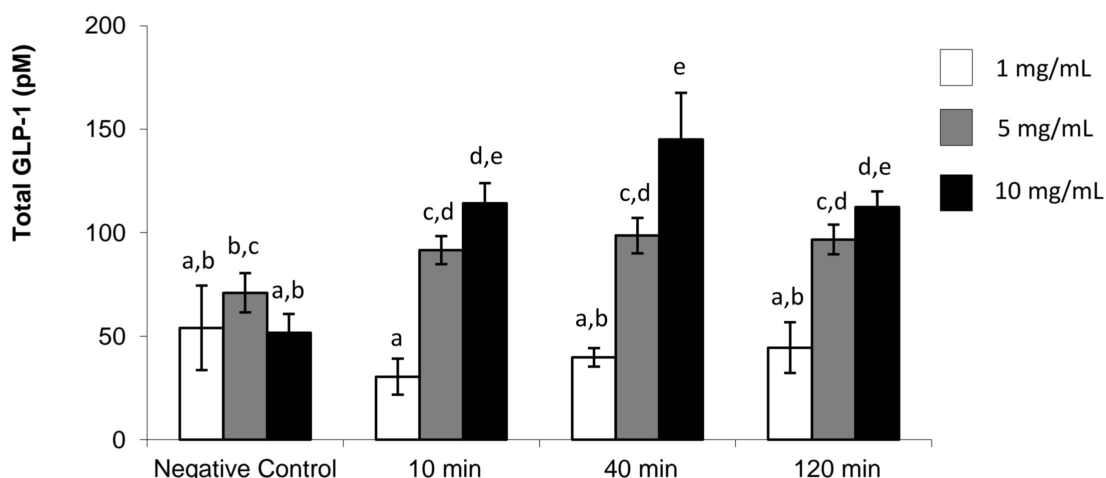

Figure 6.8 The effect of exposure of STC-1 cells to sodium caseinate (100 mg/mL), adjusted to pH 5.6 (0.7% GDL) and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 0, 10, 40 and 120 min, on GLP-1 peptide levels at 1, 5 and 10 mg/mL (lyophilised powder), as measured by MSD. Values with different superscript letters were significantly different ($p < 0.05$).

Based on GLP-1 secretion data, as shown in Figures 6.6 and 6.8, it was concluded that the hydrolysate produced in 40 min was the most potent activator of GLP-1 secretion from STC-1 cells. Therefore, it was decided to proceed with this sample with the aim of identifying the bioactive component. The effect of the 40 min time point on GLP-1 secretion was shown to be dose-dependent, i.e., incubation of STC-1 cells with increasing concentrations (0.1 to 20 mg/mL) of the sample correlated with an increase in GLP-1 secretion (Figure 6.9). The 40 min time point produced under both the original and optimised hydrolysis conditions were tested in parallel to compare the GLP-1 response of each sample. No differences in GLP-1 secretion were observed between samples, and so, the optimised hydrolysis conditions were favoured and used subsequently.

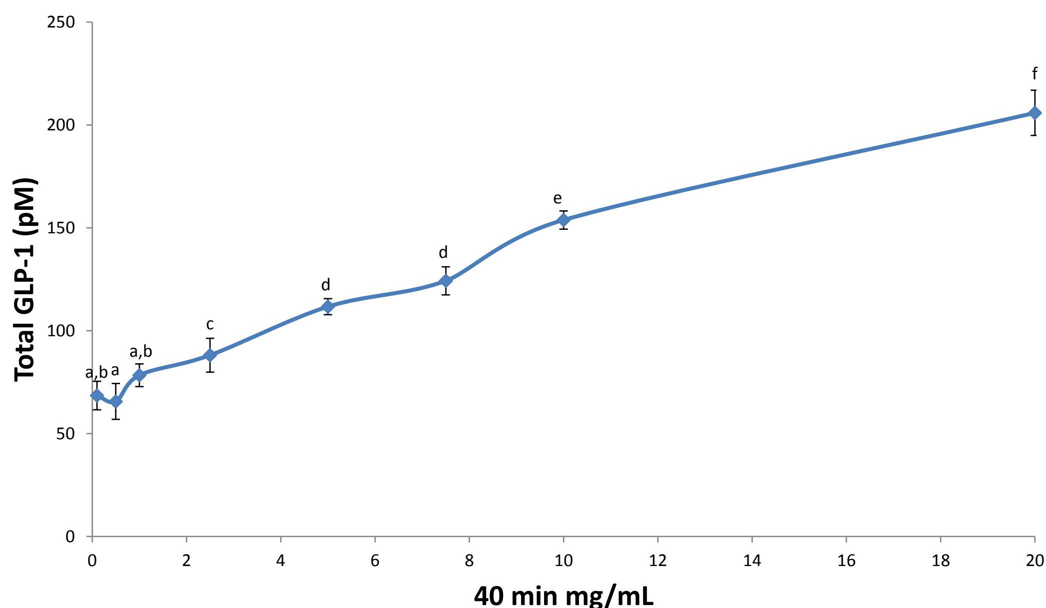

Figure 6.9 Dose-response effect of sodium caseinate (100 mg/mL), adjusted to pH 5.6 and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min at concentrations of 0.1, 0.5, 1, 2.5, 5, 7.5, 10 and 20 mg/mL on GLP-1 peptide levels as measured by MSD. Values with different superscript letters were significantly different ($p < 0.05$).

6.3.4 *Fractionation of crude hydrolysate*

In an attempt to isolate/enrich the bioactive component, the hydrolysate produced in 40 min was adjusted to pH 4.6 (pI of the caseins) to remove any intact casein and large polypeptides. The soluble fraction was further fractionated by ultrafiltration, as described in Section 6.2.3, to produce a 3 kDa retentate and permeate. These fractions are shown in the urea-PAGE electrophoretograms in Figure 6.10. The protein profile of the negative control was identical to that of the sodium caseinate standard, confirming that no hydrolysis had taken place. The unfractionated 40 min time point has already been discussed in detail. It was clear from Figure 6.10 that the predominant peptides in the hydrolysed sodium caseinate were insoluble at pH 4.6. The pH 4.6-soluble fraction and subsequent 3 kDa retentate and permeate contained trace levels of β-casein (f1-189/192 and 1-163/165) and α_{S1}-casein (f102-199 and 24-199), in addition to some peptide material in the lower half of the electrophoretogram.

The RP-UPLC chromatograms in Figure 6.11 show sodium caseinate (100 mg/mL), adjusted to pH 5.6 (0.7% GDL), hydrolysed by chymosin (180 IMCU per gram of sodium caseinate), at 37°C for 40 min, and fractions thereof. The RP-UPLC profile of the unfractionated 40 min time point produced under optimised conditions (Figure 6.11a) differed slightly to that produced under the original hydrolysis conditions (Figure 6.5b), i.e., the optimised hydrolysate contained a greater proportion of intact casein relative to peptide material than the original sample; however, the peptide profile was similar. The pH 4.6-insoluble fraction contained a large amount of intact casein; however, quite a lot of peptide material also remained (Figure 6.11b). This is in agreement with urea-PAGE analysis (Figure 6.10) where the majority of peptide material was present in the pH 4.6-insoluble fraction. The RP-UPLC profile of the pH 4.6-soluble fraction showed depletion of intact casein and enrichment in peptide material. The RP-UPLC profile of the pH 4.6-soluble 3 kDa retentate (Figure 6.11d) was virtually identical to that of the pH 4.6-soluble fraction

(Figure 6.11c), while the 3 kDa permeate was enriched in certain peptides (Figure 3.11e).

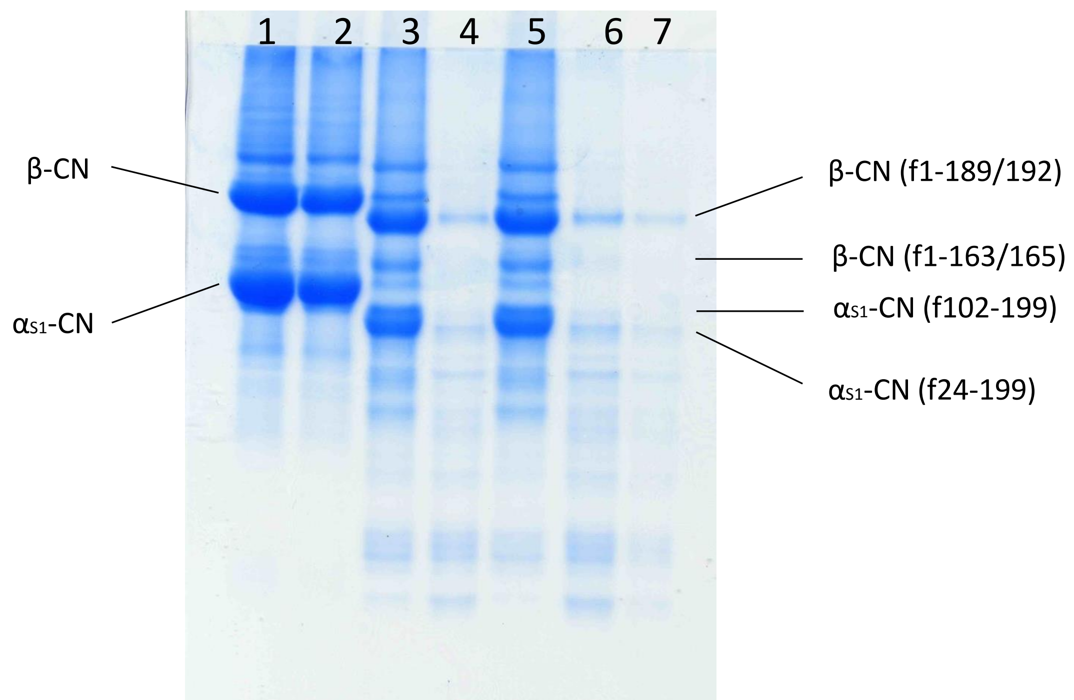

Figure 6.10 Urea-polyacrylamide gel electrophoretograms of sodium caseinate (100 mg/mL) (lane 1), adjusted to pH 5.6 (0.7% GDL), hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 0 and 40 min (lanes 2 and 3) and fractions thereof; pH 4.6-soluble fraction (lane 4), pH 4.6-insoluble fraction (lane 5), pH 4.6-soluble 3 kDa retentate (lane 6) and pH 4.6-soluble 3 kDa permeate (lane 7).

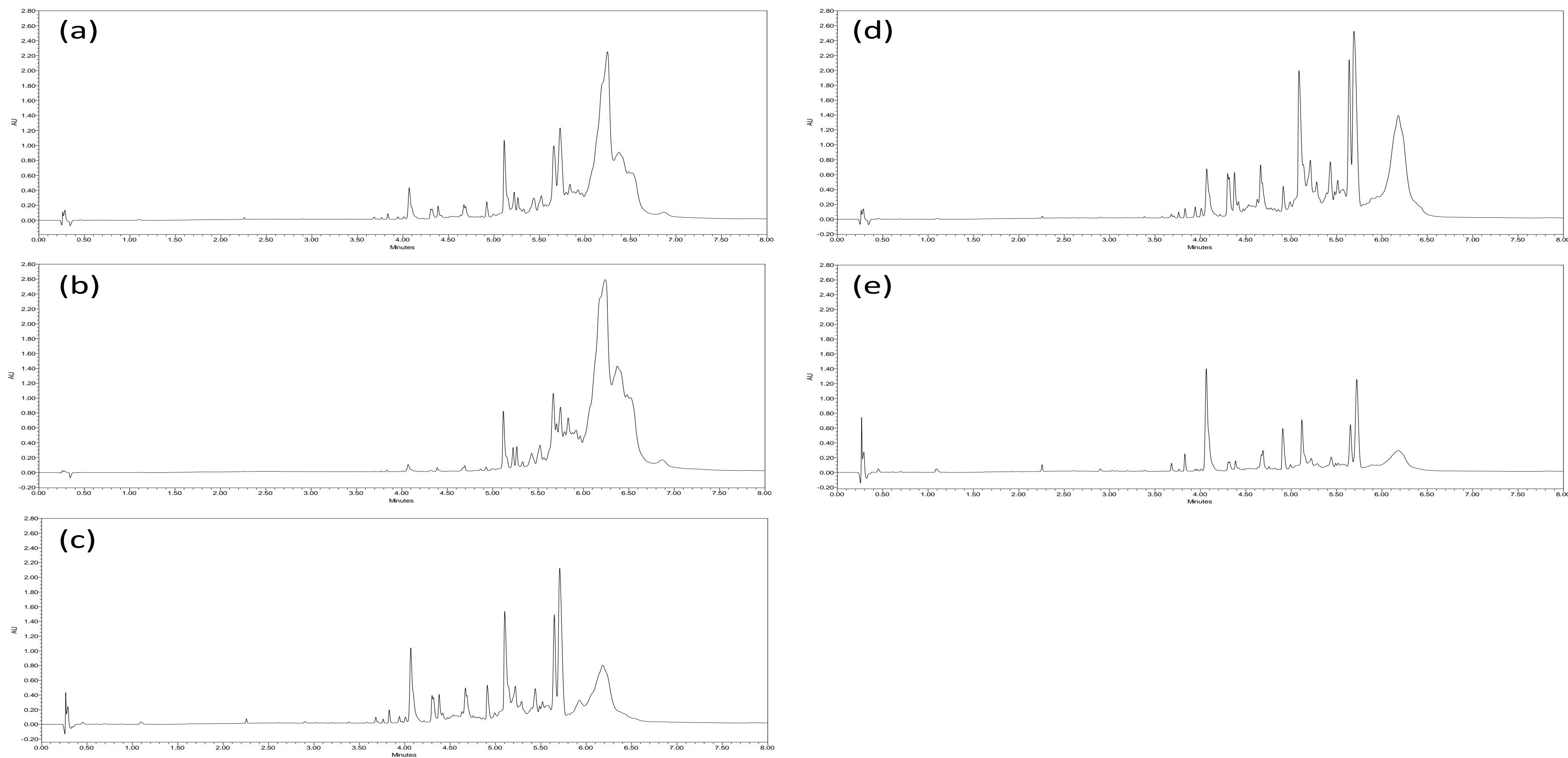

Figure 6.11 Reversed-phase UPLC profile of sodium caseinate (100 mg/ml), adjusted to pH 5.6 (0.7% GDL) and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min (a), and fractions thereof; (b) pH 4.6-insoluble, (c) pH 4.6-soluble, (d) pH 4.6-soluble 3 kDa retentate and (e) pH 4.6-soluble 3 kDa permeate.

