Targeting the glycome of major milk proteins

A Thesis Presented to National University of Ireland, Galway for the degree of Doctor of Philosophy (Science)

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!Unexpected End of Formula

Approval for examination

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not been submitted for any degree or examination at the National University of Ireland, Galway or elsewhere, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Signed: ……..

Noelle O'Riordan

Date:

Thesis Abstract

The objectives of this thesis were to 1) explore the glycosylation of major bovine milk proteins, 2) investigate the effect of lactational stage on glycan structures and 3) examine how these documented changes may influence bioactivity.

In order to achieve the first objective, we initially focused on the presence and activity of glycosidases in Irish bovine milk, which were monitored over the first three months of lactation (chapter 2). A low level of variation in enzyme activity between animals was noted and colostrum samples assayed showed the highest level of glycosidase activity. This decreased through transitional milk production to minimal but constant levels in mature milk. The most biologically relevant glycosidases in bovine milk as deteremined by this work are N-acetyl-β-D-glucosaminidase, α-L-fucosidase, α-galactosidase and N-acetyl-neuraminidase. The possibility that the elevated levels of enzymatic activity in colostrum may play a role in the digestion of bovine milk glycans in the infant mammal is discussed, focusing on their potential functionality as substitutes for bacterial glycosidases prior to colonisation by the gut microflora.

In order to fulfil the second objective, bovine lactoferrin (bLF) was isolated from milk samples (3 cows, 13 timepoints over lactation) and glycosylation changes were profiled through the use of lectin microarrays and monosaccharide analysis (chapter 3). A large number of studies have to date focussed on the structure of the glycans associated with this glycoprotein particularly when isolated from mature milk, however there is limited information available on how these structures change over lactation. Substantial profile differences between early and late lactation are detailed in this chapter and overall, the data suggests that more diverse complex-type oligosaccharides structures are present on bLF during early lactation with an abundance of oligomannose type glycans in later lactation. The impact of the differences observed in the glycoprofiles of bLF from colostrum compared to mature milk is also discussed in this chapter.

To achieve the third objective, the effect of this heterogeneous glycosylation pattern on bLF's biological activity was first investigated. Chapter 4 employed SPR technology to compare the binding of bLF from colostrum (early lactation) and mature milk (late lactation) to a panel of pathogenic bacteria

(*Staphylococcus aureus*, *Escherichia coli*, *Cronobacter sakazakii*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Salmonella typhimurium*). LF has been shown in the literature to bind to various bacteria from both commensal and pathogenic communities. This bacterial binding activity has been linked to LF's prebiotic and anti-infective activities *in vivo*. In Chapter 4, novel interactions were identified for *C. sakazakii*, *S. pneumoniae* and *P. aeruginosa* with the highest binding observed for mature milk bLF in all cases, with the exception of *S. typhimurium*. The difference in bacterial binding observed may be as a direct result of the varying glycosylation profiles. The abundance of mannose residues on mature milk bLF's glycans may play a role as decoy receptors, mimicking the high-mannose type structures present on intestinal mucins and non-specifically binding pathogenic bacteria.

Chapter 5 sought to expand on the third objective of the thesis and advance the knowledge on the role of bovine milk protein glycosylation in functionality of milk glycoproteins. Glycomacropeptide (GMP), a 64 amino acid peptide, derived from k-casein, was selected given the current interest in this glycopeptide as a prebiotic. GMP promoted the growth of *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), a prototype infant strain which has been the focus of many prebiotic studies involving free milk oligosaccharides. This activity was lost following periodate treatment of the GMP (GMP-P), which disables biological recognition of the conjugated oligosaccharides. Transcriptional analysis of *B. infantis* following growth in media supplemented with 2mg/ml GMP revealed a substantial transcriptional response to GMP relative to bacteria cultured with 2mg/ml GMP-P supplementation of the media. Exposure to intact GMP resulted in a greater number of differentially expressed transcripts and larger fold changes versus the periodate treated test. These results suggest that the *O*-linked glycosylation of GMP is intrinsic to the growth stimulation of *B. infantis* by GMP. When the pool of differentially expressed transcripts displaying up-regulation following exposure to intact GMP were considered, two duplicated genomic islands containing glycoside hydrolase (family 25) genes and fibronectin type III binding domain proteins were identified. Homologues of this genomic arrangement were present in other *Bifidobacterium* species, which suggested that it may be a conserved domain for the utilisation of glycosylated peptides/proteins. This study provides insights into the molecular basis for the prebiotic effect of

bovine milk GMP on *B. infantis* and highlights its potential as a functional food ingredient for the promotion of a beneficial gut microbiota.

Overall, this research adds to our understanding of the structural and functional importance of milk protein glycosylation. It also highlights the potential of these compositionally complex molecules as ingredients which can be exploited by the food and pharmaceutical industry. Further research in this area, including characterisation of the genetic elements in the bovine mammary gland which influence protein glycosylation and expression is required to further our knowledge on the biological importance of milk glycoproteins.

Publications

Chapter 1. O'Riordan, N., Kane, M., Joshi, L., Hickey, R.M. (2014). Structural and functional characteristics of bovine milk protein glycosylation. Glycobiology 24: 220-236.

Chapter 2. O'Riordan, N., Kane, M., Joshi, L., Hickey, R.M. (2014). Glycosidase activities in bovine milk over lactation. International dairy journal, vol. 35 (2): 116-121

Chapter 3. O'Riordan, N., Gerlach, J. Q., Kilcoyne, M., O'Callaghan, J., Kane, M., Hickey, R. M. and Joshi, L. (2014). Profiling temporal changes in bovine milk lactoferrin glycosylation using lectin microarrays. Food Chemistry 165: 388-396.

Chapter 4. O'Riordan, N., Kilcoyne, M., Joshi, L., Hickey, R.M. Investigating the influence of glycosylation variation on the pathogen binding ability of bovine lactoferrin. Submitted to Applied Microbiology for peer review.

Chapter 5. O'Riordan, N., O'Callaghan, J., Buttò, L. F., Kilcoyne, M., Kane, M., Joshi, L. and Hickey, R. M.. Bovine glycomacropeptide promotes the growth of Bifidobacterium longum subsp. infantis and modulates its gene expression. Submitted to Microbiology for peer review.

Oral Communications

O'Riordan, N., Gerlach, J. Q., Kane, M., Hickey, R. M. and Joshi, L. Lectin Arrays Demonstrate the Changes in Glycosylation of Bovine Milk Lactoferrin over Lactation. *Increasing the Impact of Glycoscience through New Tools and Technologies, International Glycomics Symposium, San Sebastian, Spain July 19-21, 2012.*

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Abstracts

O'Riordan, N., Kane, M., Joshi, L., Hickey, R.M. Glycosidases in bovine milk over lactation. *4th Annual GlycoScience Ireland meeting, Teagasc Food Research Centre, Moorepark, Ireland, October 21st, 2011.*

O'Riordan, N., Kilcoyne, M., Gerlach, J. Q., Kane, M., Hickey, R. M. and Joshi, L. Lectin Arrays Demonstrate the Changes in Glycosylation of Bovine Milk Lactoferrin over Lactation. *Increasing the Impact of Glycoscience through New Tools and Technologies, International Glycomics Symposium, San Sebastian, Spain July 19th-21st, 2012.*

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Abbreviations

°C	Degree Celsius
µ	Micro
4-MU	4-Methylumbelliferyl
Asn	Asparagine
bLF	Bovine Lactoferrin
BTN	Butyrophilin
Ca^{2+}	Calcium
CD36	Cluster Of Differentiation 36
cDNA	Complementary DNA
CFU	Colony-Forming Units
CHO cells	Chinese Hamster Ovary Cells
CMP	Caseinomacropeptide
CV	Column Volume
D	Day
Da	Dalton
DET	Differentially-Expressed Transcript
Fe^{3+}	Iron
Fuc	Fucose
g	Gram or Acceleration For Centrifugation Parameters
Gal	Galactose
GalNAc	N-Acetyl-D-Galactosamine
GH25	Glycoside Hydrolase (Family 25)
GIT	Gastrointestinal Tract
GlcNAc	N-Acetyl-D-Glucosamine
GMP	Glycomacropeptide
GMP-P	Sodium Metaperiodate Treated GMP
h	Hour
hLF	Human Lactoferrin
HMOs	Human Milk Oligosaccharides
HPAEC-PAD	High Performance Anion Exchange Chromatography With Pulsed Amperometric Detection
HPLC	High Performance Liquid Chromatography
Ig	Immunoglobulin
kDa	Kilodalton
kg	Kilogram
KOH	Potassium Hydroxide
L	Liter
LacdiNAc	N,N-Diacetyllactosediamine
LacNAc	N-Acetyl-D-Lactosamine
LF	Lactoferrin
LPS	Lipopolysaccharide
Lys	Lysine
M	Mole

m	Milli
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-Of-Flight
MS	Mass Spectrometry
Man	Mannose
MFGM	Milk Fat Globule Membrane
min	Minute
mMRS media	Minimal De Man-Rogosa-Sharpe Media
M_r	Molecular Weight
MRS media	De Man-Rogosa-Sharpe Media
MS	Mass Spectrometry
MUC	Mucin
MWCO	Molecular Weight Cut Off
n	Nano
NaCl	Sodium Chloride
$NaIO_4$	Sodium Metaperiodate
Neu5Ac	N-Acetylneuraminic Acid
Neu5GC	N-Glycolylneuraminic Acid
NMR	Nuclear Magnetic Resonance Spectroscopy
p	Pico
PAS 6/7	Lactadherin
PP3	Proteose Peptone Component 3
RP-HPLC	Reverse Phase HPLC
rpm	Revolutions Per Minute
RU	Resonance Units
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Ser	Serine
SPR	Surface Plasmon Resonance
TFA	Trifluoroacetic Acid
Thr	Threonine
v/v	Volume/Volume
κ-cn	Kappa Casein

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Thesis introduction

Bovine milk is an abundant raw material in the majority of western developed countries and constant innovation in the food sector is resulting in new products emerging on the dairy market. However, the focus has also moved to identify components of bovine milk which, when isolated or concentrated, can bring added value to applications such as infant formula or nutraceutical products. Irish dairy research in particular is expanding in this area due to the large increase in milk volumes following the abolition of milk quotas in 2015. Irish milk is globaly recognised as being of a uniquely high standard as a result of the Irish cows being grass fed for a large majority of the year versus other countries where ration feeding is more common. Irish dairy industries such as Kerry Group, Dairygold Food Ingredients and Glanbia Ingredients Ireland are investing heavily in dairy research and expanding production in anticipation of this rise in milk availability. Research bodies such as Teagasc and universities, including the National University of Ireland, Galway, have established programmes to broaden our knowledge on specific functionalities (antimicrobial activity, prebiotic activity, immuno-modulatory activity as examples) or individual components (protiens, oligosaccharides, glycoproteins etc.) associated with bovine milk. However, this topic is not specific to Ireland and there has been a global increase in the resources dedicated to bovine milk research.

Bovine milk is composed of approximately 5% lactose, 3.3% fat and 3.3% protein in addition to immunoglobulins, hormones, growth factors, enzymes and numerous other bioactive components such as vitamins and minerals. It has a long established history in the human diet as a result of its nutritional profile and perceived health benefits. As a result of these aspects and its ease of availability, bovine milk is the basis for infant formula production as a human milk substitute. However, breast-fed infants are at an advantage with respect to growth and health when compared to formula-fed babies. Therefore, infant formula manufacturers are currently focussing much effort on humanising their formulas to provide the health benefits associated with human milk.

A large of body of work exists in the literature focusing on the bioactive proteins present in bovine milk such a β-lactoglobulin and α-Lactalbumin. However, research has now moved to focus on the complex carbohydrate

structures which are inherent in bovine milk and are characterised by both free and bound (attached to glycoproteins and glycolipids) glycans. Sixteen neutral and twenty four acidic (sialylated) oligosaccharide structures have been characterised in bovine colostrum, with a number of these structures also present in human milk. When the identified biological functions of milk oligasaccharides are considered, it is obvious that the structures involved are crucial to elucidate the desired health effects. The structural homology shared between bovine and human milk oligosaccharides highlights the potential of bovine milk as a viable source of bioactive components which may mimic the effects of breastmilk. The main hurdle for isolation of bovine milk oligosaccharides at a commercial level is the difficulty in separating the components from the abundant lactose present as well as the low concentrations of these molecules found in milk.

Bovine milk glycoproteins, such as lactoferrin, kappa-casein, mucins and other milk fat globule membrane glycoproteins, offer an alternative potential source of biologically relevant glycans. In many cases, both the protein and glycan fraction of these molecules can deliver biological benefits. The larger molecular weight of these components and the more diverse chemistries facilitates ease of purification based on size exclusion or/in combination with ion exchange resins. Commercially available glycoprotein powders have found application in products such a infant formula, pharmaceutical and neutraceutical products, as discussed further in chapter 1.

This thesis aims to focus on specific gaps in the knowledge which exist in the area of bovine milk protein glycosylation and how structural changes that may occur in this glycosylation are relevant to the functionality of the intact glycoprotein. It is hoped that this information will further highlight the potential of these proteins as ingredients for human consumption.

Chapter 1

Literature review

Structural and functional characteristics of bovine milk protein glycosylation

Noelle O'Riordan, Marian Kane, Lokesh Joshi, and Rita M. Hickey

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Abstract

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 kappa-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.

1. 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, Dillard 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 (Lonnerdal 2003, Lonnerdal 2004). Biological activities associated with such proteins/peptides include immuno-stimulating, 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 (CLA), 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 capitalise 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 (48g/L), free oligosaccharides (0.05g/L) (Bode 2012) and bound glycans or glycoconjugates. Sriwilaijaroen et al. (2012) recently quantified 374.9 nM/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-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc)) residues which confer a negative charge to the structures (Barile, Marotta et al. 2010). There have been up to 40 oligosaccharide structures confirmed in bovine milks to date (Tao 2008, Barile, Marotta et al. 2010, Mariño, Lane 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 (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 milk fat globule membrane (MFGM) and extensive literature exits on milk glycolipids (Newburg and Chaturvedi 1992, Sanchez-Juanes, Alonso 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 post-translational 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 milk fat globule membrane) (figure 1). Complementary reviews have previously been published on free and bound glycans in human milk (Bode 2012, Peterson, Cheah et al. 2013).

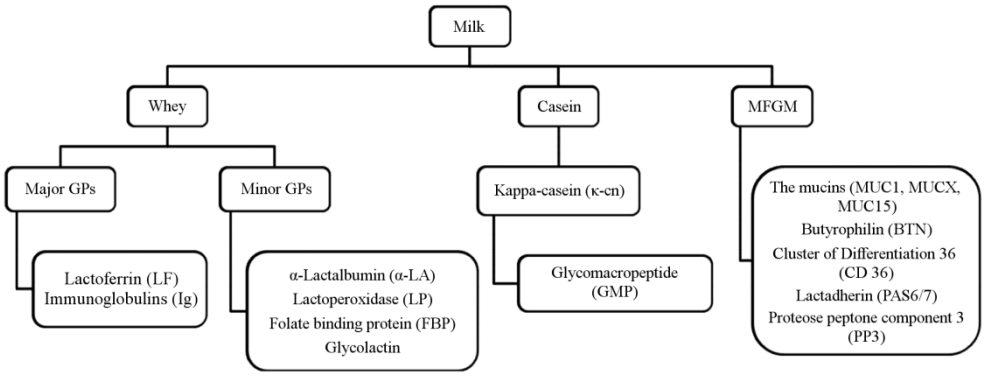

Figure 1 Overview of glycoprotein distribution in bovine milk.

2. 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 a N-acetylglucosamine 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, Wu 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. (Nwosu, Aldredge 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 2008, Aldredge, Geronimo et al. 2013), suggesting protein bound oligosaccharides are the major source of fucose in bovine milk. Such distribution patterns emphasise 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 colonise 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 to 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.

3. Bovine milk glycoproteins

3.1 Lactoferrin

Lactoferrin (LF) is a single chain, iron-binding, glycosylated (Kumar, Weber et al. 2003) member of the transferrin family (Hutchens 1997) present in the whey protein fraction of milk (Severin and Wenshui 2005) (UniPortKB/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, Sanchez 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 (0.1-0.3mg/ml) (Recio, Moreno et al. 2009). Bovine (bLF) concentration is dependent on the breed of cow, stage of lactation and daily milk production of the animal (Cheng, Wang et al. 2008, Krol, Litwinczuk et al.).

3.1.1 Lactoferrin 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 (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, Colavizza et al. 1991, Baker and Baker 2005). The bLF protein backbone (703aa) consists of two homogenous lobes (the N- and C- lobes), with 69% sequence identity with hLF. However, even though the 3-D 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, Anderson et al. 1997, Legrand, Pierce et al. 2008, Baker and Baker 2009, Gonzalez-Chavez, Arevalo-Gallegos et al. 2009).

The glycan component of bLF accounts for 6.7% (van Leeuwen, Schoemaker et al. 2012) to 11.2% (Coddeville, Strecker 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, Strecker 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 kDa and 80 kDa, respectively (Yoshida, Wei 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, Nishimura et al. 2000), which accounts for the difference in the molecular weights of the two variants. Of the total bLF present, bLF-a represents approximately 30% in colostrum and 15% in mature milk (van Veen, Geerts 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, Seipert et al. 2011). There is extensive glycan microheterogenity present in bLF, with complex, hybrid and oligomannose type glycans identified at each of the individual loci (Nwosu, Seipert et al. 2011).Van Leeuwen et al. (van Leeuwen, Schoemaker et al. 2012) identified forty two N-glycan structures while

Hua et al. (Hua, Nwosu et al. 2011) identified fifty nine distinct N-glycans. Asn-233, Asn-368 and Asn-476 showed the highest level of heterogeneity (Nwosu, Strum et al. 2010). Features such as core fucosylation, terminal sialylation and the presence of the N-acetyllactosamine (Gal-β-(1→4)-GlcNAc, LacNAc), *N*,*N*'-diacetyllactosamine (GalNAc-β-(1→4)-GlcNAc, LacdiNAc) and Gal-α-(1→3)-Gal motifs are characteristic of bLF's glycans (Coddeville, Strecker et al. 1992, Hua, Nwosu et al. 2011, van Leeuwen, Schoemaker 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, Schoemaker 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, Nwosu 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(α1-3)Gal epitope has been linked to allergen related immune responses, in particular tick bites (Commins, James et al. 2011) while Neu5Gc (which humans don't produce due to a genetic mutation) has been found to be present in both healthy (Tangvoranuntakul, Gagneux et al. 2003) and cancerous human tissue (Malykh, Schauer et al. 2001) and has been

suggested to have a role in the development and metastasis of some carcinoma cell types (Makatsori, Fermani et al. 1998, Malykh, Schauer et al. 2001). However, there is no evidence linking the consumption of bovine milk or bLF to these effects.

3.1.2 Glycosylation and protein structure

Sialic acid content determines the charge associated with an oligosaccharide chain. It is a strong acid with a pKa of 2.6 and, at physiological pH, it exists in a deprotonated form (Jaques, Brown et al. 1977), giving a negative charge to the bLF surface. It has an important role in stabilising 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, Giansanti 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, Geerts et al. 2004). BLF-a was found to be approximately 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 approximately 100-fold more resistant to proteolysis when compared to bLF, the N-glycosylation does not seem to play a role in this characteristic. Instead, the 3-D 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. (Legrand, Mazurier 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, Anderson et al. 1997). This glycan chain may contribute to interdomain interactions, stabilising the C-lobe and influencing iron release. A similar role has been proposed for the glycan chain at Asn518 in camel LF (Khan, Kumar 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 jejenum tissue (Davidson and Lonnerdal 1988) suggesting the fucosylated glycans may be involved. However, Kawakami et al. (Kawakami, Dosako 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 (0.7-2.7 pM Fuc/µg bLF (O'Riordan, Gerlach et al. 2014)) when compared to hLF, where fucosylated glycans are predominantly present (Barboza, Pinzon et al. 2012), and, therefore, it may not be as important for bLF's bioactivity.

3.1.3 Biological activities associated with Lactoferrin glycosylation

BLF has a wide variety of associated biological activities including antimicrobial (Gonzalez-Chavez, Arevalo-Gallegos et al. 2009), immunomodulatory (Debbabi, Dubarry et al. 1998), prebiotic (Kim, Ohashi et al. 2004, Rahman, Kim et al. 2009), stimulation of bone formation (Cornish, Callon et al. 2004) and anticancer (Tsuda, Sekine et al. 2002, Tsuda, Kozu et al. 2010). The bioactivities of LF have previously been reviewed by several authors (Adlerova, Bartoskova et al. 2008, Garcia-Montoya, Cendon 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 LPS Ca^{2+} binding sites on bacterial membranes and chelate loosely bound Ca^{2+}, which is involved in the stabilisation 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,

Brown 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, Giansanti et al. 2002). Sialylation of bLF is also believed to be important in preventing *Helicobacter pylori* colonisation in a mouse model (Wang, Hirmo et al. 2001) and in the inhibition of haemagglutination by the influenza virus (Kawasaki 1993).

BLF has been reported to act as a non-specific defence 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* (*E. coli*) colonisation. Members of the Enterobacteriaceae family, including *E. coli*, express type 1 fimbriae which can recognise and bind to oligomannose glycan chains on eukaryotic cell surfaces (Abraham, Sun 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. (Yoshida, Wei 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 to bLF-b. BLF-b doesn't exhibit as dramatic an effect on *E. coli* growth and the difference in the glycan chains may be responsible for this varying anti-microbial activity (Wei, Nishimura et al. 2001). The concentration of BLF-a in colostrum is higher when compared to mature milk, indicating a role for this variant in the calf's primary defence against infection. Cholera toxin is produced by *Vibrio cholerae* and causes the symptoms such as diarrhoea 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, Isoda 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.

3.2 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, Jolles et al. 1965). CMP has multiple O-glycosylation sites at various threonine (Thr) and serine (Ser) residues (Thoma-Worringer, Sorensen 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 two months of lactation and increasing again thereafter, as monitored by the release of the peptide from k-casein 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 post-translational modifications (PTMs) and vary in their level of phosphorylation and glycosylation (Thoma-Worringer, Sorensen et al. 2006). The average M_r for CMP is 7500 Da while the highest recorded M_r is 9631 Da (Mollé and Léonil 2005). Thr166 can either be phosphorylated or glycosylated (Holland, Deeth et al. 2006) and no CMP variant

has been identified which lacks a phosphate group as Ser149 is always phosphorylated (Vreeman, Visser 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, Visser 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 (Sharma, Rajput et al. 2013). At low pH, below pH4.5, GMP self-aggregates, resulting in gelatinisation and aggregate size and gel formation increases with decreasing pH, as a result of the hydrophobic side chains of sialic acid at low pH (Farías, Martinez et al. 2010). The solubility of GMP is influenced by its glycosylation (Taylor and Woonton 2009) and the emulsifying (Kreuß, Strixner et al. 2009) and foaming (Kreuß, 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, de Vries et al.).

3.2.1 GMP Glycosylation

Approximately 60% of CMP is glycosylated (Vreeman, Visser 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, Fiat et al. 1975, van Halbeek, Dorland et al. 1980, Saito, Itoh et al. 1981, Saito, Itoh et al. 1981, Saito, Itoh et al. 1982, Fiat, Chevan et al. 1988, Saito 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, Nwosu et al. 2011). Although Neu5Gc has been reported in ovine κ-casein (Addeo, Soulier et al. 1978), only Neu5Ac has been detected in the bovine form. Interestingly, no Neu5Ac-GalNAc structure was observed, suggesting completion of the core-1 disaccharide is necessary before further addition to the glycan chain can proceed (Saito 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 Molle and Leonil (1995).

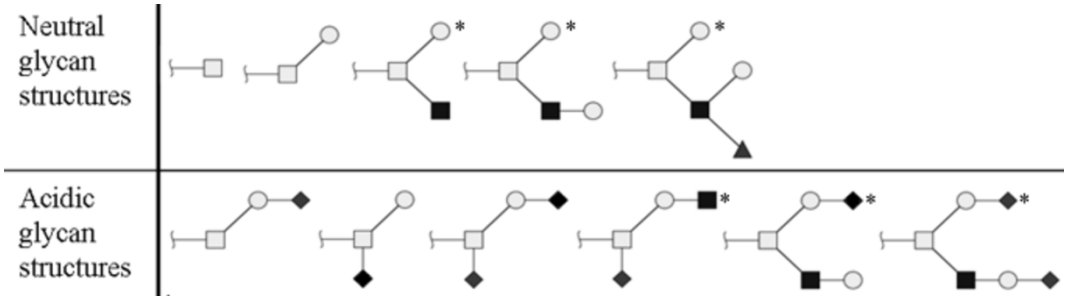

Figure 2 Neutral and acidic structures indentified in bovine GMP. * only present in GMP isolated from colostrum. □ GalNAc. ○ Gal. ■ GlcNac. ♦ Neu5Ac. ▲ Fuc. (Fournet, Fiat et al. 1975, van Halbeek, Dorland et al. 1980, Saito, Itoh et al. 1981, Saito, Itoh et al. 1981, Saito, Itoh et al. 1982, Fiat, Chevan et al. 1988, Saito 1992)

Colostrum GMP has an elevated oligosaccharide content (Guérin, Alais 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, Chevan 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 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 to the non-glycosylated peptide (Kreuß, Strixner et al. 2009), a feature which has been utilised in the separation of GMP and CMP (Kreuß 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 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. 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 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-acetylgalactosaminyltransferase (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-casein, exposing Thr163 and other additional glycosites in a stepwise 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.

3.2.2 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, Sorensen et al. 2006, Recio, Moreno 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, Yu et al. 2007) and increase Neu5Ac content of piglet saliva, influencing its viscosity and protective properties (Thoma-Worringer, Sorensen 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, emphasising the importance of Neu5Ac (Beucher, Levenez 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, Kawakami 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, Isoda et al. 1992) (Oh, Worobo 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 sialyltation (Kawasaki, Isoda et al. 1992) (Oh, Worobo 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 anti-microbial activity against a variety of pathogens. GMP binds to *E. coli* and S*almonella enteritidis* and this binding is reduced following sialidase treatment and abolished by periodate oxidation of the glycan chains (Nakajima, Tamura et al. 2005). *Actinomyces viscosus*, which has been linked to oral infections, expresses fimbriae specific for the T-antigen, Galβ(1-3)GalNAc. The core-1 structure of GMP's O-glycans therefore, can prevent haemagglutination by *A. viscosus*. Desialylation of GMP resulted in an increase in its haemagglutination inhibition activity, as the loss of terminal Neu5Ac residues exposed the core Galβ(1-3)GalNAc structure (Neeser, Chambaz et al. 1988). Desialylation of GMP also results in inhibition of haemagglutination of chick erythrocytes by the influenza virus (Kawasaki 1993). Interestingly, glycosylation was also shown to be important for human κ-cn's anti-infective activity against *Helicobacter pylori*, with fucosylation particularly vital for the inhibition of *H. pylori* adhesion (Aniansson, Andersson et al. 1990, Stromqvist, Falk et al. 1995).

κ-cn derived glycopeptides inhibited the proliferative response of mouse spleen cells to lipopolysaccharide (LPS) and phytohaemagglutinin (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, Monnai 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, Marteau et al. 1998, Thoma-Worringer, Sorensen et al. 2006). The occurrence of glycosylation limited CMP digestion by brush border membrane endopeptidases, allowing it to be absorbed into the blood (Boutrou, Jardin 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 (Boutrou, Jardin et al. 2008). The rate of CMP release from κ-cn was also reduced as Neu5Ac and the carbohydrate content increased (Doi, Kawaguchi et al. 1979, Addeo, Martin 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.

