

REVIEW

Structural and functional characteristics of bovine milk protein glycosylation

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Received on August 28, 2013; revised on December 10, 2013; accepted on December 17, 2013

Most secreted and cell membrane proteins in mammals are glycosylated. Many of these glycoproteins are also prevalent in milk and play key roles in the biomodulatory properties of milk and ultimately in determining milk’s nutritional quality. Although a significant amount of information exists on the types and roles of free oligosaccharides in milk, very little is known about the glycans associated with milk glycoproteins, in particular, the biological properties that are linked to their presence. The main glycoproteins found in bovine milk are lactoferrin, the immunoglobulins, glycomacropeptide, a glycopeptide derived from κ -casein, and the glycoproteins of the milk fat globule membrane. Here, we review the glycoproteins present in bovine milk, the information currently available on their glycosylation and the biological significance of their oligosaccharide chains.

Keywords: glycomacropeptide / glycoproteins / glycosylation / lactoferrin / milk fat globule membrane

Introduction

Milk is a fitting model for the delivery of health benefits to nourish and protect infants. Its composition within a mammalian species is suggestive of the neonatal requirements of the offspring of that species, providing the optimum levels and suitable types of nutrients required during the newborn phase of its lifecycle. Human milk components, in addition to providing nutrition, have been proposed to offer additional benefits such as stimulation of development and regulation of the newborn digestive system (German et al. 2002), enhanced absorption of iron (Fe^{3+}) and calcium (Ca^{2+}), stimulation and function of the immune system (Rivero-Urgell and Santamaria-Orleans 2001) and promotion of the development of the brain and nervous

system (Wang and Brand-Miller 2003). Indeed, a number of studies have linked newborn milk with health outcomes later in life (Morley and Lucas 2000).

Within milk, various classes of bioactive components exist (reviewed by Steijns 2001; Severin and Wenshui 2005). Research to date in this area has mainly concentrated on the biological properties of milk proteins and peptides with many studies demonstrating activity *in vitro*, in animal models and in infant and adult humans (Lönnardal 2003, 2004). Biological activities associated with such proteins/peptides include immunostimulating, antibacterial, antihypertensive and opioid-like properties (reviewed by Hayes, Ross, et al. 2007; Hayes, Stanton, et al. 2007). In addition, milk lipids possess therapeutic properties and efforts have been made to enhance the presence of certain fatty acids, such as conjugated linoleic acid, in milk through dietary manipulation (Jenkins and McGuire 2006). However, little attention has been paid to the carbohydrate fraction of milk. Until as recently as 30 years ago, the primary interest in sugars in biology was as sources of energy or in cellular structure. However, in recent years, it has become apparent that carbohydrates, whether free or bound to proteins or lipids, play essential roles as communication molecules in many intercellular and intracellular processes.

There are very few commercial products on the market that capitalize on the functions of carbohydrates within human milk. This is mainly due to the fact that the large quantities of human milk carbohydrates required for clinical trials are unavailable. In this respect, researchers are beginning to focus their attention on the milk of domestic animals, as a source for novel functional carbohydrates. Bovine milk is an ideal candidate, given its wide availability and its use in so many regularly consumed dairy products. The carbohydrate fraction of bovine milk is divided into lactose (48 g/L), free oligosaccharides (0.05 g/L) (Bode 2012) and bound glycans or glycoconjugates. Sriwilajaroen et al. (2012) recently quantified 374.9 nmol/g of *N*-glycans in dry, delipidated whey protein concentrate. Similar methods could be employed to determine the glycan content of whole milk. Lactose is a disaccharide composed of one glucose residue and one galactose residue and accounts for the majority of the carbohydrate content of bovine milk. Free oligosaccharides and glycoconjugates in contrast are less abundant. The free oligosaccharides identified in bovine milks can be divided into two classes; neutral (uncharged) oligosaccharides and acidic oligosaccharides, which are decorated with sialic acid (*N*-acetyl-neuraminic acid (Neu5Ac) and *N*-glycolyl neuraminic acid (Neu5Gc)) residues which confer a negative charge to

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the structures (Barile et al. 2010). There have been up to 40 oligosaccharide structures confirmed in bovine milks to date (Tao et al. 2008; Barile et al. 2010; Mariño et al. 2011). The most prominent acidic oligosaccharides include 3'-sialyllactose, 6'-sialyllactose and sialyllactosamine. Glycosaminoglycans/proteoglycans, such as heparan sulfate and chondroitin sulfate, are also present in the milk fat globule membrane (MFGM) (Coppa et al. 2013).

Glycoconjugates are present in two forms in bovine milk: glycolipids and glycoproteins. There are two types of glycolipids in milk, neutral glycolipids and acidic (sialic acid containing) glycolipids, also known as gangliosides. Approximately 70% of bovine milk glycolipids are associated with the MFGM and extensive literature exists on milk glycolipids (Newburg and Chaturvedi 1992; Sánchez-Juanes et al. 2009). Therefore, this review will focus specifically on glycosylation of bovine milk proteins and the biological role of the attached glycans. Protein glycosylation is a posttranslational modification which occurs in the endoplasmic reticulum and Golgi apparatus of the mammary gland through the action of various glycosyltransferases. Glycosylation is thought to be involved in receptor–ligand interactions, communication, host–pathogen interactions as well as correct protein folding, secretion of glycoproteins in their active conformation and protection of proteins from digestion. The review will focus on the structural properties and beneficial effects which are linked to the glycan chains on individual bovine milk proteins (caseins, whey proteins and those associated with the MFGM) (Figure 1). Complementary reviews have previously been published on free and bound glycans in human milk (Bode 2012; Peterson et al. 2013).

The glycome of bovine milk proteins

There are two types of protein glycosylation present in bovine milk: N-linked, where the glycan chain is covalently linked via an *N*-acetyl glucosamine molecule to the amide side chain of an asparagine residue, and O-linked, where the glycan chain is covalently attached to the hydroxyl oxygen of a serine or threonine residue (Vance et al. 1997). An analysis of the literature reveals that O-linked glycans in bovine milk are predominantly core-1 structures while oligomannose, complex and hybrid type

N-glycans have been described, as discussed in more detail later in this review. These glycan chains can be mono-, bi-, tri- or tetra-antennary structures, depending on the number of branches within the glycan chain (Nilsson 1994).

A recent study by Nwosu et al. (2012) compared the *N*-glycome of human and bovine milk. The researchers revealed an abundance of fucosylation in human glycoproteins, whereas an abundance of sialylation was observed for the glycans released from bovine milk glycoproteins. The presence of Neu5Gc was exclusive to bovine glycoproteins, with 1% of glycans containing both Neu5Ac and Neu5Gc residues. Approximately 31% of bovine milk *N*-glycans were found to be fucosylated, whereas only a small percentage of free oligosaccharides have been shown to contain fucose residues (Tao et al. 2008; Aldredge et al. 2013), suggesting that protein-bound oligosaccharides are the major source of fucose in bovine milk. Such distribution patterns emphasize the biological importance of protein-bound glycans to the young mammal. The differences between the human and bovine glycoprofiles may be indicative of the different biological needs of their offspring including nutritional and immune requirements. Indeed the profiles may reflect the requirement for protection against different pathogenic infections and the varying microflora which colonize the gut of the newborn human and calf. Interestingly, a study by Takimori et al. (2011) demonstrated dynamic glycosylation of bovine milk protein over lactation through the use of MALDI-TOF MS. The degree of sialylation and fucosylation (mono-tri) was greatest in colostrum, with a higher Neu5Gc: Neu5Ac ratio also present when compared with mature milk. Indeed, changes in sialylation were predominantly associated with immunoglobulin glycosylation. The results of this particular study will be discussed in more detail in the following sections as individual proteins are examined.

Bovine milk glycoproteins

Lactoferrin

Lactoferrin (LF) is a single chain, iron-binding, glycosylated (Kumar et al. 2003) member of the transferrin family (Hutchens and Lönnnerdal 1997) present in the whey protein fraction of milk (Severin and Wenshui 2005) (UniPortKB/

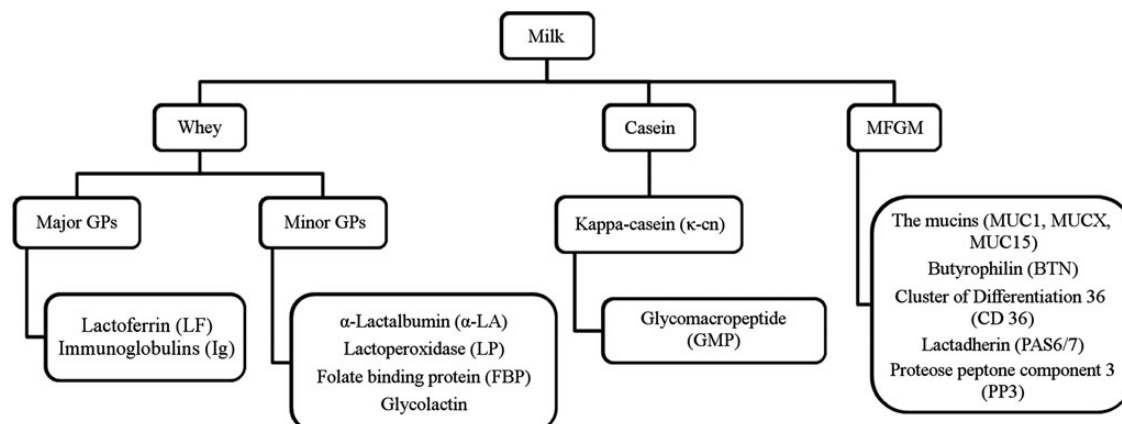


Fig. 1. Overview of glycoprotein distribution in bovine milk.