6.3.4.1 Effect of a sodium caseinate hydrolysate, and fractions thereof, on GLP-1 secretion

Sodium caseinate (100 mg/mL), adjusted to pH 5.6 (0.7% GDL), hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) for 40 min, and fractions thereof, were added to STC-1 cells to measure their effect on GLP-1 secretion (Figure 6.12). As previously shown (Figures 6.6 and 6.8), levels of GLP-1 secreted from STC-1 cells following a 4 hour exposure to hydrolysed sodium caseinate were higher compared to levels in response to the unhydrolysed sodium caseinate sample. After fractionation of the hydrolysate at pH 4.6, there was decreased secretion of GLP-1 in the response to the insoluble fraction and increased secretion of GLP-1 in response to the soluble fraction. This suggests that the bioactivity lies within the pH 4.6-soluble fraction and is not due to the abundant peptides identified in the pH 4.6-insoluble fraction by urea-PAGE (Figure 6.10), e.g., β-casein (f1-189/192 and 1-163/165) and α_{S1}-casein (f102-199 and 24-199). Subsequent ultrafiltration of the pH 4.6-soluble fraction through a 3 kDa membrane resulted in decreased secretion of GLP-1 in response to the retentate and increased secretion of GLP-1 in response to the permeate. Based on these findings, it was proposed that bioactivity lies within the 3 kDa permeate of the pH 4.6-soluble fraction.

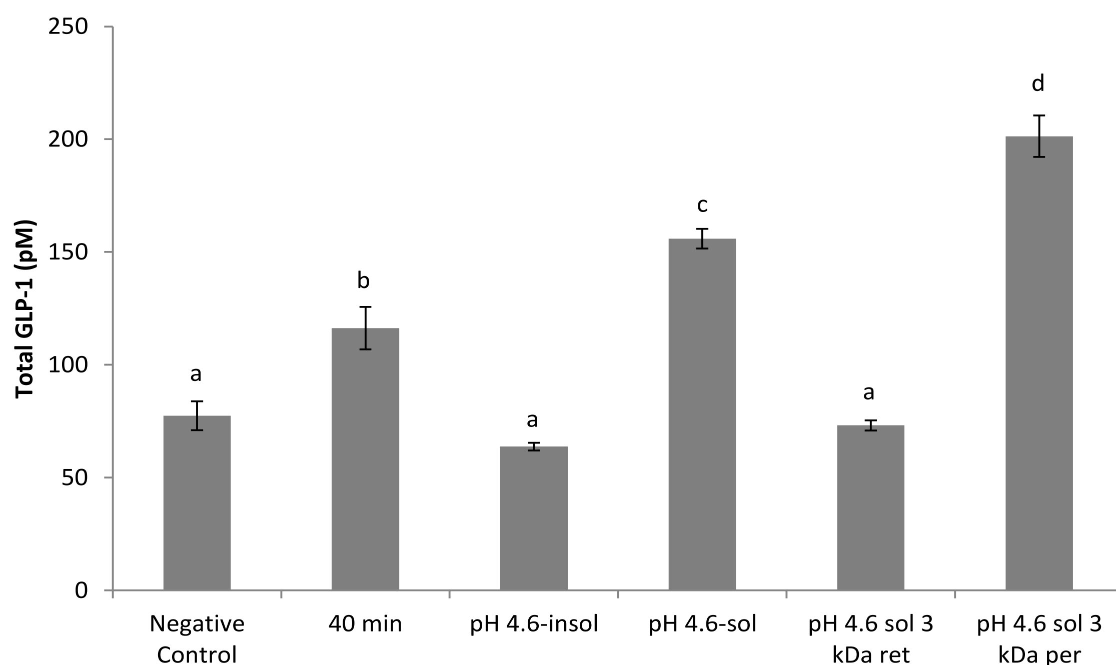

Figure 6.12 The effect of exposure of STC-1 cells to sodium caseinate (100 mg/mL) adjusted to pH 5.6 (0.7% GDL) and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min, and fractions thereof, on GLP-1 peptide levels as measured by MSD. Values with different superscript letters were significantly different ($p < 0.05$).

6.3.5 *Further optimisation of hydrolysis conditions*

6.3.5.1 pH adjustment

The optimised hydrolysis protocol described earlier was further modified in an attempt to reduce/eliminate the need for pH adjustment. Sodium caseinate (100 mg/mL) was adjusted to pH 5.6, 6 or 7, and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) for 40 min. Adjustment of pH was carried out using HCl/NaOH instead of GDL, as substantially less HCl was required to adjust the sodium caseinate solution to pH 6 and a very small quantity of NaOH was required to adjust the solution to pH 7 (sodium caseinate naturally has a pH of approximately 6.7). These hydrolysates are shown in the urea-PAGE electrophoretograms in Figure 6.13. It should be noted that hydrolysis was not maintained at the adjusted pH values, i.e., pH 5.6, 6 and 7. This was because of the relatively large quantity of enzyme preparation used, which was acidic, and so, on addition to the sodium caseinate solution, caused a change in pH, i.e., the pH of

sodium caseinate solutions adjusted to pH 5.6, 6 and 7, prior to hydrolysis, dropped to pH 5.3, 5.6 and 6.2, respectively, after addition of the enzyme. When sodium caseinate was adjusted to pH 5.6 before hydrolysis, the hydrolysis profile was similar to that shown in Figures 6.7 and 6.10. When adjusted to pH 6, the hydrolysis reaction was less extensive, as evident by more residual intact β-casein, decreased production of β-casein (f1-189/192 and 1-163/165) and decreased hydrolysis of α_{S1}-casein (f24-199), which subsequently led to decreased production of α_{S1}-casein (f102-199). Reduced levels of hydrolysis were even more pronounced when sodium caseinate was adjusted to pH 7 before addition of the enzyme. Chymosin has an acidic pH optimum; Fox (1969) reported pH 5.8 as the optimum for proteolysis of whole casein by chymosin. Adjustment of the sodium caseinate solution to pH 6 and pH 7, thus decreased the level of hydrolysis, as the pH was being moved away from its optimum.

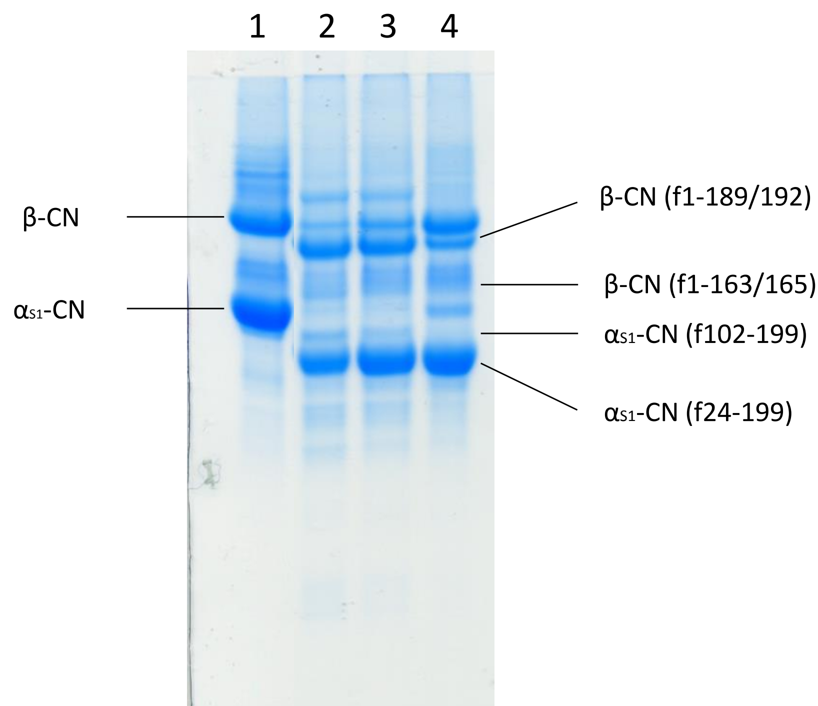

Figure 6.13 Urea-polyacrylamide gel electrophoretograms of unhydrolysed sodium caseinate (lane 1) and sodium caseinate (100 mg/mL), adjusted to pH 5.6 (lane 2), pH 6 (lane 3) or pH 7 (lane 4) and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min.

The RP-UPLC chromatograms in Figure 6.14 show sodium caseinate (100 mg/mL) adjusted to (a) pH 5.6, (b) 6 or (c) 7, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min. The results shown here are in agreement with those of Figure 6.13, i.e., moving away from the pH optimum of chymosin resulted in reduced hydrolysis of the caseins. As the pH was increased, more residual intact casein and less peptide material were observed.

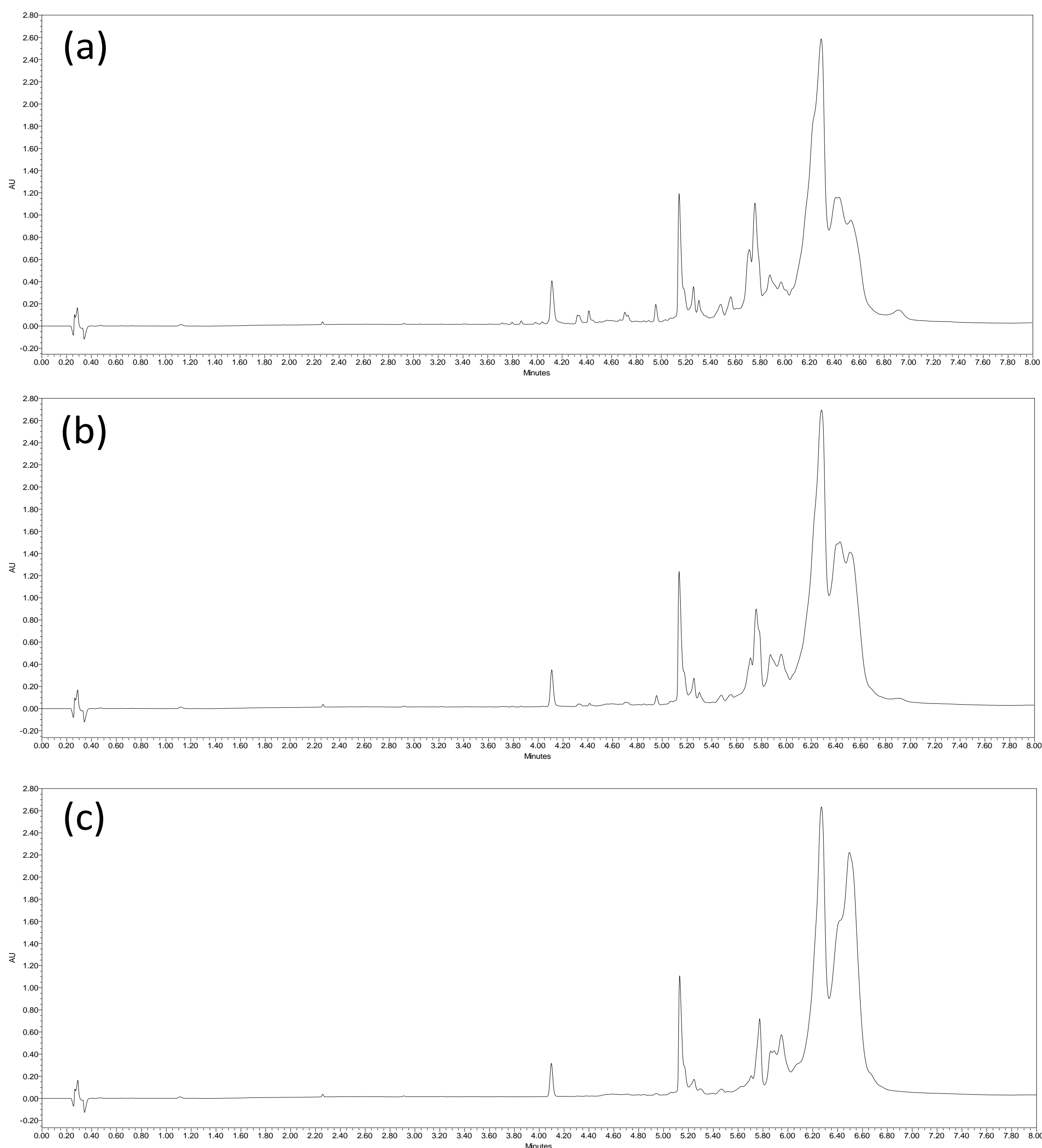

Figure 6.14 Reversed-phase UPLC profiles of sodium caseinate (100 mg/mL), adjusted to (a) pH 5.6, (b) pH 6 and (c) pH 7, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min.

Sodium caseinate (100 mg/mL) adjusted to pH 5.6, 6 or 7, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) for 40 min, were added to STC-1 cells to measure their effect on GLP-1 secretion (Figure 6.15). There were no significant differences in GLP-1 secretion in response to varying the pH of the sodium caseinate solution before hydrolysis. Based on these results, it was decided to proceed with the hydrolysis conditions which required the least amount of pH adjustment before hydrolysis, i.e., pH 7.