3.3 Milk fat globule membrane

The milk fat globule membrane (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 approximately 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), 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 sections below. In general however, bovine MFGM proteins contain predominantly mono and di sialylated core-1 O-glycans (Wilson, Robinson et al. 2008) and bi, tri and tetra antennary complex, high mannose and hybrid type N-glycans (Sato, Fumkawa et al. 1993). Significantly extended O-glycans were not detected on bovine MFGM (Wilson, Robinson et al. 2008) and sialylation is present at a high level in both N-linked 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 GalNAcβ(1-4)GlcNAc (N,N-diacetyllactosediamine; LacdiNAc), including 28% of the glycans on CD36 and 37% of the glycans on BTN, suggesting it is a common feature among MFGM glycoproteins (Sato, Fumkawa 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). Firstly, the concentration of certain glycoproteins was found to increase in milk taken at day seven of lactation when compared to colostrum. For example, MUC1, MUC15 and BTN increased by 7.7-, 7.4- and 3.2 fold, respectively in milk collected on day seven when compared to colostrum (Reinhardt and Lippolis 2008). Ujita et al. (1993) observed a decrease in MFGM sialylation in the first 5 days post-parturition, 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 characterised 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 GlcNacβ(1-6) glycosyltransferase (Wilson, Robinson et al. 2008).

3.3.1 The mucins

Mucins are a family of long, macromolecular glycoproteins which are heavily glycosylated and their carbohydrate content is usually greater than 30%. The main mucins identified in bovine milk include MUC15, MUC1 and MUCX and make up approximately 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, Berglund 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 94kDa, 65% of which is accounted for by N- and O- glycans (Pallesen, Pedersen et al. 2007). Bovine MUC15 has 14 O-glycosites sites and 15 N-glycosites on its extracellular domain (Pallesen, Berglund et al. 2002). The N-linked glycans have been characterised as hybrid type glycans, with 11 of the 15 identified N-glycosites glycosylated (Pallesen, Pedersen et al. 2007). Core fucosylation, α(2-6) sialylation and terminal N-acetyllactosamine (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, Pedersen et al. 2007).

MUC1 is predominantly O-glycosylated but N-glycosylation is also present, with 5 potential N-glycosylation sites identified. The carbohydrate content of MUC1 is approximately 57-65%, varying according to the number of tandem repeats present in the MUC1 protein (Pallesen, Andersen et al. 2001). Approximately 28% of monosaccharide content of bovine milk MUC1 is Neu5Ac (Pallesen, Andersen 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, Pearson 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, Sando 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 approximately 80% of the 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 characterised for MUCX (Liu, Erickson 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, Erickson et al. 2005), indicating 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 has been shown to inhibit *H. pylori* colonisation (Hirmo, Kelm et al. 1998, McGuckin, Every et al. 2007), with Neu5Ac identified as playing a role in *H. pylori* inhibition (Hirmo, Kelm et al. 1998). Binding to rotavirus and inhibition of HIV-1 transmission has also been attributed to human milk mucin glycosylation (Yolken, Peterson et al. 1992, Saeland, de Jong 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, Pedersen et al. 2007). The glycosylation of human MUC1 has been linked to their resistance to digestion in the stomach (Peterson, Hamosh et al. 1998), a role which may also be true for their bovine counterparts.

3.3.2 Butyrophilin

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, Takio 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, Winter et al. 1983). The glycan chains of BTN have also been found to contain Fuc and Neu5Ac residues (Sato, Takio 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, Takio et al. 1995). Bi- to tetra- antennary complex type glycans have been detected, with sialylation and fucosylation present at both N-glycosites (Sato, Takio 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 lead 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.

3.3.3 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 50kDa variant with 7.1% accounted for by its carbohydrate content and PAS-7 is a 47kDa glycoform, with a 5.5% carbohydrate content (Kim, Kanno et al. 1992). N-linked 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, Andersen et al. 1996) but only PAS-6 is N-glycosylated at Asn209, with an unsubstituted core N-glycan structure attached (Kim, Azuma et al. 1998). Sialylation and fucosylation have been detected in lactadherin glycans (Kim, Azuma et al. 1998, Seok,

Shimoda 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, Shimoda et al. 2001). The N-linked glycans of PAS-7 are more complex, with a greater level of substitution when compared to those of PAS-6. Ten N-glycan structures have been characterised for PAS-7 (Seok, Shimoda et al. 2001), but only four for PAS-6 (Kim, Azuma 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, Azuma et al. 1998). In contrast, terminal β-linked Gal residues are present in PAS-7 (Seok, Shimoda 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, Peterson 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, Hamosh et al. 1998). Further research is required to confirm activities of bovine lactadherin glycosylation.

3.3.4 Cluster of Differentiation 36

Cluster of Differentiation 36 (CD36) is a 78kDa N-glycosylated protein which accounts for 2-5% of MFGM protein content (Greenwalt, Lipsky et al. 1992, Berglund, Petersen et al. 1996). Eight N-glycosites have been identified in bovine MFGM CD36: Asn78, Asn101, Asn171, Asn204, Asn234, Asn246, Asn320 and Asn416 (Nakata, Furukawa et al. 1993). High mannose type, hybrid type and bi-, tri- and tetra- antennary complex type glycans have been isolated, and more then one type of glycan chain can be located at each glycosite. Sixteen N-linked glycan

structures have been characterised 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, Furukawa 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, Lipsky et al. 1992).

3.3.5 Proteose peptone component 3

The proteose peptone fraction of bovine milk accounts for approximately 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 28kDa phosphorylated glycoprotein also know 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 (glycam1) (Girardet, Coddeville 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, Pâquet 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 300mg/L (Sørensen and Petersen 1993).

Two O-glycosites at Thr16 and Thr 86 (Thr 16 is only approximately 50% glycosylated (Sørensen and Petersen 1993)) and one N-glycosite at Asn77 have been identified for PP3 (Girardet, Coddeville et al. 1995, Coddeville, Girardet et al. 1998). Three neutral O-glycans have been characterised, GalNAc, Galβ(1-3)GalNAc and Galβ(1-4)GlcNAcβ(1-6)[Galβ(1-3)]GalNac (Coddeville, Girardet 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, Coddeville et al. 1995, Inagaki, Nakaya et al. 2010).

PP3 has previously been shown to inhibit lipase activity in milk (Anderson 1981, Cartier, Chillard et al. 1990) by competitive adsorption (Girardet, Linden 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, Rasmussen et al. 1997). Similar activity has been observed with PP3 from yaks milk (He, Ma 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, Moulti-Mati et al. 1993). It has been suggested that the negative charge conferred by the carboxyl groups of Neu5Ac stabilises 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, Marchall et al. 1997). However, trypsin digestion of PP3 had no effect on hybridoma mitosis (Mati, Moulti-Mati et al. 1993) suggesting 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, Mathot et al. 2004) and have a prebiotic effect on *Bifidobacterium animalis* (Etienne, Girardet 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*.

3.4 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 1) (Marnila and Korhonen 2002, Abd El-Fattah, Abd Rabo et al. 2012).

Table 1 Immunoglobulin concentration in bovine colostrum and mature milk and % carbohydrate content. Adapted from Marnila et al. (2002).

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

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, Moreno et al. 2009) of the Fc region (Takimori, Shimaoka 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 post-partum. Neu5Ac was present on ~ 50% of the glycans identified in colostrum but was only present at negligible levels from one week post-partum. The heterogeneity of IgG glycosylation also decreased with a greater number of glycan structures isolated from IgG in colostrum when compared to 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, Nimmerjahn 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, Elliott et al. 2001, Recio, Moreno 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

anti-microbial ingredients in milk based products such as infant formula and yoghurt and also in immune supplements and drinks.

3.5. Minor milk glycoproteins

Minor bovine milk glycoproteins include α-lactalbumin (αLA), lactoperoxidase (LP), folate binding protein (FBP) and glycolactin. Although αLA is a major protein in whey, only a small percentage of it is glycosylated in bovine milk (table 2), 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 2) (Barman 1970, Hopper and McKenzie 1973, Luhrs 1991, Tilley, Singer et al. 1991, de Wit and Hooydonk 1996, Shen, Wang et al. 1997, Roberts, Petropavlovskaja et al. 1998, Watanabe, Varsalona et al. 1998, Slangen and Visser 1999, Wolf, Ferrari et al. 2000, Chen, Lee et al. 2006, Jaiswal, Saraswat et al. 2011, Takimori, Shimaoka 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 glycoproteins. Glycosylation may have a role secretion of these glycoproteins and protection from proteolysis (summarised table 2).

Table 2 Summary of the information available on the glycosylation of minor milk glycoproteins.

Glycoprotein	Glycosylation	Glycans & activity	References
α-Lactalbumin (αLA)	• 3-10% glycosylated • 1 N-glycosite • 14 N-glycans • GalNAc, GlcNAc, Man + low levels of Fuc, Gal and Neu5Ac • Glycoforms 15840-16690 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	Synthesised 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, Tilley, Singer et al. 1991, Slangen and Visser 1999, Takimori, Shimaoka et al. 2011)
Lactoperoxidase (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, Varsalona et al. 1998, Wolf, Ferrari et al. 2000)
Folate binding protein (FBP)	• 2 N-glycosites • Asn49 = 49% glycosylated • Asn141 = 74% glycosylated • 17 N-glycans • Complex, high mannose and hybrid type • 7 structures in common with human FBP • Low levels of Fuc and Neu5Ac	Bovine and human FBP: glycosylation has a role in stabilising the ligand binding domain	(Luhrs 1991, Shen, Wang et al. 1997, Roberts, Petropavlovskaja et al. 1998, Chen, Lee et al. 2006, Jaiswal, Saraswat et al. 2011)

4. Commercialisation 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 3) and application (table 4) of specific bovine milk glycoproteins.

LF is the most established milk glycoprotein available for use as an ingredient. The isolation of LF has been up-scaled and optimised by numerous companies and it has been granted the generally recognised as safe (GRAS) 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 lactoferrin), 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 sterilisation 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 up-scale bLF production (Synlait 2013). LF currently markets at $500-$1,000 per kg and the global market for purified LF has grown from 45,000kg in2001 to 185,000kg in 2012 and is expected to expand even further, with a projected market of 262,000kg by 2017 (Synlait 2013). Approximately 50-100 thousand litres of milk is required as starting material to produce just 1kg of LF as a by-product of the cheese process, which explains in part it's 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 a 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 (PKU) patients, including strawberry pudding, crackers and an

orange sports beverage (Lim, van Calcar et al. 2007). As a result of it's emulsifying properties (Sharma, Rajput 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 it's anti-microbial 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 sterilisation 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 (table 3 & 4) 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 realised 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 commercialisation of milk derived powders, including isolated glycoproteins, for use an food grade ingredients.

Table 3 Examples of patents related to the isolation of bovine milk glycoproteins

Patent number	Title	Company	Publication date
US20030045677	Isolation of glycoproteins from bovine milk	Davisco Foods International, Inc.	Mar 6, 2003
Lactoferrin			
US4668771 A	Method for separating bovine lactoferrin from cow's milk and purifying same	Snow Brand Milk Products Co., Ltd.	May 26, 1987
US4791193 A	Process for producing bovine lactoferrin in high purity	Morinaga Milk Industry Co., Ltd.	Dec 13, 1988
EP0348508 B1	Process for separating and purifying lactoferrin from milk using sulfate compound	Snow Brand Milk Products Co., Ltd.	Sep 9, 1992
EP0744901 B1	Isolation of lactoferrin from milk	Pharming Intellectual Property BV	Dec 5, 2001
US 20050220953 A1	Process of isolating lactoferrin	Allan Otto Fog Lihme	Oct 6, 2005
US 20090306350 A1	New purification method of lactoferrin	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	Heat-stable, aqueous lactoferrin composition and its preparation and use	Campina Nederland Holding B.V.	Jul 14, 2011
Glycomacropeptide			
EP0393850 A2	Process for producing kappa-casein glycomacropeptides	Snow Brand Milk Products Co., Ltd.	Oct 24, 1990
US5061622 A	Process for the production of κ-casein glycomacropeptide	Snow Brand Milk Products Co., Ltd.	Oct 29, 1991
US5216129 A	Production of kappa-caseino-glycomacropeptide	Nestec S.A.	Jun 1, 1993
US5968586 A	Production of κ-casein macropeptide for nutraceutical uses	Wisconsin Alumni Research Foundation	Oct 19, 1999
US6168823 B1	Production of substantially pure kappa casein macropeptide	Wisconsin Alumni Research Foundation	Jan 2, 2001
US6462181 B	Process for preparing a kappa-caseino glycomacropeptide or a derivative thereof	Arla Foods Amba	Oct 8, 2002
US20020183489 A1	Large scale production of low fat and SDS gel pure kappa-casein glycomacropeptides (GMP) from bovine deproteinzed whey	Martin Davis, Fang Ming, Sharyn Su, Mengyan Yang, Akimoto Ichinomiya	Dec 5, 2002
Milk Fat Globule Membrane components			
WO2001035760 A1	Bulk preparation of milk fat globulemembranes	Ronald C. Gorewit	May 25, 2001
WO2002062374 A2	Muc1 isolation from bovine milk and whey	Davisco Foods International, Inc.	Aug 15, 2002
EP2098122 A1	Proteose Peptone Fraction	Nestec S.A.	Sep 9, 2009

Table 4 Example of patents related to the application of bovine milk glycoproteins

Patent number	Activity	Applicant	Publication date
Lactoferrin			
JP10059865	Anti-microbial	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		Campina Nederland Holding B.V.	Apr 3, 2012
US4977137 A	Intestinal growth	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		Wyeth Llc	Jul 15, 2010
EP2385837 A2		Pfizer Italia S.r.l.	Nov 16, 2011
US6482396 B1	Dental health	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	Immune modulation	Agennix Incorporated, Federica Pericle, Atul Varadhachary	Jun 24, 2004
US20050004006 A1	Diabetes treatment	Jose Engelmayer, Atul Varadhachary	Jan 6, 2005
US20090202574 A1	Anti-cancer	Jagat Rakesh Kanwar, Neill Ward Haggarty, Kay Patricia Palmano, Geoffrey Wayne Krissansen	Aug 13, 2009
US20130039903	Eczema	Rudi Ganter, Humera Ahmad	Feb 14, 2013
Glycomacropeptide (or κ-casein)			
US5147853 A	Anti-microbial	Snow Brand Milk Products, Co., Ltd.	Sep 15, 1992
WO1994015952 A1		Per Munk Nielsen, Novo Nordisk A/S, Niels Tromholt	Jul 21, 1994
US5344820		Snow Brand Milk Products, Co., Ltd.	Sep 6, 1994
WO1996008269		Abbott Laboratories	Mar 21, 1996

US4992420 A US4994441 WO1998052524 EP1104281	Oral health	Nestec S.A. Nestec S.A. Colgate-Palmolive Company Nestle S.A.	Feb 12, 1991 Feb 19, 1991 Nov 26, 1998 Jun 6, 2001
US20020119948 US20030008810 US20030059495 WO2004034813 A2	Appetite control	Pacifichealth Laboratories, Inc. Pacifichealth Laboratories, Inc. Mcneil-PPC, Inc. Pacifichealth Laboratories, Inc.	Aug 29, 2002 Jan 9, 2003 Mar 27, 2003 Apr 29, 2004
EP1874336 US20060247153	Improved learning	Squibb Bristol Myers Co, The University Of Sydney Robert J Mcmahon, Bing Wang, Jennie Brand-Miller, Steven C Rumsey	Jan 9, 2008 Nov 2, 2011
US5260280 JP 07278013 A	Anti-toxin	Snow Brand Milk Products, Co., Ltd. Snow Brand Milk Products, Co., Ltd.	Sept 11, 1993 Oct 24, 1995
WO2012004585 EP2440232	PKU treatment	Abdulla BADAWY Wisconsin Alumni Res Found	Jun 29, 2011 Apr 18, 2012
WO2003028751 WO2005097157	Eye care	Vista Scientific Llc Milcin Therapeutics Llc	Apr 10, 2003 Oct 20, 2010
US5063203	Anti-thrombotic	Centre National De La Recherche Scientifique (CNRS), 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	Immune-modulation	Kay Patricia Palmano, Alastair Kenneth Hugh Macgibbon, Nicole Jane Moore Dalbeth	Aug 11, 2011
Milk Fat Globule Membrane components			
EP1010434 B1 US20130130970 A1	Anti-microbial	Ghen Corporation, Nisshin Pharma Inc. Rotalactis Srl	Mar 31, 2004 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

5. Future directions of glycoprotein investigation

Although in recent years a significant body of research has focussed 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 bioactivites 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 Koldovský 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 utilised 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 for their isolation at an industrial scale has

hampered their utilisation. 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→3)-Gal and Neu5Gc, which could potentially elicit allergen-like immune responses in humans (Commins et al. 2011; Nguyen et al. 2005; Padler-Karavani et al. 2008). Neu5Gc has also been suggested to be involved in cancer development and metastasis (Makatsori et al. 1998; Malykh et al. 2001). Humans cannot synthesise 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.

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Chapter 2

Glycosidase activities in bovine milk over lactation

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Abstract

The presence and activity of glycosidases in Irish bovine milk over three months of lactation was investigated. A low level of variation between animals was observed and the highest level of glycosidase activity was present in colostrum, decreasing through transitional milk production to minimal but constant levels in mature milk. N-acetyl-β-D-glucosaminidase, α-L-fucosidase, α-galactosidase and N-acetyl-neuraminidase appear to be the most biologically relevant glycosidases in bovine milk. The elevated levels of enzymatic activity in colostrum suggests the milk glycosidases may play a role in the digestion of bovine milk glycans in the infant mammal, possibly acting as substitutes for bacterial glycosidases prior to colonisation by the gut microflora, which are involved in *in vivo* oligosaccharide metabolism in the lower gastrointestinal tract.

1. Introduction

Milk is a secretion of the mammalian gland and is principally composed of water, fat, protein (largely casein and whey) and carbohydrate. Until recently, little attention has been paid to the carbohydrate fraction; however, it has become evident that carbohydrates, whether free or bound to proteins or lipids, play essential roles as communication molecules in many intercellular and intracellular processes. The concentration of free oligosaccharides in human milk (HMOs) is 12–14 g/L, higher than the total protein content (Coppa, Zampini et al. 2006), indicating their importance for the nutrition and development of the human. Some of the functions ascribed to HMOs include prebiotic activity to promote commensal growth, protecting the gut epithelium from pathogenic invasion, and stimulating development of the normal immune system (Coppa, Bruni et al. 2004, Newburg 2009, Lane, Mehra et al. 2010). While less is known about the activities associated with the glycan component of glycoconjugates, it is clear that they are prevalent in milk and play key roles in the biomodulatory properties of human milk. Indeed, many proteins in human milk are glycosylated, including lactoferrin, mucin 4, α-lactalbumin, lactadherin, κ-casein, butyrophilin, lactoperoxidase, xanthine oxidase, bile salt-stimulated lipase, α-1- antichymotrypsin, α-1-antitrypsin, a variety of immunoglobulins, and at least 26 other proteins (Dallas, Sela et al. 2012). Glycosylated lipids in milk occur in two forms, neutral glycolipids and acidic (sialic acid containing) glycolipids, also known as gangliosides. Many of these glycolipids in human milk are known to be associated with the milk fat globule membrane (MFGM) (Newburg and Chaturvedi 1992). These molecules (particularly ganglioside GM1) provide a defensive strategy, acting as decoys to prevent adhesion of enteric pathogens and viruses to the epithelial cells of the infant's gut, thereby protecting breast-fed infants against infections (Hickey 2012).

Despite the obvious value of developing glycosylated milk components as functional food ingredients, very few products are currently commercially available which capitalise on their functions. This is mainly due to the limited supply of human milk carbohydrates required for clinical trials. In this respect researchers are beginning to focus their attention on the milk of domestic animals as a source for novel functional carbohydrates (Hickey 2012). One alternative may be to use bovine milk given its wide availability and its use in so many

regularly consumed dairy products. Similar to human milk, the carbohydrate fraction of bovine milk is divided into lactose, free oligosaccharides and bound glycans. Moreover, the glycans from both species of milk share common glycan structures suggesting they may have similar biological roles. Therefore, isolation, fractionation or enrichment of fractions containing bovine milk oligosaccharides or glycoconjugates may have potential applications in infant formulae and as functional ingredients in the nutraceutical industry.

Since many of the biological activities associated with both human and bovine milk are dependent on the glycans present, for example the anti-toxin and anti-infective activity of glycomacropeptide, lactoferrin and milk mucins (Kawasaki, Isoda et al. 1992) (Teraguchi, Shin et al. 1996) (Parker, Sando et al. 2010), it becomes important to investigate the factors which influence glycan structure. Glycan structures can be altered by the glycosidic release of terminal sugars (Wiederschain and Newburg 2001), which can in turn affect biological activity. Glycoside hydrolases, or glycosidases, catalyse the hydrolysis of glycosidic linkages between monosaccharide units and are involved in the production and modification of milk oligosaccharides and glycoconjugates in mammary epithelial cells. They also occur in human and bovine milk and are mainly found in the skim fraction. Monitoring glycosidase activity may help predict glycan structures and potential bioactivities of milk. While studies examining glycosidase activity have been performed in human milk (Wiederschain and Newburg 1995, Wiederschain and Newburg 2001, Dudzik, Knas et al. 2008), little is known about the activities of such enzymes in bovine milk, particularly over lactation, although season and feed have been shown to influence glycoside hydrolase concentration in bovine milk (Jozwik, Bagnicka et al. 2004). In this study, we aim to investigate the activity of seven glycosidases in bovine milk over the first three months of lactation in order to verify their presence as well as to identify any trends in their activity in colostrum, transitional and mature milk.

2 Methods and materials

2.1 Materials

The fluorogenic substrates used to monitor glycosidase activity include 4-methylumbelliferyl (4-MU) derivatives of α-L-fucopyranoside, β-D-

galactopyranoside, α-D-galactopyranoside, β-D-glucopyranoside, β-D-glucuronide, β-D-*N*-acetylglucosaminide, and α-D-*N*-acetylneuraminic acid (NANA). All were purchased from Sigma Aldrich (Dublin, Ireland).

2.2 Milk samples

Irish milk was collected from Holstein Fresian cattle, on-site at the Teagasc, Moorepark Dairy Production Research Centre (Fermoy, Cork, Ireland). Lipids and caseins were removed by centrifugation on a Sorvall RC-5B centrifuge at 5,000 x g and 50,000 x g respectively to generate skim milk and whey. Milk was collected at day 1 to day 10, month 1, 2 and 3 (D1-D90) of lactation from three cows following calving. Fresh milk samples were used to assess enzymatic activity immediately after collection.

2.3 Glycosidase activity

The activities of α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, β-D-glucosidase, N-acetyl-β-glucosaminidase, β-D-glucuronidase, and neuraminidase were assayed with the appropriate fluorogenic substrates as previously described (Wiederschain and Newburg 2001). α-L-fucosidase activity was monitored at pH 5.0 and all other glycosidases were monitored at pH 4.5 at a final substrate concentration of 5 mmol l^{-1}. 20 μL of bovine milk was incubated with 80μL of fluorogenic substrate dissolved in 0.1 mol l^{-1} citrate-phosphate buffer, pH 4.5 or 5.0 incubated at 37°C for 1 hr and the reaction was stopped with 200 μL 0.25 mol l^{-1} glycine-KOH buffer, pH 10.4. The fluorescence of enzymatically liberated 4-methylumbelliferone was measured with excitation at 365 nm and emission measured at 480 nm. A standard curve of serially diluted 4-methylumbelliferone (0-1000 μmol l^{-1}) was constructed in order to correlate fluorescence units with substrate concentration. The specific activity of bovine milk glycosidases was expressed as μmol l^{-1} of hydrolyzed substrate per mL of milk per hour. Assays were performed in triplicate, and the standard error was reported.

3. Results

The activities of α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, β-D-glucosidase, N-acetyl-β-glucosaminidase, β-D-glucuronidase, and N-acetyl-neuraminidase in bovine milk were investigated through the use of fluorogenic substrates. Glycosidase activity was initially investigated in raw milk, skim milk

and whey to determine the optimum conditions for future enzyme assays. In some instances, loss of activity was observed following the removal of the lipid fraction during the preparation of skim milk (figure 1); therefore whole milk was used to monitor glycosidase activity for the remainder of the study. Enzymatic activity was determined in milk from three cows over the first three months of lactation and, although slight variations between each animal were observed, a common pattern was readily identifiable (figure 2). N-acetyl-β-D-glucosaminidase, α-L-fucosidase, α-galactosidase and N-acetyl-neuraminidase appear to be the most active glycosidases in bovine milk and therefore possibly the most biologically relevant. In fact, these were the most active enzymes right throughout lactation (table 1). To the best of our knowledge, this is the first time N-acetyl-neuraminidase has been identified in bovine milk, with an activity of 82 ±13 (μmol l^{-1} substrate hydrolysed)/(mL of milk)/hr in colostrum.

When total glycosidase activity was considered, it was much higher in early lactation when compared to mature milk. The highest level of activity was observed in colostrum and then a decrease was evident as lactation progressed into transitional milk production with a constant level in mature milk (D30 onwards). Of the seven enzymes investigated, four (N-acetyl-β-D-glucosaminidase, α-L-fucosidase, α-D-galactosidase and N-Acetyl-neuraminidase) showed highest activity in D1 colostrum (table 1). N-Acetyl-β-D-glucosaminidase was the most active glycosidase in early lactation (figure 2). It displayed the highest activity in the first day of lactation and reduced to 39.2% of D1 activity by D2, declining more gradually thereafter (table 1). α-L-Fucosidase and α-D-galactosidase displayed similar patterns of activity (table 1). The fourth, β-D-glucuronidase, showed only trace activity in all but the initial sample tested (table 1). N-Acetyl-neuraminidase increased in activity from D1 colostrum throughout transitional milk, decreasing in mature milk production (table 1), while β-D-galactosidase was relatively stable throughout the early days of lactation, again decreasing in mature milk (table 1). β-D-Glucosidase had minimal activity in D1 colostrum, increasing from D4 onwards, reaching its maximum level of activity in D10 milk (table 1). Thus, all glycosidases assayed exhibited reduced, but constant levels of activity in mature milk samples taken from D30 onwards in lactation.