SwissPort P24627). LF is an important bioactive molecule for the protection of a newborn in the first few days of the life (Gopal and Gill 2000) and it has been identified in the milk of a variety of animals including human, cow, pig, horse, buffalo, sheep, goat, camel and mouse (Conesa et al. 2008). The concentration of LF is lower in bovine milk when compared with human milk and is at its highest in bovine colostrum (2–5 mg/mL) decreasing sharply as lactation continues (0.1–0.3 mg/mL) (Recio et al. 2009). Bovine LF (bLF) concentration is dependent on the breed of cow, stage of lactation and daily milk production of the animal (Cheng et al. 2008; Król et al. 2010).

LF Glycosylation. LF has one to five N-glycosylation sites, depending on the LF species (Severin and Wenshui 2005). For example, mouse LF has only one potential N-glycosylation site (Asn476), human LF (hLF) has three (Asn137, Asn478 and Asn623) and bLF, caprine and ovine LF have five potential N-glycosylation sites (233, 281, 368, 476 and 545) (Pierce et al. 1991; Baker and Baker 2005). The bLF protein backbone (703 aa) consists of two homogenous lobes (the N- and C-lobes), with 69% sequence identity with hLF. However, even though the 3D structures are similar, the fully folded proteins are not entirely super-imposable (Severin and Wenshui 2005). As this review focuses on the structure of the glycan chains associated with milk glycoproteins, we refer the reader to the many excellent reviews which exist on the peptide fraction of bLF (Moore et al. 1997; Legrand et al. 2008; Baker and Baker 2009; González-Chávez et al. 2009).

The glycan component of bLF accounts for 6.7% (van Leeuwen et al. 2012) to 11.2% (Coddeville et al. 1992) of the total molecular weight of bLF and the glycan chains of bLF have a highly heterogeneous structure, containing GlcNAc, GalNAc, Gal, Fuc, Man, Neu5Ac and Neu5Gc. Prior to the identification of Neu5Gc, the molar ratios of bLF's monosaccharide content were calculated as Man 4.3: GlcNAc 2: Gal 0.4: GalNAc 0.4: Fuc 0.2: Neu5Ac 0.2 (Coddeville et al. 1992), with the abundance of mannose and GlcNAc, as a result of their presence in the N-glycan core structure. There are two bLF variants present in bovine milk, bLF-a and bLF-b. The bLF peptide has a molecular mass of 77 kDa, while the molecular masses of bLF-a and bLF-b are 84 and 80 kDa, respectively (Yoshida et al. 2000). bLF-b has four N-glycan chains attached at Asn-233, Asn-368, Asn-476 and Asn-545, while bLF-a has a fifth at Asn-281 (Wei et al. 2000), which accounts for the difference in the molecular weights of the two variants. Of the total bLF present, bLF-a represents ~30% in colostrum and 15% in mature milk (van Veen et al. 2002).

Previously, only complex and oligomannose glycans were described for bLF. However, with the development of new and more advanced analytical techniques, hybrid structures have recently been shown to be associated with bLF (Nwosu et al. 2011). There is extensive glycan microheterogeneity present in bLF, with complex, hybrid and oligomannose type glycans identified at each of the individual loci (Nwosu et al. 2011). van Leeuwen et al. (2012) identified 42 N-glycan structures, whereas Hua et al. (2011) identified 59 distinct N-glycans. Asn-233, Asn-368 and Asn-476 showed the highest level of heterogeneity (Nwosu et al. 2010). Features such as core fucosylation, terminal

sialylation and the presence of the N-acetyl lactosamine (Gal- β -(1 $\rightarrow$ 4)-GlcNAc, LacNAc), N,N'-diacetyl lactosamine (GalNAc- β -(1 $\rightarrow$ 4)-GlcNAc, LacdiNAc) and Gal- α -(1 $\rightarrow$ 3)-Gal motifs are characteristic of bLF's glycans (Coddeville et al. 1992; Hua et al. 2011; van Leeuwen et al. 2012).

In total, 76% neutral, 9% mono-sialylated and 15% di-sialylated glycan structures were detected in bLF, with 8.5% of the sialic acid detected accounted for by Neu5Gc. The glycans have also been classified as 65% oligomannose type, while the remaining 35% are complex and hybrid type (van Leeuwen et al. 2012). Multiple isomers of the high-mannose type structures containing five to nine mannose residues were observed, with 38% of the total mannose structures accounted for by Man8. The majority of these glycans were present at Asn-233 (65%), with trace levels at Asn-281 and Asn-368 (1 and 3%, respectively) (Hua et al. 2011), suggesting a prominence of complex and hybrid type structures at the latter glycosites. The abundance of high-mannose structures may be linked to the importance of bLF as a decoy receptor to prevent infection in the intestine (as discussed later).

As mentioned previously, Takimori et al. (2011) characterised total bovine milk protein glycosylation over lactation by MALDI-TOF MS. For bLF, they characterized the most highly substituted structures in colostrum, with Neu5Gc containing structures and glycans containing both sialic acid and fucose residues only present in samples taken from day one of lactation. Barboza et al. (2012) also observed distinct glycosylation patterns during the transition from colostrum to mature milk for hLF, including an increase in total glycan intensity and higher order fucosylation from week 2 onwards.

Some of the glycan structures present on bLF but absent in hLF have been linked to undesirable effects in humans. The Gal(α 1-3)Gal epitope has been linked to allergen related immune responses, in particular tick bites (Commings et al. 2011) while Neu5Gc (which humans do not produce due to a genetic mutation) has been found to be present in both healthy (Tangvoranuntakul et al. 2003) and cancerous human tissue (Malykh et al. 2001) and has been suggested to have a role in the development and metastasis of some carcinoma cell types (Makatsori et al. 1998; Malykh et al. 2001). However, there is no evidence linking the consumption of bovine milk or bLF to these effects.

Glycosylation and Protein Structure. Sialic acid content determines the charge associated with an oligosaccharide chain. It is a strong acid with a pK_a of 2.6 and, at physiological pH, it exists in a deprotonated form (Jaques et al. 1977), giving a negative charge to the bLF surface. It has an important role in stabilizing the glycoprotein structure and is responsible for the binding of Ca^{2+} ions which ensure stronger interaction between the glycans and the peptide chain. Ca^{2+} binding of the glycoprotein enhances its stability with regards to chemical and thermal denaturation. However, this characteristic is lost when the protein is desialylated (Rossi et al. 2002).

The glycan chains may also have a role in protecting the bLF protein from proteolysis. A difference in the susceptibility of bLF-a and bLF-b to trypsin digestion has been observed (van Veen et al. 2004). bLF-a was found to be ~10-fold less

susceptible to proteolysis when compared with bLF-b, which was attributed to the presence of the extra glycan chain at Asn-281 in bLF-a. The major trypsin cleavage site in bLF is at Lys282 and the glycan chain at Asn-281 may block the action of the enzyme at this site. [van Veen et al. \(2004\)](#) also concluded that although hLF was found to be ~100-fold more resistant to proteolysis when compared with bLF, the N-glycosylation does not seem to play a role in this characteristic. Instead, the 3D conformation of the hLF protein may result in its digestion sites being less accessible for trypsin degradation.

bLF is an iron-binding glycoprotein, and the structure of bLF is crucial for its unique iron-binding property. [Legrand et al. \(1990\)](#) found that complete deglycosylation of LF resulted in a decrease in the protein's iron-binding capacity. However, [Moore et al. \(1997\)](#) have suggested that glycosylation may not be directly involved in iron chelation as only 50% of bound iron was lost after removal of the glycan chains and no iron was found to bind to the released glycans. They believe the glycan moieties of bLF are involved in maintaining the stability of the Fe^{3+} saturated holoLF structure by preventing the distancing of the iron molecules from the glycoprotein and limiting the mobility of the C-lobe. It has been suggested that the glycan chain attached to Asn 545 may be involved in the release of iron from the C-lobe of bLF, as it is located near the hinge region of the iron-binding cleft ([Moore et al. 1997](#)). This glycan chain may contribute to interdomain interactions, stabilizing the C-lobe and influencing iron release. A similar role has been proposed for the glycan chain at Asn518 in camel LF ([Khan et al. 2001](#)).

As a result of its iron-binding activity, milk LF has been suggested to be involved in iron absorption through interaction with the brush-border membrane. LF binds to the intestinal membrane and then recruits free iron from the surrounding environment for absorption. The removal of fucose from the glycan chains of both human and monkey LF resulted in a significant reduction in binding of the LF to the brush-border membrane of rhesus mouse jejunum tissue ([Davidson and Lönnardal 1988](#)) suggesting that the fucosylated glycans may be involved. However, [Kawakami et al. \(1990\)](#) demonstrated that it was the peptide chain of bLF, and not the glycan moieties, that were responsible for bLF association with the brush-border membrane in the rat intestine. Fuc is present at low levels in bLF when compared with hLF, where fucosylated glycans are predominantly present ([Barboza et al. 2012](#)) and, therefore, it may not be as important for bLF's bioactivity.