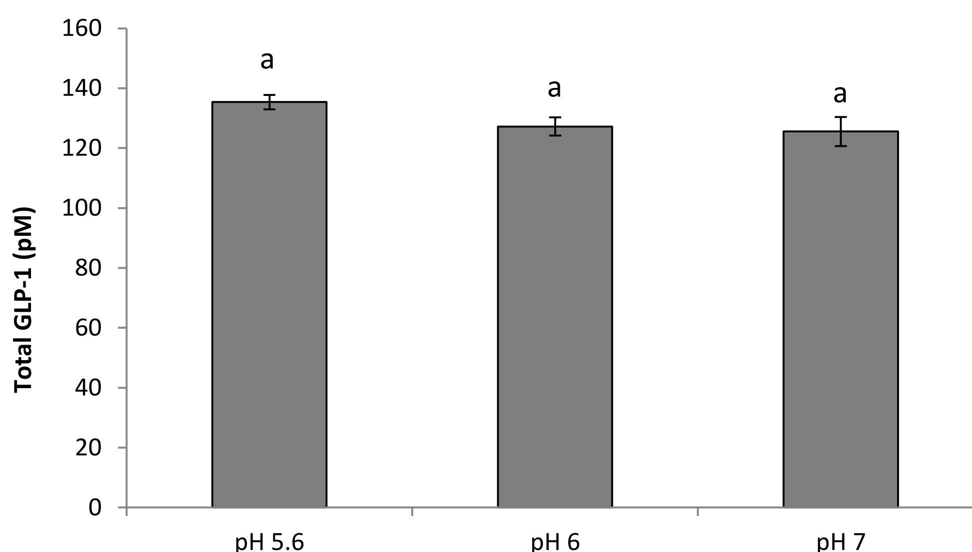

Figure 6.15 The effect of exposure of STC-1 cells to sodium caseinate (100 mg/mL) hydrolysed at 37°C by chymosin (180 IMCU per gram of sodium caseinate) at pH 5.6, 6 and 7, as described in the text, for 40 min, on GLP-1 peptide levels as measured by MSD. Values with different superscript letters were significantly different ($p < 0.05$).

The urea-PAGE electrophoretograms in Figure 6.16 show sodium caseinate (100 mg/mL), adjusted to pH 7 and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C over a period of 120 min. This experiment was carried out in order to investigate the breakdown of the caseins under the new hydrolysis conditions. Both α_{S1}- and β-casein were progressively hydrolysed, but to varying degrees. Even after 120 min, a substantial amount of β-casein was still present, while α_{S1}-casein was virtually absent. The abundance of β-casein (f1-189/192 and 1-

163/165) and α_{S1}-casein (f24-199 and 102-199) all increased with time. Hydrolysis of α_{S1}- and β-casein was comparable to that shown in Figures 6.1, 6.2 and 6.3, but to a lesser extent, because, as already mentioned, at pH 7, chymosin was moving away from its pH optimum. The purity of the enzyme preparation was also investigated, as shown in Figure 6.16 (lane 14), i.e., the protein profile of Maxiren 180 was dominated by the presence of a single electrophoretic band.

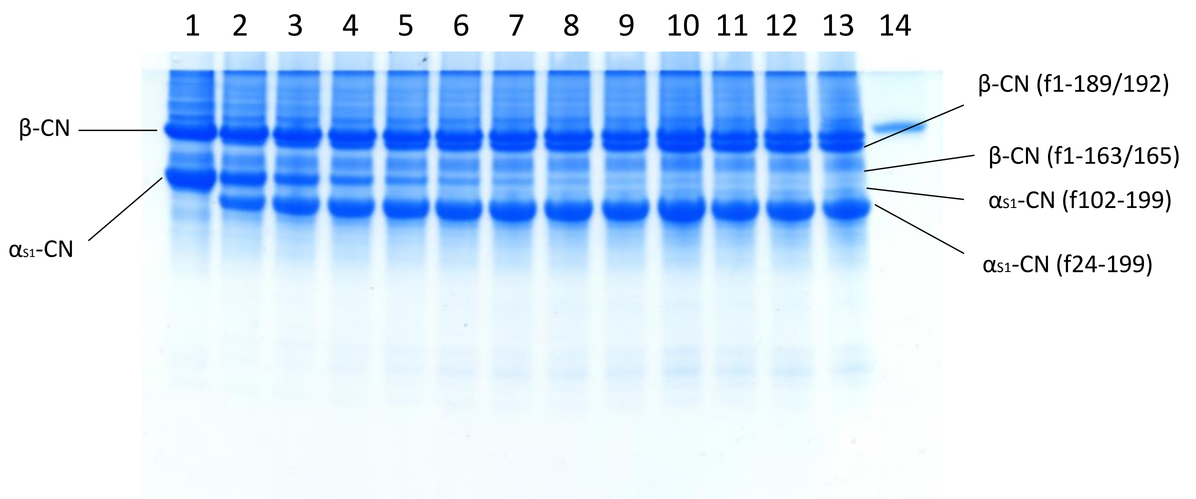

Figure 6.16 Urea-polyacrylamide gel electrophoretograms of sodium caeseinate (100 mg/mL), adjusted to pH 7, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min (lanes 1-13 respectively) and the enzyme preparation (lane 14).

6.3.5.2 In vivo *mice study*

Based on the *in vitro* effects of the sodium caseinate hydrolysates described thus far, it was decided to proceed with an *in vivo* study measuring cumulative feed intake with 20 male mice of the common inbred strain, C57BL/6 (Figure 6.17). Sodium caseinate (100 mg/mL), adjusted to pH 7, and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min, was injected intraperitoneally into ten mice at a dosage of 750 mg/kg. In parallel, 10 mice were injected intraperitoneally with Hank's Balanced Salt Solution (HBSS) which acted as a vehicle control. The mice which were given the hydrolysed sodium caseinate

demonstrated significantly lower feed intake when compared with control mice at all time points from 1 to 8 hours post-injection. No significant differences in cumulative feed intake (30 min post-injection) were observed between mice which were given the hydrolysed sodium caseinate and control.

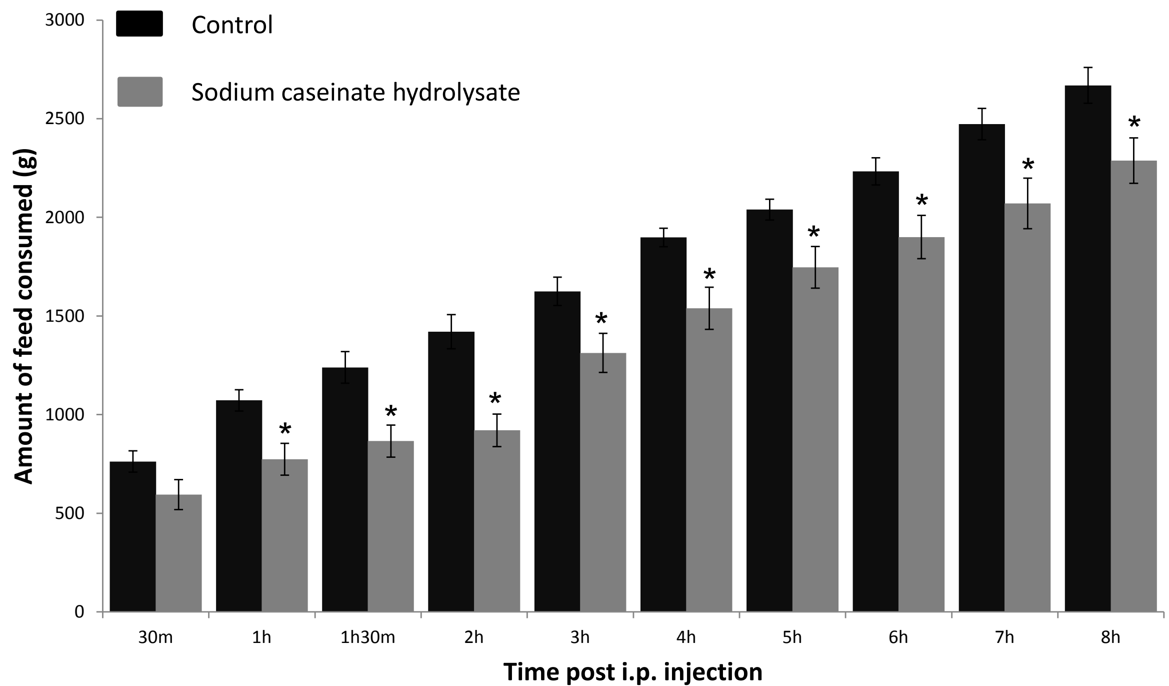

Figure 6.17 Effects of intra peritoneal (i.p.) administration of sodium caseinate (100 mg/mL), adjusted to pH 7, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min, on cumulative feed intake in male C57/BL6 mice over 8 hours. Each value represents mean ± SEM (n=20). Values with a superscript symbol (*) were significantly different ($p < 0.05$) from corresponding control groups.

6.3.5.3 Enzyme:substrate ratio

In an effort to optimise the hydrolysis conditions further, the significance of enzyme:substrate ratio was investigated. To this point, 180 IMCU per gram of sodium caseinate had been used. This ratio was by far in excess of that traditionally used in cheese making (approximately 180 IMCU per 100 g casein); however, very little hydrolysis, other than cleavage of the Phe(105)-Met(106) bond of κ-casein, occurs in milk during rennet coagulation. The quantity of enzyme used was reduced

20-fold so that 9 IMCU enzyme was now added per gram of sodium caseinate. As previously discussed, the enzyme used was an acidic preparation which caused a drop in pH when added to the sodium caseinate solution. Reducing the quantity of enzyme used greatly reduced its effect on the pH of the sodium caseinate solution. It was found that chymosin had no effect on the hydrolysis of α_{S1}- or β-casein at pH 7 under these conditions, i.e., 20-fold enzyme reduction (results not shown), and so, hydrolysis was carried out at pH 6 and 6.5 with the 20-fold enzyme reduction, with the aim of maintaining the same degree of hydrolysis achieved with the original enzyme:substrate ratio. It was found that, at pH 6, when using 9 IMCU per gram of sodium caseinate, a reaction time of 100 min was required to achieve to same degree of hydrolysis as with the original conditions. Similarly, at pH 6.5, when using 9 IMCU per gram of sodium caseinate, a reaction time of 220 min was required to achieve the same degree of hydrolysis as with the original conditions. The RP-UPLC chromatograms in Figure 6.18 show the hydrolysis of sodium caseinate (100 mg/mL) hydrolysed at 37°C by chymosin (a) at pH 7 for 40 min (180 IMCU per gram of sodium caseinate), (b) at pH 6 for 100 min (9 IMCU per gram of sodium caseinate) and (c) at pH 6.5 for 220 min (9 IMCU per gram of sodium caseinate).

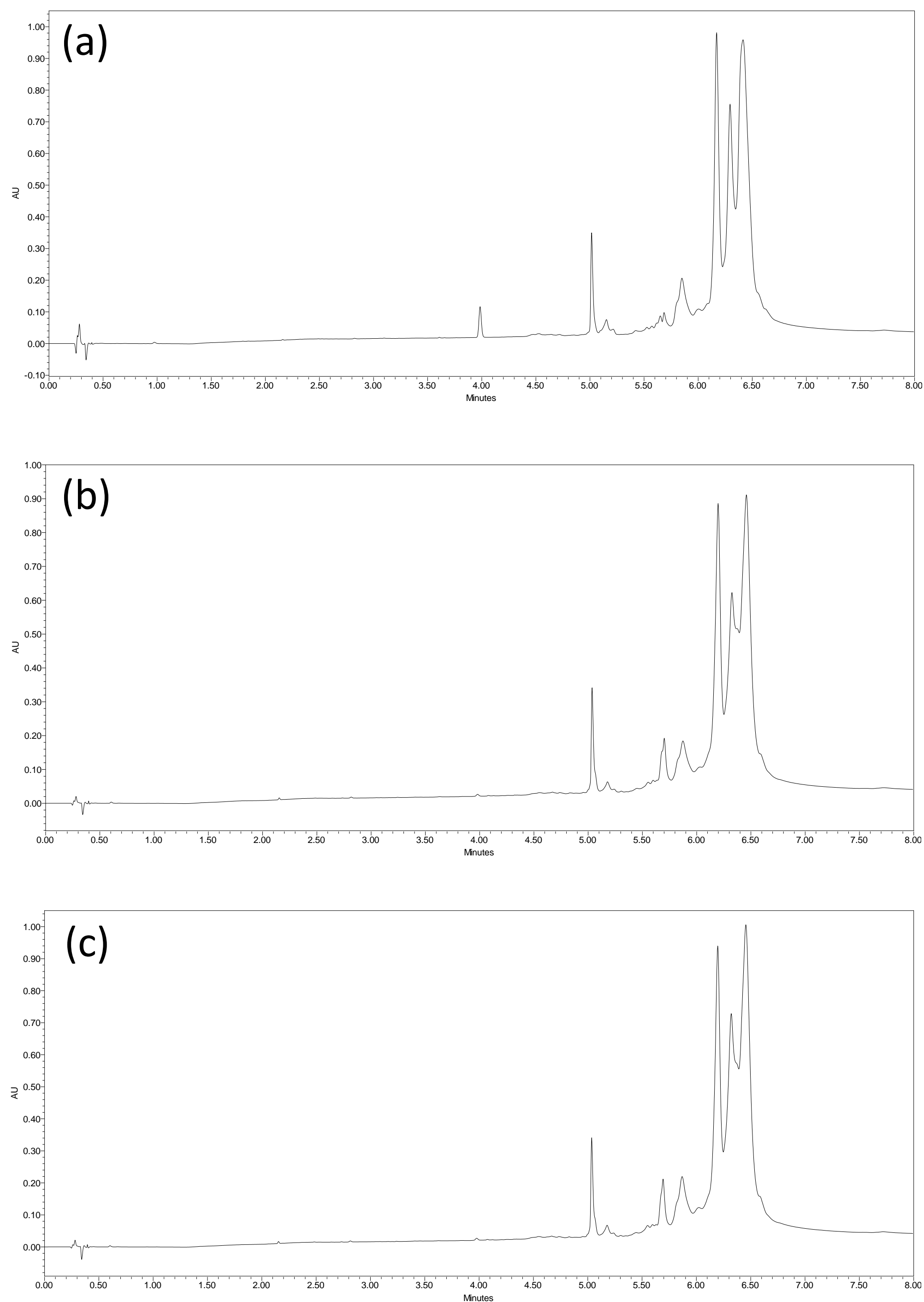

Figure 6.18 Reversed-phase UPLC profile of sodium caseinate (100 mg/mL), adjusted to (a) pH 7, (b) pH 6 and (c) pH 6.5, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate at pH 7; 9 IMCU per gram of sodium caseinate at pH 6 and pH 6.5) at 37°C for 40, 100 and 220 min, respectively.