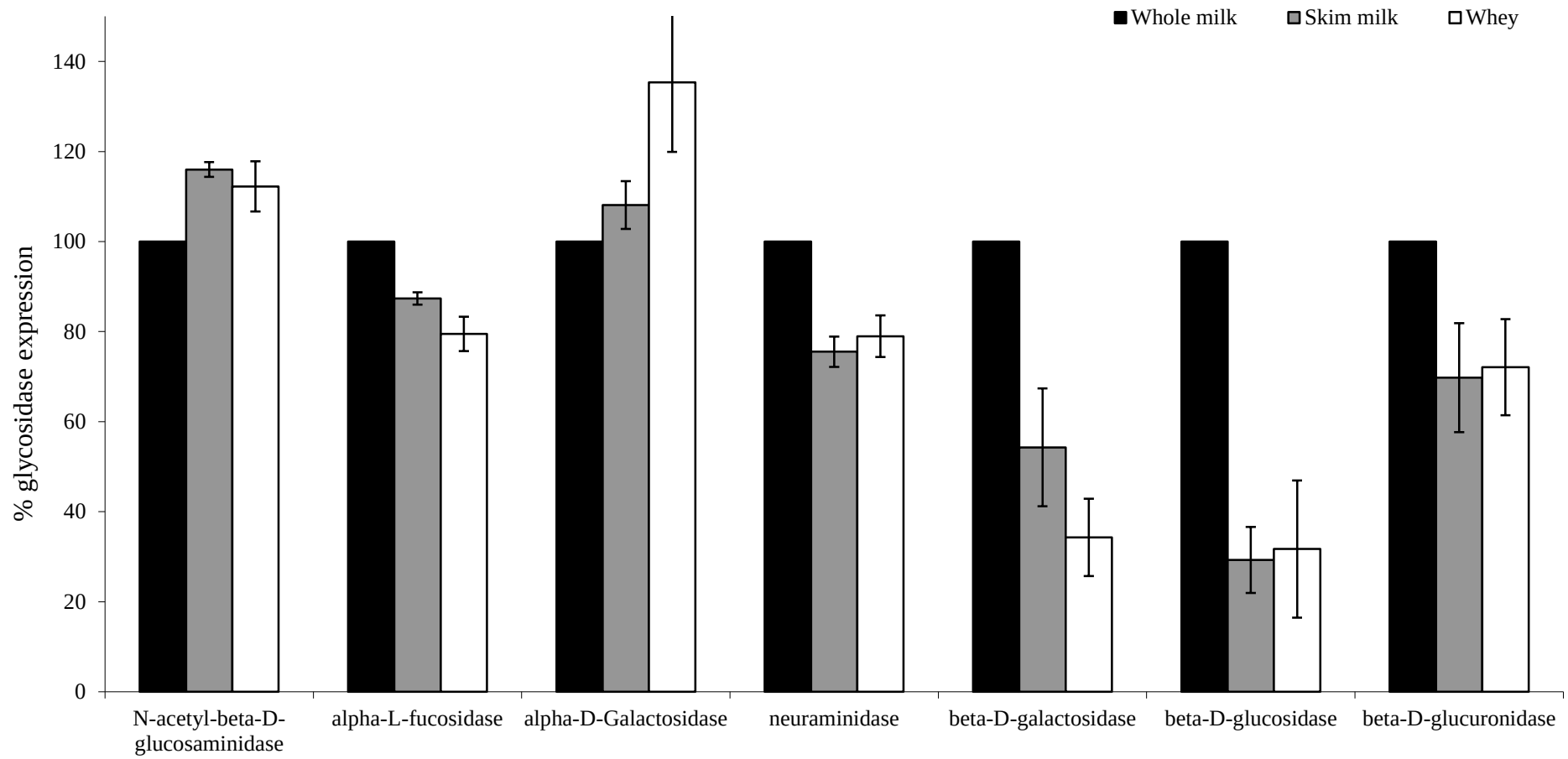

Figure 1 Percentage of glycosidase expression (%) present in whole milk which was detected in skim milk and whey. The data represent means +/- standard deviation of three replicates.

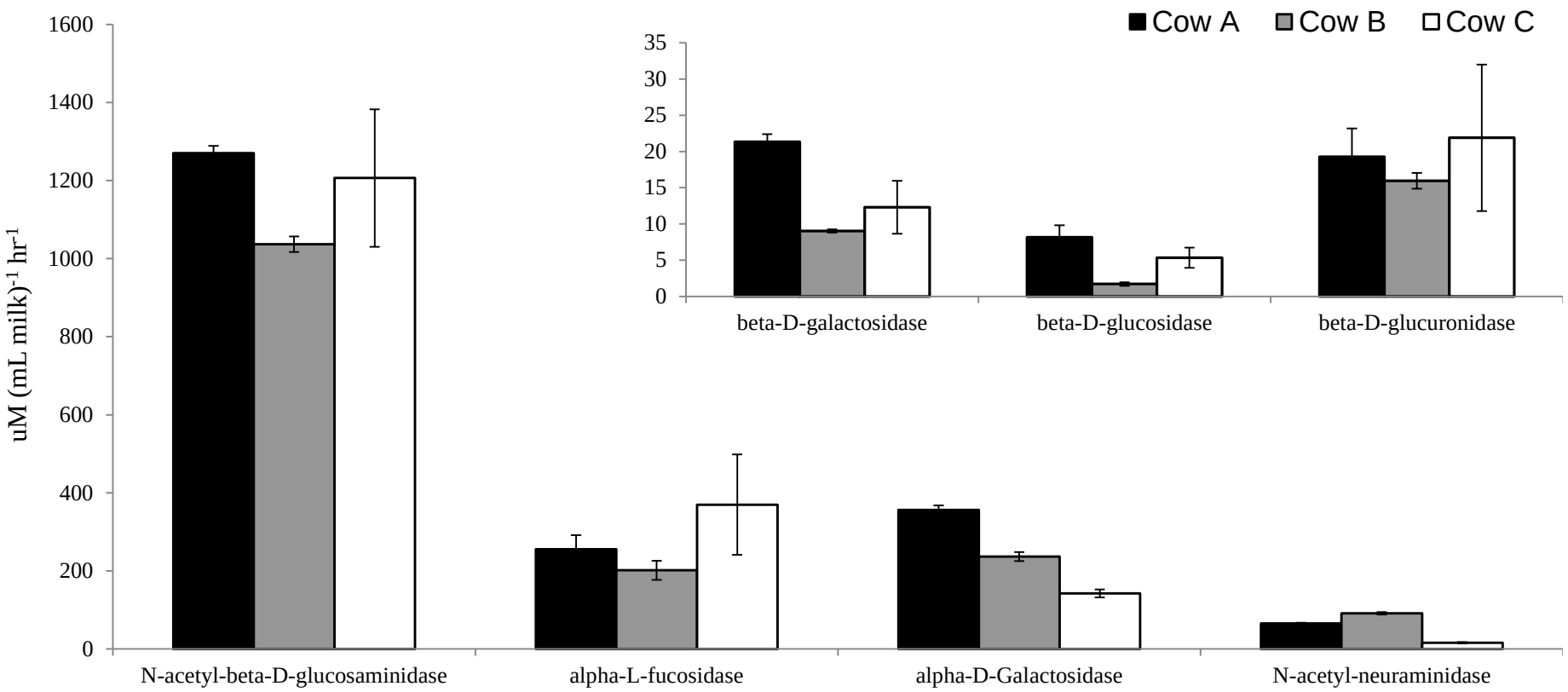

Figure 2 Glycosidase activities in bovine colostrum (D1 milk) from three cows, expressed as (μmol l-1 substrate hydrolysed)/(mL of milk)/hr. The data represent means +/- standard deviation of three replicates.

Table 1 Average glycosidase activities in milk from three cows in triplicate (n=3) expressed as (μmol l^{-1} substrate hydrolysed)/(mL of milk)/hr over the first three months of lactation. Standard deviation (±) is reported.

Day	N-acetyl-β-D-hexosaminidase	α-L-fucosidase	α-D-Galactosidase	N-acetyl-neuraminidase	β-D-galactosidase	β-D-glucosidase	β-D-glucuronidase
1	1171.2 ± 120.6	275.7 ± 85.7	198.8 ± 75.4	81.8 ± 12.8	14.2 ± 6.4	5.1 ± 3.2	19.0 ± 3.0
2	460.4 ± 252.5	180.5 ± 4.34	82.7 ± 44.0	89.8 ± 6.0	11.9 ± 3.6	10.2 ± 5.9	3.4 ± 1.9
3	332.3 ± 160.5	132.0 ± 18.4	60.2 ± 27.0	82.9 ± 11.4	8.5 ± 4.6	7.2 ± 5.4	2.9 ± 1.6
4	307.6 ± 129.6	199.0 ± 38.5	48.6 ± 9.5	114.0 ± 5.3	13.3 ± 6.7	17.7 ± 4.3	1.8 ± 0.5
5	251.8 ± 126.7	120.5 ± 19.4	43.5 ± 13.4	103.4 ± 17.8	9.8 ± 3.8	23.1 ±2.8	1.0 ± 0.3
6	266.8 ± 76.8	159.6 ± 40.7	39.8 ± 12.3	124.5 ± 7.2	13.7 ± 0.1	32.4 ± 7.0	1.33 ± 0.3
7	217.4 ± 98.0	119.4 ± 26.8	34.6 ± 3.6	116.5 ± 1.9	13.8 ± 1.0	26.1 ± 2.7	0.9 ± 0.3
8	237.4 ± 131.9	168.5 ± 9.4	39.1 ± 22.8	138.1 ± 5.9	12.4 ± 4.6	29.3 ± 13.3	1.2 ± 0.5
9	166.4 ± 81.6	131.8 ± 3.3	33.4 ± 15.4	110.6 ± 9.1	7.3 ± 0.9	21.1 ± 7.1	0.9 ± 0.7
10	60.2 ±12.5	104.1 ± 32.8	25.0 ± 15.0	119.5 ± 24.9	7.9 ± 4.9	10.4 ± 6.0	0.9 ± 0.7
30	50.7 ± 11.8	77.6 ± 10.7	14.1 ± 6.3	37.8 ± 0.1	7.1 ± 3.7	20.4 ± 7.5	1.0 ± 0.6
60	25.5 ± 18.7	82.1 ± 20.5	17.9 ± 7.4	27.9 ± 8.8	6.9 ± 3.2	19.7 ± 9.0	0.8 ± 0.3
90	1.6 ± 0.3	68.3 ± 22.7	19.2 ± 4.4	28.0 ± 8.7	8.0 ± 3.4	17.2 ± 6.0	0.9 ± 0.5

4. Discussion

Glycosidase expression was highest in whole milk, with the exception of N-acetyl-β-glucosaminidase and α-D-galactosidase, whose activity increased following the removal of the lipid and casein fractions. This suggests the fat globules and casein micelles present in bovine milk may be preventing access of these enzymes to their substrates.

Following the generation of skim milk and whey, a reduction was observed in the activity of β-D-glucuronidase and N-acetyl-neuraminidase, but most significantly in β-D-galactosidase and β-D-glucosidase activity. Keenan & Huang (1972) previously characterised the ability of the bovine milk fat globule membrane (MFGM) to hydrolyse uridine diphosphogalactose and uridine diphosphoglucose. The work presented here supports the hypothesis that a number of glycosidase activities are associated with the milk fat globule fraction of bovine milk. 46% of β-D-galactosidase activity and 71% of β-D-glucosidase activity in whole milk was lost when the MFGM was removed. Also, N-acetyl-neuraminidase reduced to 76.5% of the original activity following the generation of skim milk. A similar distribution was observed for β-D-glucuronidase, with approximately 30% of its activity lost upon removal of the lipid fraction of bovine milk. It should be noted, however, that enzyme activities were not assayed directly in the lipid fraction of milk in this study and it is possible that some enzyme-activating factor is lost upon the removal of the lipid layer, resulting in a reduction in the assayed activity of these enzymes. Of the seven glycosidases tested in this study, β-D-galactosidase was the only one to show any association with the casein fraction of bovine milk, with 20% of its activity lost as a result of the removal of casein during the preparation of whey.

Seven glycosidases were monitored in the milk of three cows over three months of lactation with N-acetyl-β-D-glucosaminidase, α-L-fucosidase, α-galactosidase and N-acetyl-neuraminidase displaying the highest activity throughout the sampling period. The highest levels of enzyme expression were observed in D1 colostrum. Glycosidase levels in mature milk were greatly reduced and remained constant from D30 onwards. Similar expression patterns have been documented for glycosidases in the milk of other species. Hurley & Grieve (1988) characterised N-acetyl-β-D-glucosaminidase expression in sow's milk and, as shown in this study, observed an initial rapid decrease in expression

between D1 and D3, with a slower decline to D14, after which enzyme levels remained constant. This decline in N-acetyl-β-D-glucosaminidase activity has also been observed previously in bovine milk over lactation (Timms and Schultz 1985). Such findings suggest the presence of glycosidases in bovine milk is more biologically important in the initial days following parturition, with the requirement for these enzymes decreasing as lactation progresses.

Several studies have investigated glycosidase levels in human milk following parturition. Similar to the results presented here for bovine milk, N-acetyl-β-D-glucosaminidase and α-L-fucosidase have been identified as the most active glycosidases in human milk (Wiederschain and Newburg 2001). Using comparable methods to those employed in the current study, Wiederschain & Newburg *et al.* (2001) were unable to detect other glycosidases in human milk, in contrast to separate studies which previously identified neuraminidase activity (Schauer, Veh et al. 1976). However, upon further incubation of the milk samples at 37^{o}C (Wiederschain and Newburg 2001), an increase in free N-acetyl-neuraminic acid was observed, suggesting the presence of neuraminidase activity which may not have been detected through the use of fluorogenic substrates. In this study, the 4-MU labeled monosaccharide derivatives were used at a concentration of 5mM in order to detect glycosidases which may be present at a low concentration. Wiederschain *et al.* (2001) used similar substrates but at the lower concentration of 1mM, which may account for the absence of neuraminidase activity when using the fluorogenic assay. Human milk N-acetyl-β-D-hexosaminidase (Dudzik, Knas et al. 2008) and α-L-fucosidase (Wiederschain and Newburg 1995) activities were reported to decrease during the initial days post parturition, as in our study, but increased again as lactation progresses from week 3 and week 4, respectively. Overall, the glycosidase expression pattern characterised in this study for bovine milk is similar to that found in the milk of other mammalian species. The only difference noted was an increase in N-acetyl-β-D-hexosaminidase (Dudzik, Knas et al. 2008) and α-L-fucosidase (Wiederschain and Newburg 1995) activity in human mature milk which was not detectable in bovine milk in this study.

The carbohydrate components of bovine milk are present as free oligosaccharides and bound glycans, in the form of glycoconjugates such as glycoproteins and glycolipids. Lactose is the most dominant glycan structure

present in bovine milk at 46g/L (Jenness, 1974). Free oligosaccharides are present at 0.7-1.2g/L (Tao, et al., 2008) in bovine colostrum and the concentration decreases rapidly as lactation progresses to 0.03-0.06g/L (Urashima and Taufik 2010) in mature milk. Bound glycans are more challenging to quantify. However, (Sriwilaijaroen, Kondo et al. 2012) recently quantified 374.9nmol l^{-1}/g of N-glycans in dry, delipidated whey protein concentrate. Similar methods could be employed to determine the glycan content of whole milk. A heterogeneous mix of acidic and neutral free oligosaccharides have been previously characterised (Tao, DePeters et al. 2008, Mariño, Lane et al. 2011) and a similarly complex mixture of glycan structures has been identified in the bovine milk glycoprotein fraction, with a total of 51 N-glycan structures identified (Nwosu, Aldredge et al. 2012). O-glycosylation is also present on bovine milk glycoproteins in proteins such as glycomacropeptide (Hua, Nwosu et al. 2011) and proteins of the MFGM (Sato, Fumkawa et al. 1993, Wilson, Robinson et al. 2008). The major glycolipids of milk which reside in the MFGM include lactosylceramide and glucosylceramide (Newburg and Chaturvedi 1992) with lactose and glucose modifications, respectively. Bovine milk glycans, be they in free form or in glycoconjugate form, have a wide range of associated bioactivities, including antimicrobial, prebiotic and immunoregulatory activities (Recio, Moreno et al. 2009, Lane, Mehra et al. 2010) and these bioactivities are closely linked to glycan structure.

Many of the glycan structures identified in milk differ from one another by one monosaccharide residue, e.g. a terminal sialic acid, fucose (in glycoproteins only) or galactose moiety. The greatest number of these structures has been identified in colostrum, with glycan heterogeneity decreasing and lactose content increasing in the initial days post parturition (Nakamura, Kawase et al. 2003, Tao 2009, Takimori, Shimaoka et al. 2011). This decrease in glycan heterogeneity may influence enzyme expression, as less substrate would be available for the glycosidases. For example, α-L-fucosidase activity decreases from colostrum onward, as does the level of glycoprotein fucosylation in bovine milk (Takimori, et al., 2011). In other words, a reduction in the presence of α-linked fucose residues is observed which correlates with a reduction in the presence of the enzyme required for hydrolysis, suggesting possible co-regulation of the release of glycan structures and glycosidase expression in the mammary gland may be occurring.

N-acetyl-neuraminidase activity does not appear to follow the pattern observed for the other glycosidases assayed, with less fluctuation over lactation, reaching peak expression in transitional milk and decreasing significantly in mature milk. A decrease in the sialylation of bovine milk oligosaccharides and glycoproteins over lactation has been well documented (Martin, Martin-Sosa et al. 2001, Martin-Sosa, Martin et al. 2003, Nakamura, Kawase et al. 2003, Wilson, Robinson et al. 2008, Tao 2009, Barile, Marotta et al. 2010, Takimori, Shimaoka et al. 2011). However, a corresponding pattern in N-acetyl-neuraminidase activity is not evident. Glycosidases therefore may have another role in milk, possibly in the *in vivo* digestion of bovine milk oligosaccharides. Sialic acid has many biological functions including antimicrobial (Hirmo, Kelm et al. 1998) and prebiotic (Idota, Kawakami et al. 1994) activities and may be incorporated into brain gangliosides in the infant mammal (Wang, McVeagh et al. 2003). The constant level of N-acetyl-neuraminidase expression during the initial days of life may be important for the release of diminishing amounts of this bioactive monosaccharide for the benefit of the newborn calf.

Milk glycan digestion by glycosidases may have a role in the release or exposure of bioactive saccharides that have immuno-modulatory functions early on in the development of the GI tract and in the promotion of beneficial microflora in the lower digestive tract. As it takes seven days post-parturition for the establishment of a bifidobacterium-rich gut flora in the newborn calf (Vlkova, Trojanova et al. 2006), the elevated level of glycosidase activity in colostrum may be linked to the necessity for the release of terminal monosaccharides *in vivo* for utilisation as a carbon source by intestinal bacteria. Intestinal bacteria, in particular bifidobacteria, have been previously shown to be involved in the digestion of milk glycans (LoCascio, Ninonuevo et al. 2007, Sela, Chapman et al. 2008, Sela, Li et al. 2011, Garrido, Nwosu et al. 2012, Garrido, Ruiz-Moyano et al. 2012), and in their absence in the immature mammalian gut, greater glycosidase activity in early lactation may be required to assist in the digestion of milk glycans.

There are some questions which must be addressed before the activity of glycosidases *in vivo* can be confirmed. Firstly, the survival and activity of the enzymes throughout the gastrointestinal tract (GIT) must be investigated, possibly by performing feed trials and sampling gastric and intestinal content. Similar

experiments were performed to investigate the activity exogenously fed human milk bile salt-stimulated lipase activity *in vivo* in infants, in order to demonstrate it's role in the catalysis of short chain fatty acids from dietary triacylglycerides in the human duodenum (Fredrikzon, Hernell et al. 1978, Wang, Kuksis et al. 1983). Secondly, for milk glycosidases to have an effective role in the digestion of milk glycans, they must be present *in vivo* at biologically relevant concentrations. In order to function as a substitute for bacterial glycosidases, milk enzymes must have similar activity to the total activity of glycosidases expressed by the entire gut microbiota. Once survival and activity of milk glycosidases *in vivo* has been confirmed and duration of activity has been calculated, total milk glycosidase activity ingested by the calf can be quantified by considering daily milk intake. Milk glycosidase concentrations could possibly be quantified through the use of an enzyme standard and appropriate chromatography techniques. However, the presence of the enzyme does not necessarily infer functionality as factors such as pH influence activity *in vivo*. Therefore a method to monitor glycosidase activity *in vivo* would be more informative. Feeding calves fluorescently labelled monosaccharides, e.g. 4MU- NANA, and monitoring the presence of the released fluorophore in excreted urine and faecal samples may provide some insight. Quantifying total glycosidase activity expressed by gut bacteria however is complicated by the number of bacterial strains present and variety of glycosidases expressed.

Finally, the presence of a variety of glycosidases in bovine milk, and their distribution throughout all fractions of the milk, suggests the continuous secretion of these enzymes into the milk may be of biological significance to the infant calf. No studies to date have identified a biological role for these enzymes *in vivo*. Feeding trials using a bovine model could be employed to investigate the biological effect of glycosidases. Parameters such as the intestinal microbiota, levels of immune factors and the glycan content of faecal samples could be compared between animals fed heat-treated milk, for the inactivation of milk glycosidases under the conditions described by Andrews *et al.* (1987), and animals which consumed un-treated milk to determine the possible biological significance of bovine milk glycosidases. Faecal fermentations using similar treatments may give insight into the role of milk glycosidases in the release of bioactive structures for the promotion of bifidobacterial growth.

5. Conclusions

A substantial decrease in the activity of glycosidases in bovine milk as lactation progresses was observed. The elevated presence of glycosidases in early lactation suggests they may be more biologically important to the infant mammal in the early days following birth. These enzymes have the ability to cleave terminal monosaccharides from bovine milk glycans, therefore altering their structure and, potentially, their biological value. They may possibly be involved in the digestion of milk oligosaccharides, mediating oligosaccharide metabolism *in vivo* in order to harness their biological potential. However, further investigation is required to confirm this activity and to confirm survival of milk glycosidases during transit through the mammal gastrointestinal tract.

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Chapter 3

Profiling temporal changes in bovine milk lactoferrin glycosylation using lectin microarrays

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Abstract

Bovine milk glycoprotein bovine lactoferrin (bLF) has a variety of biological activities related to its constituent glycans. However, little is known about bLF's oligosaccharide structural changes over the course of lactation. BLF was isolated at 13 time points during the first three months of lactation from three individual cows and glycosylation changes were profiled by lectin microarray. Substantial profile differences between early and late lactation were observed and accompanying monosaccharide analysis revealed that the occurrence of the non-human sialic acid, *N*-glycolylneuraminic acid, was greater during early stage milk production. Overall, the data suggested that more diverse complex-type oligosaccharides structures were present on bLF during early lactation with an abundance of oligomannose type glycans in later lactation. The differences in the glycoprofiles of bLF from colostrum to mature milk suggest that these may have different functionality *in vivo*.

1. Introduction

Glycosylation is one of the most abundant and diverse forms of protein post-translational modification. Approximately 50% of eukaryotic proteins are glycosylated and this is normally a requirement for their correct biological function. Bovine milk has a large number of constituent glycoproteins, including lactoferrin (bLF), κ-casein, immunoglobulins, mucins, butyrophilin, lactadherin, CD36, PP3, α-lactalbumin, lactoperoxidase, folate-binding protein and glycolactin, all of which play key roles in the biomodulatory properties and nutritional quality of milk (Recio, Moreno et al. 2009). BLF is of particular interest as it has a wide range of bioactivities including anti-microbial, anti-adhesive, prebiotic, immunomodulatory and anti-cancer properties (Garcia-Montoya, Cendon et al. 2012), resulting in its use as an ingredient in infant formula and functional foods such as prebiotic drinks and immune supplements. The glycans of bLF contribute to its anti-adhesive (Teraguchi, Shin et al. 1996) and iron-binding activities (Legrand, Mazurier et al. 1990), as well as protecting the protein backbone from proteolysis (van Veen, Geerts et al. 2004). The concentration of bLF in milk is highest in colostrum (2-5 mg/mL), which is produced in the days immediately following birth, and decreases sharply to 0.1-0.3 mg/mL (Recio, Moreno et al. 2009) by day 5 post-partum (Abd El-Fattah, Abd Rabo et al. 2012). BLF in mature milk has up to five *N*-linked glycans, which are a mixture of the high-mannose, complex and hybrid types, and up to 59 different structures have been identified to date (Hua, Nwosu et al. 2011). Two isoforms of bLF have been identified; bLF-b (containing four *N*-linked oligosaccharides) is the prevalent glycoform in milk while bLF-a (containing five *N*-linked glycans) accounts for 30% of bLF in colostrum (Yoshida, Wei et al. 2000) but only 15% of bLF in mature milk (Kolodziejczyk, Tedeschi et al. 2010). As the glycosylation of bLF has been shown to play a role in its bioactivity and affect its digestion profile (O'Riordan, Kane et al. 2014), structural changes of constituent glycans may result in variations in both the biological function and rate of digestion of the protein in the infant gut. Thus, it is important to determine any glycosylation changes of glycoproteins at multiple points during lactation.

Changes in protein glycosylation throughout lactation have previously been investigated for total bovine milk glycoproteins (Takimori, Shimaoka et al.

2011), human LF (hLF) (Barboza, Pinzon et al. 2012) and bovine milk fat globule membrane (MFGM) (Wilson, Robinson et al. 2008). Takimori, *et al.* (2011) characterised total bovine milk protein glycosylation at day 1 and week 1 to 4 of lactation *via* chemoselective glyco-blotting and mass spectrometric (MS) analysis and, for bLF, the most highly substituted glycans containing both fucosylation and sialylation were characterised for colostrum only. However, only five timepoints were sampled in that study and sampling was only done up to the first month. Traditional methods of analysis such as MS, high performance liquid chromatography (HPLC) with enzymatic digestions and nuclear magnetic resonance spectroscopy (NMR) yield very detailed structural data but require extensive sample processing, lengthy analysis time, high levels of expertise and, in some cases, relatively large quantities of biological samples. This has meant that in the past only a small number of time points and only one individual cow or pooled samples have been examined. Thus, a high throughput method of carbohydrate structural profiling of glycoproteins would make sampling a greater number of time points for multiple animals more feasible and enable the monitoring of potential temporal structural variation more effectively.

Lectins are proteins of non-immune origin which recognise specific carbohydrate determinants and lectin microarrays allow rapid glycan profiling without the need to release glycans from the glycoprotein (Gerlach, Kilcoyne et al. 2014). As the analysis of bLF glycosylation is excessively complicated compared to hLF due to the presence of unusual motifs including *N*,*N*'-diacetyllactosamine (GalNAc-β-(1→4)-GlcNAc, LacdiNAc) (van Leeuwen, Schoemaker et al. 2012), lectin microarray profiling represents a relatively high throughput method which can identify important carbohydrate motifs in a large number of samples. Lectins have been previously used for analysing milk glycans (Gustafsson, Kacskovics et al. 2005) and a limited selection of lectins has been employed to characterise the LF glycosylation from mature milk from a variety of mammalian species (Yen, Wu et al. 2011, Zinger-Yosovich, Sudakevitz et al. 2011), but not in the microarray format.

To assess the applicability of employing lectin microarrays for profiling complex glycosylation of multiple bLF samples from more than one cow, milk was collected each day from three individual cows for the first 10 days of

lactation and at months 1, 2 and 3 thereafter for a total of 13 time points to focus on temporal glycosylation changes in the initial days post-partum, where the most dynamic glycosylation has been observed for other milk glycoproteins (Wilson, Robinson et al. 2008, Froehlich, Dodds et al. 2010). A microarray consisting of 43 lectins, each with their own individual carbohydrate motif specificity was used to profile changes in bLF glycosylation over lactation for each individual animal (i.e. for 39 samples in total). This resulted in a characteristic profile for each glycoform at a particular time point. The monosaccharide composition of each bLF sample at each time point was determined and correlated well with the predicted structural components based on the generated glycoprofiles and known structural characterisations. Temporal trends of bLF glycosylation were also mapped. From these data, specific time points for maximum or minimum expression of desirable carbohydrate motifs can be quickly identified and aid in future strategies for enrichment or depletion. In addition, lectin microarrays were shown to be a feasible and attractive approach for detailed temporal study of milk glycoproteins of multiple animals.

2 Methods and materials

2.1 Materials

The Sartobind S SingleSep nano 1 mL capsule was from Sartorius Stedim Biotech (Germany). Nexterion® Slide H microarray slides were purchased from Schott AG (Germany). Pure, unlabelled lectins (table S1) were acquired from EY Laboratories, Inc. (San Mateo, CA, USA), Vector Laboratories, Ltd. (Orton Southgate, U.K.) or Sigma-Aldrich Co. (Dublin, Ireland). Pure monosaccharides (Fuc, Gal, glucose (Glc), GlcNAc, GalNAc and Man) and Bradford reagent were also from Sigma-Aldrich Co. Carboxylic acid succinimidyl ester AlexaFluor 647 fluorescent label was purchased from Life Technologies (Carlsbad, CA, USA). Zeba Spin desalting centrifugal columns (0.5 mL, 7 kDa molecular weight cut off (MWCO)) were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Sialic acids standards *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) were from Dextra (Reading, U.K.). All other reagents were from Sigma-Aldrich Co. unless otherwise noted and were of the highest grade available.