Biological Activities Associated with LF Glycosylation. bLF has a wide variety of associated biological activities including antimicrobial ([Gonzalez-Chavez et al. 2009](#)), immunomodulatory ([Debbabi et al. 1998](#)), prebiotic ([Kim et al. 2004](#); [Rahman et al. 2009](#)), stimulation of bone formation ([Cornish et al. 2004](#)) and anticancer ([Tsuda et al. 2002, 2010](#)). The bioactivities of LF have previously been reviewed by several authors ([Adlerova et al. 2008](#); [García-Montoya et al. 2012](#)). Here, we will focus specifically on the information currently available on the role glycan chains play in bLF bioactivities.

bLF has been found to compete with lipopolysaccharide (LPS) Ca^{2+} -binding sites on bacterial membranes and chelate

loosely bound Ca^{2+} , which is involved in the stabilization of the anionic LPS in the outer membrane of bacterial cells. Sialic acid is thought to play a role in this activity considering its calcium-binding properties ([Jaques et al. 1977](#)) and the fact that desialylated bLF was found to lose its Ca^{2+} chelating characteristic. The carbonate ion (COO^-) on the deprotonated sialic acid competes for the loosely bound Ca^{2+} ions, resulting in the release of LPS and destabilization of the bacterial membrane ([Rossi et al. 2002](#)). Sialylation of bLF is also believed to be important in preventing *Helicobacter pylori* colonization in a mouse model ([Wang et al. 2001](#)) and in the inhibition of haem-agglutination by the influenza virus ([Kawasaki et al. 1993](#)).

bLF has been reported to act as a non-specific defense molecule against invading pathogens and the glycan chains are important in this activity. [Teraguchi et al. \(1996\)](#) have identified a role for the oligomannose glycans of bLF in preventing *Escherichia coli* colonization. Members of the Enterobacteriaceae family, including *E. coli*, express type 1 fimbriae which can recognize and bind to oligomannose glycan chains on eukaryotic cell surfaces ([Abraham et al. 1988](#)) which can facilitate bacterial adhesion to and invasion of the cells. [Teraguchi et al. \(1996\)](#) demonstrated that the high-mannose-type glycans on bLF acted as receptors for the mannose-specific type 1 fimbriae, therefore preventing *E. coli* interaction with the eukaryotic cell by acting as a decoy receptor. When hLF was tested, this activity was not observed. This may be due to the presence of only complex type glycans on the human protein. The same group went on to demonstrate that bLF caused the agglutination of type 1 fimbriated *E. coli* cells as a result of the specific interaction between the mannose residues on the glycoprotein and the type 1 fimbriae of the bacteria.

[Yoshida et al. \(2000\)](#) compared the antimicrobial activity of the two bLF variants against *E. coli* and found the bLF-a displayed a greater antibacterial activity when compared with bLF-b. bLF-b does not exhibit as dramatic an effect on *E. coli* growth and the difference in the glycan chains may be responsible for this varying antimicrobial activity ([Wei et al. 2001](#)). The concentration of bLF-a in colostrum is higher when compared with mature milk, indicating a role for this variant in the calf's primary defense against infection. Cholera toxin is produced by *Vibrio cholerae* and causes the symptoms such as diarrhea and in some cases death ([Holmgren 1981](#)). bLF inhibited the binding of this toxin to Chinese hamster ovary (CHO)-k1 cells and ganglioside G_{M1} by interacting with the cholera toxin given its homology to the oligosaccharide receptor ([Kawasaki et al. 1992](#)). This activity is dependent on the glycan chains of bLF, as treatment with sialidase resulted in the complete loss of cholera toxin binding.

The continued presence of bLF in bovine colostrum through to mature milk suggests that the antimicrobial activity is important for the infant mammal. As research in the area advances, the role of the glycan chains as direct antimicrobial agents and as decoy receptors to prevent infection is becoming more apparent. The high-mannose glycans of bLF in particular make it an attractive potential ingredient in the functional food industry.

The glycan moieties of bLF, and LF from other animals such as sheep and goat which may have similar bioactive glycan structures, as compared by [Zinger-Yosovich et al. \(2011\)](#), could play an important role in promoting consumer health as nutraceutical

ingredients, as discussed later in this review. However, many of the mechanisms underlying the bioactivities associated with LF are still not fully understood and warrant further investigation.

Kappa-casein and caseinomacropeptide

Caseinomacropeptide (CMP) is a phosphoglycoprotein formed by chymosin hydrolysis of milk κ -casein (κ -cn) and released into the whey during cheese production. The remaining casein portion is precipitated in the cheese curd (Brody 2000). CMP is a 64 amino acid hydrophilic peptide and is cleaved from the C terminal of κ -cn at methionine (106) and terminates at valine (169) (Delfour et al. 1965). CMP has multiple O-glycosylation sites at various threonine (Thr) and serine (Ser) residues (Thoma-Worringer et al. 2006) and when in its glycosylated form, it is referred to as glycomacropeptide (GMP). The concentration of GMP in bovine milk has been shown to fluctuate over lactation, decreasing from colostrum for the first 2 months of lactation and increasing again thereafter, as monitored by the release of the peptide from κ -cn in milk by the action of rennin (Furlanetti and Prata 2003).

Up to 16 genetic variants of bovine κ -cn have been identified which have different post-translational modifications (PTMs) and vary in their level of phosphorylation and glycosylation (Thoma-Worringer et al. 2006). The average M_r for CMP is 7500 Da, whereas the highest recorded M_r 9631 Da (Molle and Leonil 2005). Thr166 can either be phosphorylated or glycosylated (Holland et al. 2006) and no CMP variant has been identified which lacks a phosphate group as Ser149 is always phosphorylated (Vreeman et al. 1986). When a CMP peptide has multiple phosphate molecules attached, glycosylation is not observed. This may be due to inhibition of the glycosylation pathway *in vivo* through phosphorylation at Ser127 (Vreeman et al. 1986) or restricted access of the glycosyltransferases to the O-glycosylation site as a result of the phosphate groups.

Glycosylation has an effect on the physical properties of GMP (as reviewed by Neelima et al. 2013). At low pH, below pH 4.5, GMP self-aggregates, resulting in gelatinization and aggregate size and gel formation increases with decreasing pH, as a result of the hydrophobic side chains of sialic acid at low pH (Fariás et al. 2010). The solubility of GMP is influenced by its glycosylation (Taylor and Woonton 2009) and the emulsifying (Kreuz, Strixner, et al. 2009) and foaming (Kreuz, Krause, et al. 2009) properties of GMP are not as stable as that of the unglycosylated CMP. Casein micelle size has also been correlated with the presence of glycosylation on κ -cn (Bijl et al. 2014).

GMP glycosylation. Approximately 60% of CMP is glycosylated (Vreeman et al. 1986) (referred to as GMP) with exclusively O-linked glycans and this glycosylation is variable and influenced by stage of lactation and the genetic phenotype of κ -cn (Dziuba and Minkiewicz 1996). Several groups have elucidated the glycan structures of bovine GMP (Fournet et al. 1975; van Halbeek et al. 1980; Saito, Itoh, Adachi, et al. 1981; Saito, Itoh, Adachi, Suzuki, et al. 1981, 1982; Fiat et al. 1988; Saito and Itoh 1992). Both neutral and acidic core-1, O-glycans have been identified (Figure 2), with sialylation a prominent feature; 82% of glycans from bovine GMP were either mono- or di-sialylated (Hua et al. 2011). Although Neu5Gc has been reported in ovine κ -cn (Addeo et al. 1978), only Neu5Ac has been detected in the bovine form. Interestingly, no Neu5Ac-GalNAc structure was observed, suggesting that completion of the core-1 disaccharide is necessary before further addition to the glycan chain can precede (Saito and Itoh 1992). Several potential glycosites have been identified in GMP; however, only three are substituted in the most highly glycosylated forms (Molle and Leonil 1995). It is possible that trace glycosylation is present at the other glycosites but at levels outside the limits of the assay sensitivity used by Molle and Leonil (1995).

Colostrum GMP has an elevated oligosaccharide content (Guérin et al. 1974). Only GalNAc, Gal and Neu5Ac have been identified in GMP glycans from mature milk, but glycans from colostrum samples in addition contain GlcNAc and Fuc. Furthermore, a greater number of glycans and more complex structures have been identified in colostrum GMP (Fiat et al. 1988), as can be seen in Figure 2. A disialylated tetrasaccharide is the most abundant glycan present in mature GMP, accounting for 56% of the glycan structures (Saito and Itoh 1992), and this high level of sialylation is vital for GMP's biological activities, as will be discussed later. As a result of the high level of sialylation, GMP has a lower pI when compared with the non-glycosylated peptide (Kreuz, Strixner et al. 2009), a feature which has been utilized in the separation of GMP and CMP (Kreuz and Kulozik 2009).