6.3.5.4 Effect of sodium caseinate hydrolysates, produced at different pH values, on GLP-1 secretion

Sodium caseinate (100 mg/mL), adjusted to pH 6, pH 6.5 and pH 7, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate at pH 7; 9 IMCU per gram of sodium caseinate at pH 6 and pH 6.5) at 37°C for 100, 220 and 40 min, respectively, were added to STC-1 cells to measure their effect on GLP-1 secretion (Figure 6.19). There were no significant differences in GLP-1 secretion in response to varying the enzyme:substrate ratio and pH. Based on these results, it was decided to proceed with the hydrolysis conditions whereby sodium caseinate was adjusted to pH 6.5 before hydrolysis and hydrolysed using 9 IMCU per gram of sodium caseinate, as this was the most effective approach.

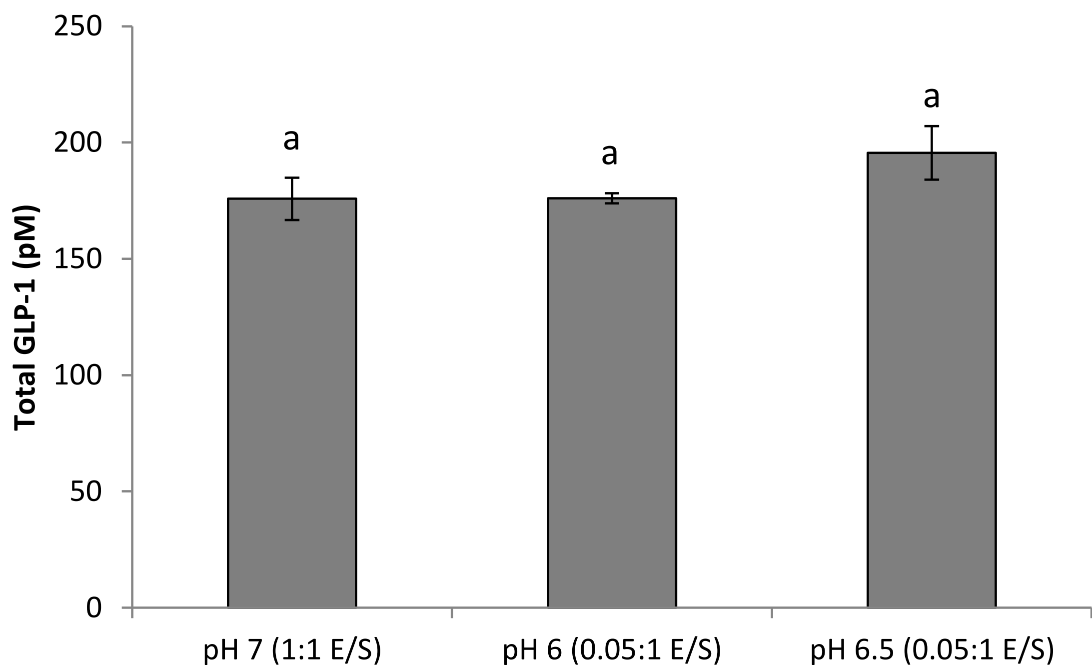

Figure 6.19 The effect of exposure of STC-1 cells to sodium caseinate (100 mg/mL), adjusted to pH 7, pH 6 and pH 6.5, hydrolysed by chymosin (180 IMCU per gram of sodium caseinate at pH 7; 9 IMCU per gram of sodium caseinate at pH 6 and pH 6.5) at 37°C for 40, 100 and 220 min, respectively, on GLP-1 peptide levels as measured by MSD. Values with different superscript letters were significantly different ($p < 0.05$).

6.3.5.5 Fractionation of a sodium caseinate hydrolysate, produced under optimised conditions

The RP-UPLC chromatograms of Figure 6.20 show the hydrolysis of sodium caseinate (100 mg/mL), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min, and fractions thereof. The profile of the unfractionated hydrolysate was similar to that shown in Figure 6.18c. The pH 4.6-insoluble fraction was virtually identical to the unfractionated hydrolysate. The pH 4.6-soluble 1 kDa retentate is enriched in peptide material (3.6 to 6 min) and depleted with respect to intact casein, while the pH 4.6-soluble 1 kDa permeate is a very simple hydrolysate, containing only 3 major peaks. This suggests that the majority of peptide material in the pH 4.6-soluble fraction is greater than 1 kDa in size.

6.3.5.6 Effect of a sodium caseinate hydrolysate, produced under optimised conditions, and fractions thereof, on GLP-1 secretion

Sodium caseinate (100 mg/mL), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min and fractions thereof; pH 4.6-soluble, pH 4.6-insoluble, pH 4.6-soluble 1 kDa retentate and pH 4.6-soluble 1 kDa permeate were exposed to STC-1 cells to measure their effect on GLP-1 secretion (Figure 6.21). Adjustment of the hydrolysate to pH 4.6 resulted in increased secretion of GLP-1 in response to the soluble fraction, this was also seen in Figure 6.12. No difference in GLP-1 secretion was observed with the pH 4.6-insoluble fraction compared to the unfractionated hydrolysate. The 1 kDa retentate and permeate had higher levels of GLP-1 secretion compared with the unfractionated hydrolysate, but lower levels than the pH 4.6-soluble fraction. When the protein concentration of each sample was taken into account, bioactivity was in the order: pH 4.6-soluble 1 kDa permeate > pH 4.6-soluble > pH 4.6-soluble 1 kDa retentate > unfractionated hydrolysate > pH 4.6-insoluble fraction. This order of bioactivity was a reasonable prediction, assuming that the bioactivity was protein-based.

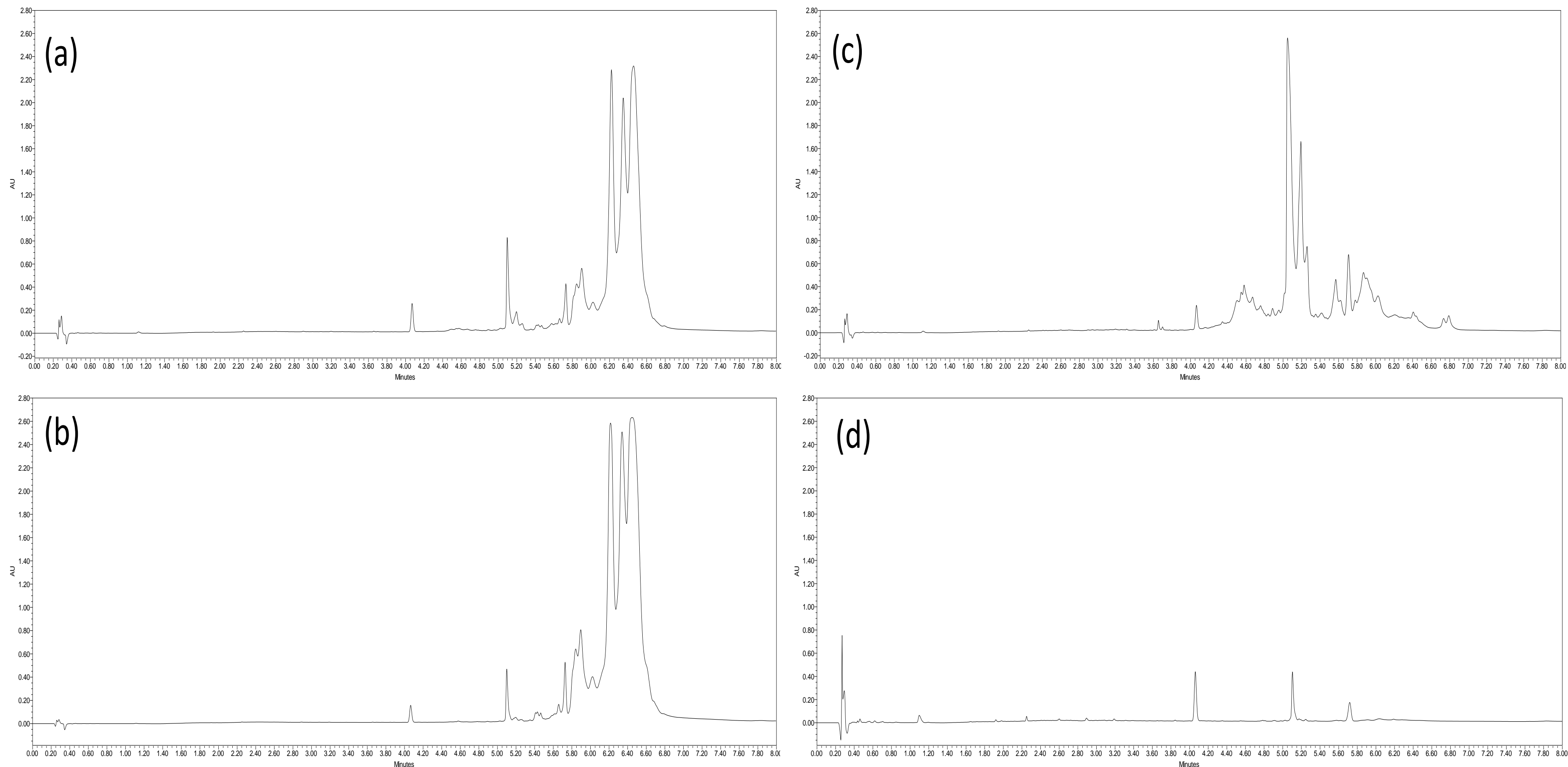

Figure 6.20 Reversed-phase UPLC profile of sodium caseinate (100 mg/ml), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min (a) and fractions thereof; (b) pH 4.6-insoluble, (c) pH 4.6-soluble 1 kDa retentate and (d) pH 4.6-soluble 1 kDa permeate.

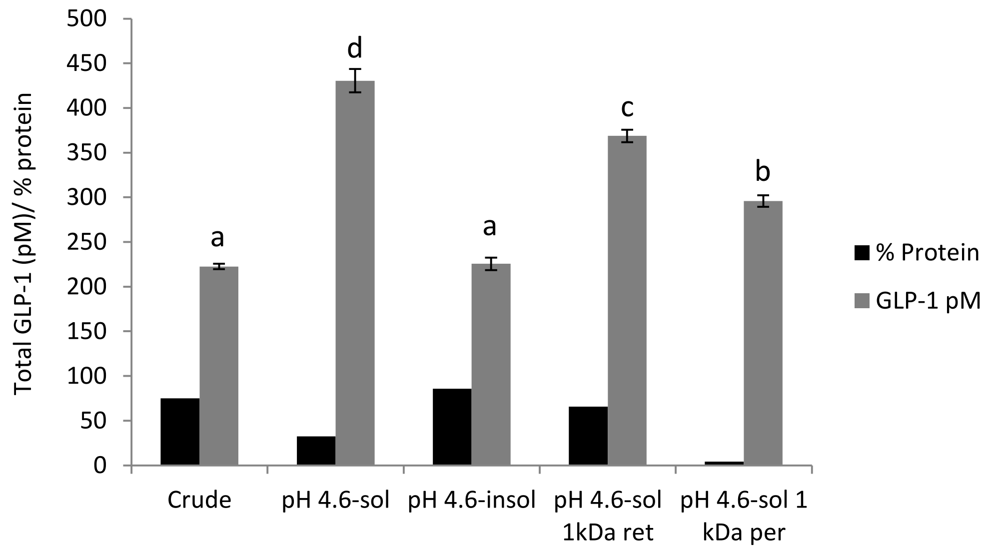

Figure 6.21 The effect of exposure of STC-1 cells to sodium caseinate (100 mg/mL), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min, and fractions thereof, on GLP-1 peptide levels as measured by MSD. Also shown is the protein content of each lyophilised sample, as determined by the Kjeldahl method. Values with different superscript letters were significantly different ($p < 0.05$).

6.3.6 *Free amino acid analysis of sodium caseinate hydrolysate*

GLP-1 is released from intestinal cells in response to several components of the diet, including carbohydrates, fats and proteins (Tolhurst *et al.*, 2009). The effect of protein on GLP-1 secretion has been investigated by several authors (Elliot *et al.,* 1993; Herrmann *et al.,* 1995); however, the underlying mechanism has yet to be elucidated. Amino acids are thought to play a role in GLP-1 secretion; Reimer (2006) reported a dose-dependant increase in GLP-1 secretion in response to a mixture of essential amino acids (EAA), while Reimann *et al.* (2004) observed that glutamine is a more potent GLP-1 secretagogue than glucose or other amino acids. To investigate the possibility of free amino acids causing a GLP-1 response in the current study, sodium caseinate (100 mg/mL), adjusted to pH 7, was hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 40 min. Aliquots were taken at 5 min intervals, the hydrolysis reaction stopped by heat inactivation of the enzyme, and the lyophilised samples analysed for their free amino acid (FAA)

content (Table 6.1). Histidine and lysine were the only free amino acids present in the unhydrolysed sodium caseinate (T_0).

Aspartic acid and phenylalanine were present after 5 min at concentrations of 8.5 and 35.7 µg/g, respectively, but were not present in any subsequent samples. The concentrations of glutamic acid, alanine and methionine after 5 min were 38.5, 29.4 and 585.2 µg/g, respectively, and did not increase by more than 4% over 40 min. The concentrations of histidine and lysine increased from 10.5 and 31.0 µg/g to 103.8 and 45.3 µg/g, respectively, over 40 min. As no free amino acids, with the exception of histidine and lysine, were present in the unhydrolysed sodium caseinate, it is likely that any free amino acids present in hydrolysed samples were introduced by the enzyme preparation, either naturally present or produced by the yeast strain used to produce chymosin recombinantly, i.e., *Kluyveromyces lactis.* This is a fair assumption as chymosin itself does not produce any free amino acids (Visser and Slangen, 1977; McSweeney *et al.,* 1993; Upadhyay *et al.,* 2004). It was thus concluded to be very unlikely that the increased GLP-1 secretion associated with the sodium caseinate hydrolysate was due to the presence of free amino acids, as they were present at extremely low concentrations.