2.2 Sample collection

Milk was collected at evening milking daily from three non-related, multiparous Holstein-Friesian cows (cows A, B and C) from day one of calving to day ten post-parturition (D1-D10) and at three more time points on the last day of months one, two and three (D30, D60, and D90) for a total of 13 sample time points. All samples were frozen at -20 °C immediately after collection.

2.3 Lactoferrin isolation

Thawed milk samples were centrifuged at 5,000 rpm at 4 °C for 10 min and the fat layer, which floated to the top of the milk sample, was removed. The pH of the resultant skim milk was adjusted to 4.5 to precipitate the casein, and whey was prepared by centrifugation at 13,000 rpm at 4 °C for 30 min followed by filtration of the supernatant through a 0.45 μm filter. BLF was isolated from the prepared whey, based on an optimised adaptation of isolation techniques previously described for LF (Plate, Beutel et al. 2006). In brief, membrane adsorption cation exchange chromatography was performed using a Sartobind S SingleSep nano 1 mL capsule (36 cm^2) on an Äkta Purifier equipped with a Monitor UV-900 detector (GE Healthcare Life Sciences, Uppsala, Sweden). Eluent A was 20 mM sodium phosphate (pH 7) and eluent B was 2 M NaCl, 20 mM sodium phosphate (pH 7). The membrane was equilibrated with two column volumes (CV) of eluent A and 150 mL of the filtered whey was injected at a flow rate of 3 mL/min. The membrane was washed with 2 CVs of eluent A and bound proteins were subsequently eluted with a three-step salt gradient; I, 0-10% eluent B linear gradient over 4 CVs, II, 10-50% eluent B linear gradient over 4 CVs, and III, 50-100% eluent B linear gradient over 1 CV. The eluate was monitored by absorbance at 214 and 280 nm. Fractions from step II containing purified bLF were pooled, dialysed in 18.2 MΩ water and lyophilised to dryness. To verify purity of bLF, reverse phase HPLC (RP-HPLC) was performed on a Source^{TM} 5RPC ST 4.6 x 150 mm column (GE Healthcare Life Sciences) with a Waters 2695 HPLC instrument equipped with a 2487 Dual λ absorbance detector (Waters, Milford, MA, USA). Eluent A was 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC-grade water and B was 90% acetonitrile, 0.1% TFA in HPLC-grade water. Separation was performed with a gradient (table S2) at a flow rate of 0.8 mL/min. Eluate was monitored by absorbance at 214 and 280 nm and only a

single peak was observed as expected. A commercially available bLF standard was also chromatographed for comparison. Isolated bLF and a commercially available bLF standard were also analysed by SDS-PAGE (10% acrylamide gels) and coomassie brilliant blue and periodic acid Schiff stained to investigate sample purity.

2.4 Fluorescent labeling of glycoproteins

The bLF protein samples (300 µg) and the asialofetuin (ASF) standard were labeled with Alexa Fluor® 647 (carboxylic acid succinimidyl ester, λ_{ex} 650 nm, λ_{em} 665 nm) in 100 mM sodium bicarbonate, pH 8.0 for 1 h in the dark and samples were kept in the dark after this point. Excess dye was removed from the labelled bLF samples using a centrifugal desalting column (7 kDa MWCO). Absorbance at 650 and 280 nm for each sample was measured and the protein concentration and degree of substitution was determined according to manufacturer's instructions using the extinction coefficient 85,700 M^{-1} cm^{-1} for bLF (Gifford, Ishida et al. 2012) (table S3).

2.5 Construction and incubation of lectin microarrays

A panel of 43 lectins were prepared at a concentration of 0.5 mg/mL in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4, and appropriate mixture of 10 mM Na_2HPO_4 and NaH_2PO_4 for correct pH), pH 7.4, supplemented with 1 mM of the appropriate haptenic sugar (table S1) to ensure preservation of carbohydrate recognition domains during printing. Lectins were printed at approximately 1 nL per feature on Nexterion® Slide H microarray slides using a SciFLEXARRAYER S3 piezoelectric printer (Scienion AG, Berlin, Germany) essentially as previously described (Kilcoyne, Gerlach et al. 2012, Gerlach, Kilcoyne et al. 2014). The lectins were maintained at 10 °C during printing to minimise evaporation. Eight replicate subarrays were printed per microarray slide, with each lectin spotted in replicates of six per subarray. Slides were then incubated in a humidity chamber overnight at room temperature to ensure completion of conjugation. Remaining functional groups were deactivated by incubation in 100 mM ethanolamine in 50 mM sodium borate, pH 8, for 1 h at room temperature. The slides were washed with PBS, pH 7.4, containing 0.05% Tween-20 (PBS-T) three times for 3 min each wash, once with PBS, centrifuged dry (450 x *g*, 5 min) and stored at 4 °C with desiccant until use.

Just before use, the lectin microarray slides were allowed to equilibrate to room temperature for 30 min with desiccant. All microarray slides were incubated using an 8-well gasket slide and incubation cassette system (Agilent Technologies Ireland, Ltd., Cork, Ireland) and were protected from light throughout the procedure. Fluorescently-labelled bLF samples and ASF control were diluted to 0.5 μg/mL in Tris-buffered saline supplemented with Ca^{2+} and Mg^{2+} ions (TBS; 20 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) pH 7.2 with 0.05% Tween-20 (TBS-T). 70 μL of each diluted sample was applied to each well of the gasket and incubations (1 h, 23 °C, 4 rpm) and slide washes were carried out as previously described (Kilcoyne, Gerlach et al. 2012, Gerlach, Kilcoyne et al. 2014). The slides were dried by centrifugation and imaged immediately in an Agilent G2505 microarray scanner using the Cy5 channel (633 nm excitation, 80% PMT, 5 μm resolution).

2.6 Data extraction

Microarray data extraction was performed as previously described (Kilcoyne, Gerlach et al. 2012, Kilcoyne, Gerlach et al. 2012). In brief, raw intensity values were extracted from the image files using GenePix Pro v6.1.0.4 (Molecular Devices, Berkshire, U.K.) and a proprietary *.gal file to identify printed lectin positions using adaptive diameter (70-130%) circular alignment based on 230 μm features and exported as text to Excel (version 2007, Microsoft). Local background-corrected median feature intensity data (F543median-B543) was selected and the median of six replicate spots per subarray was handled as a single data point for graphical and statistical analysis (n=3). Binding data was presented in histogram form as the mean intensity with standard deviation of three experimental replicates (18 data points in total).

2.7 Statistical analysis

The lectin microarray data were subjected to multivariate analysis as follows: the data were scale normalised in R by firstly centring the dataset by subtracting the mean of each set of variables from each data point in the set and then dividing each datapoint in the centred dataset by the standard deviation of the centred data. This created a dataset with a mean of 0 and a standard deviation of 1 to prevent distortion due to the different magnitudes of the fluorescence between different lectins. Cluster plotting based on p-value (Suzuki and Shimodaira 2013) was

performed on the scaled dataset using the 'pvclust' function in the 'pvclust' library; this allowed a p-value to be calculated on a multi-scale bootstrapped data set to estimate the uncertainty of the clustering analysis. Hierarchical clustering in pvclust was done using the 'hclust' function using Ward's minimum variance method and 10,000 bootstraps. Clusters indicated to be significant ($p<0.05$) were enclosed by rectangles in the final plot.

2.8 Monosaccharide analysis

For the release of sialic acids, 1 mg bLF from each time-point was hydrolysed in 0.1 M HCl at 80 ^{o}C for 1 h (Gallagher, Morris et al. 1985). A blank control (water) and bovine fetuin were hydrolysed in parallel for background subtraction and hydrolysis controls, respectively. Samples were dried from water three times in a centrifugal evaporator (miVac Quattro concentrator, miVac, UK), the hydrosylate was resuspended in 500 µL 18.2 MΩ water and sialic acid content was analysed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS3000 system equipped with an electrochemical detector (Dionex, Sunnyvale, CA, USA) with comparison to Neu5Ac and Neu5Gc standards. In brief, 25 µl of each reconstituted sample was injected onto a CarboPac PA20 column (3 x 150 mm, Dionex) equipped with an amino trap column (3 x 30 mm) at a flow rate of 0.35 mL/min. Eluent A was 100 mM NaOH and B was 100 mM NaOH with 500 mM sodium acetate (NaOAc). Neu5Ac and Neu5Gc were separated by a gradient elution (0-10 min, 14%-60% eluent B), followed by a washing step at 60% eluent B for 1 min. The column was then re-equilibrated at 14% eluent B for 6 min. Each sample was injected in triplicate, Neu5Ac and Neu5Gc were quantified by reference to the standard curve and average values were reported.

For the analysis of monosaccharides, 1 mg of each sample was hydrolysed in 2 N TFA at 100 ^{o}C for 4 h and analysed essentially as previously described (Kilcoyne, Gerlach et al. 2012). Water and fetuin were hydrolysed in parallel as above for background subtraction and ensure consistent hydrolysis conditions, respectively. Each sample was injected in triplicate, the monosaccharide content of each sample was quantified from standard curves of Fuc, Gal, Man, Glc, GalN and GlcN and average values were reported.

3. Results and Discussion

3.1 Purification of bLF from milk

BLF was isolated from bovine milk at 13 time points from three animals over the first three months of lactation using a scaled down optimised method based on cation exchange chromatography. BLF was separated successfully from whey in one step using a cation exchange capsule and a salt gradient elution. BLF eluted at 0.6 M NaCl and was collected for further characterisation (figure 1). Comparison of sample RP-HPLC elution profile with a commercially available bLF standard confirmed the elution concentration of bLF from the second gradient step**.** BLF purity was calculated from the RP-HPLC profile by ((area of bLF peak)/(total area under the curve))*100 and this method resulted in >90% bLF purity. SDS-PAGE also confirmed isolated bLF purity (figure S1). Other methods for bLF isolation must include pre-incubation of the milk or whey sample with the chromatographic resin prior to elution with the salt gradient. For example, methods based on heparin-Sepharose resin have a 3 h incubation (Barboza, Pinzon et al. 2012) while SP-Sepharose methods (Conesa, Sanchez et al. 2008) require an overnight incubation. Similar to Wei *et al.* (2000), bLF eluted from the adsorption membrane at a lower salt concentration in comparison to other methods which employ up to 1 M NaCl (Barboza, Pinzon et al. 2012) to elute bLF. Previously, bLF has been isolated on a larger scale based on a similar principle to that described in this study (Plate, Beutel et al. 2006) and thus, the one-step purification technique described here may be suitable for up-scaling.

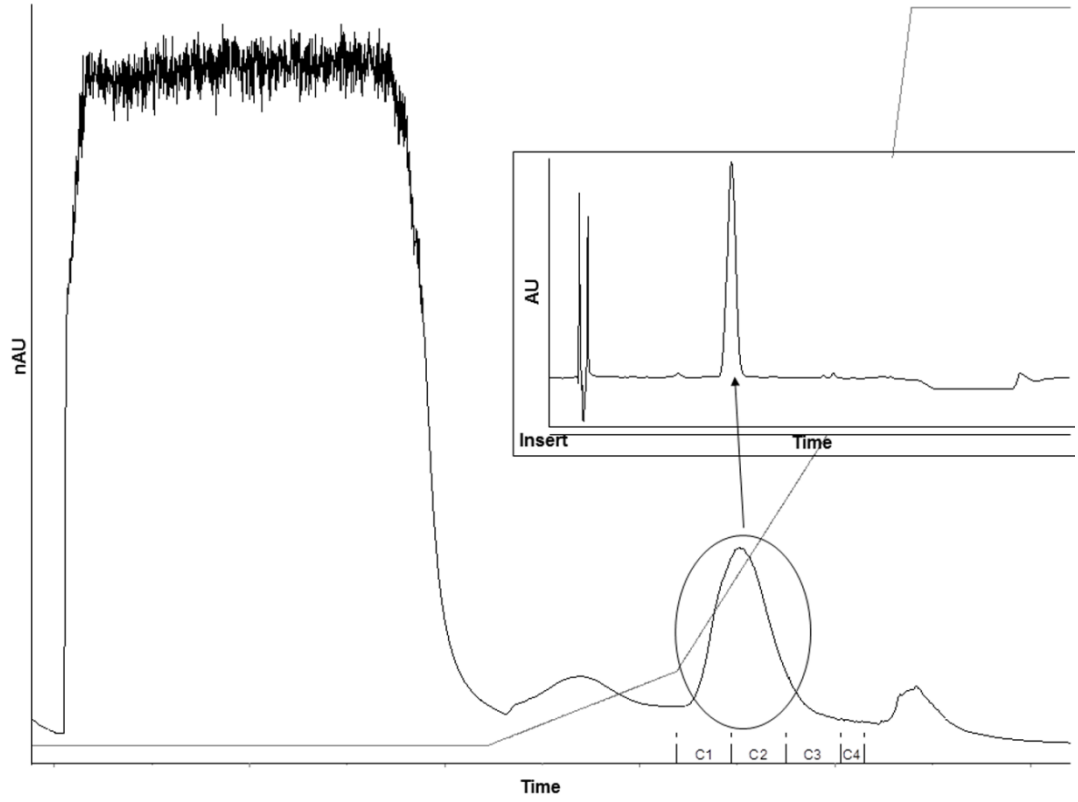

Figure 1 Profile of whey injection onto Sartobind S SingleSep nano 1 mL capsule followed by elution with NaCl gradient. Black line, absorbance at 280 nm; grey line, NaCl concentration; C1-C4, fractions collected from gradient step II containing isolated bLF. Insert RP-HPLC profile of pooled C1-C3 fractions following dialysis to ascertain purity of isolated bLF.

3.2 Lectin microarray profiling bLF glycosylation

All fluorescently labelled bLF samples were profiled on a microarray consisting of 43 lectins (figure 2). Binding to the lectins LEL, PHA-E and Con A are taken as indicators of the presence of *N*-linked glycosylation. The lectin LEL binds to the GlcNAc residues of the chitobiose (GlcNAc-β-(1→4)-GlcNAc) disaccharide in the *N*-linked glycan core structure and the intense signal observed from LEL was expected considering that the occurrence of exclusively *N*-linked glycosylation was previously reported for bLF (Spik, Coddeville et al. 1988). PHA-E predominantly binds to biantennary *N*-linked glycans, while Con A has an affinity for Man, which is present in the core of all types of *N*-linked glycans. The lack of binding to PNA, which has a binding affinity for the *O*-glycan core-1 structure (T-antigen, table S1), indicated that *O*-glycosylation was unlikely, in agreement with only *N*-linked structures known for bLF.

While binding to Con A indicated the presence of *N*-linked glycans in all samples, binding to the other Man-specific lectins HHA, NPA and GNA further revealed the presence of high mannose type structures. The predominance of high mannose structures in mature bLF has been well characterised (Hua, Nwosu et al. 2011, van Leeuwen, Schoemaker et al. 2012), and $Man_8GlcNAc_2$ has been reported as the most abundant glycan structure present in mature bLF, accounting for 38% of the total glycan structures (Hua, Nwosu et al. 2011). The lectins PSA and Lch-B did not interact with any of the bLF samples, which implies the absence of fucosylated high mannose structures.

The presence of biantennary complex type structures was evidenced by bLF binding to the lectins PCA, which has affinity for GlcNAc residues in complex glycans, and PHA-E. The *N*-linked glycan core structure of bLF can be substituted on both antennae by *N*-acetyllactosamine (Gal-β-(1→4)-GlcNAc, LacNAc) and/or LacdiNAc motifs (Plate, Beutel et al. 2006, van Leeuwen, Schoemaker et al. 2012) (figure S2), which can be further modified with sialic acid residues. The lectins SNA-II and AIA bind to either terminal Gal or GalNAc residues, indicating the presence of terminating LacNAc and LacdiNAc structures. The presence of non-sialylated LacNAc was also indicated by persistant binding of bLF with the lectins RCA-I, CAA and ECA (table S1 and figure 2) which recognise the terminating Gal residue. Binding was also observed

for PHA-L, which binds to tri- and tetra-antennary *N*-linked glycans and tri-antennary hybrid and high-mannose type structures have been described for mature bLF (van Leeuwen, Schoemaker et al. 2012). The lectins ABL and ACA are typically characterised as having affinity for the mucin-type core-1 *O*-linked glycan structure (Boland, Chen et al. 1991, Irazoqui, Vides et al. 1999) but they may also have affinity for LacNAc motifs, which shares a similar structure of a Gal residue β-linked to an acetlyated sugar. The presence of the T-antigen on bLF was not supported by PNA binding nor any previous reports of *O*-linked glycosylation and thus, it is more probable that ACA and ABL interacted with the LacNAc motifs in these samples. Binding to GlcNAc-specific lectins including DSA, STA, sWGA and WGA suggested the occurrence of GlcNAc terminated hybrid and complex structures, which also agreed with previous literature (van Leeuwen, Schoemaker et al. 2012).

As well as binding to GlcNAc, WGA also interacts with sialic acid (table S1). The linkage of the sialic acids responsible for the intense binding of WGA with bLF can be further distinguished by other sialic acid-specific lectins present on the microarray. The lectins SNA-I and MAA are specific for terminal α-(2→6)- and α-(2→3)-linked sialic acids, respectively, and bLF binding was only observed with SNA-I, which suggested that the sialic acid present in bLF was exclusively α-(2→6)-linked in agreement with structural analysis previously described for bLF (Spik, Coddeville et al. 1988).

The lectins AAL, LTA and UEA-I have binding affinities for α-(1→6)-, α-(1→3)- and α-(1→2)-linked Fuc, respectively (table S1). All bLF samples only interacted with AAL of the Fuc-binding lectins on the microarray which suggested that fucosylation was present in the α-(1→6)-linkage only in all structures, in agreement with previous structural analysis (Spik, Coddeville et al. 1988, van Leeuwen, Schoemaker et al. 2012). In addition, the presence of terminal α-linked Gal residues was indicated by binding to the lectins VRA, EEA, MOA, MPA and GSL-I (table S1). EEA in particular has a preference for binding to Gal-α-(1→3)-Gal (Teneberg, Alsén et al. 2003). The Gal-α-(1→3)-Gal epitope, which is not produced in humans, has been shown to be present on bLF in minor amounts as part of three complex and hybrid type structures (van Leeuwen, Schoemaker et al. 2012). The Gal-α-(1→3)-Gal epitope has been linked to

allergen-related immune responses in humans (Commins, James et al. 2011), but there is no evidence linking the consumption of bovine milk or bLF to these effects.

3.3 Monosaccharide analysis of bLF samples

Acid hydrolysis and HPAEC-PAD analysis was performed and the residues GlcN, Man, GalN, Gal, Fuc, Neu5Ac and Neu5Gc were identified in all samples (table 1) which supported the previous conclusions of the presence of complex and high-mannose type *N*-linked glycosylation. As lactation time progressed, an increase in the intensity of the Man-binding lectins (Con A, HHA, NPA and GNA) in mature milk for all three cows was observed (figure 2). The most abundant monosaccharide in mature milk bLF was Man, with concentration almost doubling over the sampled time range. Man increased from 45.8 ± 17.7 pM/µg bLF in colostrum to 88.3 ± 18.5 pM/µg bLF at month 3 (D90) (figure S2, table 1). However, the highest concentration of Man was at D7 and D8, which did not correlate with the lectin binding data. An increase in the number of Man residues present on each high-mannose type glycan already present on the glycoprotein would not be detected by the Man-specific lectins on the lectin microarray. The high-mannose type glycans of bLF (Teraguchi, Shin et al. 1996) may act as decoy receptors for bacterial adhesins and the abundance of these structures on mature bLF may be linked to its role in protection of the infant from pathogenic adhesion to the intestinal mucus layer.

As stated above, bLF complex type glycans contain extended structures consisting of Gal, GlcNAc and GalNAc residues in LacNAc and LacdiNAc motifs (van Leeuwen, Schoemaker et al. 2012) (figure S2). Lectins specific for these monosaccharides displayed the highest intensity binding at D1 for all three cows, and decreased over milk production time (figure 2), which implied a decrease in the occurrence of these monosaccharides. This observation was confirmed by monosaccharide analysis (table 1) and probably signified a decrease in the heterogeneity of bLF complex-type glycans as lactation progressed. The concentration of GalN was higher in early lactation in comparison to Gal, which suggested a predominance of LacdiNAc motifs (table 1). However, from D6 onwards, both Gal and GalN were present at comparable concentrations (also previously reported (van Leeuwen, Schoemaker et al. 2012), which suggested an

equal concentration of LacNAc and LacdiNAc motifs in the complex-type glycan population of bLF. The decrease of LacNAc and LacdiNAc over lactation confirmed the same conclusion indicated by the trend of generally decreased binding of bLF over time to lectins AIA, RCA-I, CAA, ACA and PHA-L. LacNAc containing glycoproteins have been shown to inhibit the adherence of *E. coli* by acting as a competitive inhibitor for host cell receptors (Hyland, Griener et al. 2006). In addition to LacNAc, a proportion of the Gal quantified in the samples was also from the non-human Gal-α-(1→3)-Gal epitope. The lectins VRA and EEA, which indicated the presence of Gal-α-(1→3)-Gal, had the highest intensity binding with colostrum bLF samples and decreased to lower levels as lactation progressed. This suggested that complex glycans containing Gal-α-(1→3)-Gal were present mainly in early lactation.

Fuc was detected at low concentrations at all timepoints, with its highest concentration in colostrum (table 1), in agreement with previous reports (van Leeuwen, *et al*., 2012), which suggested that fucosylation was an infrequent modification of the glycans of bLF. The binding of AAL, specific for α-(1→6)-linked Fuc (table S1), remained consistent from D2 throughout the production of transitional milk and decreased in intensity at the later time points (figure 2). This was verified by the monosaccharide analysis (table 1) and may suggest a decrease in the biological pressure for the preservation of Fuc residues on bLF as lactation progresses. Fucosylated oligosaccharides have a role in hLF binding to the brush border membrane (Davidson and Lonnerdal 1988) and the prebiotic and the anti-microbial effects of milk oligosaccharides (Newburg 2009). However, the specific role of bLF fucosylation remains unclear, although it may not be as diverse as that observed for human milk fucosylated structures due to the lower overall Fuc concentration in bovine milk.

Overall, the monosaccharide analysis corroborated the results of the lectin microarray and further, lectin profiles and monosaccharide analysis could be correlated with previously published glycan structural motifs. This demonstrated the reliability of the lectin microarray method for profiling structural changes of bLF over lactation.

Table 1 Average concentration (± standard deviation) of monosaccharides (pM) per µg bLF collected from three cows over the first three months of lactation, based on three separate analyses of bLF isolated from each animal (n=3).

Day	GlcN	Man	GalN	Gal	Neu5Ac	Neu5Gc	Fuc
1	98.4 ± 30.1	45.8 ± 17.7	44.8 ± 9.6	26.6 ± 13.2	87.7 ± 16.8	13.9 ± 1.0	4.2 ± 2.4
2	95.5 ± 18.2	48.1 ± 6.4	22.1 ± 9.7	23.2 ± 12.7	67.3 ± 13.5	7.0 ± 1.1	2.8 ± 1.9
3	78.1 ± 2.2	50.3 ± 44.8	23.0 ± 7.8	14.7 ± 0.2	34.0 ± 9.6	4.7 ± 1.9	2.4 ± 1.0
4	89.8 ± 15.1	59.8 ± 46.9	23.6 ± 4.1	12.7 ± 10.5	61.2 ± 3.2	4.3 ± 1.7	3.0 ±1.4
5	87.6 ± 51.2	74.5 ± 32.9	23.3 ± 8.7	15.1 ±1.4	61.2 ± 8.4	7.0 ± 2.7	2.3 ± 0.8
6	80.8 ± 42.3	62.8 ± 16.4	17.2 ± 6.2	18.8 ± 18.2	61.5 ± 19.0	6.7 ± 2.7	2.9 ± 2.2
7	65.4 ± 27.8	119.9 ± 47.2	17.3 ± 3.5	17.8 ± 13.8	49.4 ± 13.3	4.5 ± 1.6	2.6 ± 1.3
8	71.8 ± 41.2	127.5 ± 12.1	17.9 ± 5.4	19.3 ± 10.8	49.9 ± 7.3	3.7 ± 0.8	2.2 ± 0.7
9	91.2 ± 27.9	88.4 ± 16.5	15.9 ± 0.2	12.8 ± 8.1	37.8 ± 18.7	3.3 ± 2.2	2.6 ± 1.7
10	53.1 ± 6.4	59.3 ± 26.4	15.4 ± 8.5	19.1 ± 9.9	59.7 ± 8.3	4.5 ± 1.4	1.1 ± 0.2
30	74.9 ± 0.1	89.4 ± 18.3	22.6 ± 4.1	14.8 ± 2.6	41.0 ± 21.2	2.8 ± 2.6	2.7 ± 0.5
60	60.5 ± 48.8	74.7 ± 13.5	20.1 ± 3.6	12.1 ± 5.7	49.3 ± 29.1	4.1 ± 2.8	1.2 ± 1.3
90	69.0 ± 8.9	88.3 ± 18.5	13.6 ± 3.0	13.9 ± 1.0	22.2 ± 6.6	1.4 ± 1.1	0.7 ± 0.4

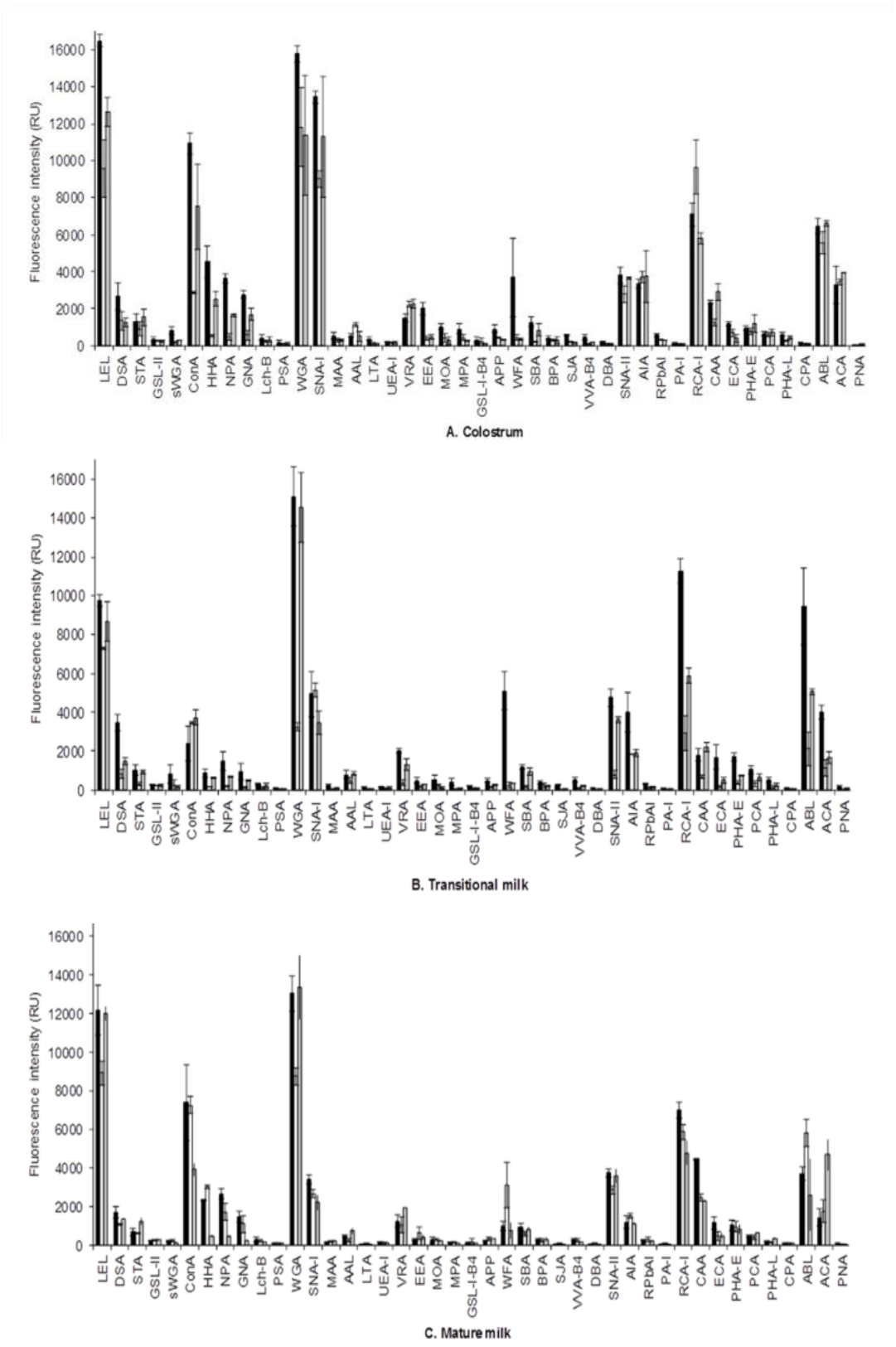

Figure 2 Lectin microarray profiles of bLF from three cows from A colostrum (D1) B transitional milk (D6) and C mature milk (D90). Cow A: blue, Cow B: purple, Cow C: green.