Previously, glycosylation of GMP was thought to be a random event, with glycan chains attached to Thr or Ser residues with no obvious pattern (Saito and Itoh 1992). However, with techniques in glycoscience evolving, Holland et al. (2005) were able to provide evidence that this was not the case. Through the use of 2D gel electrophoresis and tandem mass spectrometry, they described a glycosylation model for GMP, with a hierarchy of glycan addition to individual glycosites.

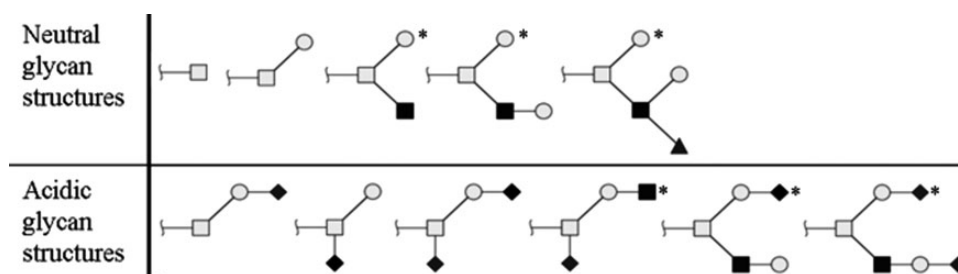


Fig. 2. Neutral and acidic structures identified in bovine GMP. Asterisk denotes only present in GMP isolated from colostrum. Open square, GalNAc; open circle, Gal; filled square, GlcNAc; filled diamond, Neu5Ac; filled triangle, Fuc (Fournet et al. 1975; van Halbeek et al. 1980; Saito, Itoh, Adachi, et al. 1981; Saito, Itoh, Adachi, Suzuki, et al. 1981, 1982; Fiat et al. 1988; Saito and Itoh 1992).

The first glycan chain of GMP is always attached to Thr152, the second to Thr163 and the third to Thr154. In the absence of a glycan chain at Thr152, no further glycosylation appears to occur, suggesting that other sites remain latent until Thr152 is occupied. Further glycosylation may have been present at other glycosites, but at levels which were too low to be detected by the methods used by Holland et al. (2005).

This hierarchal system may be as a result of changes to the casein micelle structure following glycosylation at an individual glycosite (Holland et al. 2005). *N*-Acetylgalactosaminyl transferase (GalNAcT) is the enzyme responsible for the initial steps of O-glycosylation and exposure of the target glycosite on the surface of the folded protein is a requirement for enzyme activity. Attachment of the initial glycan chain to Thr152 may cause changes in the nearby secondary and tertiary structure of κ -cn, exposing Thr163 and other additional glycosites in a step-wise manner for GalNAcT accessibility.

In contrast with the model described by Holland et al. (2005), Hua et al. (2011) assigned 41% of the GMP's glycan chains to Thr154 and only 14% to Thr152, with 29% to Thr163. The remaining 16% were either associated with Thr142 or Thr157, or could not be assigned to a specific amino acid location due to the proximity of the glycosites. Further analysis is required to resolve the biological pathway for GMP glycosylation.

Biological Activities Associated with GMP Glycosylation. The biological activities of bovine GMP, including its prebiotic, antimicrobial and immunomodulatory effects, have previously been extensively reviewed (Brody 2000; Thoma-Worringer et al. 2006; Recio et al. 2009). Therefore, this section shall specifically focus on the information available on the biological role of the glycosylation of GMP.

The sialic acid content of GMP is vital for most of the biological roles ascribed to this molecule. Feeding GMP as a source of sialic acid has been shown to improve learning in piglets during early development (Wang et al. 2007) and increase Neu5Ac content of piglet saliva, influencing its viscosity and protective properties (Thoma-Worringer et al. 2006). Both the GMP peptide and glycan chains together increased cholecystokinin (CCK) release in rats, an intestinal peptide hormone which stimulates the digestion of fat and proteins. The effect was only observed for a slightly glycosylated form of GMP and was lost following neuraminidase treatment, emphasizing the importance of Neu5Ac (Beucher et al. 1994). Sialylated oligosaccharides and glycopeptides, including bovine GMP, have been shown to promote the growth of the bifidobacteria strains such as *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Bifidobacterium infantis* (Idota et al. 1994). The ability of GMP to support and promote the growth of healthy gut microflora suggests there may be potential to use this milk phosphoglycoprotein as a prebiotic in functional foods.

GMP has been shown to interact with toxins such as cholera toxin (Kawasaki et al. 1992; Oh et al. 2000) and *E. coli* heat labile enterotoxin (Isoda et al. 1994) via its glycans chains. As these and other toxins adhere to cells by interacting with carbohydrate receptors on the cell surface, this means GMP can act as a decoy because its glycans are similar in structure to

epithelial cell surface toxin receptors. Similar to bLF GMP inhibited the binding of cholera toxin to Chinese hamster ovary (CHO)-k1 cells and ganglioside G_{M1} and, again, this activity is dependent on GMP sialylation (Kawasaki et al. 1992; Oh et al. 2000). Isoda et al. (1994) obtained similar inhibitory results in the presence of GMP for *E. coli* heat labile enterotoxins LT-I and LT-II using the CHO-K1 model.

The glycan chains of GMP have also been shown to have antimicrobial activity against a variety of pathogens. GMP binds to *E. coli* and *Salmonella enteritidis* and this binding is reduced following sialidase treatment and abolished by periodate oxidation of the glycan chains (Nakajima et al. 2005). *Actinomyces viscosus*, which has been linked to oral infections, expresses fimbriae specific for the T-antigen, $\text{Gal}\beta(1-3)\text{GalNAc}$. The core-1 structure of GMP's O-glycans, therefore, can prevent hemagglutination by *A. viscosus*. Desialylation of GMP resulted in an increase in its hemagglutination inhibition activity, as the loss of terminal Neu5Ac residues exposed the core $\text{Gal}\beta(1-3)\text{GalNAc}$ structure (Neeser et al. 1988). Desialylation of GMP also results in inhibition of hemagglutination of chick erythrocytes by the influenza virus (Kawasaki et al. 1993). Interestingly, glycosylation was also shown to be important for human κ -cn's anti-infective activity against *H. pylori*, with fucosylation particularly vital for the inhibition of *H. pylori* adhesion (Aniansson et al. 1990; Strömqvist et al. 1995).

κ -cn derived glycopeptides inhibited the proliferative response of mouse spleen cells to LPS and phytohemagglutinin (PHA). This inhibitory activity was influenced by the level of sialylation. Inhibition of PHA-induced proliferation increased as the presence of Neu5Ac residues increased. The role of Neu5Ac was validated by the decrease in inhibition observed following neuraminidase treatment (Otani et al. 1995). In contrast, GMP has also been shown to enhance the proliferative and phagocytic activity of human macrophage-like cell line, U937, as a result of both its Neu5Ac content and its peptide backbone (Li and Mine 2004).

To perform its biological functions *in vivo*, GMP must first reach its target area of action. GMP can be liberated from κ -cn *in vivo* by the action of digestive enzymes following the ingestion of milk. Intact CMP, released from κ -cn in the stomach, has been detected in the plasma of humans at physiologically significant concentrations (Chabance et al. 1998; Thoma-Worringer et al. 2006). The occurrence of glycosylation limited CMP digestion by brush-border membrane endopeptidases, allowing it to be absorbed into the blood (Boutroux et al. 2008). The half-life of GMP was twice that of CMP and the degree of glycosylation directly influenced digestion as the most heavily glycosylated forms were digested more slowly. The attached glycans may block the action of the endopeptidases by steric hindrance, blocking access of the enzymes to the peptide backbone (Boutroux et al. 2008). The rate of CMP release from κ -cn was also reduced as Neu5Ac and the carbohydrate content increased (Doi et al. 1979; Addeo et al. 1984). Overall, the glycosylation of κ -cn and GMP protect the protein from digestive enzymes, ensuring the active forms reach the intestine and are absorbed into the blood stream. The release of GMP from κ -cn *in vivo* highlights its biological importance for the promotion of intestinal health.

Milk fat globule membrane

The MFGM is a trilayer consisting of proteins and phospholipids which surrounds the lipid droplets that are secreted by the lactating mammary gland, ensuring they remain dispersed throughout the milk rather than aggregating.

Proteins of the MFGM only account for ~1–2% of the total bovine milk protein content (Riccio 2004) and anything from 50 (Spitsberg 2005) to 120 (Reinhardt and Lippolis 2006) proteins have been identified on the bovine MFGM, varying depending on isolation methods, and a large number of these are glycopeptides (Spitsberg 2005). Some of the most researched glycosylated proteins of the MFGM are the mucins, butyrophilin (BTN), cluster of differentiation 36 (CD36) and lactadherin (PAS-6/7). The “PAS” name given to the MFGM glycoproteins relates to their positive reaction with periodic acid schiff (PAS) stain as a result of their glycosylation. The glycosylation patterns of each of these glycoproteins will be discussed individually in the following sections. In general however, bovine MFGM proteins contain predominantly mono- and di-sialylated core-1 *O*-glycans (Wilson et al. 2008) and bi-, tri- and tetra-antennary complex, high-mannose and hybrid type *N*-glycans (Sato et al. 1993). Significantly extended *O*-glycans were not detected on bovine MFGM (Wilson et al. 2008) and sialylation is present at a high level in both *N*- and *O*-linked glycan chains. It is worth noting the uniquely high level of *N*-acetylgalactosamine in the *N*-linked glycan chains associated with bovine MFGM, a feature absent in MFGM glycans from other species examined to date. In total, 29% of MFGM *N*-linked oligosaccharides contain terminal GalNAc in the form of GalNAc β (1–4) GlcNAc (*N,N*-diacetyllactosediamine; LacdiNAc), including 28% of the glycans on CD36 and 37% of the glycans on BTN, suggesting that it is a common feature among MFGM glycoproteins (Sato et al. 1993).