Table 6.1 Free amino acid (µg/g) analysis of sodium caseinate (100 mg/mL), adjusted to pH 7, and hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 0, 5, 10, 15, 20, 25, 30, 35 and 40 min.

Amino Acid	T0	T5	T10	T15	T20	T25	T30	T35	T40
Cysteine	0.0	22.3	21.9	0.0	54.0	21.0	21.4	47.3	48.1
Aspartic Acid	0.0	8.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glutamic Acid	0.0	38.5	41.7	40.5	40.0	41.4	43.0	38.1	35.3
Alanine	0.0	29.4	36.5	33.6	31.9	30.0	32.4	29.1	29.5
Methionine	0.0	585.2	622.3	623.0	637.3	611.3	623.3	644.5	606.1
Phenylalanine	0.0	35.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Histidine	10.5	63.4	77.6	76.1	83.3	79.0	105.2	107.1	103.8
Lysine	31.0	31.8	52.9	56.7	59.1	43.9	51.6	65.2	45.3

6.3.7 *Analysis of chymosin solution*

Figure 6.22 shows the RP-UPLC profile of the enzyme preparation. The chromatogram is dominated by one large peak which eluted at 4 min, which is most likely chymosin. Several minor peaks were present from 0.5 to 4.0 min; it is possible that these peaks were peptide material or free amino acids.

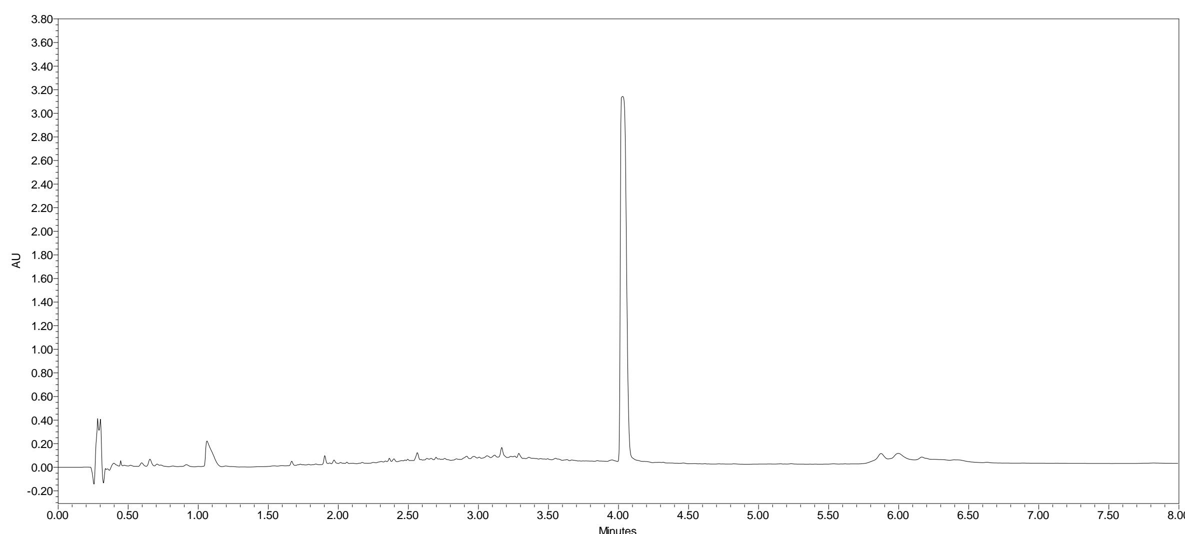

Figure 6.22 RP-UPLC profile of Maxiren 180.

6.3.8 *Fractionation of pH 4.6-soluble peptide material*

In an attempt to identify the bioactive component responsible for increased GLP-1 secretion in STC-1 cells, sodium caseinate (100 mg/mL), adjusted to pH 6.5, was hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min. The crude hydrolysate was adjusted to pH 4.6, as the soluble fraction was shown to have increased bioactivity compared with the crude hydrolysate and pH 4.6-insoluble fraction (Figures 6.12 and 6.21). The pH 4.6-soluble fraction was further fractionated by filtering through a 0.2 µm membrane. This 0.2 µm permeate was then filtered through a series of ultrafiltration membranes which were set up in such a way that the permeate from each membrane became the feed solution for the proceeding membrane, i.e., 10 kDa, 5 kDa, 3 kDa, and 1 kDa.

Figure 6.23 shows the RP-UPLC profiles of the crude hydrolysate, pH 4.6-soluble fraction, and subsequent fractions. The pH 4.6-soluble fraction was dominated by two peptide peaks, at approximately 5 and 5.5 min, with several minor peaks visible from 2.0 to 6.0 min. These two abundant peaks were also present in the 0.2 µm retentate, but at much lower levels. The function of the 0.2 µm membrane was to remove any large aggregated peptide material. As can be seen from Figure 6.23 (c), only a very small proportion of the peptide material in the pH 4.6-soluble fraction existed in an aggregated form. The peptide which eluted at 5 min in the pH 4.6-soluble fraction was present in the 10 kDa retentate, as was a peptide at 5.2 min; however, the peptide at 5.5 min was not present. The peptide profiles of the 5, 3 and 1 kDa retentates and 1 kDa permeate were very similar, which suggests that UF was not successful in the separation of peptides.

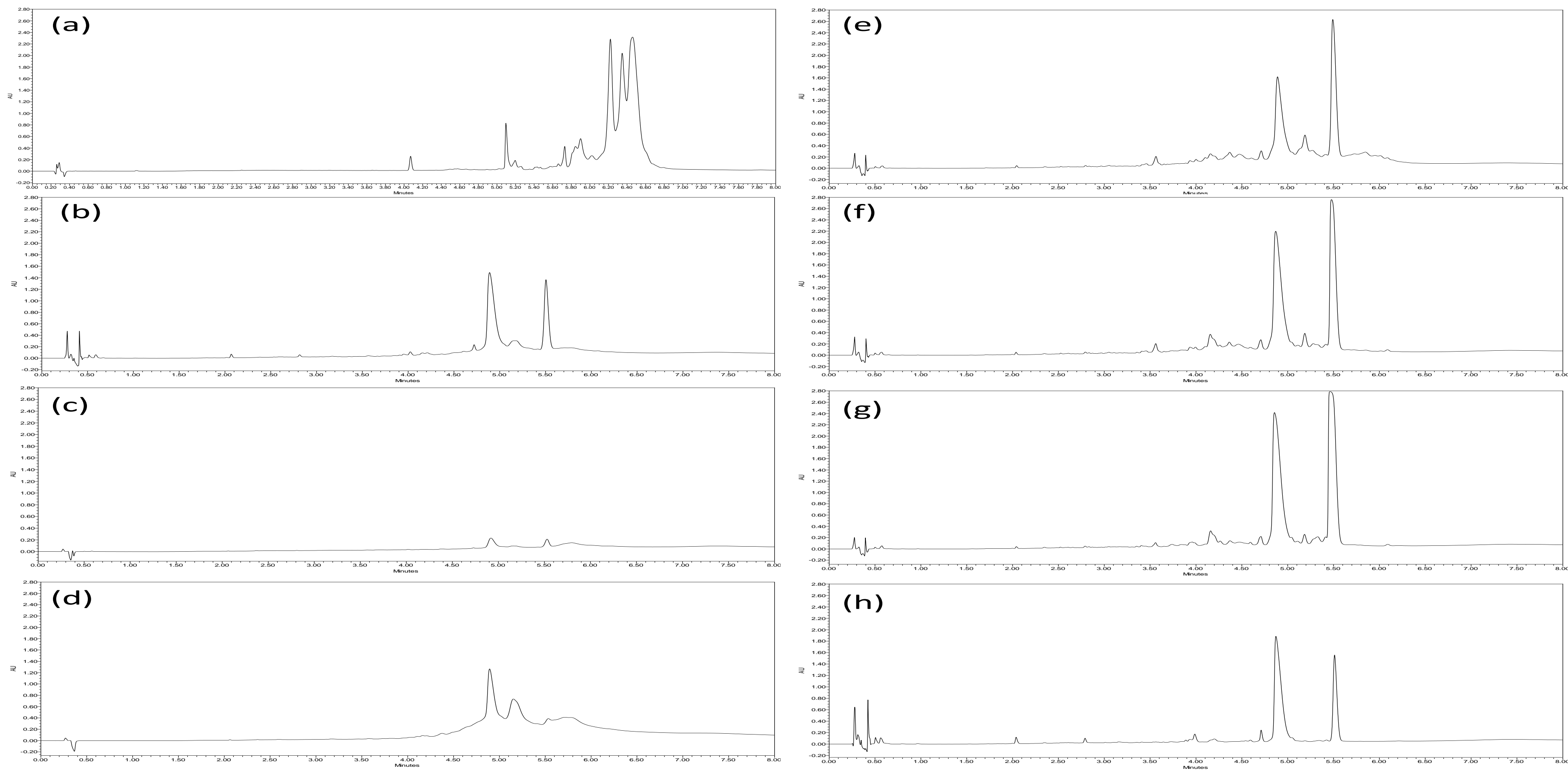

Figure 6.23 Reversed-phase UPLC profiles of sodium caseinate (100 mg/ml), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min, and fractions thereof; (a) crude hydrolysate, (b) pH 4.6-soluble, (c) 0.2 µm retentate, (d) 10 kDa retentate, (e) 5 kDa retentate), (f) 3 kDa retentate, (g) 1 kDa retentate and (h) 1 kDa permeate.

6.3.8.1 Effect of a pH 4.6-soluble fraction of a sodium caseinate hydrolysate, and fractions thereof, on GLP-1 secretion

Sodium caseinate (100 mg/mL), adjusted to pH 6.5 and hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min, and fractions thereof, were added to STC-1 cells to measure their effect on GLP-1 secretion (Figure 6.24). Adjustment of the hydrolysate to pH 4.6 resulted in increased secretion of GLP-1 in response to the soluble fraction, as also seen in Figures 6.12 and 6.21; however, the effect was not as pronounced in this case. Filtration of the pH 4.6-soluble fraction through a 0.2 µm membrane resulted in a large increase in GLP-1 secretion in response to the retentate. Overall, bioactivity was in the order: 0.2 µm retentate > 1 kDa retentate = 5 kDa retentate = 3 kDa retentate > pH 4.6-soluble fraction > 1 kDa permeate > crude hydrolysate > 10 kDa retentate. These samples were tested for their effect on GLP-1 secretion on an equal solids basis. When the protein concentration was taken into account, assuming the bioactivity was protein-based, bioactivity was in the order 1 kDa permeate > 5 kDa retentate > 1 kDa retentate > 3 kDa retentate > 0.2 µm retentate > pH 4.6-soluble fraction > 10 kDa retentate > crude hydrolysate.

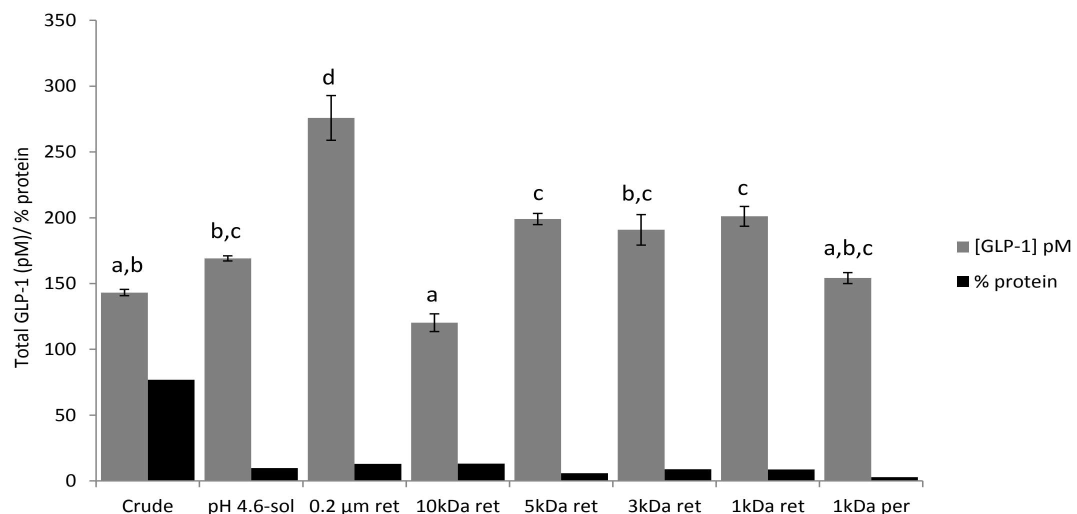

Figure 6.24 The effect of exposure of STC-1 cells to sodium caseinate (100 mg/mL), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min, and fractions thereof, on GLP-1 peptide levels as measured by MSD. Also shown is the protein content of each lyophilised sample, as determined by the Bradford method for protein determination. Values with different superscript letters were significantly different ($p < 0.05$).