3.4 Temporal changes in glycosylation

Changes in bLF glycosylation were monitored over the first three months of lactation by lectin microarray profiling and monosaccharide analysis. Monosaccharide analysis revealed that the carbohydrate content of bLF was at its highest in D1 colostrum (table 1). Similarly, the highest binding intensities were observed for the lectin array in the initial days postpartum, indicating that glycans in bLF were substituted with more complex structures immediately following parturition. The intensities of lectin binding decreased from D4 onwards, with fewer lectins displaying a binding response. This suggested that D4 coincided with the end of colostrum production, with less structural diversity occurring in the glycosylation thereafter. Indeed, these data also suggested that there were three distinct phases of bLF glycosylation during bovine lactation, (i) colostrum (D1-D4), (ii) transitional milk (D5-D30) and (iii) mature milk (month one onwards). Interestingly, it has been reported that bLF concentration remains relatively high in bovine colostrum for the initial three days following birth and decreases rapidly thereafter (Abd El-Fattah, Abd Rabo et al. 2012).

When the profiles of lectin binding over lactation were compared (figure 2), a reduction in the binding intensities for the majority of lectins was observed as lactation progressed, suggesting a decrease in the heterogeneity of the glycan structures present. Complex-type bLF glycans contain extended structures consisting of sialic acid, Gal, GlcNAc and GalNAc residues (van Leeuwen, Schoemaker et al. 2012). Lectins specific for these monosaccharides displayed the highest intensity binding at D1 for all three cows, and decreased through transitional milk production (figure 2), which implied a decrease in the occurrence of these motifs. This observation was confirmed by monosaccharide analysis (table 1) and may signify a decrease in the heterogeneity of bLF complex-type glycans as lactation progresses. Unsupervised hierarchical clustering of the lectin binding data identified four clusters and, interestingly, all mature milk samples (D30-D90) clustered closely together (figure 3, cluster A). This indicated that a common glycoprofile was present in mature bLF. The wider distancing of samples from earlier time points was likely as a result of more heterogeneous structures present in early lactation, as suggested by the binding of a larger number of lectins to bLF from the preliminary stages of lactation. This

glycan heterogeneity may provide the infant with a wider range of biological benefits in the initial days following birth.

As lactation progressed from transitional milk production onwards, an increase in the intensity of the Man-binding lectins (Con A, HHA, NPA and GNA) in mature milk for all three cows was observed (figure 2). When the relative abundances of the individual monosaccharides present on bLF's glycans were considered (figure S2), an obvious shift from a mainly complex structure composition at D1 (Man = 14%) to a high mannose structure-rich environment in mature milk (Man = 42%) at D90 was evident. A common Man-rich glycan profile in mature milk bLF explains the clustering observed in the pvclust.

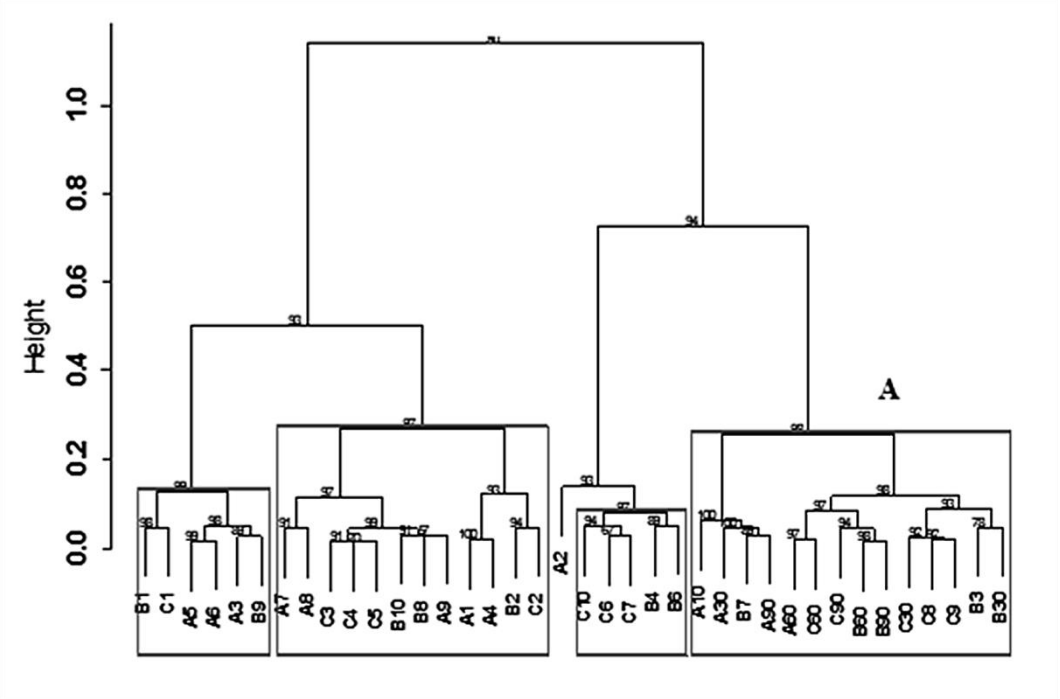

Figure 3 Cluster dendrogram of lectin binding profiles for bLF samples from three cows (A-C) from D1 to D90 of lactation. Clusters with a P value ≥ 0.95 were selected. A Timepoints from mature milk cluster closely together**.**

3.5 Temporal changes in sialylation

The responses for SNA-I, which has binding specificity for α-(2→6)-linked sialic acid, and monosaccharide analysis (table 1) revealed an overall decrease in sialic acid content over time from D1 colostrum. The reduced sialic acid content may be attributed to a change from di-sialylated glycans in colostrum to mono-sialylated glycans in later lactation or as a result of a shift from the presence of predominantly protein-bound sialic acid to an increase in the proportion of sialylated free oligosaccharides present in milk (Martin, Martin-Sosa et al. 2001). Sialic acid is important for stabilisation of LF due to its calcium binding ability, which affects the antibacterial activity of bLF (Rossi, Giansanti et al. 2002).

Two forms of sialic acids were detected, Neu5Ac and Neu5Gc. Neu5Ac was the most abundant form of sialic acid detected and its concentration was relatively high, even in mature milk (figure 4, table 1). At D90, it accounted for 10.6% of the monosaccharides present, which may suggest that it has a nutritionally important role as part of bLF (figure S2). Neu5Ac has been found to have a bifidogenic activity (Idota, Kawakami et al. 1994) and therefore, it may contribute to the prebiotic effect observed for bLF (Rahman, Kim et al. 2009). The feeding of protein-bound sialic acid has also been linked to cognitive development and enhanced learning during early development in pigs (Wang, et al., 2007). Neu5Gc had the highest concentration in D1 colostrum with decreasing concentration thereafter. Neu5Gc accounted for 13.6% of sialic acid in colostrum (figure 4) (4.3% of all monosaccharide content (figure S2)), and remained present in bLF at a low concentration even until the final time point in this study at D90 (5.7% of total sialic acid content) (table 1 and figure 4), which is similar to a previous report of 8.5% Neu5Gc of total sialic acid in mature bLF (van Leeuwen, Schoemaker et al. 2012).

As a result of a mutation in the cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase gene in the sialic acid pathway, humans are incapable of synthesising Neu5Gc, but human cells and tissues have the capacity to absorb Neu5Gc from external sources (Padler-Karavani, Yu et al. 2008), such as ingested bovine milk.

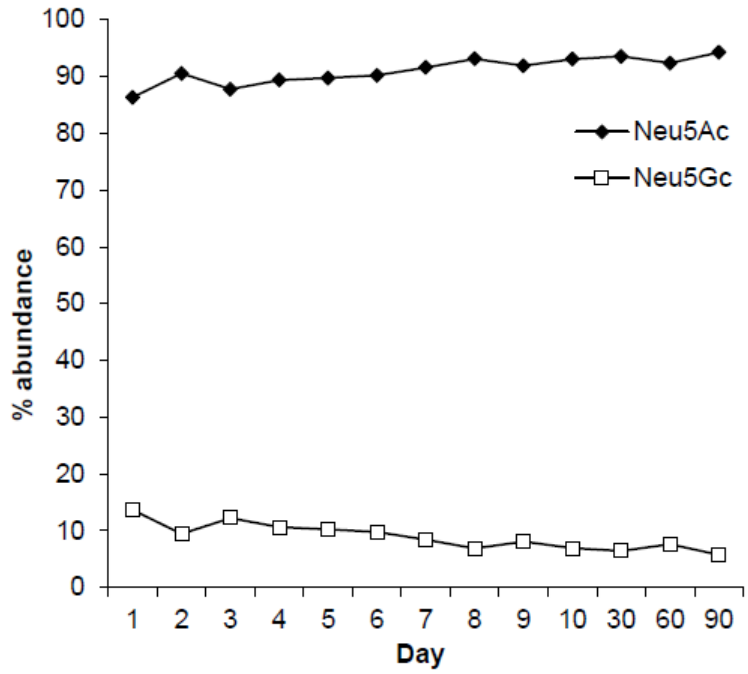

Figure 4 Percentage abundance of Neu5Ac and Neu5Gc (pM monosaccharide per µg bLF) from the milk of three cows over the first three months of lactation.

The absorption of dietary Neu5Gc may elicit an immune response in humans (Padler-Karavani, Yu et al. 2008) and it has been suggested it may have a role in cancer development and metastasis, as various carcinoma cell types have been shown to express Neu5Gc containing glycoconjugates (Malykh, Schauer et al. 2001). However, the link between dietary Neu5Gc and human health is yet to be understood.

It is clear from the data presented in this study that major changes to the glycoprofile of bLF occurred during the initial days post-parturition. At birth, calves lack blood immunoglobulins (Ig) (Abd El-Fattah, Abd Rabo et al. 2012). The glycan structures present on bLF, as well as other glycoconjugates and free oligosaccharides, may act as an innate immune system passed from mother to calf to prevent infection and promote the colonisation of the calf's gut by beneficial bacteria. Changes to the bovine milk glycome over lactation may be an evolutionary adaptation to meet the changing biological requirements of the calf as it matures and the calf's own immune system and gut microbiota develops.

4. Conclusions

Lectin microarrays offer a platform which can monitor glycan structural characteristics of bLF and these glycoprofiles can be well correlated with detailed glycan structures previously reported. At an industrial scale, lectin microarrays would also offer a more convenient approach to monitoring protein glycosylation as the need for glycan release prior to analysis is eliminated and the time required for sample analysis is dramatically reduced. The extent of the temporal changes in bLF glycosylation over lactation suggested that bLF from colostrum and bLF from mature milk could be considered as two distinct ingredients to be exploited by the functional food industry. Colostrum-derived LF offers a diverse range of LF glycosylation variants abundant in complex-type structures whereas mature milk LF is a source rich in high mannose *N*-linked structures. Mature bovine milk is available in large quantities worldwide and, therefore, mature bLF is readily available and widely used as a functional ingredient in commercial products, as outlined in the introduction. In contrast, bovine colostrum is available in more limited quantities and, thus, bLF isolated from early lactation may have potential as an ingredient for more functionally specific products e.g. in infant formulae for babies suffering from immune disorders, during and following infection or as a prebiotic supplement for patients who have experienced perturbations to their intestinal microbiota following treatment with medications such as broad-spectrum antibiotics. The majority of products available from bovine colostrum to date are derived from unfractionated colostrum with little information available on the beneficial contribution of its purified components, such as LF. It is clear that *in vivo* studies are required to provide more insight into how the glycoforms of lactoferrin translate into specific health benefits and/or bioactive functions and how the presence of non-human, potentially immunogenic structures such as the Gal-α-(1→3)-Gal epitope and Neu5Gc may manifest *in vivo*. The results reported here indicate that bLF offers immense potential as a feedstock for the isolation of functional food ingredients by exploiting the temporal changes in lactoferrin glycosylation as source of novel bioactives. Commercial development and production of bioactive bLF variants with broad application in the area of functional food is therefore a realistic ambition for the future.

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6. Supplementary figures and tables

Table S1 Lectins employed to monitor the dynamic glycosylation over lactation.

Abbreviation	Origin	Species	Common name	Major Ligand(s)
AIA, Jacalin	Plant	*Artocarpus integrifolia*	Jack fruit lectin	Gal (~sialylation independent)
RPbAI	Plant	*Robinia pseudoacacia*	Black locust lectin	Gal, GalNAc
PA-I	Bacteria	*Pseudomonas aeruginosa*	Pseudomonas lectin	Gal, Gal derivatives
SNA-II	Plant	*Sambucus nigra*	Sambucus lectin-II	Gal/GalNAc
SJA	Plant	*Sophora japonica*	Pagoda tree lectin	βGalNAc
DBA	Plant	*Dolichos biflorus*	Horse gram lectin	GalNAc
APP	Plant	*Aegopodium podagraria*	Ground elder lectin	GalNAc
SBA	Plant	*Glycine max*	Soy bean lectin	GalNAc
VVA-B4	Plant	*Vicia villosa*	Hairy vetch lectin	GalNAc
BPA	Plant	*Bauhinia purpurea*	Camels foot tree lectin	GalNAc/Gal
WFA	Plant	*Wisteria floribunda*	Japanese wisteria lectin	GalNAc/Sulfated GalNAc
ACA	Plant	*Amaranthus caudatus*	Amaranthin	Sialylated/Gal-β(1,3)-GalNAc
ABL	Fungi	*Agaricus bisporus*	Edible mushroom lectin	Gal-β(1,3)-GalNAc, GlcNAc
PNA	Plant	*Arachis hypogaea*	Peanut lectin	Gal-β(1,3)-GalNAc
GSL-II	Plant	*Griffonia simplicifolia (aka Bandeiraea simplicifolia)*	Griffonia/Bandeiraea lectin-II	GlcNAc
sWGA	Plant	*Triticum vulgaris*	Succinyl WGA	GlcNAc
DSA	Plant	*Datura stramonium*	Jimson weed lectin	GlcNAc
STA	Plant	*Solanum tuberosum*	Potato lectin	GlcNAc oligomers
LEL	Plant	*Lycopersicum eculentum*	Tomato lectin	GlcNAcβ1-4GlcNAc
NPA	Plant	*Narcissus pseudonarcissus*	Daffodil lectin	α(1,6)Man
GNA	Plant	*Galanthus nivalis*	Snowdrop lectin	Man-α(1,3)-
HHA	Plant	*Hippeastrum hybrid*	Amaryllis agglutinin	Man-α(1,3)-Man-α(1,6)-
ConA	Plant	*Canavalia ensiformis*	Jack bean lectin	Man, Glc, GlcNAc
Lch-B	Plant	*Lens culinaris*	Lentil isolectin B	Man, fucose dependent
PSA	Plant	*Pisum sativum*	Pea lectin	Man, fucose dependent
WGA	Plant	*Triticum vulgaris*	Wheat germ agglutinin	NeuAc/GlcNAc
MAA	Plant	*Maackia amurensis*	Maackia agglutinin	Sialic acid-α(2,3)-
SNA-I	Plant	*Sambucus nigra*	Sambucus lectin-I	Sialic acid-α(2,6)-
PHA-L	Plant	*Phaseolus vulgaris*	Kidney bean leukoagglutinin	tri-tetraantennary βGal/Gal-β(1,4)-GlcNAc
PCA	Plant	*Phaseolus coccineus*	Scarlet runner bean lectin	GlcNAc in complex glycans
PHA-E	Plant	*Phaseolus vulgaris*	Kidney bean erythroagglutinin	biantennary, bisecting GlcNAc,βGal/Gal-β(1,4)GlcNAc
RCA-I/120	Plant	*Ricinus communis*	Castor bean lectin-I	Gal-β-(1, 4)-GlcNAc
CPA	Plant	*Cicer arietinum*	Chickpea lectin	Complex glycopeptides
CAA	Plant	*Caragana arborescens*	Pea tree lectin	Gal-β-(1, 4)-GlcNAc
ECA	Plant	*Erythrina cristagalli*	Cocks comb/coral tree lectin	Gal-β-(1, 4)-GlcNAc oligomers
AAL	Fungi	*Aleuria aurantia*	Orange peel fungus lectin	α-Fuc (1-6)
LTA	Plant	*Lotus tetragonolobus*	Lotus lectin	α-Fuc (1-3)
UEA-I	Plant	*Ulex europaeus*	Gorse lectin-I	α-Fuc (1-2)
EEA	Plant	*Euonymous europaeus*	Spindle tree lectin	α-Gal
GSL-I-B4	Plant	*Griffonia simplicifolia (aka Bandeiraea simplicifolia)*	Griffonia/Bandeiraea lectin-I	α-Gal
MPA	Plant	*Maclura pomifera*	Osage orange lectin	α-Gal
VRA	Plant	*Vigna radiata*		α-Gal
MOA	Fungi	*Marasmius oreades*	Fairy ring mushroom lectin	α-Gal

Table S2 Stepwise gradient separation for RP-HLPC. Eluent A: 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC water; eluent B: 90% acetonitrile, 0.1% TFA in HPLC water.

Time (mins)	**Eluent A**	**Eluent B**
0	60	40
3	60	40
19	48	52
22	30	70
23	0	100
28	0	100
29.5	60	40
34.1	60	40

Table S3 Protein concentration and degree of substitution by Alexa Fluor 647 of bovine lactoferrin samples.

Timepoint	Protein concentration (µM)			Degree of labelling (M of Alexa Fluor 647 dye/mole protein)		
	Cow A	**Cow B**	**Cow C**	**Cow A**	**Cow B**	**Cow C**
D1	10.798	14.635	11.16	3.418	3.571	4.582
D2	11.155	15.028	10.4	3.751	2.442	4.64
D3	12.119	13.847	8.425	2.928	1.151	5.314
D4	6.951	12.469	9.434	3.257	4.258	4.812
D5	10.540	N/A	8.144	3.092	N/A	6.099
D6	6.824	8.5017	8.817	4.991	6.388	5.828
D7	12.48	12.865	7.249	3.235	3.733	6.5
D8	9.364	12.648	10.915	4.67	1.356	5.068
D9	10.425	10.322	11.389	3.379	4.978	4.776
D10	9.984	10.203	9.352	4.392	4.273	5.973
D30	13.576	8.292	10.768	3.075	4.531	4.406
D60	9.734	11.277	5.932	5.184	3.978	6.644
D90	9.175	11.236	10.859	5.149	4.309	4.462

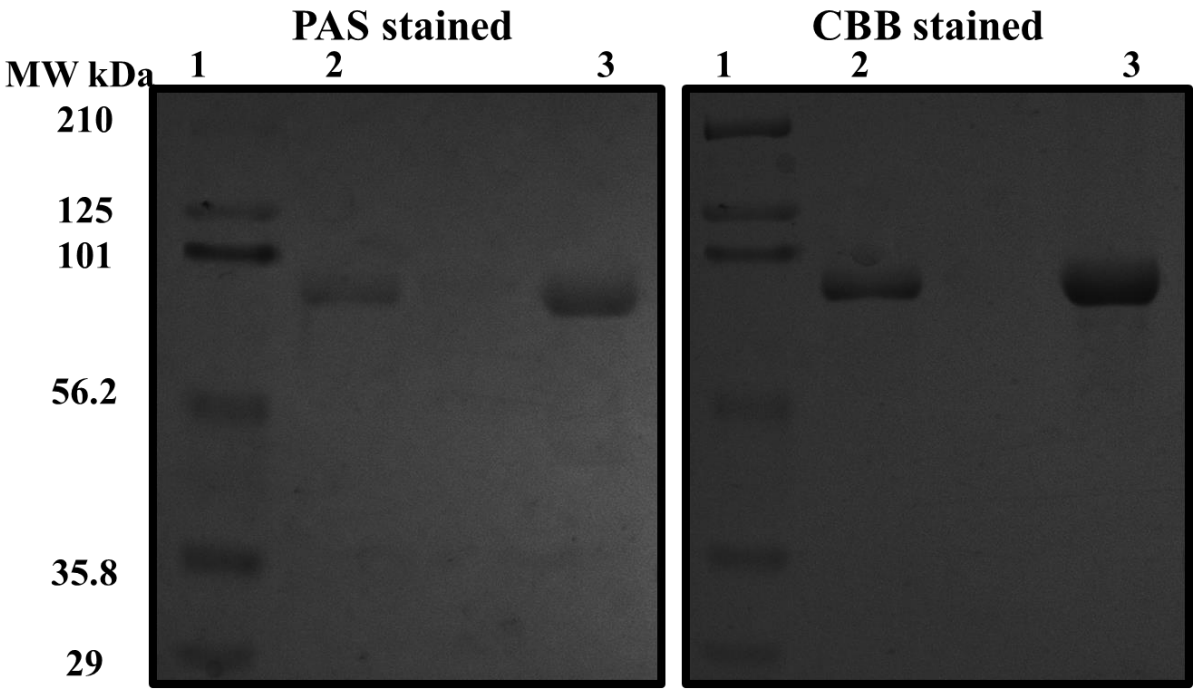

Figure S1 Separation of isolated bovine lactoferrin and a commercially available bovine lactoferrin standard on a 10% acrylamide SDS-PAGE and periodic acid schiff (PAS) and Coomassie brilliant blue (CBB) stained. Lane 1: Molecular weight ladder (band size indicated on the y-axis of the image). Lane 2: Bovine lactoferrin isolated by the methods described in this study. Lane 3: commercially available bovine lactoferrin standard.

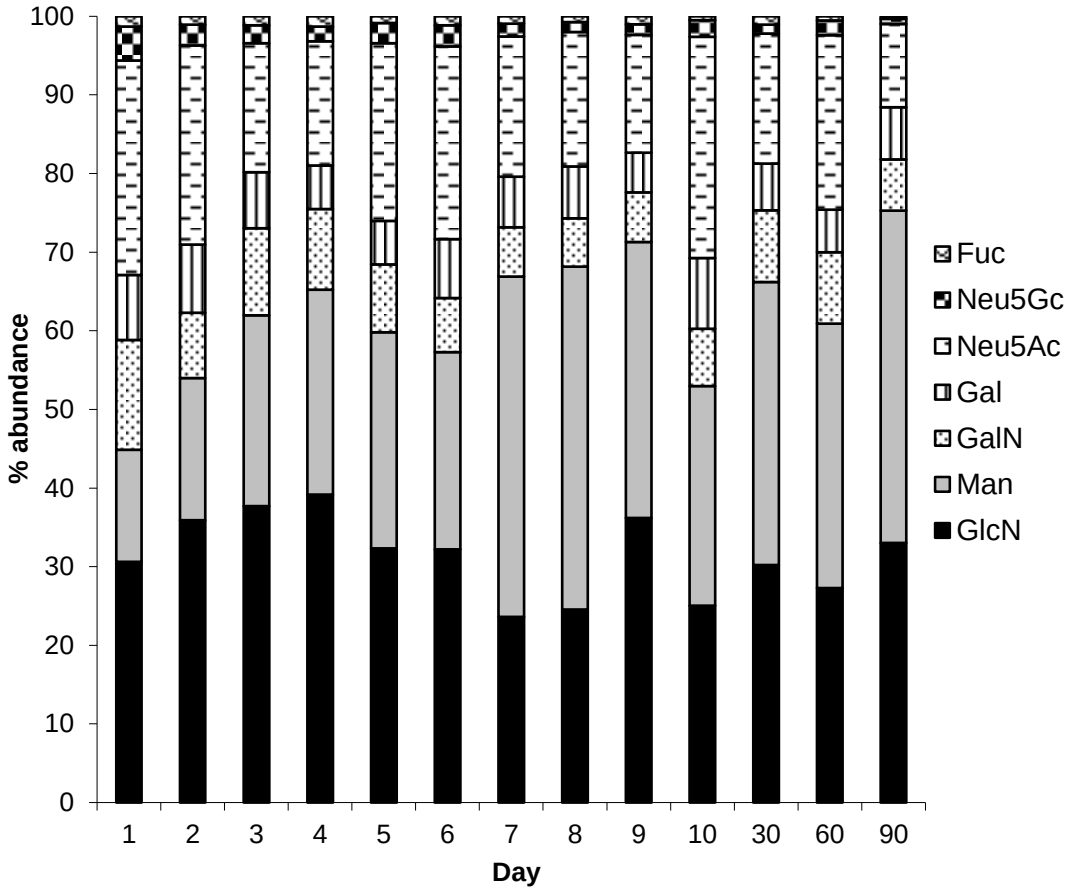

Figure S2 Average percentage abundance of monosaccharides based on total concentration (pM monosaccharide per µg bovine lactoferrin) from bovine lactoferrin purified from the milk of three cows sampled over the first three months of lactation.

Chapter 4

Investigating the influence of glycosylation variation on the pathogen binding ability of bovine lactoferrin.

Noelle O'Riordan, Michelle Kilcoyne, Lokesh Joshi, and Rita M. Hickey

Manuscript to be submitted to FEMS for peer review

Investigating the influence of glycosylation on pathogen binding

Abstract

The glycans attached to certain milk proteins are known to act as soluble receptor analogs of epithelial cell surface carbohydrates. These glycans display structural homology to host cell receptors and thus, function as receptor decoys to which pathogens can bind to instead of the host. Bovine lactoferrin (LF) has been shown to prevent adhesion to and invasion of mammalian cell lines by pathogenic bacteria, with evidence for direct bacterial binding by the milk glycoprotein. However, the glycosylation pattern of LF changes over the lactation cycle. The effect this variation has on the milk glycoprotein's ability to interact with pathogens is largely unknown. In this study, surface plasmon resonance technology was employed to compare the binding of LF from colostrum (early lactation) and mature milk (late lactation) to a panel of pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Cronobacter sakazakii*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Salmonella typhimurium*). Novel interactions with LF were identified for *C. sakazakii*, *S. pneumoniae* and *P. aeruginosa* with the highest binding ability observed for mature milk LF in all cases, with the exception of *S. typhimurium*. The difference in bacterial binding observed may be as a result of the varying glycosylation profiles.