Studies examining MFGM glycoproteins over lactation have found that significant differences are present in colostrum samples when compared with MFGM samples from later in lactation (Reinhardt and Lippolis 2008). First, the concentration of certain glycoproteins was found to increase in milk taken at day 7 of lactation when compared with colostrum. For example, MUC1, MUC15 and BTN increased by 7.7-, 7.4- and 3.2-fold, respectively, in milk collected on day 7 when compared with colostrum (Reinhardt and Lippolis 2008). Ujita et al. (1993) observed a decrease in MFGM sialylation in the first 5 days postparturition, with a decrease in acidic oligosaccharide structures from 74% at day 1 to 59% at day 5. A similar pattern was observed by Wilson et al. (2008), who characterized the decline of a mono-sialylated core-2 *O*-glycans over lactation, which was a dominant structure at day 3 of lactation but reduced from then onwards. A general decrease in core-2 *O*-glycans was observed as lactation progressed, suggesting a regulation of the core-2 GlcNAc β (1–6) glycosyltransferase (Wilson et al. 2008).

The Mucins. Mucins are a family of long, macromolecular glycoproteins which are heavily glycosylated and their carbohydrate content is usually >30%. The main mucins identified in bovine milk include MUC15, MUC1 and MUCX and make up ~5–10% of the MFGM protein content (Patton 1999). The dominance of *O*-linked core-1 glycans, with

N-glycans present to a lesser extent, and high levels of sialylation are features of mucin glycosylation. These similarities in structure may be as a result of their origins on the apical plasma membrane of the lactating epithelial cell, which subsequently forms MFGM.

MUC15 (also known as PASIII, PAS3, glycoprotein C and glycoprotein 4, based on its mobility when using SDS–PAGE and positive PAS staining (Pallesen et al. 2002)) accounts for 0.08% of the total protein in raw milk and 1.5% of MFGM protein content. MUC15 has a molecular weight of 94 kDa, 65% of which is accounted for by *N*- and *O*-glycans (Pallesen et al. 2007). Bovine MUC15 has 14 *O*- and 15 *N*-glycosites on its extracellular domain (Pallesen et al. 2002). The *N*-linked glycans have been characterized as hybrid type glycans, with 11 of the 15 identified *N*-glycosites glycosylated (Pallesen et al. 2007). Core fucosylation, α (2–6) sialylation and terminal *N*-acetyl lactosamine (LacNAc) are characteristic of the *N*-glycans in MUC15. The *O*-linked glycans are core-1 structures, with both sialylated and unsubstituted structures identified. The ratio of monosaccharides on MUC15 was calculated as Fuc:GalNAc:GlcNAc:Gal:Man:Neu5Ac 1:4:6:5:4:5. Following PNGase treatment, equal quantities of GalNAc, GlcNAc, Gal and Neu5Ac were associated with the *O*-linked glycans (Pallesen et al. 2007).

MUC1 is predominantly *O*-glycosylated but *N*-glycosylation is also present, with five potential *N*-glycosylation sites identified. The carbohydrate content of MUC1 is ~57–65%, varying according to the number of tandem repeats present in the MUC1 protein (Pallesen et al. 2001). Approximately 28% of monosaccharide content of bovine milk MUC1 is Neu5Ac (Pallesen et al. 2001), due to the high level of sialylation of the *O*-linked core-1 glycans. The *N*-glycans are also sialylated, with trace levels of fucosylation detected (Sando et al. 2009). MUC1 interacted preferentially with Gram-negative bacteria, binding to *E. coli* and *S. typhimurium*, and preventing their adhesion to Caco-2 intestinal cells (Parker et al. 2010). Sialidase treatment significantly reduced this effect, identifying Neu5Ac as a key component in binding of MUC1 to bacteria.

It was originally thought that MUCX was simply a more glycosylated form of MUC1 but it is now believed that MUCX is in fact a unique mucin in itself. In comparison to MUC1, MUCX contains more glycosylation sites and the carbohydrate content makes up ~80% of its molecular weight (Patton 1999). MUCX is very loosely bound to the fat globule membrane and is easier to remove than MUC1. As seen in MUC1, MUCX is mainly *O*-glycosylated, but low levels of *N*-linked glycans were also identified. Again, core-1 *O*-glycans and an abundance of terminal Neu5Ac residues have been characterized for MUCX (Liu et al. 2005). However, when the lectin-binding profiles of both mucins were compared, MUCX interacted with a greater number of the lectins tested. This suggests that the glycans present on this mucin are of a more complex nature (Liu et al. 2005), indicating that the potential MUCX may have in binding to a wide spectrum of pathogenic microorganisms and thereby possibly preventing certain infectious diseases.

However, the biological role of bovine milk mucins remains unclear and, in particular, the importance *in vivo* of their dense glycosylation. The mucins may function as an element of an innate immune system present in milk to prevent against

bacterial invasion. Human and mouse mucins have been shown to inhibit *H. pylori* colonization (Hirno et al. 1998; McGuckin et al. 2007), with Neu5Ac identified as playing a role in *H. pylori* inhibition (Hirno et al. 1998). Binding to rotavirus and inhibition of HIV-1 transmission has also been attributed to human milk mucin glycosylation (Yolken et al. 1992; Saeland et al. 2009). Therefore, bovine mucins may potentially have similar antimicrobial activity when their high level of sialylation is considered. The dense glycosylation gives the mucins a strong water holding capacity and, therefore, the surrounding aqueous phase is extremely viscous and protects the peptide chain from degradation (Pallesen et al. 2007). The glycosylation of human MUC1 has been linked to their resistance to digestion in the stomach (Peterson et al. 1998), a role which may also be true for their bovine counterparts.

Butyrophilin. BTN accounts for 40% of the total protein in bovine MFGM (Mather and Jack 1993) and is exclusively N-glycosylated (Valivullah and Keenan 1989) at Asn55 and Asn215, with oligomannose, complex and hybrid type glycans identified (Sato et al. 1995). The carbohydrate content of BTN accounts for 4–6% of its molecular mass (Mather and Jack 1993) and the main monosaccharide residues include GlcNAc, Man and Gal (Heid et al. 1983). The glycan chains of BTN have also been found to contain Fuc and Neu5Ac residues (Sato et al. 1995). Nine structures have been identified at BTN. Hybrid type N-glycans have only been found at Asn215 and glycans containing the LacdiNAc motif were only detected at Asn55 (Sato et al. 1995). Bi- to tetra-antennary complex type glycans have been detected, with sialylation and fucosylation present at both N-glycosites (Sato et al. 1995).

BTN is believed to have a role in anchoring xanthine oxidase to the cytoplasmic surface in the MFGM structure and the proteins have been found to cross link with each other (Valivullah and Keenan 1989). Its presence at the apical surface of secretory cells in the mammary gland, as well as being conserved in a number of species, has led to the theory that BTN is involved in milk lipid secretion (Mather and Jack 1993). However, these theories have yet to be confirmed but a role for BTN in maintenance of the MFGM structure is suggested and no doubt the negative charge conferred by BTN's sialylated glycans could have a role in this activity.

Lactadherin. Lactadherin, also known as PAS-6/7 or milk fat globule-EGF factor 8 (MFG-E8), is present as two glycosylated variants with the same protein core on bovine MFGM. PAS-6 is a 50 kDa variant with 7.1% accounted for by its carbohydrate content and PAS-7 is a 47 kDa glycoform, with a 5.5% carbohydrate content (Kim et al. 1992). N- and O-linked glycan chains have been found on both variants, with Asn41 a shared N-glycosite between the two glycoforms. O-glycosylation is present at Ser9 in PAS-6 and at Thr16 in PAS-7. Biantennary N-linked glycans have been identified at Asn41 (Hvarregaard et al. 1996) but only PAS-6 is N-glycosylated at Asn209, with an unsubstituted core N-glycan structure attached (Kim et al. 1998). Sialylation and fucosylation have been detected in lactadherin glycans (Kim et al. 1998; Seok et al. 2001). For instance in relation to PAS-7, 33.3% of the glycans identified were sialylated, 64% were fucosylated and 18.7%

were modified with both Neu5Ac and Fuc residues (Seok et al. 2001). The N-linked glycans of PAS-7 are more complex, with a greater level of substitution when compared with those of PAS-6. Ten N-glycan structures have been characterized for PAS-7 (Seok et al. 2001), but only four for PAS-6 (Kim et al. 1998). A novel feature of the glycan chains in PAS-6 is the presence of α -linked Gal at the non-reducing end of the N-linked core (Kim et al. 1998). In contrast, terminal β -linked Gal residues are present in PAS-7 (Seok et al. 2001). This would suggest different pathways exist *in vivo* for the glycosylation of lactadherin leading to PAS-6 and PAS-7.