6.3.9 I*dentification of peptides by LC-MS*

The LC-MS profiles in Figure 6.25 show sodium caseinate (100 mg/mL), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram sodium caseinate) at 37°C for 220 min, and fractions thereof. The ten most abundant peptides present in each of these samples, selected according to relative peak intensity, were identified by LC-MS (Table 6.2). With the exception of the 10 kDa retentate (Figure 6.25d), the LC-MS profiles of all samples were quite similar. β-Casein (f193-209) was the most abundant peptide present in all samples, except for the 10 kDa retentate. This peptide has previously been isolated and identified in yoghurt, fermented milk and cheese (Dionysius *et al.,* 2000; Gagnaire *et al.,* 2001; Hernandez-Ledesma *et al.,* 2004) and is a major cause of bitterness in Gouda (Visser *et al.,* 1983) and Cheddar (Kelly *et al.,* 1996) cheese. It is a hydrophobic peptide composed of 17 amino acids, with a molecular mass of 1881.2 Da (Singh *et al.,* 2005), and has been shown by several authors to have immunomodulatory properties: mitogenic activity on primed lymph node cells and unprimed rat spleen cells (Coste *et al.,* 1992), chemotactic activity on L14 lymphoblastoid cell line (Frontenau *et al.,* 1998), and enhanced phagocytosis in rat macrophages (Sandre *et al.,* 2001). Regazzo *et al.* (2010) provided evidence for the bioavailability of this peptide, showing that it is transported across the Caco-2 cell monolayer by resisting the action of brush-border membrane-peptidases. β-Casein (f193-209) has also been shown to have antimicrobial activity against *Escherichia coli* DH5α (Birkemo *et al.,* 2009); Lee *et al.* (1987) investigated the emulsifying properties of casein-derived peptides and found a synergistic effect in emulsion activity between β-casein (f193-209) and κ-casein (f106-169) (glycomacropeptide).

The primary site of chymosin action on α_{S1}-casein is Phe(23)-Phe(24) (McSweeney *et al.,* 1993). The second most abundant peptide identified in all samples, again with the exception of the 10 kDa retentate, was α_{S1}-casein (f1-23). This is a hydrophilic peptide composed of 23 amino acids with a molecular mass of 2764.2 Da. α_{S1}-Casein (f1-23) is a positively charged antimicrobial peptide which has a broad spectrum of activity against Gram-positive bacteria (Hill *et al.,* 1974) and the pathogenic strains *Enterobacter sakazakii* ATCC 12868 and *Escherichia coli* DPC5063

(Hayes *et al.,* 2006). This peptide is both therapeutic and prophylactic and responses to its therapeutic effect can produce long-term immune responses (Lahov and Regelson, 1996).

The third most abundant peptide identified in all samples, with the exception of the 10 kDa retentate, was α_{S1}-casein (f3-23). This is a hydrophilic peptide composed of 21 amino acids with a molecular mass of 2510.9 Da. To the best of our knowledge, this peptide has not previously been reported as a product of chymosin action on α_{S1}-casein. Nor is it produced by the indigenous milk proteinases, plasmin, elastase, cathepsin D or cathepsin G (Upadhyay *et al.,* 2004). This peptide is, however, produced by the action of cathepsin B on α_{S1}-casein (Considine *et al.,* 2004). In the current study, it was most likely the result of cleavage of the Pro(2)-Lys(3) bond of α_{S1}-casein (f1-23) by chymosin.

In general, the ten most abundant peptides identified across all samples were derived from individual caseins in the order α_{S1}- > β- > κ- > α_{S2}-casein. Peptides ranged from 3 amino acids with a molecular mass of 373.5 Da (β-casein f190-192) to 59 amino acids with a molecular mass of 6758.4 Da (β-CN f15-73). Peptides which were identified in multiple samples, besides β-casein (f193-209), α_{S1}-casein (f1-23) and α_{S1}-casein (f3-23), were α_{S1}-casein (f1-20), α_{S1}-casein (f1-22), α_{S1}-casein (f17-23), α_{S1}-casein (f19-23), α_{S1}-casein (f21-23), α_{S1}-casein (f25-32), α_{S1}-casein (f176-199), β-casein (f74-89), β-casein (f166-189) and β-casein (f190-209). α_{S1}-Casein-derived peptides originated mainly from the N-terminal region of the protein, and in most cases were products of α_{S1}-casein (f1-23). The only peptide identified derived from α_{S2}-casein was α_{S2}-casein (f164-168). The Phe(163)-Leu(164) bond of α_{S2}-casein is a recognised cleavage site of chymosin (McSweeney *et al.,* 1994).

During cheesemaking, κ-casein is hydrolysed at its Phe(105)-Met(106) bond to produce *para*-κ-casein, which remains attached to the micelle core, and glycomacropeptide (GMP), which diffuses into the aqueous phase (Crabbe, 2004). GMP is unique in its amino acid composition as it lacks aromatic amino acids and is rich in branched chain amino acids (Silva and Malcatta, 2005). GMP has several

reported biological activities: ability to bind cholera toxins (Kawasaki *et al.,* 1992) and *E. coli* enterotoxins (Isoda *et al.,* 1994), inhibition of bacterial and viral adhesion (Dosaka *et al.,* 1992), suppression of gastric secretions (Yvon *et al.,* 1994), promotion of bifidobacterial growth (Proulx *et al.,* 1992) and modulation of immune system responses (Monnai and Otani, 1997). The Phe(105)-Met(106) bond of κ-casein is the primary cleavage site of chymosin in the milk protein system (Reid *et al.*, 1997) and is many times more susceptible than any other bond in milk proteins (Upadhyay *et al.,* 2004). Interestingly, GMP was not identified in the current study by LC-MS; however, several peptides within its sequence were found, i.e., κ-casein (f106-131, f106-129, f161-169). Of these peptides, κ-casein (f161-169) has previously been identified as a secondary hydrolysis product of the action of chymosin on κ-casein (Reid *et al.,* 1997).

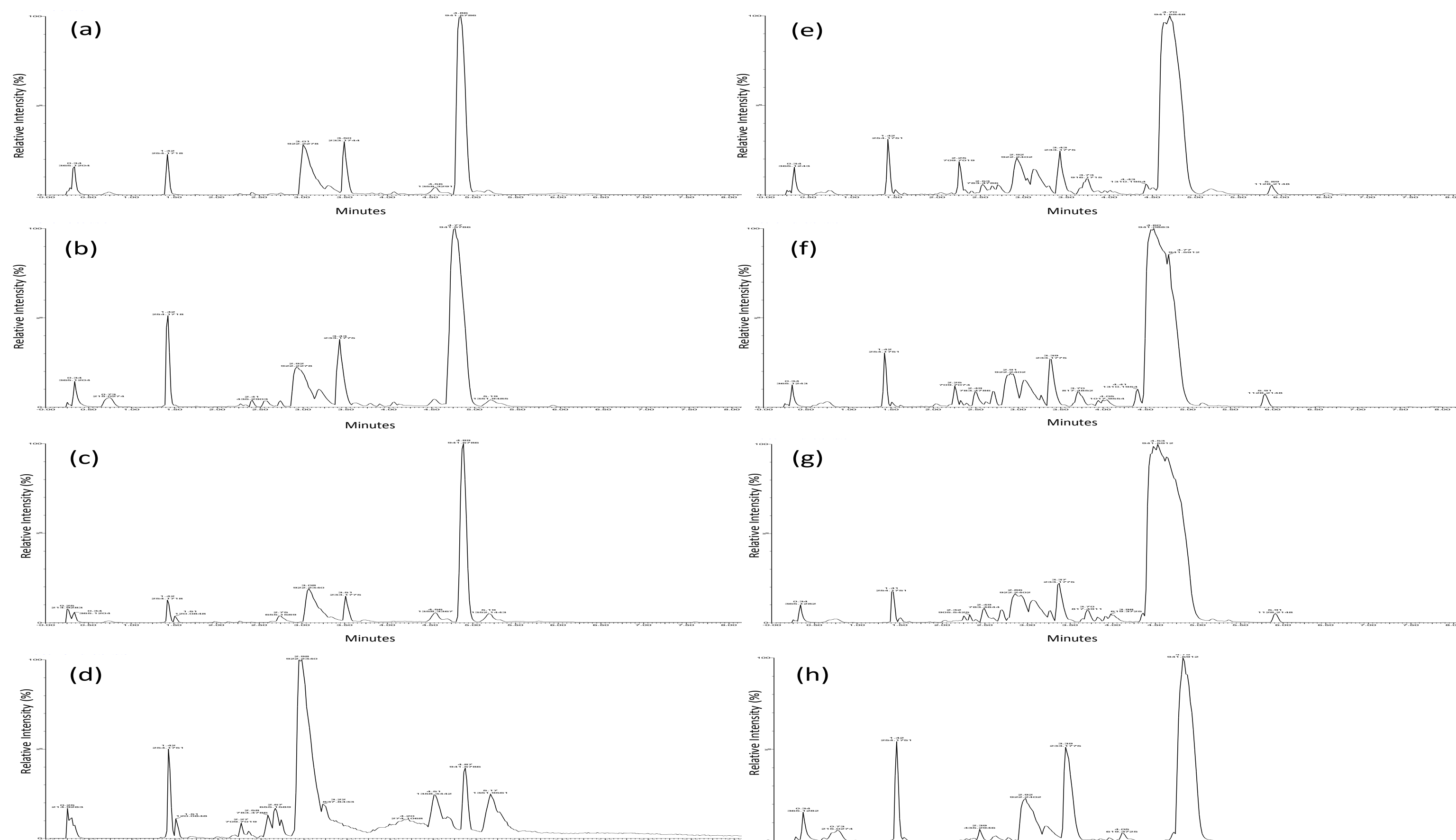

Figure 6.25 LC-MS profiles of sodium caseinate (100 mg/ml), adjusted to pH 6.5 and hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min and fractions thereof; (a) crude hydrolysate, (b) pH 4.6-soluble fraction, (c) pH 4.6-soluble 0.2 µm retentate, (d) pH 4.6-soluble 10 kDa retentate, (e) pH 4.6-soluble 5 kDa retentate, (f) pH 4.6-soluble 3 kDa retentate, (g) pH 4.6-soluble 1 kDa retentate, and (h) pH 4.6-soluble 1 kDa permeate.

Table 6.2 Identification of the ten most abundant peptides present in sodium caseinate (100 mg/mL), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min and fractions thereof measured by Q-TOF LC-MS; pH 4.6-soluble fraction, pH 4.6-soluble 0.2 μm retentate, pH 4.6-soluble 10 kDa retentate, pH 4.6-soluble 5 kDa retentate, pH 4.6-soluble 3 kDa retentate, pH 4.6-soluble 1 kDa retentate, and pH 4.6-soluble 1 kDa permeate

Sample	Peptide	Sequence	Retention Time	Relative Intensity
Crude hydrolysate	β-CN f(193-209)	YQEPVLGPVRGPFPIIV	4.83	1818581
	α_{s1}-CN f(1-23)	RPKHPIKHQGLPQEVLNENLLRF	3.02	1187726
	α_{s1}-CN f(3-23)	KHPIKHQGLPQEVLNENLLRF	3.28	145204
	α_{s1}-CN f(1-11)	RPKHPIKHQGL	3.02	80936
	α_{s1}-CN f(17-23)	NENLLRF	3.01	64446
	α_{s1}-CN f(31-42)	VFGKEKVNELSK	3.28	36391
	α_{s1}-CN f(19-23)	NLLRF	3.01	24403
	α_{s1}-CN f(21-23)	LRF	3.01	23630
	α_{s1}-CN f(5-13)	PIKHQGLPQ	3.01	22906
	β-CN f(64-89)	GPIPNSLPQNIPPLTQTPVVVPPFLQ	3.60	21914
pH 4.6-soluble fraction	β-CN f(193-209)	YQEPVLGPVRGPFPIIV	4.79	4476712
	α_{s1}-CN f(1-23)	RPKHPIKHQGLPQEVLNENLLRF	2.96	2448382
	α_{s1}-CN f(3-23)	KHPIKHQGLPQEVLNENLLRF	3.21	522308
	β-CN f(74-89)	IPPLTQTPVVVPPFLQ	2.96	333647
	α_{s1}-CN f(25-32)	VAPFPEVF	2.95	111752
	β-CN f(15-73)	SLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQN	4.58	100581
	β-CN f(190-192)	FLL	3.44	98828
	α_{s1}-CN f(1-20)	RPKHPIKHQGLPQEVLNENL	2.58	96670
	α_{s2}-CN f(164-184)	LKKISQRYQKFALPQYLKTVY	2.89	84559
	α_{s1}-CN f(14-24)	EVLNENLLRFF	3.21	79417
pH 4.6-sol 0.2 μm retentate	β-CN f(193-209)	YQEPVLGPVRGPFPIIV	4.79	689653
	α_{s1}-CN f(1-23)	RPKHPIKHQGLPQEVLNENLLRF	3.00	475033
	α_{s1}-CN f(3-23)	KHPIKHQGLPQEVLNENLLRF	3.23	47688
	α_{s1}-CN f(1-22)	RPKHPIKHQGLPQEVLNENLLR	2.70	46716
	α_{s1}-CN f(25-32)	VAPFPEVF	3.00	30205
	α_{s1}-CN f(1-18)	RPKHPIKHQGLPQEVLNE	3.00	18732
	α_{s1}-CN f(21-23)	LRF	2.99	16444
	β-CN f(104-161)	PKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQS	5.14	15381
	α_{s1}-CN f(19-23)	NLLRF	3.00	14937
	α_{s1}-CN f(1-14)	RPKHPIKHQGLPQE	3.36	14528
pH 4.6-sol 10 kDa retentate	α_{s1}-CN f(1-23)	RPKHPIKHQGLPQEVLNENLLRF	2.91	1389943
	β-CN f(69-85)	SLPQNIPPLTQTPVVVP	2.97	261351
	β-CN f(193-209)	YQEPVLGPVRGPFPIIV	4.86	144784
	α_{s1}-CN f(3-23)	KHPIKHQGLPQEVLNENLLRF	3.13	142366
	α_{s1}-CN f(1-22)	RPKHPIKHQGLPQEVLNENLLR	2.62	91819
	κ-CN f(106-129)	MAIPPKKNQDKTEIPTINTIASGE	5.13	89757
	α_{s1}-CN f(1-13)	RPKHPIKHQGLPQ	2.91	88294
	β-CN f(75-103)	PPLTQTPVVVPPFLQPEVMGVSKVKEAMA	2.87	79093
	κ-CN f(161-169)	TVQVTSTAV	2.91	73201
	α_{s1}-CN f(1-20)	RPKHPIKHQGLPQEVLNENL	2.53	42602

(continued)