1. Introduction

Bovine milk lactoferrin (LF) is a single chain, iron-binding, glycosylated protein (Kumar, Weber et al. 2003) present in the whey protein fraction of milk (Severin and Wenshui 2005) (UniPortKB/SwissPort P24627). Five glycosylation sites are present on LF, which may have high-mannose type, complex type or hybrid type N-linked glycans attached. The associated glycan chains are composed of N-acetyl-glucosamine (GlcNAc), galactose (Gal), N-acetyl-galactosamine (GalNAc), fucose (Fuc), mannose (Man), N-acetyl-neuraminic acid (Neu5Ac) and N-glycolyl-neuraminic acid (Neu5Gc) (as previously reviewed (O'Riordan, Kane et al. 2014)) and variation in the glycosylation pattern of LF has been described over the lactation cycle (O'Riordan, Gerlach et al. 2014). A greater degree of heterogeneity in glycan structure has been reported for LF from early lactation, with an abundance of high-mannose type glycans present on the glycoprotein in mature milk (O'Riordan, Gerlach et al. 2014). The glycan chains in mature milk LF are 65% oligomannose type, consisting of multiple isomers of high-mannose type glycans, containing five to nine mannose residues, 38% of the total mannose structures accounted for by Man8 (van Leeuwen, Schoemaker et al. 2012). Neu5Gc was shown to be present on bovine LF only in the initial days postpartum and the total sialic acid content decreased as lactation progressed (O'Riordan, Gerlach et al. 2014).

LF has a wide variety of associated biological activities including antimicrobial (Gonzalez-Chavez, Arevalo-Gallegos et al. 2009), immunomodulatory (Debbabi, Dubarry et al. 1998), prebiotic (Kim, Ohashi et al. 2004, Rahman, Kim et al. 2009), stimulation of bone formation (Cornish, Callon et al. 2004) and anticancer (Tsuda, Sekine et al. 2002, Tsuda, Kozu et al. 2010). These bioactivities have previously been reviewed by several authors (Adlerova, Bartoskova et al. 2008, Garcia-Montoya, Cendon et al. 2012) and the glycan component of LF has an important role to play in many of these properties (O'Riordan, Kane et al. 2014). The presence of the glycan chains influence the tertiary structure of the protein, enhance its ion binding ability (Legrand, Mazurier et al. 1990, Moore, Anderson et al. 1997, Rossi, Giansanti et al. 2002) and its resistance to enzyme digestion (van Veen, Geerts et al. 2004). The attached glycans are also believed to have a role in LF's antibacterial (Teraguchi, Shin et al. 1996, Yoshida, Wei et al. 2000, Wang, Hirmo et al. 2001) and antiviral

(Kawasaki, Isoda et al. 1992, Kawasaki 1993) activities. However, to date, information on how glycosylation variation over lactation influences bioactivity is limited.

Biomolecular interaction analysis (Biacore) involves the use of surface plasmon resonance (SPR) to measure the binding of candidate compounds or cells to specific ligands. SPR measures the changes in the refractive index near a planar chip surface induced by binding of soluble molecules to immobilized counterpart molecules on the sensor chip (Huber and Mueller 2006). It has been widely used for the quantification and kinetic analysis of receptor–ligand interactions (Estmer Nilsson, Abbas et al. 2010, Schlick and Cloninger 2010, Šípová, Zhang et al. 2010, Lane, Mehra et al. 2011, Lin, Chen et al. 2011, Milkani, Lambert et al. 2011). Recently, this methodology has been identified as a reliable, high throughput method for profiling the interaction of whole bacterial cells with an immobilised glycans (Lane, Mehra et al. 2011).The authors validated the Biacore assay by investigating the interaction between *Campylobacter jejuni* and the milk oligosaccharide, 2'-fucosyllactose, to which it is known to bind (Morrow, Ruiz-Palacios et al. 2004, Newburg, Ruiz-Palacios et al. 2005). The assay monitors interactions dynamically over time in a continuous flow system and may mimic natural conditions in a more realistic manner when compared with static adhesion assays (Lane, Mehra et al. 2011). A number of similar studies to monitor whole bacterial cell interactions with immobilised compounds of interest have also been documented (Holmes, May et al. 1997, Oli, McArthur et al. 2006, Kinoshita, Uchida et al. 2007, Huang, Okawara et al. 2013).

The current study aims to exploit SPR to investigate the importance of the glycan chains in the interaction between LF and bacteria. Commercially available LF from colostrum and mature bovine milk was biotinylated and immobilised on the streptavidin coated surface of a SA chip. A number of pathogenic bacterial strains were selected and screened for interactions with both glycovariants.

2. Methods and materials

2.1 Materials

LF from colostrum and mature milk was purchased from Sigma-Aldrich Co. (Dublin, Ireland). Mueller–Hinton broth and Brain Heart Infusion broth were purchased from Oxoid (Basingstoke, Hampshire, UK). The Biacore X instrument, SA chip, biotin CAPture kit, HBS–EP buffer (10 mMHepes, 150 mM NaCl, 3.8 mM ethylenediaminetetraacetic acid (EDTA), 0.05% (v/v) Tween 20), and amine coupling kit were purchased from GE Healthcare (Buckinghamshire, UK). The EZ-Link biotin–PEG4–hydrazide kit, sodium acetate buffer (pH 5.5), and Zeba desalt spin column were purchased from Pierce (Rockford, IL, USA). Sodium meta-periodate was purchased from Sigma-Aldrich Co. (Dublin, Ireland).

2.2 Bacterial strains and culture conditions

Bacterial strains used in this study and their respective growth media are listed in table 1. Bacterial culture stocks were maintained in their respective growth media containing 50% glycerol at −80°C in the culture collection at Teagasc Food Research Centre, Moorepark and propagated twice prior to use. All bacterial strains were grown aerobically for 12–24 h at 37°C. Bacterial cells were washed three times in HBS–EP buffer and re-suspended to a concentration of $1x10^8$ colony-forming units (CFU)/ml for screening studies unless otherwise stated.

Table 1 List of bacterial strains

Stain	Media
Staphylococcus aureus DPC 5971	Muller–Hinton
Escherichia coli O157:H7 P1432 (DPC 6053)	Muller–Hinton
Escherichia coli O157:H7 NCTC 12900 (DPC 6055)	Muller–Hinton
Cronobacter sakazakii NCTC 8155 (DPC 6440)	Brain Heart Infusion
Cronobacter sakazakii DPC 6531	Brain Heart Infusion
Streptococcus pneumoniae ATCC BAA-255	Todd Hewitt + 0.5% yeast extract
Pseudomonas aeruginosa ATCC 33354	Tryptic soy broth
Salmonella enterica subsp. enterica serovar *Typhimurium ATCC BAA-185 (DPC 6047)*	Brain Heart Infusion
Listeria monocytogenes DPC 3437	Brain Heart Infusion
Listeria monocytogenes NCTC 11994 (DPC 3563)	Brain Heart Infusion

2.3 Biotinylation of Lactoferrin

LF samples were biotinylated using EZLink biotin–PEG4–hydrazide as per the manufacturer's instructions. Briefly, 2 mg of LF was dissolved in 1 ml of 0.1 M sodium acetate buffer (pH 5.5). 1 ml of cold sodium meta-periodate solution (20 mM periodate) was added and the solution was mixed well. The mixture was protected from light and incubated for 30 min at room temperature. Excess periodate was removed using a Zeba desalt spin column equilibrated with 0.1 M sodium acetate buffer (pH 5.5). One part of 50 mM biotin–hydrazide solution was added to nine parts of the treated sample and incubated for three-four hours at room temperature. Unbound biotin remaining in the sample was removed by passing the solution sequentially through two Zeba desalt spin columns. The sample was then stored at 4°C until use.

2.5 Biacore assay

All analysis was carried out on a Biacore X instrument at a constant temperature (25°C) and flow rate (10 µl/min), unless otherwise stated, using HBS–EP as the run buffer and a SA chip. The streptavidin-coated SA chip surface was primed with a short injection of 1 M NaCl and 50 mM NaOH (filtered and degassed). Whole bacterial cells were harvested and resuspended in HBS–EP running buffer as described in section 2.2. In order to confirm the absence of non-specific binding of the selected bacterial strains to the SA chip surface, cell suspensions ($1x10^8$ CFU/ml) were injected over the chip surface at 10µl/min, and the binding signal was measured. Signal change was reported in response units (RU). The chip surface was washed with HBS–EP running buffer between bacterial injections to ensure full removal of microbial cells.

Thereafter, biotin-labelled LF (from either colostrum or mature milk) was diluted in HBS–EP buffer (50 µg/ml), and 100 µl of this solution was injected over the surface at a flow rate of 10 µl/min. The chip surface was then washed with HBS–EP buffer to ensure the removal of any non-immobilized molecules. Subsequently, bacterial injections were repeated to evaluate bacterial binding to immobilised colostrum and mature milk LF.

2.6 Statistical analysis

All experiments were performed in triplicate and results are presented as mean values ± standard deviations of three replicate experiments. Nonspecific binding

of the analyte to the test surface was eliminated from all experiments through the use of a reference surface.

3. Results

In the current study, SPR was used to investigate the effect of changes in LF glycosylation over lactation on its ability to bind to pathogenic bacteria. A panel of pathogenic bacteria were initially exposed to the surface of the SA chip, minus the immobilised analyte, to ensure the absence of non-specific binding to the chip surface, which could result in false results during the test runs. For all bacteria screened, non-specific binding was not observed (data not shown). Concentrations of bacteria at approximately 1 $x10^8$ CFU ml^{-1} were used in the assays as previous studies have shown that concentrations in this range are generally required to generate a measurable signal (Lane, Mehra et al. 2011).

Based on previous studies (Naidu, Andersson et al. 1991, Teraguchi, Shin et al. 1996, Roe, Currie et al. 2001) and the results generated in the initial phase of this work, *S. aureus* DPC 5971 and *E. coli* P1432 were identified as positive and negative controls for binding to LF respectively. Exposure of bacterial suspensions with increasing cell number resulted in an increase in the RU value with *S. aureus* DPC 5971, confirming the injected bacterial cells were interacting with the immobilised LF (Figure 1). No increase in RU value was observed for *E. coli* P1432 following exposure of increasing concentrations of bacterial cells to immobilised LF (Figure 1), confirming the lack of binding of this strain to the analyte. These strains were exposed to the surface of the SA chip with immobilised LF at the beginning of each experimental set to ensure consistent performance of the chip. The response of *E. coli* P1432 (colostrum: 16.8 ± 4.3RU; mature milk: 17 ± 7.1) was considered a baseline minimum RU value and used as a reference for screening the other pathogens for positive interactions.

RU changes following exposure of the bacterial strains to the immobilised colostrum and mature milk LF are shown in Figure. 2. Of the positive interactions profiled, the strongest signal was observed for *C. sakazakii* NCTC 8155, *S. pneumoniae* ATCC BAA-255 and *P. aeruginosa* ATCC 33354. Neither *L. moncytogenes* strain tested displayed binding to either LF glycovariant. For the majority of strains which displayed positive binding, a stronger interaction was observed for mature milk LF versus colostrum LF. *S. typhimurium* ATCC BAA-

185 was the sole exception to this, interacting only with the glycovariant of the protein isolated from colostrum.

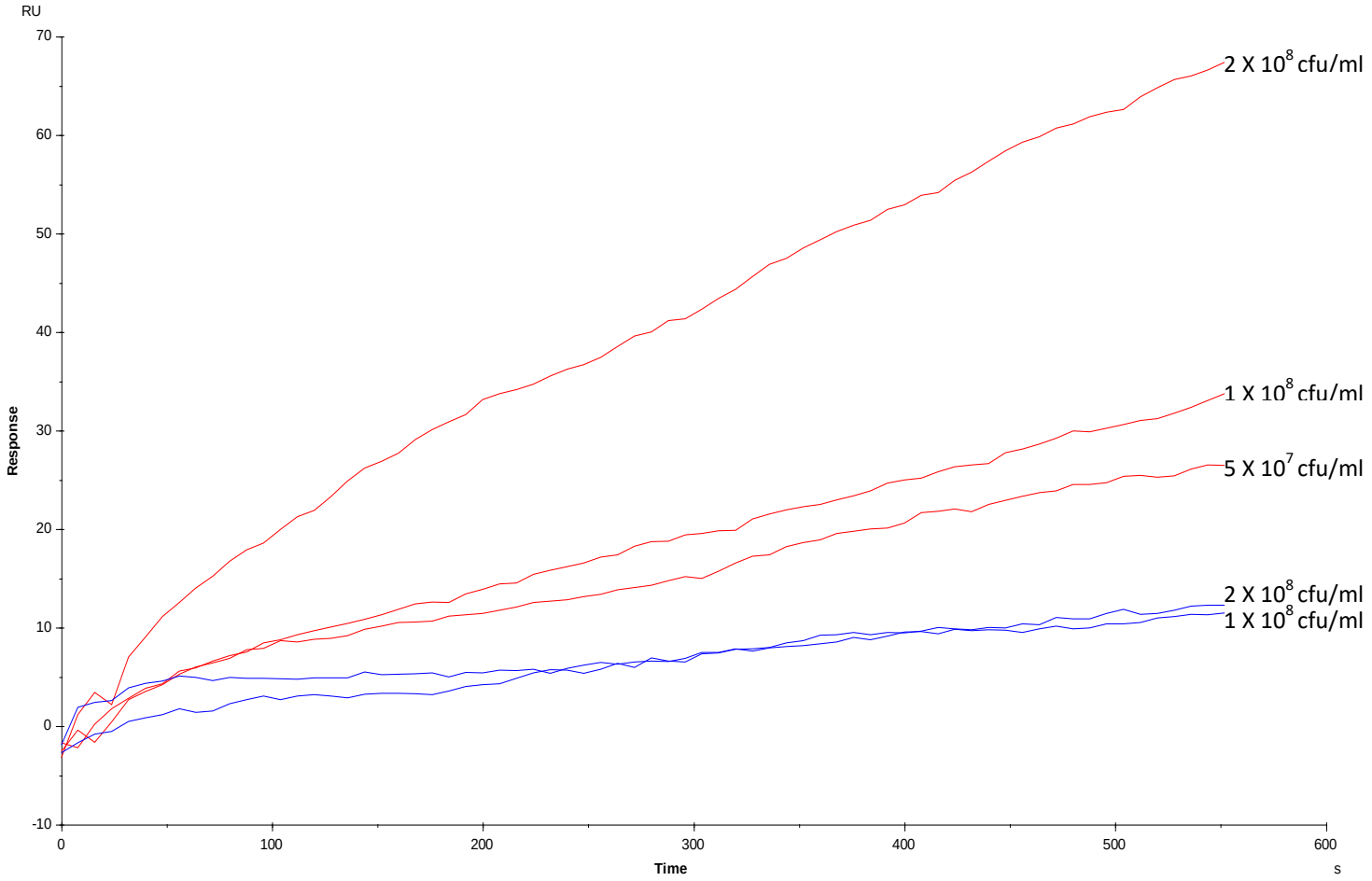

Figure 1 Increasing concentrations of *Staphylococcus aureus* DPC 5971 and *Escherichia coli* O157:H7 P1432 (DPC 6053) injected over the surface of an SA chip immobilised with mature LF to evaluate effect of increasing bacterial numbers on the RU response.

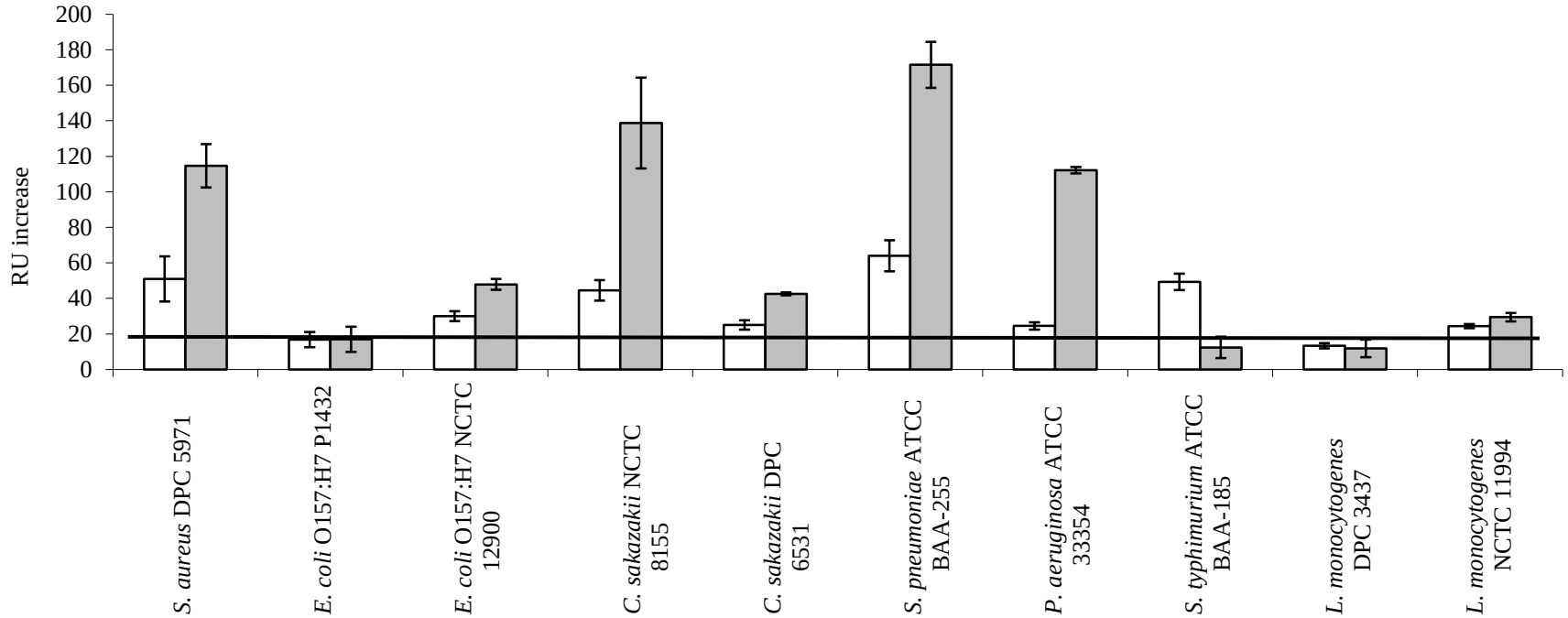

Figure 2 RU change follow exposure of a panel of pathogenic bacteria to LF from colostrum (white) or mature milk (grey) immobilised on a Biacore SA chip. *E. coli* P1432 was identified as a negative control and its response marked as the minimum requirement for consideration as a positive interaction (black horizontal line).

4. Discussion

The greater interaction with mature LF may be linked to the abundance of high-mannose type glycans on this glycovariant. Teraguchi, Shin et al. (1996) described the relationship between LF and members of the Escherichia family and the dependence of the observed interaction on the presence of high-mannose type glycans on the milk protein. Members of this family which express type 1 fimbriae can recognise and bind to oligomannose glycan chains on eukaryotic cell surfaces (Abraham, Sun 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 bovine LF acted as receptors for the mannose specific type 1 fimbriae, therefore preventing bacterial interaction with the eukaryotic cell by acting as a decoy receptor. When human LF was tested, this activity was not observed, potentially as a result of the presence of only complex type glycans on the human protein variant. The same group went on to demonstrate that bovine LF 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. The results presented here suggest that bovine LF may also have similar interactions with other pathogenic bacteria as a result of its direct bacterial interaction via its oligomannose type glycans. The glycoprotein may have a role as a more non-specific defence mechanism, inhibiting bacterial adhesion to mammalian cells.

The lower binding observed for LF isolated from early lactation may also be linked to the presence of more diverse antimicrobial elements in colostrum such as immunoglobulins and oligosaccharides. Therefore, there may be less of a biological requirement for LF to contribute to the inhibition of pathogenic infection. Also, the presence of sialic acid on colostrum LF glycans suggests this glycovariant may have an alternative method of anti-bacterial activity in early lactation. Sialylation has previously been linked to LF's antimicrobial activity as a result of its calcium chelation activity, competing for loosely bound calcium ions involved in the stabilization of lipopolysaccharides in the outer membrane of bacterial cells (Jaques, Brown et al. 1977, Rossi, Giansanti et al. 2002).

S. aureus DPC 5971 is a bovine mastitis isolate (O'Flaherty, Flynn et al. 2005) and was selected as the positive control for this study as receptors for LF

have previously been identified in *S. aureus* strains associated with mastitis infection (Naidu, Andersson et al. 1991). The LF concentration in milk has been shown to increase in cows suffering from subclinical mastitis (Hagiwara, Kawai et al. 2003), possibly as part of an immune response to infection. The results of this study demonstrate that although *S. aureus* DPC 5971 binds to both colostrum and mature milk LF, the glycoprofile present on mature milk LF is more favourable for bacterial binding, suggesting that the *S. aureus* receptors which interact with LF are regulated by the glycosylation present on the protein.

Two *E. coli* O157:H7 strains were included in this study, *E. coli* P1432 and *E. coli* NCTC 12900, the former included as the negative control. O157 strains have previously been shown to be unable to produce the type 1 fimbriae (Roe, Currie et al. 2001) described by Teraguchi, Shin et al. (1996) as the mode of binding for *E. coli* cells to LF mannose residues. Minimal binding was observed for *E. coli* NCTC 12900 with mature milk LF. LF has been shown to bind to different *E. coli* strains with varying levels of efficiency, for both the human and bovine variants of the protein (Naidu, Erdei et al. 1991, Erdei, Forsgren et al. 1994).

Two *C. sakazakii* strains were exposed to immobilised LF in this study; C. *sakazakii* NCTC 8155, a strain isolated from dried milk powder, and *C. sakazakii* DPC 6531, a brain tumour isolate. The most significant binding was observed for C. *sakazakii* NCTC 8155. LF has been shown to display anti-bacterial (Wakabayashi, Yamauchi et al. 2008, Harouna, Carramiñana et al. 2015) and anti-infective (Quintero-Villegas, Wittke et al. 2014) activity against *C. sakazakii*. This study confirms the direct binding of LF to *C. sakazakii*, which may give further insight into its mode of antibacterial action, which to date is believed to be dependent on the protein's iron chelating ability. Galactoligosaccharides (Quintero, Maldonado et al. 2011) and LF containing bovine whey powders (Halpin, Brady et al. 2010) have previously been shown to inhibit the adherence of *Cronobacter* strains to gastrointestinal cells and in comes cases prevent invasion.

For both strains, a higher RU value was noted when the immobilised analyte was the mature milk glycovariant, again highlighting the importance of the mature milk glycoprofile in bacterial binding. In relation to the strain

specificity observed for *C. sakazakii*, LF binds more intensely to the strain isolated from milk powder. Milk powder is a common source of *C. sakazakii* contamination and the WHO has issued guidelines relating to the recommended minimum temperature for reconstitution to reduce infection risk. Fortification of milk powders and infant milk formulas with LF as a secondary hurdle to infection may further reduce the annual incidences of *C. sakazakii* infection, which are most common in neonates, infant and immune compromised adults.

Novel interactions for *S. pneumoniae* ATCC BAA-255 and *P. aeruginosa* ATCC 33354 with LF were observed in this study, both interacting to a greater extent with the glycoprotein from mature milk. Two proteins from *S. pneumoniae* which bind to human LF have previously been identified (Hammerschmidt, Bethe et al. 1999). These proteins have been suggested to be elements of a novel virulence mechanism, harnessing LF's iron binding activity to overcome iron limitation at mucosal surfaces. Antibacterial activity against *P. aeruginosa* has been observed upon exposure to a peptide derived from LF, lactoferricin B (Bellamy, Takase et al. 1992). However, this is the first study to the best of our knowledge that demonstrates binding of bovine milk derived LF to the whole bacterial cells. Both *S. pneumoniae* (Andersson, Dahmen et al. 1983) and *P. aeruginosa* (Lesman-Movshovich, Lerrer et al. 2003) have previously been shown to have affinity for neutral oligosaccharides with terminal D-Gal residues suggesting the complex type glycans in mature milk have a role to play in binding of these strains. Complex glycans have a higher level of sialylation in colostrum (O'Riordan, Gerlach et al. 2014) inhibiting recognition of terminal Gal residues by bacterial lectins. Therefore, the mature milk glycoprofile offers terminal D-Gal residues as targets for bacterial binding.

S. typhimurium was the sole bacterial strain to show greater binding to colostrum LF. *S. typhimurium* adhesion to intestinal cells has previously been shown to be linked to cellular glycosylation and can be inhibited by lectins PNA, AIA, ECA, RCA I and WGA which compete for the glycan receptors on the epithelial cell surface (Giannasca, Giannasca et al. 1996). Binding patterns of these lectins to LF over lactation was previously profiled and all lectins displayed the highest interaction with colostrum LF (O'Riordan, Gerlach et al. 2014) suggesting the glycosylation profile of LF from early lactation is homologous to

the binding sites on Caco-2 cells for *S. typhimurium*. Although cows of all ages be infected with salmonella bacteria, serious infections and deaths are most often seen in young calves (Robertsson, Lindberg et al. 1983). The glycosylation pattern of LF in early lactation may contribute to the immune protection delivered to the calf via its mother's milk and may have evolved to offer protection against infections most likely to occur in early life.

Colostrum LF has a higher N-acetylneuraminic acid (Neu5Ac) content versus mature milk LF (O'Riordan, Gerlach et al. 2014) and this monosaccharide is closely linked to LF's antibacterial activity, as previously reviewed (O'Riordan, Kane et al. 2014). However, minimal bacterial binding to colostrum LF was observed in this study. This would suggest LF's glycosylation may be relgualted to deliver the optimimum protection to the life-stage of the offspring.

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Chapter 5

Bovine glycomacropeptide promotes the growth of Bifidobacterium longum subsp. infantis and modulates its gene expression

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Abstract

Bovine milk glycomacropeptide (GMP) is a 64 amino acid peptide, derived from k-casein, with exclusively *O*-linked glycosylation. GMP promoted the growth of *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) in a concentration dependent manner and this activity was lost following periodate treatment of the GMP (GMP-P), which disables biological recognition of the conjugated oligosaccharides. Microarray analysis of gene transcription in *B. infantis* following exposure to GMP revealed a substantial transcriptional response to GMP relative to bacteria treated with GMP-P, with a greater number of differentially expressed transcripts and larger fold changes versus the control. Thus, the *O*-linked glycosylation of GMP is intrinsic to the growth stimulation of *B. infantis* by GMP. The pool of differentially expressed transcripts included two glycoside hydrolase (family 25) genes, which were substantially up-regulated following exposure to GMP but not GMP-P. These GH25 genes were present in duplicated genomic islands which also contained up-regulated genes encoding for fibronectin type III binding domain proteins and numerous phage-related proteins. Homologues of this genomic arrangement were present in other *Bifidobacterium* species, which suggested that it may be a conserved domain for the utilisation of glycosylated peptides. This study provides insights into the molecular basis for the prebiotic effect of bovine milk GMP on *B. infantis* and highlights its potential as a functional food ingredient for the promotion of a beneficial gut microbiota.