Again, the biological roles of the oligosaccharides on bovine lactadherin remain unclear. Human lactadherin, however, may inhibit rotavirus infection in a dose dependent manner, by blocking its entry into the host cell. In one study, the antiviral activity was lost upon the removal of sialic acid, suggesting that in this case the glycan chains, especially those which contain sialic acid, were vital for inhibiting this strain of rotavirus (Yolken et al. 1992). However, it should be noted that human and most animal rotavirus strains do not require the presence of sialic acid on the cell surface for efficient infectivity (Ciarlet and Estes 1999). Bovine lactadherin has also been shown to inhibit rotavirus replication in MA104 cells; however, the role of the glycan chains was not investigated (Inagaki, Nagai, et al. 2010). As described previously for other glycoproteins, glycosylation protects the lactadherin peptide chain in the acidic environment of the stomach (Peterson et al. 1998). Further research is required to confirm activities of bovine lactadherin glycosylation.

Cluster of differentiation 36. CD36 is a 78 kDa N-glycosylated protein which accounts for 2–5% of MFGM protein content (Greenwalt et al. 1992; Berglund et al. 1996). Eight N-glycosites have been identified in bovine MFGM CD36: Asn78, Asn101, Asn171, Asn204, Asn234, Asn246, Asn320 and Asn416 (Nakata et al. 1993). High-mannose type, hybrid type and bi-, tri- and tetra-antennary complex type glycans have been isolated, and more than one type of glycan chain can be located at each glycosite. Sixteen N-linked glycan structures have been characterized for CD36, with 61% neutral and 39% acidic structures. CD36 shares similar glycan features with the other MFGM glycoproteins including sialylation, fucosylation and terminal LacdiNAc (Nakata et al. 1993).

To date, no biological activity has been ascribed to the oligosaccharides on CD36. However, as seen for the other glycoproteins of the MFGM, the attached glycan chains are believed to protect the protein backbone from proteolysis (Greenwalt et al. 1992).

Proteose Peptone Component 3. The proteose peptone fraction of bovine milk accounts for ~10% of total whey protein and consists of a complex mix of low-molecular-weight heat-stable, acid soluble proteins. Proteose peptone component 3 (PP3), a 28-kDa phosphorylated glycoprotein also known as lactophorin (Girardet and Linden 1996), was previously suggested to be derived from the MFGM and is the bovine homologue of murine glycosylation-dependent cell adhesion molecule-1 (Girardet et al. 1995). It shares common glycan structures with the MFGM glycoproteins and an antigenic relationship between

MFGM and the proteose peptone fraction has been established (Kanno and Yamauchi 1979; Kester and Brunner 1982; Nejjar et al. 1986). It was Sørensen et al. (1997) who first confirmed the presence of PP3 on the MFGM. However, the group also detected PP3 in the whey fraction suggesting PP3 is weakly associated with the MFGM and is easily lost into the whey fraction of milk. PP3 is not present in human milk and is present at a high concentration in bovine milk at 300 mg/L (Sørensen and Petersen 1993).

Two *O*-glycosites at Thr16 and Thr 86 (Thr 16 is only ~50% glycosylated (Sørensen and Petersen 1993)) and one N-glycosite at Asn77 have been identified for PP3 (Girardet et al. 1995; Coddeville et al. 1998). Three neutral *O*-glycans have been characterized, GalNAc, Gal β (1–3)GalNAc and Gal β (1–4)GlcNAc β (1–6)[Gal β (1–3)]GalNAc (Coddeville et al. 1998) with 19 bi-, tri- and tetra-antennary complex *N*-glycan structures with LacNAc and LacdiNAc motifs, core fucosylation and terminal sialylation observed (Girardet et al. 1995; Inagaki, Nakaya, et al. 2010).

PP3 has previously been shown to inhibit lipase activity in milk (Anderson 1981; Cartier and Chillard 1990) by competitive adsorption (Girardet et al. 1993). Its presence on the MFGM may have a role in limiting access of lipases to the milk fat globule lipid core and preventing spontaneous lipolysis in milk (Sørensen et al. 1997). Similar activity has been observed with PP3 from yaks milk (He et al. 2012).

PP3 has been shown to have mitogenic activity in DNA synthesis in MARK3 hybridomas, a feature which is lost following neuraminidase treatment (Mati et al. 1993). It has been suggested that the negative charge conferred by the carboxyl groups of Neu5Ac stabilizes the active conformation of the PP3 and the loss of Neu5Ac following neuraminidase treatment results in conformation changes leading to the loss of its mitogenic activity (Guimont et al. 1997). However, trypsin digestion of PP3 had no effect on hybridoma mitosis (Mati et al. 1993), suggesting that the PP3 glycans may be directly influencing hybridoma mitosis. The peptide backbone of PP3 on the other hand has been shown to have antibacterial activity against *Streptococcus thermophilus* (Campagna et al. 2004) and have a prebiotic effect on *Bifidobacterium animalis* (Etienne et al. 1994). A study incorporating more bacterial strains may identify further antimicrobial and prebiotic activities for bovine PP3 and a role for its sialylated glycans. PP3 is an ideal candidate for the functional food industry as a result of its high concentration in bovine milk. However, further research is first required to fully understand its biological roles and the importance of its glycosylation *in vivo*.

Immunoglobulins

Bovine milk contains the immunoglobulins (Ig) IgG, IgA and IgM and, in contrast to human milk where IgA is the dominant Ig present, IgG is dominant in bovine milk. Bovine colostrum is a rich source of Igs but the concentration decreases rapidly thereafter (Table I) (Marnila and Korhonen 2002; Abd El-Fattah et al. 2012).

Ig glycosylation is conserved at particular glycosites in the constant region of the Ig heavy chain. In IgG, N-glycosylation is conserved at Asn297 (Recio et al. 2009) of the Fc region (Takimori et al. 2011). In fact, the constant regions of the Fc

stem interact via their attached glycan chains (Deisenhofer 1981). Takimori et al. (2011) investigated IgG glycosylation over lactation and identified complex type *N*-glycans with core fucosylation throughout lactation. However, there was a notable decrease in the level of IgG sialylation postpartum. Neu5Ac was present on ~50% of the glycans identified in colostrum but was only present at negligible levels from 1 week postpartum. The heterogeneity of IgG glycosylation also decreased with a greater number of glycan structures isolated from IgG in colostrum when compared with later in lactation. The authors suggest that this change in glycosylation is linked to the changing biological needs of the calf. Sialylation of IgG is important for its anti-inflammatory activity (Kaneko et al. 2006), which may be important in the early life of the calf.

The role of glycosylation in the function of Igs from non-bovine sources has previously been reviewed (Wright and Morrison 1997; Krotkiewski 1999; Rudd et al. 2001; Recio et al. 2009). The glycan chains of bovine milk Igs may have similar biological roles and protect the Ig protein from digestion by proteolytic enzymes, allowing the intact or only partially digested Ig to reach the intestine for absorption into the blood. Non-glycosylated Igs, generated by site-directed mutagenesis of the N-glycosites of IgA, have altered secretion levels, suggesting a role for the glycan chains in Ig secretion (Taylor and Wall 1988). Very few studies have focused on the glycosylation of bovine Igs and, therefore, further research is required to fully understand the biological importance of Ig glycosylation in bovine milk. Bovine milk Igs offer potential as antimicrobial ingredients in milk-based products such as infant formula and yoghurt and also in immune supplements and drinks.

Minor milk glycoproteins

Minor bovine milk glycoproteins include α -lactalbumin (α LA), lactoperoxidase (LP), folate-binding protein (FBP) and glycolactin. Although α LA is a major protein in whey, only a small percentage of it is glycosylated in bovine milk (Table II), which is why this glycoprotein is discussed in this section. With the exception of glycolactin, detailed structures have been described for the attached glycan chains of these glycoproteins (Table II) (Barman 1970; Hopper and McKenzie 1973; Luhers 1991; Tilley et al. 1991; de Wit and Hooydonk 1996; Shen et al. 1997; Roberts et al. 1998; Watanabe et al. 1998; Slangen and Visser 1999; Wolf et al. 2000; Chen et al. 2006; Jaiswal et al. 2011; Takimori et al. 2011). They are mainly *N*-glycosylated fucosylated and sialylated proteins. However, little information is available on the biological importance of glycosylation in the bioactivity of these

Table I. Immunoglobulin concentration in bovine colostrum and mature milk and % carbohydrate content

Ig	Average (g/L)		Carbohydrate content (%)
	Colostrum	Mature milk	
IgG	60	0.47	2.6–3.1
IgA	3.5	0.075	6–10
IgM	5	0.52	10–12

Adapted from Marnila and Korhonen (2002).

glycoproteins. Glycosylation may have a role secretion of these glycoproteins and protection from proteolysis (Table II).

Commercialization and current patents relating to bovine milk glycoproteins

The biological value of bovine milk glycoproteins has resulted in their use as ingredients in the functional food industry. A search of online patent databases (GooglePatents W.I.P.O.) shows a significant number of patents currently exist for both the isolation (Table III) and application (Table IV) of specific bovine milk glycoproteins.