Table 6.2 *(continued)*

Sample	Peptide	Sequence	Retention Time	Relative Intensity
pH 4.6-sol 5 kDa retentate	β-CN f(193-209)	YQEPVLGPVRGPFPIIV	4.55	4801312
	α_{s1}-CN f(1-23)	RPKHPIKHQGLPQEVLNENLLRF	2.87	849292
	α_{s1}-CN f(3-23)	KHPIKHQGLPQEVLNENLLRF	3.04	464084
	α_{s1}-CN f(81-100)	IQKEDVPSERYLGYLEQLLR	3.65	163756
	α_{s1}-CN f(176-199)	APSFSDIPNPIGSENSEKTTMPLW	4.37	146975
	β-CN f(74-89)	IPPLTQTPVVVPPFLQ	2.86	134994
	α_{s1}-CN f(1-20)	RPKHPIKHQGLPQEVLNENL	2.48	102315
	β-CN f(190-209)	FLLYQEPVLGPVRGPFPIIV	5.79	83489
	α_{s1}-CN f(3-14)	KHPIKHQGLPQE	3.12	79367
	κ-CN f(106-131)	MAIPPKKNQDKTEIPTINTIASGEPT	2.68	70691
pH 4.6-sol 3 kDa retentate	β-CN f(193-209)	YQEPVLGPVRGPFPIIV	4.44	8796597
	α_{s1}-CN f(1-23)	RPKHPIKHQGLPQEVLNENLLRF	2.81	1120830
	α_{s1}-CN f(3-23)	KHPIKHQGLPQEVLNENLLRF	2.99	682015
	α_{s1}-CN f(176-199)	APSFSDIPNPIGSENSEKTTMPLW	4.33	229825
	α_{s1}-CN f(1-20)	RPKHPIKHQGLPQEVLNENL	2.44	195561
	α_{s1}-CN f(143-174)	AYFYPELFRQFYQLDAYPSGAWYYVPLGTQYT	5.11	178529
	β-CN f(73-88)	NIPPLTQTPVVVPPFL	2.80	176486
	β-CN f(166-189)	SQSKVLPVPQKAVPYPQRDMPIQA	2.67	157004
	β-CN f(190-209)	FLLYQEPVLGPVRGPFPIIV	5.81	148505
	κ-CN f(77-94)	QVLSNTVPAKSCQAQPTT	3.71	123410
pH 4.6-sol 1 kDa retentate	β-CN f(193-209)	YQEPVLGPVRGPFPIIV	4.35	10605850
	α_{s1}-CN f(1-23)	RPKHPIKHQGLPQEVLNENLLRF	2.77	1304473
	α_{s1}-CN f(3-23)	KHPIKHQGLPQEVLNENLLRF	2.98	765448
	α_{s1}-CN f(81-100)	IQKEDVPSERYLGYLEQLLR	3.64	217304
	α_{s1}-CN f(1-20)	RPKHPIKHQGLPQEVLNENL	2.43	196019
	κ-CN f(102-113)	HLSFMAIPPKKN	2.86	171744
	β-CN f(166-192)	SQSKVLPVPQKAVPYPQRDMPIQAFLL	3.88	163985
	β-CN f(166-189)	SQSKVLPVPQKAVPYPQRDMPIQA	2.65	159020
	α_{s1}-CN f(185-199)	PIGSENSEKTTMPLW	3.22	127558
	β-CN f(190-209)	FLLYQEPVLGPVRGPFPIIV	5.81	118345
pH 4.6-sol 1 kDa permeate	β-CN f(193-209)	YQEPVLGPVRGPFPIIV	4.78	5459459
	α_{s1}-CN f(1-23)	RPKHPIKHQGLPQEVLNENLLRF	2.94	2330794
	α_{s1}-CN f(3-23)	KHPIKHQGLPQEVLNENLLRF	3.19	518411
	β-CN f(193-208)	YQEPVLGPVRGPFPII	4.65	93850
	α_{s1}-CN f(17-23)	NENLLRF	2.94	85820
	α_{s1}-CN f(1-20)	RPKHPIKHQGLPQEVLNENL	2.59	81329
	κ-CN f(62-77)	AKPAAVRSPAQILQWQ	4.78	63904
	α_{s1}-CN f(24-34)	FVAPFPEVFGK	4.07	58253
	α_{s1}-CN f(21-23)	LRF	2.93	47130
	α_{s1}-CN f(19-23)	NLLRF	2.94	42963

6.3.10 *Synthetic peptides*

Based on the LC-MS data shown in Table 6.2, four of the most abundant peptides identified in the sodium caseinate hydrolysate were artificially synthesised. Figures 6.26 and 6.27 show the RP-UPLC profiles of these peptides, i.e., β-casein (f193-209 and f190-209) and α_{S1}-casein (f1-23 and f3-23). These peptides were exposed to STC-1 cells at 0.01, 0.1 and 0.5 mg/mL to measure their effect on GLP-1 secretion (Figure 6.28). Sodium caseinate (100 mg/mL), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram sodium caseinate) at 37°C for 220 min was used as a positive control for GLP-1 secretion. None of the synthetic peptides were found to cause an increase in the secretion of GLP-1 from STC-1 cells.

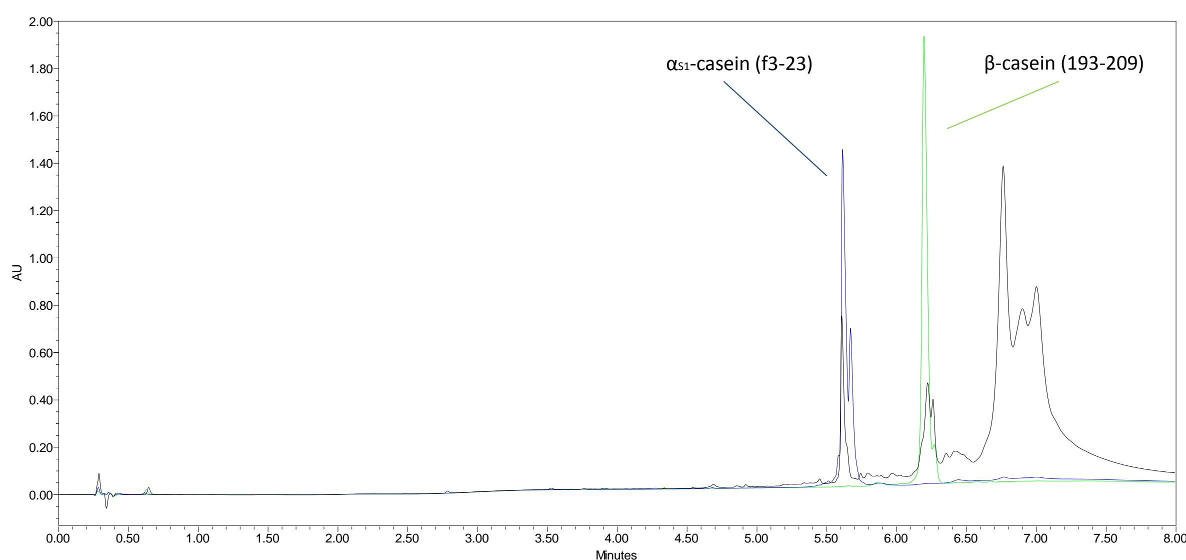

Figure 6.26 RP-UPLC chromatograms of α_{S1}-casein (f3-23), β-casein (f193-209) and sodium caseinate (100 mg/mL), adjusted to pH 6.5 and hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min.

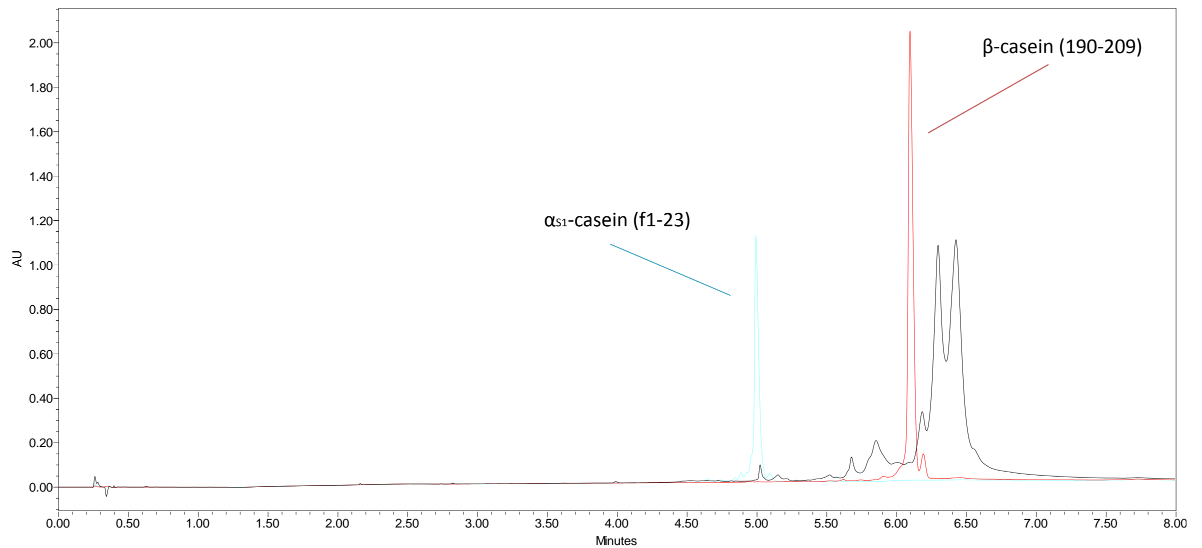

Figure 6.27 RP-UPLC chromatograms of α_{S1}-casein (f1-23), β-casein (f190-209) and sodium caseinate (100 mg/mL), adjusted to pH 6.5, hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min.

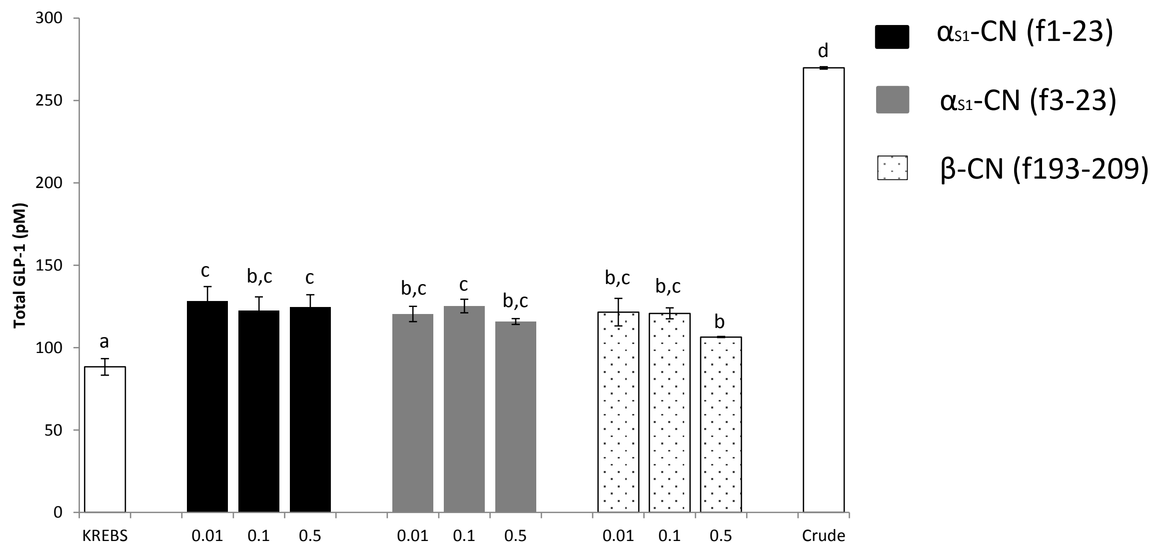

Figure 6.28 The effect of exposure of STC-1 cells to sodium caseinate (100 mg/mL), adjusted to pH 6.5 and hydrolysed by chymosin (9 IMCU per gram of sodium caseinate) at 37°C for 220 min, α_{S1}-CN (f3-23 and f3-23) and β-CN (f193-209), on GLP-1 peptide levels as measured by MSD. Values with different superscript letters were significantly different ($p < 0.05$).

6.4 Conclusion

The current study focussed on the generation of a sodium caseinate and chymosin hydrolysate which targets the gut-derived satiety hormone glucagon-like peptide-1 (GLP-1). Initially, sodium caseinate (30 mg/mL), adjusted to pH 5.6, was hydrolysed by chymosin (180 IMCU per gram of sodium caseinate) at 37°C for 10, 40 or 120 min. When exposed to STC-1 cells, hydrolysed sodium caseinate samples caused a significant increase in the secretion of GLP-1 compared to the unhydrolysed control. Sodium caseinate, hydrolysed by chymosin for 40 min, was found to be the most potent promoter of GLP-1 secretion *in vitro*, suggesting that the bioactive component was produced during the initial stages of the hydrolysis reaction. The unfractionated hydrolysate was used in a mice feeding study and was found to significantly lower feed intake when compared with control mice.

The hydrolysis protocol was optimised in several ways in order to make it more suitable for large scale commercial production. Total solids level of the sodium caseinate solution was increased from 3 to 10%, the pH of which sodium caseinate was adjusted to before hydrolysis was varied in the range 5.6 to 7.0, and the enzyme:substrate ratio was reduced 20-fold. The optimised hydrolysis protocol was considered to be industrially feasible and economically viable.

Fractionation of the crude hydrolysate was carried out by isoelectric precipitation of the caseins and ultrafiltration of the soluble fraction in an attempt to identify the bioactive component/s. The pH 4.6-insoluble fraction, which was composed predominantly of intact casein and large polypeptides, had no effect on GLP-1 secretion. Bioactivity was associated with the pH 4.6-soluble fraction and was most concentrated in the < 1 kDa size range, suggesting that the bioactive component/s was a peptide of approximately 7 amino acids in length (assuming the mean molecular mass of an amino acid to be 136.75 Da). The three most abundant 2% TCA-soluble peptides produced during hydrolysis of sodium caseinate by chymosin were identified by LC-MS, synthesised, and tested for their effect on GLP-1 secretion, however, no effects were observed. Hence, the bioactive component/s was most likely present at extremely low concentrations.