1. Introduction

The human gastrointestinal tract (Nakajima, Tamura et al.) is colonised by a large number of microorganisms (400-500 bacterial species from 40-50 different genera (Savage 2005)) and bifidobacteria are a bacterial group frequently present, colonising the GIT in the initial days following birth (Favier, de Vos et al. 2003, Turroni, Peano et al. 2012). A bifidobacteria-rich microbiome has numerous biological benefits to the host, including maintenance of a healthy GIT, inhibition of microbial infection and alleviating symptoms associated with digestive illness (Leahy, Higgins et al. 2005, Picard, Fioramonti et al. 2005). Therefore, there is growing interest in enhancing the bifidobacterial population of the GIT. Human milk oligosaccharides (HMOs) represent the main impetus for bacterial colonization of the distal large intestine of the breast-fed infant (Scholtens, Oozeer et al. 2012). The high concentrations of HMOs and oligosaccharides either *N*- or *O*-linked to proteins processed after intestinal digestion are thought to be the main contributors to the predominance of *Bifidobacterium* species in the infant gut (Garrido, Dallas et al. 2013). Indeed, the genome of one particular strain, *Bifidobacterium longum* subsp *infantis* ATCC15697 (*B. infantis*), a common member of the gastrointestinal microbiota of breast-fed infants, has revealed particular adaptations for the metabolism of HMOs (LoCascio, Ninonuevo et al. 2007, Sela, Chapman et al. 2008, LoCascio, Desai et al. 2010, Garrido, Kim et al. 2011, Sela, Li et al. 2011, Garrido, Ruiz-Moyano et al. 2012, Sela, Garrido et al. 2012, Kim, An et al. 2013), milk glycoconjugates (Garrido, Dallas et al. 2013) and even commercial oligosaccharides such as galacto-oligosaccharides (GOS) (Garrido, Ruiz-Moyano et al. 2013) and fructo-oligosaccharides (FOS) (Perrin, Warchol et al. 2001). In particular, the strain consumes these various carbohydrates using a variety of glycosyl hydrolases and ABC transporters (Sela, Chapman et al. 2008, Garrido, Dallas et al. 2013). In addition to their bifidogenic properties, HMOs also enhance the adhesion of *B. infantis* to human intestinal cell lines (Chichlowski, De Lartigue et al. 2012, Kavanaugh, O'Callaghan et al. 2013) and prevent pathogenic invasion (Newburg 2000, Coppa, Zampini et al. 2006, Bode 2009).

However, the large quantities of HMOs required for use as functional ingredients are unavailable, while commercial oligosaccharides such as GOS and FOS cannot match the complexity of the HMO structures and biological benefits.

Bovine milk is potentially an alternative source of complex oligosaccharides with associated biological activity. Bovine milk oligosaccharides (BMOs) have the desirable structural complexity (Mariño, Lane et al. 2011, Aldredge, Geronimo et al. 2013) similar to that of HMOs and have been shown to possess beneficial biological activities (Idota, Kawakami et al. 1994, Lane, Mehra et al. 2011). However, the large quantities required are currently commercially unavailable. Certain glycoconjugates present in bovine milk, such as lactoferrin and glycomacropetide (GMP), have been purified at a large scale, as previously reviewed (O'Riordan, Kane et al. 2014) and may offer an alternative source of functional oligosaccharides.

Of the glycosylated proteins present in bovine milk, caseinomacropeptide (CMP), a 64 amino acid peptide derived from κ-casein (κ-cn) (Delfour, Jolles et al. 1965), offers great potential as a functional food ingredient due to its associated bifidogenic activity (Idota, Kawakami et al. 1994, Janer, Pelaez et al. 2004, Chen, Cao et al. 2012, Robitaille 2013). The bactericidal effect of CMP against *Escherichia coli* in an acidic environment has been reported (Robitaille, Lapointe et al. 2012), while also enhancing the survival of *Lactobacillus rhamnosus* under similar conditions. Importantly, CMP can be produced in the quantities required for use as a food ingredient (Tullio, Karkle et al. 2007) and it is commercially available as an ingredient for application in functional foods, beverages, cosmetics and supplements (O'Riordan, Kane et al. 2014). This peptide has numerous O-linked glycosylation sites (but no *N*-linked), and approximately 60% of CMP found in bovine milk is in the glycosylated form and is referred to as GMP (Vreeman, Visser et al. 1986).

GMP has been shown to improve the growth of lactobacilli and bifidobacteria, both *in vitro* (Janer, Pelaez et al. 2004, Robitaille 2013) and *in vivo* (Chen, Cao et al. 2012), and also possesses a number of other biological benefits including anti-infective (Nakajima, Tamura et al. 2005, Hermes, Molist et al. 2013) and anti-toxigenic (Kawasaki, Isoda et al. 1992, Isoda, Kawasaki et al. 1994) activities. The carbohydrate content of bovine GMP plays a role in its associated biological benefits including immuno-regulatory (Otani and Hata 1995, Li and Mine 2004), bacterial cell binding (Nakajima, Tamura et al. 2005), anti-toxigenic (Oh, Worobo et al. 2000), and anti-viral (Kawasaki 1993) activities. Improved learning ability (Wang, Yu et al. 2007) and increased salivary sialic

acid content (Thoma-Worringer, Sorensen et al. 2006) was also observed in piglets following feeding with a GMP-supplemented diet. The oligosaccharides present on GMP are highly sialylated as a result of the predominance of a di-sialylated tetrasaccharide, which accounts for 56% of the oligosaccharide structures present (Saito 1992). This relatively high level of sialylation may be vital for GMP's bifidogenic properties as the prebiotic activity of a number of milk oligosaccharides and glycopeptides has been previously been linked to their sialic acid content (Idota, Kawakami et al. 1994).

However, there are conflicting reports on the importance of the carbohydrate content of GMP in relation to the growth promotion of bifidobacteria, with recent reporting that only the peptide backbone of CMP was essential for prebiotic activity (Robitaille 2013). In the present work, the growth- and metabolic-related transcriptomic changes of an archetypical infant *B. infantis* strain following transient exposure to GMP is reported. The oligosaccharides on GMP were degraded using sodium metaperiodate to investigate the role of GMP glycosylation. The observed phenotypic changes were subsequently correlated with transcriptional changes as determined by microarray analysis.

2.Materials and methods

2.1 Materials

GMP, with a maximum assayed lactose content of 1%, was kindly provided by Davisco Foods International Inc. (Eden Prairie, MN, USA). Vivaspin 6 3 kDa molecular weight cut off (MWCO) centrifugal filters were from Sartorius Stedim Biotech GmbH (Göttingen, Germany). *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (*B. infantis*) was purchased from DSMZ (Germany). De Man-Rogosa-Sharpe (MRS) broth was purchased from Oxoid Ltd. (Basingstoke, England). The Anaerocult A system was purchased from Merck (Dannstadt, Germany). All other reagents were from Sigma-Aldrich Co. (Dublin, Ireland) unless otherwise stated and were of the highest grade available.

2.2 Periodate treatment of GMP

Sodium metaperiodate ($NaIO_4$) treatment of GMP to produce GMP-P was performed as previously described (Alemka, Whelan et al. 2010). Briefly, GMP (2 mg/ml) was incubated with 0.011 mM $NaIO_4$ (Alemka, Whelan et al. 2010) at 4 °C for 30 min. Excess $NaIO_4$ was removed by centrifugal filtration using 3 kDa

MWCO with three phosphate buffered saline, pH 7.4 (PBS) washes and the retentate containing GMP-P was lyophilised.

2.3 Bacterial culture and growth experiments

B. infantis was routinely cultured in MRS supplemented with 0.05% w/v L-cysteine (mMRS) at 37°C under anaerobic conditions generated using the Anaerocult A system (Kavanaugh, O'Callaghan et al. 2013). Bacterial culture stocks were maintained in mMRS containing 50% glycerol at −80°C and propagated twice in mMRS media prior to use. Bacterial growth assays were performed in mMRS supplemented with increasing concentrations of GMP (0.5, 1, 2, 4, and 8 mg/ml) filter sterilised through a 0.22 μM membrane. The medium was inoculated with a 1% inoculation of an overnight culture with an optical density at 600 nm (OD_{600nm}) of approximately 1.0 (corresponding to approximately 2 x 10^8 cfu/ml). Cultures entered mid-exponential phase of growth after 16 h and OD_{600nm} values at this timepoint were taken for comparison between each treatment. Growth experiments were performed in triplicate and the data presented is the average of three independent replicate assays. OD600nm was measured on a PharmaSpec UV-1700 UV-visible spectrophotometer.

2.4 RNA Isolation

B. infantis was cultured to mid-exponential phase in mMRS as a control and mMRS supplemented with GMP or GMP-P (2 mg/ml) and bacterial pellets were harvested by centrifugation at 4,500 x g for 8 min. The supernatant was removed and the bacterial pellets were resuspended in RNAprotect bacteria reagent (Qiagen, Hilden, Germany) and incubated at room temperature for 10 min. Bacteria were again pelleted at 5,000 x g for 10 min and the supernatant was discarded. Bacterial RNA was isolated using the RNA isolate mini kit (Bioline, London, UK) with modifications, as previously described [16]. RNA quantity and quality was determined using a NanoDrop 1000 (Wilmington, DE, USA) and products with 260/280 nm readings >1.9 and 230/260 nm readings ≥1.00 were selected for further analysis. Sample quality was further assessed by agarose gel electrophoresis. RNA samples were subsequently shipped to IMGM laboratories (Martinsried, Germany) for labelling and DNA microarray analysis. To ensure the absence of RNA degradation during transit, RNA was again assessed using an

Agilent Bioanalyser (RNA600 Chip) and only samples with a RNA integrity number (RIN) of 10 were approved for microarray analysis.

2.5 Microarray analysis

The RNA was spiked with synthetic polyadenylated transcripts (Agilent spike-in controls) and labelled by a protocol for reverse transcription followed by *in vitro* transcription (RT-IVT). In brief, 500 ng of RNA was used for each reaction which was reversed transcribed into complementary DNA (cDNA) using random priming (Full SpectrumTM MultiStart Primers for T7 IVT, System Biosciences (SBI)). This was then converted into labelled cRNA by *in vitro* transcription (Quick-Amp Labelling Kit One-Color, Agilent Technologies, Co. Cork, Ireland) incorporating cyanine 3- (Cy3-) conjugated CTP. Manufacturer's instructions were followed for both the reverse transcription and labelling reactions.

A NanoDrop analyser and an Agilent 2100 Bioanalyser with a 6000 Nano LabChip Kit (Agilent Technologies) were employed to determine the labelling efficacy and samples with cRNA yields >825 ng and a specific activity of 9.0 pM Cy3 per µg of cRNA were selected for array analysis. Following cRNA clean-up and quantification (NanoDrop), 600 ng of each Cy3-labeled cRNA sample was fragmented and prepared for One-Color based hybridization (Gene Expression Hybridization Kit, Agilent Technologies). Hybridization was performed at 65 °C for 17 h on custom Bifidobacterium GE Microarrays (8X15K format) (Kavanaugh, O'Callaghan et al. 2013) which were then washed with increasing stringency using Gene Expression Wash Buffers (Agilent Technologies) followed by drying with acetonitrile. Fluorescent signal intensities were detected with Scan Control A.8.4.1 Software (Agilent Technologies) on the Agilent DNA Microarray Scanner and extracted from the images using Feature Extraction 10.7.3.1 Software (Agilent Technologies) and the design file 033172_D_F_20110831.xml.

2.6 qPCR Analysis

cDNA was synthesized from 1 µg of RNA (isolated as described above) with a reaction mixture (final volume 20 µl) consisting of 3.2 µg of random hexamers, 0.5 µl of Transcript Reverse Transcriptase (Roche, West Sussex, UK), 0.5 µl of Protector RNAse inhibitor, 1 mM dNTPs mix and 4 µl of Transcriptor RT Reaction Buffer (Roche). Template and random primers were incubated at 65 °C for 10 min, followed by addition of the remaining components. The reaction

mixture was then incubated at 55 °C for 30 min and inactivated by heating to 85 °C for 5 min. PCR primers and probes were designed using the Universal Probe Library Assay Design Centre (Roche). Primer sequences and probe combinations are listed in table S1. 16S rRNA (Blon_R0085) was used as an endogenous control to correct for variability in the starting total RNA and provide a stable expression marker against which relative levels of expression were determined. Amplification reactions contained 2.5 µl of cDNA, 5 µl of 2X SensiMix II Probe Buffer (Bioline), 5 pmol/µl of each primer and probe mix and were brought up to a total of 10 µl by the addition of RNase-free water. All reactions were performed in duplicate in 384-well plates on the LightCyclerH480 System (Roche) and positive and negative controls were included in each run. Thermal cycling conditions applied were 95 °C for 10 min, [95 °C for 10 s, 60 °C for 45 s, and 72 °C for 1 s to allow for fluorescence acquisition] (55 cycles), 40 °C for 10 s as recommended by the manufacturer. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was employed to calculate relative changes in gene expression.

2.7 Statistical analysis

The microarray data were processed using the Limma package of the R statistics suite. Background correction was done using the normexp method with offset= 16. Inter-array normalisation was not done. The microarray data were clustered using the heatmap.2 package in R to identify any experimental samples as outliers. The fold change values presented are actual fold-change values for the test samples relative to the controls. Genes with a fold-change of >1.5 where the p-value was ≤0.05 were considered to be differentially transcribed. Transcriptional changes were displayed graphically through the use of Volcano plots (-Log10 p-value versus fold-change) and whole genome plots, where the fold change for each locus tag was plotted as a bar-chart.

The statistical treatment of the qPCR data was by Student's **t**-test and one-way ANOVA for pairwise or multiple comparisons, respectively, to determine statistically significant results, where $p<0.05$ was considered significant.

3 Results and discussion

3.1 Effect of GMP on *B. infantis* growth and intestinal adhesion

To investigate if GMP had a prebiotic effect on the archetypal infant associated bifidobacterial strain, *B. infantis*, which has previously been shown to have

adaptations for the utilisation of milk glycans (Sela, Chapman et al. 2008), *B. infantis* was cultured in mMRS supplemented with 2mg/ml of GMP, as this concentration has been proven to positively influence the growth of probiotic strains *Lactobacillus rhamnosus* RW-9595-M and *B. thermophilum* RBL67 . A 20.6 ± 3.6% increase in OD_{600nm} was observed in the mid-exponential phase of *B. infantis* cultures supplemented with GMP, indicating a growth promoting effect (figure S1)**.** Assays were repeated with increasing GMP concentrations and OD_{600nm} was recorded after 16 hours incubation, at mid-exponential phase. A concentration dependent growth promoting effect was observed (figure 1A) with the most significant effect (an increase of 44 ± 9.9% versus the control) observed using 8 mg/ml GMP.

Similar stimulation on *B. lactis* growth has been reported when cultured in milk supplemented with bovine GMP (Janer, Pelaez et al. 2004). That GMP can influence the growth of *Bifidobacterium* in a nutrient-rich media such as mMRS suggests it may also have an effect *in vivo,* in the complex environment that is the lower GIT. To perform its biological functions *in vivo,* GMP must first reach the lower GIT. Intact GMP has been detected in the plasma of humans at physiologically significant concentrations (Chabance, Marteau et al. 1998, Thoma-Worringer, Sorensen et al. 2006) confirming that intact GMP reaches the lower GIT where it can influence the growth of the microbiota and be absorbed into the bloodstream. All additional work for this study was performed with 2mg/ml of GMP as this is in line with concentrations used in previous studies (Robitaille 2013) and concentrations are unlikely to occur in biological conditions *in vivo* due to the concentration of the peptide present in bovine milk (average 38.8mg/L +/- 12.11mg/L) (Furlanetti and Prata 2003).

Addition of 2 mg/ml oxidised GMP-P resulted in a substantially lower (5.5%) increase in growth compared to that following addition of 2 mg/ml GMP (20.6%, figure 1B). Periodate oxidation of glycoproteins converts vicinal diols of oligosaccharide residues to two aldehyde groups, making the carbohydrate structures biologically unrecognisable (Kilcoyne, Gerlach et al. 2011). Following periodate treatment of GMP, a decrease of approximately 75% in the growth promotion effect of the glycopeptide was observed. A slight growth increase was still evident in the GMP-P group, indicating that the peptide portion may also

influence bacterial growth. Overall, these data suggested that GMP glycosylation has a very important role to play in the promotion of *B. infantis* growth.

GMP is exclusively *O*-glycosylated with both neutral and acidic oligosaccharides (Kawasaki 1993). Sialic acid is a prominent feature of these glycans, with 82% of GMP oligosaccharides being either mono- or di-sialylated (Hua, Nwosu et al. 2012). This high sialic acid content may be the basis for the prebiotic activity described for GMP, as prebiotic activity of other sialic acid containing compounds have previously been demonstrated (Idota, Kawakami et al. 1994). The prebiotic potential of GMP was previously tested on the probiotic strains Lactobacillus rhamnosus RW-9595-M and B. thermophilum RBL67 and it was concluded that this activity was not dependent on the presence of GMP glycosylation (Robitaille 2013). *B. infantis* is genetically predisposed to utilise HMOs (Sela, Chapman et al. 2008, LoCascio, Desai et al. 2010, Sela, Li et al. 2011, Garrido, Dallas et al. 2013) and this genetic characteristic may explain its response to GMP glycosylation. The observations from the present study suggest that different bacteria may utilise different components of GMP, and so the prebiotic effect may be strain dependent.

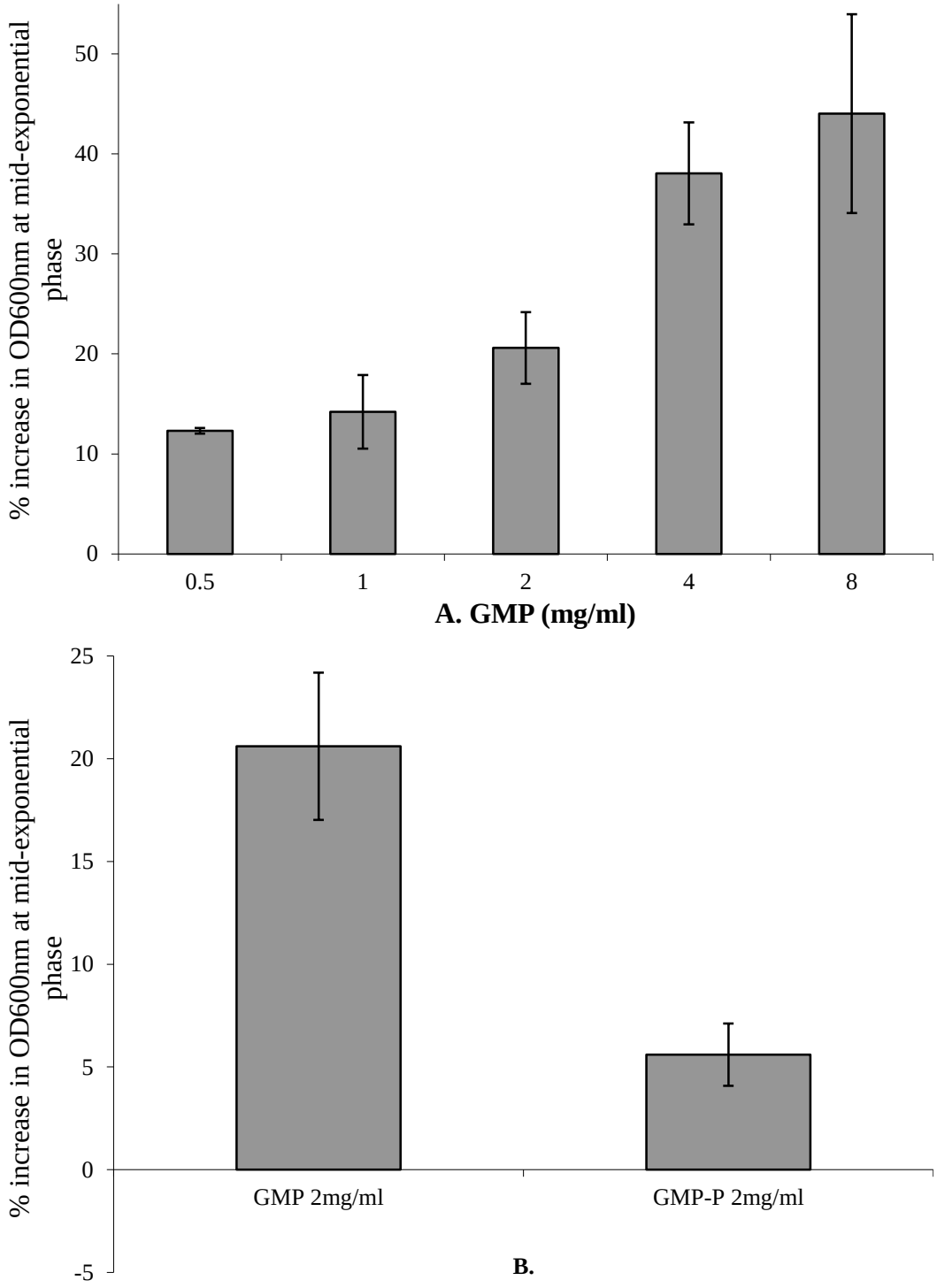

Figure 1 A % increase in OD_{600nm} of *B. infantis* cultured in the presence of increasing concentrations of GMP versus unsupplemented mMRS at mid-exponential phase. **B** Comprison of the % increase in OD_{600nm} of *B. infantis* cultured in the presence of 2mg/ml GMP or GMP-P versus unsupplemented mMRS at mid-exponential phase.

3.2 Gene expression analysis

B. infanits was cultured to mid-exponential phase in MRS, MRS + GMP (2mg/ml) and MRS + GMP-P (2mg/ml) and bacteria were harvested for microarray analysis to profile gene expression in response to the presence of GMP or GMP-P in the growth media versus the control (MRS only). The overall number of genes differentially expressed following GMP supplementation is summarised in figure 2A and a complete list of differentially expressed transcripts (DETs) is provided in table S2. The largest collection of DETs were up-regulated by GMP (264 genes), while considerably fewer genes were down-regulated (47 genes). 153 DETs were up-regulated by GMP-P and none were down-regulated. DETs were categorised into groups based on their annotated function as follows: genes involved in glycan metabolism, cellular transport, protein metabolism, genes involved in cell division and growth, hypothetical or proteins of unknown function and other (figure 2B). With the exception of the hypothetical protein category, genes involved in cell division were the largest population of DETs, which is not surprising considering the observed growth promotion effect of GMP.

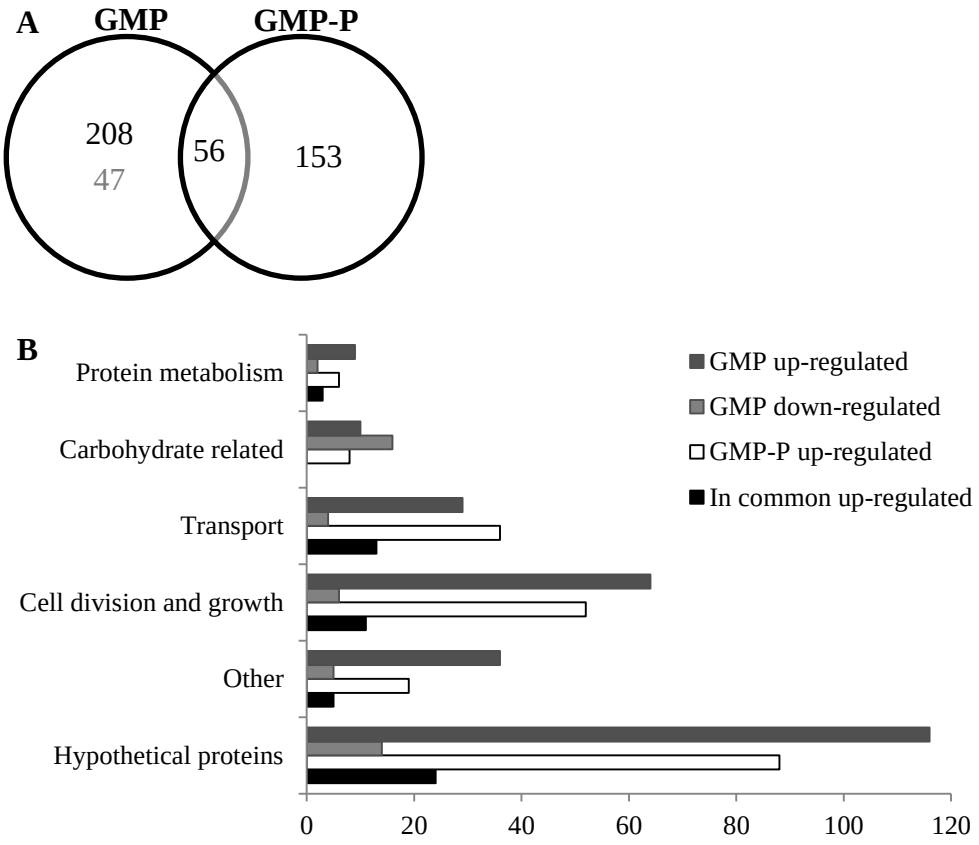

Figure 2 A Number of differentially expressed transcripts following exposure to GMP or oxidised GMP-P. The cut-off point for inclusion was fold change ≥1.5 and p-value ≤ 0.05. Black = up-regulated versus control. Grey = down-regulated versus control. **B** Numbers of *B. infantis* genes, grouped according to functional category, which were differentially regulated following growth in the presence of GMP or GMP-P. Black bars indicated the number of genes that were common to both treatments.

In total, 56 genes were shared between both GMP and GMP-P supplements (figure 2A), with the majority of the annotated genes in this population involved in non-carbohydrate related transport and cell growth, as outlined in table S2. A cluster of phosphonate ABC transporter genes (Blon_0020-Blon_0025) as well as numerous other transport related genes were up-regulated by both treatments and may give insight into the effect of GMP on nutrient uptake by *B. infantis* from the environment. When the genome of *B. infantis* was first published in 2008, 35% of the ORFs in *B. infantis* were annotated as hypothetical proteins, which comprised a total of 845 proteins of unknown function (Sela, Chapman et al. 2008). The majority of these genes are still currently annotated as hypothetical and this category represented the largest group of genes influenced by exposure to GMP and GMP-P, indicating the need for further functional genomic studies to elucidate the role of specific genes.

Volcano plots (figure 3) provide a graphic representation of the greater magnitude of the transcriptomic response to GMP treatment when compared with GMP-P. Although both treatments resulted in a substantial number of DETs in comparison to the control, 311 and 209 genes respectively (figure 2), the Volcano plots illustrated that the response to GMP was more extensive, with larger fold changes observed in both up- and down-regulated genes. This suggested that the oxidation of the GMP oligosaccharides reduced the effect of GMP on *B. infantis* gene expression.