LF is the most established milk glycoprotein available for use as an ingredient. The isolation of LF has been upscaled and optimized by numerous companies and it has been granted the generally recognized as safe status, qualifying it for use in the food industry. bLF is available from several suppliers under various brand names, e.g. Glanbia (Bioferrin®), Erie Foods International (NatraFerrin), FreislandCampina (Vivinal LF), Ingredia nutritional (Premium LF), Morinaga Milk and Nexira. To date, bLF has been used as a nutraceutical in infant formulae, fortified milks, iron supplements and drinks, chewing gum, immune-enhancing nutraceuticals, cosmetic formulae, animal feed and pet care supplements (Severin and Wenshui 2005). New technologies have allowed for the sterilization of bLF for use in food products such as infant formula and yogurt (Cao

and Maas 2009) and Australian and New Zealand-based companies recently invested \$14 and \$15 million, respectively, to upscale bLF production (<http://www.theaustralian.com.au> 2013; Synlait 2013). LF currently markets at \$500–\$1000 per kg and the global market for purified LF has grown from 45,000 kg in 2001 to 185,000 kg in 2012 and is expected to expand even further, with a projected market of 262,000 kg by 2017 (Synlait 2013). Approximately 50–100 thousand liters of milk is required as starting material to produce just 1 kg of LF as a by-product of the cheese process, which explains in part its high price (<http://www.theaustralian.com.au> 2013).

In terms of the other bioactive glycoproteins, Davisco Foods International, Erie Foods International (NatraPep) and Arla Food Ingredients (lacprodan CGMP-20) produce GMP powders, while Arla Food Ingredients also produces an MFGM enriched product (lacprodan MFGM-10). These powders are marketed for use as ingredients in functional foods, beverages, cosmetics and supplements. A range of GMP supplemented foods have previously been produced for phenylketonuria patients, including strawberry pudding, crackers and an orange sports beverage (Lim et al. 2007). As a result of its emulsifying properties (Neelima et al. 2013), GMP offers the potential for use in a wide range of products such as yoghurts and fermented milks, with the added biological benefit of its antimicrobial activity. However, heat treatment has been shown to affect the level of GMP’s glycosylation, in particular its Neu5Ac content (Taylor and Woonton 2009). This is a main concern when the

Table II. Summary of the information available on the glycosylation of minor milk glycoproteins α-LA, LP and FBP

Glycoprotein	Glycosylation	Glycans and activity	References
αLA	• 3–10% glycosylated • 1 N-glycosite • 14 N-glycans • GalNAc, GlcNAc, man + low levels of Fuc, Gal and Neu5Ac • Glycoforms 15,840–16,690 Da • Major structure = biantennary complex type glycan with core fucosylation, LacdiNAc motif on both antenna, ± terminal sialylation • Increase of non-sialylated form over lactation • Increase of second sialylated glycan over lactation	Synthesized as a glycoprotein but glycan tail lost upon exposure to milk hydrolases. Possible role of glycosylation in α-LA secretion from the mammary gland.	Barman (1970), Hopper and McKenzie (1973), Slangen and Visser (1999), Takimori et al. (2011), Tilley et al. (1991)
LP	• Carbohydrate content: 6.4–11.5% • 5 N-glycosites, Asn6, Asn222, Asn 258 and Asn349 always glycosylated, Asn112 85% glycosylated • 20 N-glycans • Complex and high-mannose type glycans • 50% glycans = high-mannose type • Mono- + di-fucosylation • Sialylation	Protection of protein backbone from digestion	de Wit and Hooydonk (1996), Watanabe et al. (1998), Wolf et al. (2000)
FBP	• 2 N-glycosites • Asn49 = 49% glycosylated • Asn141 = 74% glycosylated • 17 N-glycans • Complex, high-mannose and hybrid type • Seven structures in common with human FBP • Low levels of Fuc and Neu5Ac	Bovine and human FBP: glycosylation has a role in stabilizing the ligand-binding domain	Chen et al. (2006), Jaiswal et al. (2011), Luhrs (1991), Roberts et al. (1998), Shen et al. (1997)

No information on glycolactin was available for inclusion.

Table III. Examples of patents related to the isolation of bovine milk glycoproteins

Patent number	Title	Company	Publication date
US20030045677 <i>LF</i>	Isolation of glycoproteins from bovine milk	Davisco Foods International, Inc.	Mar 6, 2003
US4668771 A	Method for separating bovine LF from cow's milk and purifying same	Snow Brand Milk Products Co., Ltd.	May 26, 1987
US4791193 A	Process for producing bovine LF in high purity	Morinaga Milk Industry Co., Ltd.	Dec 13, 1988
EP0348508 B1	Process for separating and purifying LF from milk using sulfate compound	Snow Brand Milk Products Co., Ltd.	Sep 9, 1992
EP0744901 B1	Isolation of LF from milk	Pharming Intellectual Property BV	Dec 5, 2001
US 20050220953 A1	Process of isolating LF	Allan Otto Fog Lihme	Oct 6, 2005
US 20090306350 A1	New purification method of LF	Crea Biopharma Inc.	Dec 10, 2009
US20100121037 A1	Method for selective fractionation of growth factors from dairy products	Universite Laval	May 13, 2010
US20110172160 A1 <i>GMP</i>	Heat-stable, aqueous LF composition and its preparation and use	Campina Nederland Holding B.V.	Jul 14, 2011
EP0393850 A2	Process for producing κ -cn GMPs	Snow Brand Milk Products Co., Ltd.	Oct 24, 1990
US5061622 A	Process for the production of κ -cn GMP	Snow Brand Milk Products Co., Ltd.	Oct 29, 1991
US5216129 A	Production of κ -cn-GMP	Nestec S.A.	Jun 1, 1993
US5968586 A	Production of κ -cn macropeptide for nutraceutical uses	Wisconsin Alumni Research Foundation	Oct 19, 1999
US6168823 B1	Production of substantially pure κ -cn macropeptide	Wisconsin Alumni Research Foundation	Jan 2, 2001
US6462181 B	Process for preparing a κ -cn GMP or a derivative thereof	Arla Foods Amba	Oct 8, 2002
US20020183489 A1	Large-scale production of low fat and SDS gel pure κ -cn GMPs from bovine deproteinized whey	Martin Davis, Fang Ming, Sharyn Su, Mengyan Yang, Akimoto Ichinomiya	Dec 5, 2002
<i>MFGM components</i>			
WO2001035760 A1	Bulk preparation of MFGMs	Ronald C. Gorewit	May 25, 2001
WO2002062374 A2	Muc1 isolation from bovine milk and whey	Davisco Foods International, Inc.	Aug 15, 2002
EP2098122 A1	Protease peptone fraction	Nestec S.A.	Sep 9, 2009

sterilization of GMP for use in food products is considered as these processes can involve heat exposure and therefore affect the glycoprofile, and therefore bioactivity, of the peptide.

When the large number of patents for the production and use of individual bovine milk glycoproteins (Tables III and IV) are considered, there is obviously still much scope for their further exploitation as functional ingredients. The market value of these proteins is beginning to be realized in recent years as a result of the development of methods for their large-scale isolation. However, potential still exists for the development of novel products incorporating these glycoproteins and, in particular, for industrial scale isolation of the bovine milk glycoprotein fraction as a whole, which would offer a wider range of nutraceutical benefits when compared with individual glycoproteins. Recent occurrences of whey protein and LF powder contamination by microbial and chemical pollutants (Gray 2013; Wade and Theunissen 2013) highlight the importance of good manufacturing practice and quality control in the isolation and commercialization of milk derived powders, including isolated glycoproteins, for use as food grade ingredients.

Future directions of glycoprotein investigation

Although in recent years a significant body of research has focused on bovine milk glycoproteins, their structure and biological activities, major gaps in the knowledge still exist. Much of this shortfall relates to the digestion of glycoproteins *in vivo* and their survival while transiting the gastrointestinal tract (GIT) where they encounter harsh pH changes and proteolytic enzymes. As discussed earlier in this review, GMP can survive gastric transit and has been shown to be absorbed into the

bloodstream. However, GMP is a relatively small glycopeptide when compared with the other proteins mentioned, therefore similar studies investigating their metabolism is essential. The bioavailability of glycoproteins and their end products of digestion will impact their nutraceutical value as the majority of their associated bioactivities would occur in the lower GIT *in vivo*, such as prebiotic and anti-infective activities. Digestion of the glycoprotein structure may result in the active form not being present in the colon. Various studies have attempted to mimic *in vivo* digestion through the use of proteolytic enzymes prior to examining the glycoproteins in bioassays (Britton and Koldovsky 1989; Furlund et al. 2013); however, these models do not match the true complexity of the digestive system. *In vivo* feeding trials are required to fully understand the glycoprotein's journey from ingestion, through digestion, to bioactivity.