Even though the bioactive component/s was not identified, this sodium caseinate hydrolysate has proven *in vitro* and *in vivo* effects on GLP-1 secretion and could potentially be used in a functional food product which targets gastric satiety signals. Identification of cellular targets and mechanisms involved in GLP-1 release using this *in vitro* model could provide important insights for the design of future human trials aimed at designing novel nutritional strategies for the treatment of obesity and its related diseases.

6.5 Acknowledgements

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6.6 References

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Chapter 7

7.1 General discussion

The major role of milk proteins is to supply young mammals with amino acids and organic nitrogen. In recent years, extensive research has been carried out on the health benefits of milk proteins and peptides. Biologically active peptides are defined as specific protein fragments which have a positive impact on the physiological functions of the body. These peptides are inactive within the parent protein, but can be released by gastrointestinal digestion of milk, fermentation of milk with proteolytic starter cultures or hydrolysis by proteolytic enzymes. Milk-derived biologically active peptides have a wide range of reported health benefits which include opioid, antihypertensive, antithrombotic, mineral binding, immunomodulating and antimicrobial properties. The aim of this thesis was to investigate not only the traditional methods used for the generation of bioactive peptides, but also novel processes such as heat treatment, and the role of indigenous milk proteases, e.g., in mastitic milk, in the production of such peptides. In addition, colostrum was characterised as a source of bioactive proteins and peptides.

Bovine colostrum is the initial milk secreted by cows and is an excellent source of nutrients, antibodies and growth factors for the suckling calf. Colostrum was therefore identified as a rich source of bioactive material which was most likely present in mature milk but at significantly reduced levels. There are few reports in the literature on the composition and physico-chemical properties of colostrum throughout the early-lactation period, and so a comprehensive study of these factors was undertaken (**Chapter 2**). Distinct differences were observed, not only compared with mature milk, but also between individual cows and consecutive milkings. The most notable differences were observed during the transition from the first to second milking; however, even at the sixth milking *post partum*,

secretions were not comparable to mature milk. The pH of colostrum was very low initially and increased with time *post partum*, while the protein concentration was highest initially, due mainly to elevated levels of whey proteins, and decreased with time *post partum*. The concentration of α-lactalbumin, β-lactoglobulin, immunoglobulins, serum albumin and lactoferrin were all higher in colostrum than in mature milk. The concentration of casein was also higher in colostrum than in mature milk, but not to the same extent as some of the whey proteins. It was found that a significant proportion of casein micelles in colostrum, especially in earlier milkings, were non-sedimentable at 100,000 g x 1 h. The reason for this is unclear, but it was speculated that the high viscosity of certain colostrum samples resulted in poor sedimentability of casein micelles. The average diameter of casein micelles in first milkings were larger than in subsequent milkings. The concentrations of Na, Mg, P, K and Ca were higher in colostrum than in mature milk while the concentration of lactose was lower. The concentration of lactose in colostrum was inversely related to the concentration of oligosaccharides. The colour of colostrum was highly variable; early milkings had a reddish-yellow colour due to the presence of carotenoid pigments and red blood cells. Colostrum had poor heat stability and early milkings were the most heat-labile. Colostrum was found to have good rennet coagulation properties but poor acid gelation properties. In several colostrum samples, casein did not precipitate at pH 4.6. These samples seem to have experienced a shift in pI, possibly due to high levels of glycosylated κ-casein.

In recent years, colostrum has attracted considerable interest as a functional food ingredient due to its superior nutritional and protective value compared to mature milk. Colostrum has reported benefits for the treatment of a wide range of conditions, e.g., various gastrointestinal disorders, respiratory tract infection, rheumatoid arthiritis and healing injured tissues of the body. In **Chapter 3**, various fractions of colostrum were characterised and tested for their effect on the secretion of pro- and anti-inflammatory cytokines from a macrophage cell line and bone marrow dendritic cells, as well as insulin secretion from a pancreatic beta cell line. Buttermilk from colostrum caused a significant reduction in the secretion of

the pro-infammatory cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-12, and a significant increase in the secretion of the anti-inflammatory cytokine, IL-10. Based on these findings, buttermilk from colostrum may represent a functional food product/ingredient which targets low-grade systemic inflammation and consequently the development and progression of several multifactorial disorders including artherosclerosis, metabolic syndrome, type 2 diabetes, and cardiovascular diseases. It is speculated that the reduction in the secretion of pro-inflammatory cytokines and increase in the secretion of anti-inflammatory cytokines observed in **Chapter 3** may have been due to milk fat globule membrane (MFGM) associated proteins which were present in higher concentrations in buttermilk from colostrum compared with buttermilk from mature milk, i.e., xanthine oxidoreductase, periodic acid Schiff 6/7, butyrophilin and adipophilin. The inflammatory potential of buttermilk from colostrum may also be due to its carbohydrate and/or lipid components, several of which are also up-regulated in buttermilk from colostrum compared with buttermilk from mature milk. The serum phase of colostrum caused a significant increase in insulin secretion when administered to a pancreatic β-cell line. These results suggest that the serum phase of colostrum may be of benefit in the treatment of type 2 diabetes, where the glucose-sensing capacity of pancreatic β-cells is reduced. The insulinotrophic potential of the serum phase of colostrum observed in **Chapter 3** may have been due to essential amino acids and/or free fatty acids.

Mastitis is an inflammation of the mammary gland which is characterised by an increase in the somatic cell count (SCC) and level of proteolysis in milk. In **Chapter 4**, proteomic changes in the milk of 8 cows in response to infusion with the endotoxin lipopolysaccharide (LPS) at quarter level in a model mastitic system were examined. A significant increase in the SCC of milk from LPS-challenged cows was observed, which was reflected by marked differences in the corresponding protein and peptide profiles. Generally, milk samples from LPS-challenged quarters showed an increase in the level of hydrolysis of the caseins and an increased concentration of serum proteins. Production of biologically active proteins and peptides in this

manner would require an intramammary injection of LPS to mimic bacterial invasion without an actual live bacterial infection and may represent a novel alternative to traditional methods; however, ethical issues regarding animal welfare need to be investigated. The pH 4.6-soluble fraction of this milk was found to cause a significant induction in the secretion of IL-10 from a murine macrophage cell line. IL-10 is an anti-inflammatory cytokine, the primary function of which appears to be the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells. As mentioned in **Chapter 3**, development of a functional food product/ingredient which targets IL-10 may have potential in treating low-grade systemic inflammation and several associated disorders.

One aspect which most of the methods currently being used for the generation of bioactive peptides have in common is that they all use protease activity. In **Chapter 5**, heat-induced hydrolysis of sodium caseinate was investigated as an alternative to enzymatic hydrolysis of the caseins from the viewpoints of protein breakdown and peptide formation. Results showed that at 130°C and pH 7, extensive hydrolysis of α_{S1}-, α_{S2}-, β- and κ-caseins occurred over a 120 min period, after which time very little intact protein remained. In total, 1023 peptides were identified and characterised in the 2% trichloroacetic acid (TCA)-soluble fraction of heat-hydrolysed sodium caseinate, of which 326 originated from α_{S1}-casein, 125 from α_{S2}-casein, 353 from β-casein and 219 from κ-casein. With the exception of κ-casein, peptides were uniformly dispersed throughout the entire sequence of the each protein. The vast majority of the 1023 peptides identified were present at trace levels, but eleven peptides were produced in abundance. Of these, 7 originated from α_{S1}-casein, 1 from β-casein and 3 from κ-casein. Three of the peptides identified in **Chapter 5** have previously been reported to have biological activities relating to treatment of high blood pressure, modulation of the immune system and inhibition of microbial growth. A peptide fraction produced during heat-induced hydrolysis of sodium caseinate was found to cause a significant increase in the secretion of the anti-inflammatory cytokine, IL-10, from a murine macrophage

cell line. As discussed in **Chapters 3** and **4**, peptides which regulate IL-10 *in vivo* may be used for the treatment of various inflammatory disorders. Results from **Chapter 5** provide a good body of evidence to suggest that heat-induced hydrolysis represents an alternative to enzymatic or microbial hydrolysis of milk proteins for the generation of bioactive peptides.

Chymosin is the most commonly used enzyme in the food industry. Proteolysis in cheese is extremely complex as it is catalysed by proteinases and peptidases from five sources, i.e., the coagulant, the milk, starter lactic acid bacteria, non-starter lactic acid bacteria and secondary starter. In **Chapter 6**, sodium caseinate was hydrolysed by chymosin in an attempt to reduce the complexity of proteolysis which occurs in cheese and the effects of sodium caseinate hydrolysed by chymosin on the gut-derived satiety hormone glucagon-like peptide-1 (GLP-1) were investigated. Initially, three sodium caseinate hydrolysates were produced with varying degrees of hydrolysis, i.e., low, medium and high, of which the sample with the medium degree of hydrolysis caused the greatest secretion of GLP-1 from STC-1 cells. In addition, this sample significantly reduced feed intake in mice when injected intraperitoneally when compared with control mice. Several optimisation steps were investigated to make the hydrolysis protocol more suitable for large scale commercial production, i.e., increasing total solids level, varying the pH at which hydrolysis was carried out, and reduction of the enzyme:substrate ratio. Fractionation of the crude hydrolysate by isoelectric precipitation of casein and ultrafiltration of the soluble fraction showed that the bioactive component(s) was enriched in the < 1 kDa fraction. The three most abundant peptides produced during hydrolysis of sodium caseinate by chymosin were identified and characterised by LC-MS and subsequently synthesised and tested for their effect on GLP-1 secretion; however, no effect was observed. Even though the bioactive component/s was not identified, this sodium caseinate hydrolysate had good *in vitro* and *in vivo* effects on GLP-1 secretion and feed intake, and hence, would make a suitable candidate for a functional food product/ingredient for the treatment of obesity and its related diseases by targeting gastric satiety signals.

Overall, the studies described in this thesis expand on current knowledge and provide good evidence for the use of novel methods for the isolation, generation and characterisation of bioactive proteins and/or peptides.

7.2 General conclusion

Functional foods are those which possess characteristics that help in maintaining or achieving good health, in addition to providing basic nutrition. In recent years, the demand for functional food products has risen considerably due to a number of factors, i.e., increased awareness by the public of the links between diet and disease, increased interest in healthier lifestyles, and rising healthcare costs. Milk, and its various components, are a rich source of bioactive compounds with the potential to promote human health and wellbeing.

In particular, the results in this thesis indicate that colostrum represents a potential functional food product/ingredient which is rich in naturally occurring bioactive components, e.g., immunoglobulins, MFGM proteins, lactoferrin, cytokines, growth factors, oligosaccharides and lipid components. Special care is needed when processing colostrum, as its composition and physico-chemical properties differ markedly from that of mature milk, e.g., low pH, high concentration of serum components, non-sedimentability of a large proportion of casein micelles at 100,000 g for 1 h and non-precipitability of casein micelles at pH 4.6.

Numerous studies have reported the health benefits of milk-derived bioactive peptides which, in most cases, are not active within the native protein molecule. Enzymatic hydrolysis is the most commonly used method in the generation of bioactive peptides. The results described in this thesis provide information on a number of alternative methods in the generation of such peptides, i.e., heat-induced hydrolysis, and amplification of the activity of indigenous milk proteases in a model mastitic system. Peptides produced by these routes may have potential in

the treatment of various inflammatory and immune-related disorders. In addition, a hydrolysate of sodium caseinate was shown to increase the secretion of the gut-derived satiety hormone, GLP-1, which may represent a functional food product/ingredient which targets obesity and its associated complications. Further work is needed to evaluate the commercial feasibility of these methods as well as the mechanisms involved in both their production and biological activities.

7.3 Suggested future work

Chapter 2

- The high viscosity of certain colostrum samples was considered the cause of non-sedimentability at 100,000 g x 1 h of a high proportion of casein micelles. This hypothesis could be tested by investigating the effect of viscosity of mature milk on sedimentability at 100,000 g x 1 h of casein micelles. This would involve increasing the viscosity of mature milk using a thickening agent, e.g., starch.
- The reason for the non-precipitability of casein micelles at pH 4.6 in certain colostrum samples was unclear, although high levels of glycosylated κ-casein were thought to be responsible. This hypothesis could be tested by investigating the effect of varying the level of glycosylation of κ-casein in mature milk on isoelectric precipitability of casein micelles.

Chapter 3

- Buttermilk and the serum phase of colostrum displayed *in vitro* anti-inflammatory and insulinotrophic properties. Further work is needed to determine if these *in vitro* bioactive effects can be replicated *in vivo*. This is also true for Chapters 4 and 5.

Chapter 4

- The protein and peptide profile of LPS-induced mastitic milk were analysed by polyacrylamide gel electrophoresis and reversed phase-ultra performance liquid chromatography. A more detailed proteomic characterisation by mass spectrometry would enhance understanding of changes which occur in mastitic milk.
- A pH 4.6-soluble fraction of LPS-induced mastitic milk showed good anti-inflammatory properties. The next step in identifying the bioactive component(s) would involve a more comprehensive fractionation of these samples.

Chapter 5

- Heat-induced hydrolysis of sodium caseinate was investigated at 130°C and pH 7 over 120 min. It would be interesting to investigate the effect of altering the time, temperature and pH on heat-induced hydrolysis of sodium caseinate and possibly other milk proteins.
- A peptide fraction of heat-hydrolysed sodium caseinate showed good anti-inflammatory properties, most likely due to one of eleven major peptides. Isolation/synthesis and testing of these peptides would confirm this.

Chapter 6

- Following on from this study, an animal trial was performed looking at the effects of a sodium caseinate and chymosin hydrolysate on feed intake in pigs (data not shown). No significant differences were observed compared with pigs fed an unhydrolysed control. Based on these results, the bioactive component(s) do not appear to survive gastric transit. Therefore, encapsulation of the hydrolysate may be required in the future.