In recent years, methods for the analysis of milk glycoproteins have advanced rapidly, with improvements in mass spectrometry methods, as well as the establishment of methods such as lectin arrays for glycoprotein analysis. As a result, a large body of information is now available on the glycan structures present on bovine milk proteins (as discussed in this review). However, the role of the attached glycans in their biological functions is yet to be as fully understood. Future research must focus on this area in order to fully understand the biological role of protein glycosylation and, in turn, harness this activity for inclusion in commercial products in order to enhance human health. To date, bLF and GMP have been the focus of much research and are most commonly utilized as functional ingredients. Other bovine milk glycoproteins, such as the mucins and the immunoglobulins, offer similar potential but the absence of information on their bioactivities and methods

Table IV. Example of patents related to the application of bovine milk glycoproteins

Patent number	Activity	Applicant	Publication date	
LF				
JP10059865	Antimicrobial	Morinaga Milk Industry Co., Ltd.	Mar 3, 1998	
WO1998021231 A2		University of Saskatchewan	May 22, 1998	
US6258383 B1		Lactoferrin Products Company	Jul 10, 2001	
JP2004292462		T Cell Research Institute Ltd	Oct 21, 2004	
WO2006121507 A1		Bristol-Myers Squibb Company, Thomas Cleary, Robert J. McMahon, Theresa Ochoa	Nov 16, 2006	
US20070191264 A1		Bristol-Myers Squibb Company, A Delaware Corporation	Aug 16, 2007	
JP2009102370		Aziende Chimiche Riunite Angelini Francesco	May 14, 2009	
US20130034542		Rudi Ganter, Humera Ahmad	Feb 7, 2013	
US20080193551 A1		Skin care	Campina Nederland Holding B.V.	Oct 28, 2004
WO2006098625 A1			Campina Nederland Holding B.V., Waard Rick De, Angela Loriann Walter	Sep 21, 2006
US20040214750 A1	Izolda Georgiades		Aug 14, 2008	
US 8147875 B2	Intestinal growth	Campina Nederland Holding B.V.	Apr 3, 2012	
US4977137 A		Baylor College Of Medicine	Dec 11, 1990	
EP2251031 A1		Nestec S.A.	Nov 17, 2010	
EP0385279 A2	Prebiotic	Morinaga Milk Industry Co., Ltd.	Sep 5, 1990	
US5322836		Morinaga Milk Industry Co., Ltd.	Jun 21, 1994	
WO2010081126 A2	Dental health	Wyeth Llc	Jul 15, 2010	
EP2385837 A2		Pfizer Italia S.r.l.	Nov 16, 2011	
US6482396 B1		Campina Melkunie B.V.	Nov 19, 2002	
WO2005072261 A2		A. Satyanarayan Naidu	Aug 11, 2005	
EP2039342 A1		Snow Brand Milk Products, Co., Ltd.	Mar 25, 2009	
WO2013068020 A1		Coswell S.P.A.	May 16, 2013	
WO2001089553 A1	Lipid digestion	Barnes Jewish Hospital, Curtis A. Spilburg, William F. Stenson	Nov 29, 2001	
WO2004052305 A2	Immunomodulation	Agennix Incorporated, Federica Pericle, Atul Varadhachary	Jun 24, 2004	
US20050004006 A1	Diabetes treatment	Jose Engelmayer, Atul Varadhachary	Jan 6, 2005	
US20090202574 A1	Anticancer	Jagat Rakesh Kanwar, Neill Ward Haggarty, Kay Patricia Palmano, Geoffrey Wayne Krissansen	Aug 13, 2009	
US20130039903	Eczema	Rudi Ganter, Humera Ahmad	Feb 14, 2013	
GMP (or κ-cn)				
US5147853 A	Antimicrobial	Snow Brand Milk Products, Co., Ltd.	Sep 15, 1992	
WO1994015952 A1		Per Munk Nielsen, Novo Nordisk A/S, Niels Tromholt	Jul 21, 1994	
US5344820	Oral health	Snow Brand Milk Products, Co., Ltd.	Sep 6, 1994	
WO1996008269		Abbott Laboratories	Mar 21, 1996	
US4992420 A		Nestec S.A.	Feb 12, 1991	
US4994441		Nestec S.A.	Feb 19, 1991	
WO1998052524		Colgate-Palmolive Company	Nov 26, 1998	
EP1104281		Nestle S.A.	Jun 6, 2001	
US20020119948	Appetite control	Pacifichealth Laboratories, Inc.	Aug 29, 2002	
US20030008810		Pacifichealth Laboratories, Inc.	Jan 9, 2003	
US20030059495		Mcneil-PPC, Inc.	Mar 27, 2003	
WO2004034813 A2	Improved learning	Pacifichealth Laboratories, Inc.	Apr 29, 2004	
EP1874336		Squibb Bristol Myers Co, The University Of Sydney	Jan 9, 2008	
US20060247153		Robert J McMahon, Bing Wang, Jennie Brand-Miller, Steven C Rumsey	Nov 2, 2011	
US5260280	Anti-toxin	Snow Brand Milk Products, Co., Ltd.	Sept 11, 1993	
JP 07278013 A		Snow Brand Milk Products, Co., Ltd.	Oct 24, 1995	
WO2012004585	PKU treatment	Abdulla BADAWY	Jun 29, 2011	
EP2440232		Wisconsin Alumni Res Found	Apr 18, 2012	
WO2003028751	Eye care	Vista Scientific LLCc	Apr 10, 2003	
WO2005097157		Milcin Therapeutics LLC	Oct 20, 2010	
US5063203	Anti-thrombotic	Centre National De La Recherche Scientifique, IVS Institut Des Vaisseaux Et Du Sang	Nov 5, 1991	
JP2000270812	Mineral absorption	Snow Brand Milk Products, Co., Ltd.	Oct 3, 2000	
WO2005037248	Wound healing	Milcin Therapeutics LLC	Apr 28, 2005	
US20060246146	Salivary	Bristol-Myers Squibb Company	Nov 2, 2006	
US20110195128	Immunomodulation	Kay Patricia Palmano, Alastair Kenneth Hugh Macgibbon, Nicole Jane Moore Dalbeth	Aug 11, 2011	
MFGM components				
EP1010434 B1	Antimicrobial	Ghen Corporation, Nisshin Pharma Inc.	Mar 31, 2004	
US20130130970 A1		Rotalactis Srl	May 23, 2013	
WO2003103700 A1	Blood coagulation	Gary E. Gilbert, Jialan Shi, The Brigham & Women's Hospital, Inc., U.S. Department Of Veterans Affairs	Dec 18, 2003	
WO2011020736A1	Lipid uptake	Nestec S.A.	Jun 27, 2012	
WO2012170021 A1	Nutritional composition	Nestec S.A.	Dec 13, 2012	

for their isolation at an industrial scale has hampered their utilization. This is an area that must first be addressed before products integrating these glycoproteins can be developed.

Another factor which must be considered is the specific glycan features present on bovine milk glycoproteins which have been linked to undesirable effects *in vivo*, such as the allergenic motifs Gal- α -(1 $\rightarrow$ 3)-Gal and Neu5Gc, which could potentially elicit allergen-like immune responses in humans (Nguyen et al. 2005; Padler-Karavani et al. 2008; Commins et al. 2011). Neu5Gc has also been suggested to be involved in cancer development and metastasis (Makatsori et al. 1998; Malykh et al. 2001). Humans cannot synthesize Neu5Gc *in vivo* as a result of a genetic mutation in the sialic acid pathway (Nguyen et al. 2005); however, dietary Neu5Gc can be absorbed, which could potentially result in adverse health effects. To date, a correlation between milk consumption and these negative health outcomes has not been established. In contrast, Neu5Gc present in bovine colostrum may have a protective role for the new born calf, acting as a decoy receptor to prevent enterotoxigenic *E. coli* adhering to the epithelium of the small intestine (Puente and Hueso 1993).

A fascinating area at present is the investigation of changes in protein glycosylation as a result of stage of lactation, feed, breed, mastitis infection (as discussed above). Of these, lactation appears to have the greatest influence on the structure of the attached glycan chains. Bovine colostrum is an excellent source of glycoproteins with a distinct glycosylation profile to milk from later in lactation; however, China has recently banned the use of bovine colostrum and dairy products derived from it in infant formula (<http://www.chinadaily.com.cn> 2012), possibly as a result of the lack of scientific evidence to substantiate their health effects. This area requires further investigation in order to elucidate the biological effect of the varying glycoprofiles in colostrum, although the relatively low quantities of colostrum available in comparison to milk from later in lactation may limit its use as a functional ingredient.

Funding

Noelle O'Riordan is in receipt of a Teagasc Walsh fellowship.

Conflict of interest

None declared.

Abbreviations

bLF, bovine lactoferrin; BTN, butyrophilin; BTN, butyrophilin; CD36, cluster of differentiation 36; CMP, caseinomacropptide; FBP, folate-binding protein; GalNAcT, *N*-acetylgalactosaminyl transferase; GIT, gastrointestinal tract; GMP, glycomacropptide; hLF, human; Ig, immunoglobulin; LF, lactoferrin; LP, lactoperoxidase; LPS, lipopolysaccharide; MFGM, milk fat globule membrane; Neu5Ac, *N*-acetyl neuraminic acid; Neu5Gc, *N*-glycolyl neuraminic acid; PAS, periodic acid schiff; PHA, phytohemagglutinin; PP3, protease peptone component 3; PTMs, posttranslational modifications; κ -cn, κ -casein; α LA, α -lactalbumin

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