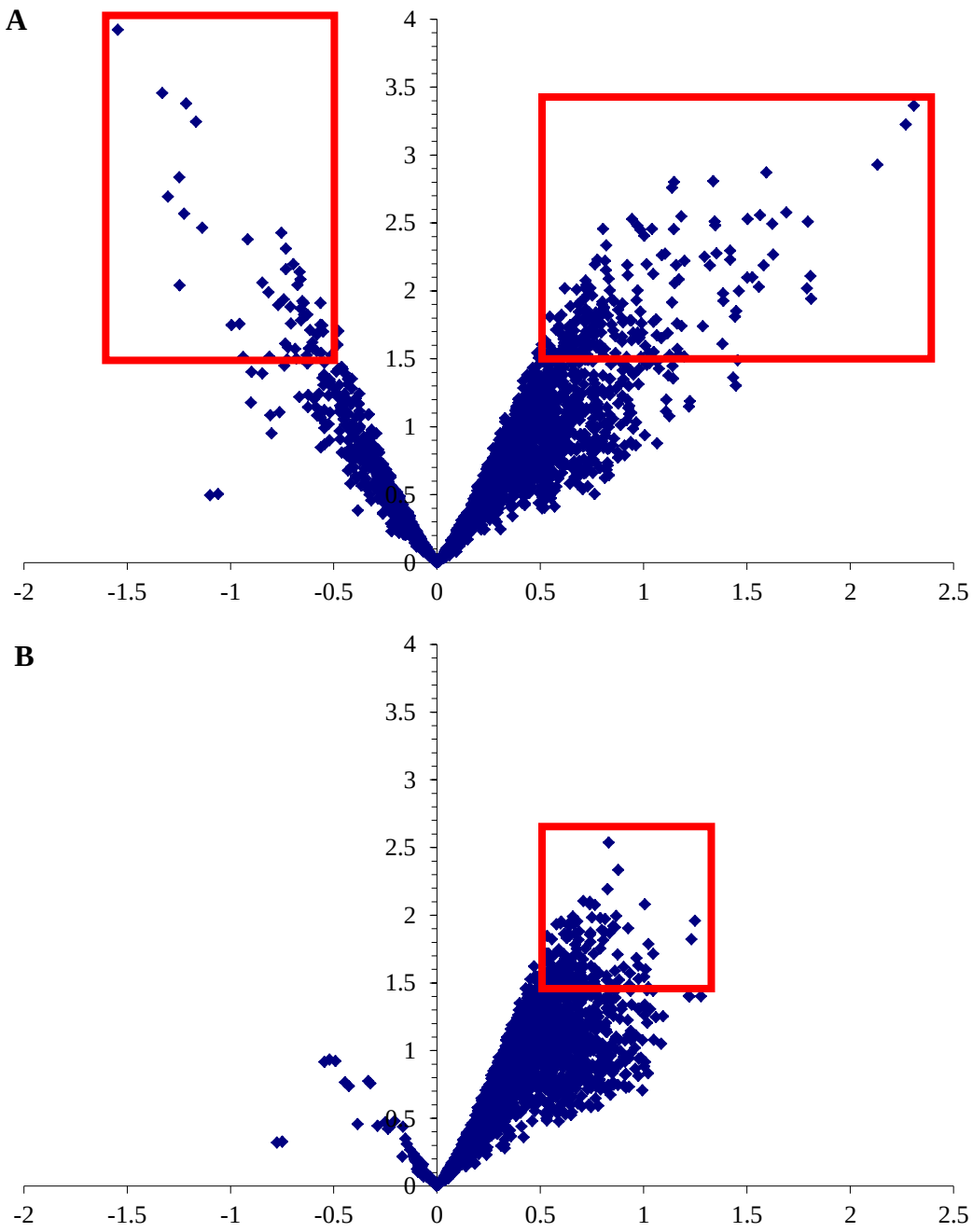

Figure 3 Volcano plots (-Log10 p-value (x-axis) vs fold-change (y-axis)) demonstrating transcriptional changes following *B. infantis* exposure to A GMP and B GMP-P. Red boxes highlight genes which were deemed as significantly changed following each treatment (fold change ≥1.5 and p-value ≤ 0.05).

3.3 Effect of GMP treatment on carbohydrate-related genes

GMP treatment resulted in the up- and down-regulation of genes involved in carbohydrate transport and metabolism (table 1). Although a number of carbohydrate metabolism genes were also differentially expressed in response to GMP-P, the genes were different to those up-regulated in response to GMP-P and the fold-change in expression was generally lower (table 1). A number of genes annotated as LacI regulatory genes were up-regulated by both GMP and GMP-P. LacI proteins can interact directly with DNA and inhibit expression of genes involved in lactose metabolism and the genes annotated as LacI genes in table 1 may have a similar role in regulating sugar metabolism in *B. infantis* if functional. Although unique LacI regulatory genes were differentially expressed in response to both treatments, those up-regulated in the GMP-P group were induced to a much lesser extent. This differential induction of regulatory genes suggested that *B. infantis* may modulate its carbohydrate metabolism pathways in response to the GMP oligosaccharides.

Table 1 Differentially regulated genes associated with carbohydrate metabolism

Locus tag	Annotated function	Treatment	FC	p-value
Blon_1571	glycoside hydrolase, family 25	GMP	3.51	0.01
Blon_1831	glycoside hydrolase, family 25	GMP	3.46	0.01
Blon_2415	regulatory protein, LacI	GMP	2.02	0.01
Blon_0139	4-alpha-glucanotransferase	GMP	1.98	0.02
Blon_1480	Extracellular solute-binding protein, family 5 (potential O-linked glycan utilisation cluster)	GMP	1.87	0.04
Blon_0070	glycogen/starch/alpha-glucan phosphorylase	GMP	1.73	0.02
Blon_0874	regulatory protein, LacI	GMP	1.6	0.02
Blon_0137	alpha amylase, catalytic region	GMP	1.59	0.05
Blon_1019	O-sialoglycoprotein endopeptidase	GMP	1.56	0.02
Blon_2145	NLP/P60 protein (cell wall associated glycoside hydrolase)	GMP	1.56	0.02
Blon_2171	UDP-glucose 4-epimerase	GMP	-1.53	0.02
Blon_0268	glycoside hydrolase family 2, sugar binding	GMP	-1.54	0.03
Blon_1722	Fructose-6-phosphate phosphoketolase	GMP	-1.56	0.01
Blon_1745	Pyruvate kinase	GMP	-1.58	0.01
Blon_1905	Beta-glucosidase	GMP	-1.58	0.02
Blon_2063	galactose-1-phosphate uridylyltransferase	GMP	-1.63	0.02
Blon_2380	extracellular solute-binding protein, family 1 (SBP for mannose oligosaccahrides)	GMP	-1.65	0.03
Blon_0881	glucosamine-6-phosphate isomerase (nagB)	GMP	-1.67	0.04
Blon_2175	binding-protein-dependent transport systems inner membrane component (LNB/GNB metabolism cluster)	GMP	-1.75	0.03
Blon_0540	lactaldehyde reductase	GMP	-1.8	0.01
Blon_0646	glycosyl hydrolase, BNR repeat-containing protein (sialic acid utilisation cluster)	GMP	-1.92	0.03
Blon_2176	binding-protein-dependent transport systems inner membrane component (LNB/GNB metabolism cluster)	GMP	-1.99	0.02
Blon_1714	pyruvate formate-lyase activating enzyme	GMP	-2.2	0
Blon_0640	PfkB domain protein (sialic acid utilisation cluster)	GMP	-2.24	0
Blon_1715	formate acetyltransferase	GMP	-2.32	0
Blon_2174	hypothetical protein Blon_2174 (LNB/GNB metabolism cluster)	GMP	-2.34	0.00

Locus tag	**Annotated function**	**Treatment**	**FC**	**p-value**
Blon_2416	Beta-galactosidase	GMP-P	1.91	0.03
Blon_0248	Alpha-L-fucosidase	GMP-P	1.8	0.05
Blon_0821	carbohydrate kinase, FGGY	GMP-P	1.6	0.02
Blon_0431	sugar transporter	GMP-P	1.58	0.01
Blon_2057	periplasmic binding protein/LacI transcriptional regulator	GMP-P	1.56	0.03
Blon_0208	6-phosphogluconolactonase	GMP-P	1.55	0.02
Blon_0621	Glucan 1,3-beta-glucosidase	GMP-P	1.54	0.02
Blon_0516	regulatory protein, LacI	GMP-P	1.51	0.03

Of the carbohydrate related genes up-regulated solely in response to GMP-P, the most significant were a β-galactosidase (Blon_2416) and an α-L-fucosidase (Blon_0248). The increased expression of a fucosidase gene was unexpected as fucosylated GMP oligosaccharides have only been detected in GMP isolated from colostrum to date (Fournet, Fiat et al. 1975) and mature milk was the source of the GMP used here. In addition, GMP-P was periodate treated, so intact fucose residues could not have survived, regardless of whether they were present or not before periodate treatment. The expression of an α-L-fucosidase in *B. infanits* during growth on inulin, a non-fucose containing substrate, has previously been reported also (Kim, An et al. 2013). The expression of β-galactosidase (Blon_2416) was also increased in the GMP group (fold change = +1.65) but was not statistically significant ($p = 0.07$). However, numerous genes in close proximity to Blon_2416 on the chromosome were also up-regulated following growth in GMP (Blon_2415, Blon_2419-Blon_2423) (table S2), including a LacI regulatory gene.Three genes related to sucrose and starch metabolism were up-regulated following exposure to GMP only; Blon_0070, Blon_0137 and Blon_0139. These genes may be involved in the metabolism of glucose present in the growth media. Their increased expression following exposure to GMP could result in an increased metabolism of glucose from the environment and contribute to the observed growth increase. Interestingly, Blon_0139 was also up-regulated following exposure of *B. infantis* to a mixture of BMOs 3'- and 6'-sialyllactose (Kavanaugh, O'Callaghan et al. 2013).

The presence of a possible *O*-linked oligosaccharide utilisation gene cluster on the *B. infantis* genome was previously described (Garrido, Dallas et al. 2013) and comprised Blon_1479, an α-*N*-acetylgalactosaminidase homologue, and Blon_1480, an ABC transporter for peptide importation. In the present study, Blon_1480 was highly up-regulated in response to growth in the presence of GMP while Blon_1479 expression was unaffected. As GMP itself is a glycopeptide with highly sialylated *O*-linked oligosaccharides, it may be recognised by Blon_1480 for import. Furthermore, the gene Blon_1019 was also up-regulated following growth in the presence of GMP and it has a predicted function related to the metabolism of *O*-glycosylated peptides, as an *O*-sialoglycoprotein endopeptidase.

In contrast, a sialidase previously characterised as metabolising α(2-6) linked sialylated oligosaccharides, Blon_0646 (Sela, Li et al. 2011), was down

regulated in the GMP group. Sela et al. (Sela, Li et al. 2011) reported lack of functionality of this enzyme on sialylated milk oligosacchardies also. The combination of these findings demonstrate that *B. infantis* has glycosidic genes which are not activated in the presence of milk substrates, suggesting glycan digestion by this strain may be more tightly regulated than previously understood. In the same region of the chromosome as Blon_0646, Blon_0640, a PfkB domain-containing protein involved in the pentose phosphate pathway, was also down-regulated in the presence of GMP when compared to mMRS alone. Interestingly, a number of genes related to simple sugar and free oligosaccharide metabolism, such as members of the lacto-*N*-biose (LNB) and galacto-*N*-biose (GNB) metabolism cluster (Blon_2171-Blon_2177) (LoCascio, Desai et al. 2010), were down-regulated in the GMP treated group. The down-regulation of the same genes in the LNB/GNB cluster [Blon_2174-Blon-2176] was previously described following exposure of *B. infantis* to 3'- and 6'-sialyllactose (Kavanaugh, O'Callaghan et al. 2013). No significant transcriptional changes were observed on the 43-kbp cluster at Blon_2331-Blon_2361 which are dedicated to the import and processing of HMOs (Sela, Chapman et al. 2008). Downstream of this genome section, a large number of genes were differentially expressed in the GMP treated group, including up-regulation of Blon_2370-Blon_2373, involved in bacterial growth, and down-regulation of transport-related Blon_2379-Blon_2380. Again, a similar effect was previously observed following exposure to 3'- and 6'-sialyllactose (Kavanaugh, O'Callaghan et al. 2013). It is possible that the presence or absence sialylation is more important than whether the glycans are in the free or bound form.

Two genes encoding glycoside hydrolases from family 25 (GH25), Blon_1571 and Blon_1831, were up-regulated in response to GMP. GH25 has only one documented function to date, as a lysozyme which is involved in cell wall and peptidoglycan catabolism and may thus have a role in facilitating cell division and in defence. *B. infantis* possesses two other GH25 hydrolases, Blon_1100, which was unaffected by growth in the presence of GMP, and Blon_1654, which had a fold change increase of +1.55 but was not statistically significant. It is important to note that assigning functions to glycoside hydrolases primarily on the basis of sequence similarity is an approach that is error prone due to the presence of conserved domains in proteins with quite different functions.

When the whole genome transcription plots were considered, large peaks in the GMP exposed cells were observed in the vicinity of both significant GH25s (figure S2). When these gene loci were studied in more detail, clusters of substantial homology were identified. These clusters contained tRNAs and some phage related proteins, suggesting they may have been acquired by horizontal gene transfer. The cluster at Blon_1546 – Blon_1571 (cluster A) had a larger number of genes up-regulated, with the highest overall up-regulation observed for hypothetical genes at this locus. Fibronectin type III domain proteins (Fn3) were also present in both clusters downstream of the GH25 (Blon_1563 and Blon_1825). Both Fn3 proteins were also up-regulated in response to GMP, as were several other hypothetical genes in the clusters. Fn3 domains have previously been described in a number of bacterial glycoside hydrolases (Little, Bork et al. 1994, da Silva, García-Fraga et al. 2014). These domains may have a role in the degradation of insoluble substrates and may have been acquired by bacteria through horizontal gene transfer from animals (da Silva, García-Fraga et al. 2014). The presence of these genes containing Fn3 domains downstream of the GH25 genes and their up-regulation upon exposure to GMP, implied that they may play an important role in GMP's bifidogenic effect particularly in response to relatively complex substrates such as GMP.

A BLASTX search for similar Fn3 proteins identified two other bifidobacteria containing similar gene arrangements to cluster A of *B. infantis*. *Bifidobacterium breve* DSM20213 had just one area of homology with a Fn3 protein at locus BIFBRE_01730 while *Bifidobacterium adolescentis* L2-32 contained two duplicates of the cluster with Fn3 proteins at loci BIFADO_01393 and BIFADO_01092. GH25 hydrolases were only present in the clusters identified in *B. infantis* and only the genes encoding for Fn3 protein and both flanking genes showed homology across all five domains (figure 4). BlastX analysis revealed all Fn3 proteins shared ≥50% identity (table S3). Clustal analysis revealed large areas of homology between all five Fn3 proteins at the N- and C- termini, including the fibronectin type III domain. These genome comparisons suggest that some bifidobacteria may contain gene clusters that allow them to utilise complex glycopeptide substrates such as GMP and that these may have been acquired by horizontal gene transfer.

3.4 Gene Expression Validation by qPCR

Due to the high similarity between the genes in the both GH25 clusters, qPCR was performed to verify both clusters were up-regulated and eliminate the possibility of cross-hybridisation on the array, resulting in false positives. The high similarity between both GH25 genes made it difficult to use primer based methods to selectively analyse their expression. Therefore, flanking genes, including the genes encoding for the Fn3 proteins, were selected for qPCR analysis and confirmed the up-regulation of cluster A as observed in the microarray (table S4). QPCR also confirmed the up-regulation of the Fn3 gene (Blon_1825) in the smaller cluster (B).

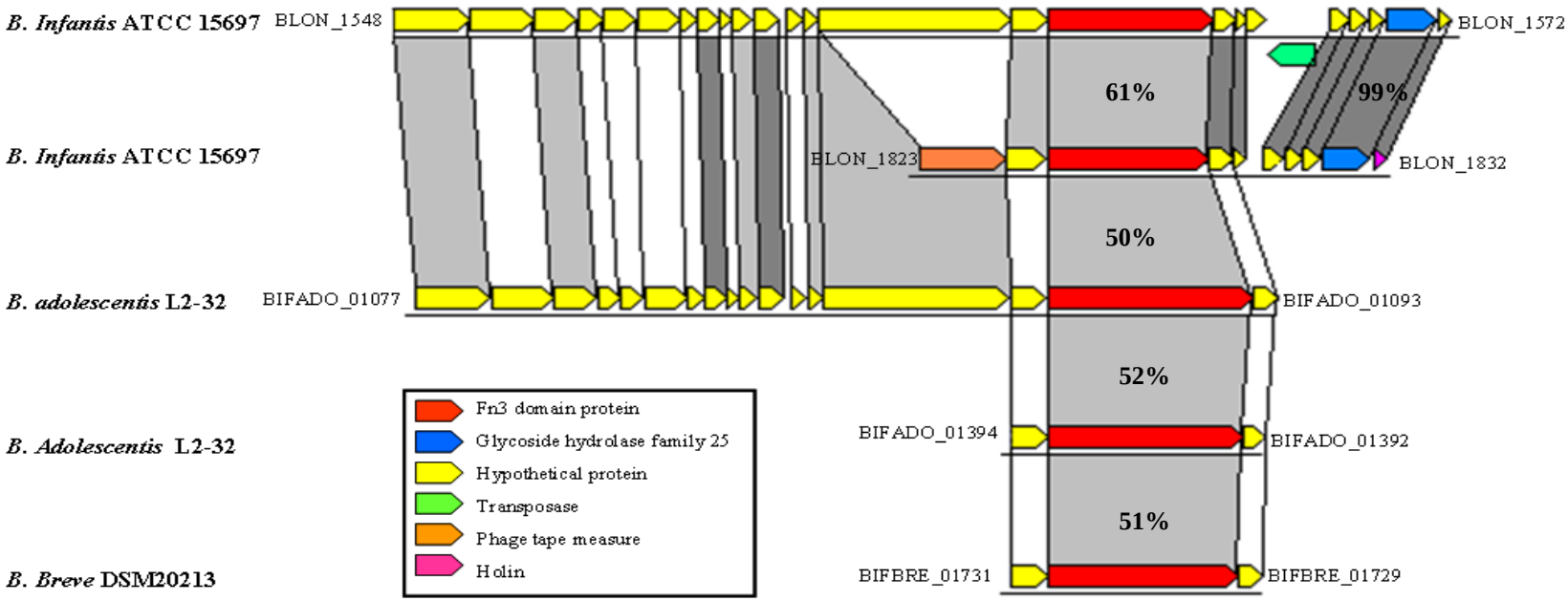

Figure 4 Comparison of clusters identified in bifidobacterium containing fibronectin type III domain proteins. Each solid arrow indicates an open reading frame (ORF) and arrow length is proportional to the length of the predicted ORF. Locus tags are indicated at the beginning and end of areas of homology and arrow colour indicates annotated function. The amino acid identity of each predicted protein is indicated as a percentage relative to its equivalent protein encoded by *B. infantis* ATCC 15697 in the cluster present at locus tags blon_1548 – blon_1572 (white shading = 25-50% identity, light grey shading = 50-70% identity, dark grey shading = >70% identity).

4. Conclusions

The introduction of bovine GMP into the growth environment resulted in an increase in the growth of infant associated strain, *B. infantis*. This effect was substantially reduced following periodate oxidation of the conjugated oligosaccharides. The inclusion of GMP as a functional ingredient may influence the predominance of benefical bacteria in the gut. Treatment with GMP induced some glycogenes while appearing to repress others and these data suggested that the response of *B. infantis* to glycosylated substrates is a tightly regulated response and exposure to a specific glycan rpesentation elicits a highly specialised response. The transcriptional response may be divided into three quite distinct components; transport of extracellular molecules, glycan metabolism, and growth and cell division. It is likely that the transport and metabolism genes are specific to the GMP oligosaccharides while the growth and cell division response is likely to be a general response to increased growth rate. The presence of two gene clusters that were upregulated in response to GMP exposure indicated that the ability to utilise GMP and similar substrates was likely a trait acquired during adaptation to a specific environmental niches, most probably by horizontal gene transfer. This hypothesis was supported by the identification of similar clusters in other *Bifidobacterium* species. Genomic variability in the genus *Bifidobacterium* is extensive as, while the core genome of essential genes numbers 967, the entire pan-genome is >5000 genes (Bottacini, Medini et al. 2010). *B. infantis* ATCC15697 has a large genomic island involved in oligosaccharide breakdown (Sela, Chapman et al. 2008) and the present results indicate that there are other (smaller) gene clusters, possibly with a narrower range of function, that mediate utilisation of glycosylated substrates.

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6. Supplementary figures and tables

Table S1 Selected genes, primers, and probes for qPCR.

Gene/annotated function	Primer (5' – 3')	Roche probe #
Blon_0139 4-alpha-glucanotransferase	F: cgccggacttctataaccag R: ccagatagcgtgggctga	28
Blon_0646 glycosyl hydrolase, BNR repeat-containing protein	F: gatcaaggaggattggatgc R: gtagaacgggacgagcagac	1
Blon_1555 hypothetical protein	F: ccggtgaaggtcgtgtgt R: cgccactgatggagaacat	40
Blon_1562 hypothetical protein	F: cgacctgtcaccatcaacaa R: tccgccaacagtgaggtact	7
Blon_1563 Fibronectin, type III domain protein	F: gggcaagtgggagacgta R: atagagggcggcggtatc	3
Blon_1571 and Blon_1831 glycoside hydrolase, family 25	F: ctcgacctcaactacttcagagg R: cctgtaggtcgatggtctgg	59
Blon_1825 Fibronectin, type III domain protein	F: gtgcagaccgacgcctat R: actgcagggtagccagacc	52
Blon_2421 binding-protein-dependent transport systems inner membrane component	F: gattctcttggcgattttcg R: gaaatcgggctcgtcgtag	14
Blon_2423 Bleomycin hydrolase	F: ggttcgatcaccaatcagga R: gtaacgcagcgtgttcagg	42
Blon_R0085 16S rRNA	F: acgggtgagtaatgcgtga R: acccgtttccaggagctatt	43

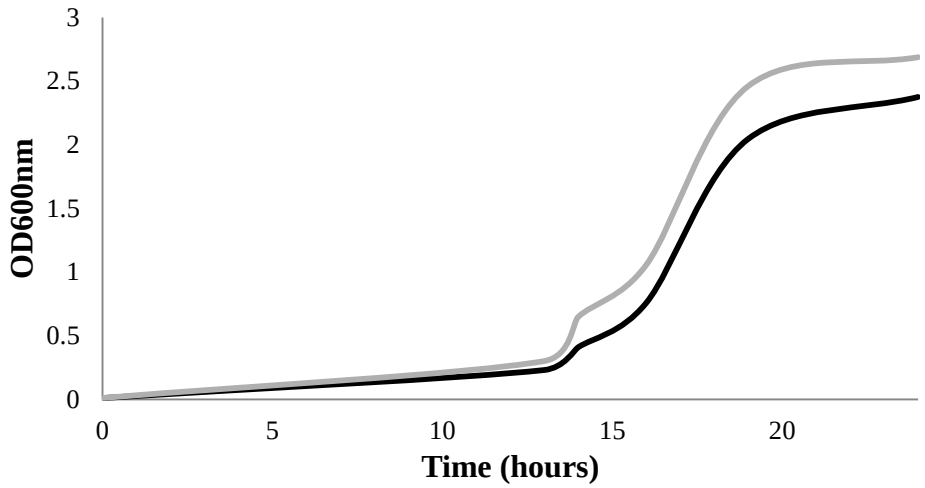

Figure S1 Growth of *B. infantis* in mMRS with (grey) and without (black) supplementation with 2mg/ml bovine GMP over 24 hours.

Table S2 Complete list of genes differentially regulated following **A** Both GMP and GMP-P, **B** GMP and **C** GMP-P treatment.

A. In common to both treatments		**GMP**		**GMP-P**	
Locus tag	**Annotated function**	**FC**	**p-val**	**FC**	**p-val**
Blon_0020	phosphonate ABC transporter, inner membrane subunit	1.67	0.05	1.87	0.02
Blon_0021	phosphonate ABC transporter, inner membrane subunit	1.53	0.03	1.77	0.01
Blon_0022	phosphonate ABC transporter, ATPase subunit	1.58	0.03	1.67	0.01
Blon_0023	phosphonate ABC transporter, periplasmic phosphonate-binding protein	1.59	0.02	1.70	0.01
Blon_0024	transcriptional regulator, RpiR family	1.76	0.01	1.84	0.00
Blon_0025	Haloacid dehalogenase domain protein hydrolase	2.20	0.00	1.69	0.02
Blon_0050	hypothetical protein Blon_0050	1.59	0.03	1.53	0.05
Blon_0061	SSS sodium solute transporter superfamily	2.53	0.00	1.83	0.02
Blon_0068	major facilitator superfamily MFS_1	1.64	0.02	1.54	0.03
Blon_0084	peptidase S9, prolyl oligopeptidase active site domain protein	1.63	0.02	1.53	0.04
Blon_0113	Urease accessory protein UreF	1.52	0.03	1.53	0.03
Blon_0114	urease accessory protein UreG	1.54	0.05	1.59	0.04
Blon_0148	short-chain dehydrogenase/reductase SDR	1.66	0.01	1.54	0.02
Blon_0169	conserved hypothetical protein	2.07	0.02	1.82	0.04
Blon_0201	ABC transporter related	1.51	0.04	1.53	0.04
Blon_0252	protein of unknown function DUF156	1.65	0.01	1.59	0.02
Blon_0263	histidine acid phosphatase	1.65	0.01	1.52	0.03
Blon_0277	endonuclease III	1.66	0.01	1.67	0.01
Blon_0298	hypothetical protein Blon_0298	1.58	0.03	1.63	0.02
Blon_0324	ABC transporter related	1.97	0.05	1.96	0.05
Blon_0407	putative transcriptional regulator, MerR family	1.77	0.02	1.82	0.01
Blon_0419	binding-protein-dependent transport systems inner membrane component	1.54	0.05	1.54	0.05
Blon_0472	histidine kinase, dimerisation and phosphoacceptor region	1.71	0.02	1.59	0.05
Blon_0491	aminotransferase, class I and II	1.54	0.01	1.51	0.01
Blon_0495	major facilitator superfamily MFS_1	1.57	0.01	1.63	0.01
Blon_0497	conserved hypothetical protein	1.75	0.00	1.55	0.01
Blon_0533	hypothetical protein Blon_0533	2.01	0.03	2.02	0.03
Blon_0662	conserved hypothetical protein	1.80	0.02	1.62	0.04
Blon_0761	polar amino acid ABC transporter, inner membrane subunit	1.58	0.03	1.67	0.02
Blon_0765	hypothetical protein Blon_0765	1.82	0.03	1.81	0.04
Blon_0816	Transposase and inactivated derivatives-like protein	1.70	0.05	1.72	0.04
Blon_0916	hypothetical protein Blon_0916	1.68	0.03	1.62	0.04
Blon_1013	competence protein ComEA helix-hairpin-helix repeat protein	1.53	0.04	1.63	0.02
Blon_1093	MATE efflux family protein	1.65	0.01	1.67	0.01
Blon_1098	chaperone DnaJ domain protein	1.92	0.00	1.51	0.03
Blon_1149	phage integrase family protein	1.63	0.02	1.64	0.02
Blon_1175	hypothetical protein Blon_1175	1.87	0.04	2.00	0.03
Blon_1290	hypothetical protein Blon_1290	1.85	0.05	1.86	0.05
Blon_1295	hypothetical protein Blon_1295	1.90	0.01	1.56	0.05

Blon_1326	hypothetical protein Blon_1326	1.73	0.02	1.81	0.01
Blon_1327	hypothetical protein Blon_1327	1.86	0.02	2.01	0.01
Blon_1328	hypothetical protein Blon_1328	1.79	0.04	1.80	0.04
Blon_1329	hypothetical protein Blon_1329	1.75	0.04	1.81	0.03
Blon_1334	hypothetical protein Blon_1334	1.65	0.03	1.53	0.05
Blon_1351	hypothetical protein Blon_1351	1.51	0.02	1.56	0.01
Blon_1387	conserved hypothetical protein	1.69	0.03	1.90	0.01
Blon_1435	Death-on-curing protein	1.81	0.02	1.68	0.04
Blon_1436	putative oxidoreductase	1.62	0.03	1.82	0.01
Blon_1656	hypothetical protein Blon_1656	1.61	0.03	1.57	0.04
Blon_1664	GCN5-related N-acetyltransferase	1.59	0.04	1.60	0.04
Blon_1918	Camphor resistance CrcB protein	1.65	0.02	1.76	0.01
Blon_1934	Bile acid:sodium symporter	2.25	0.01	1.83	0.04
Blon_1991	hypothetical protein Blon_1991	1.70	0.02	1.75	0.02
Blon_2276	hypothetical protein Blon_2276	1.59	0.05	1.68	0.03
Blon_2287	hypothetical protein Blon_2287	2.55	0.01	1.97	0.03
Blon_2450	hypothetical protein Blon_2450	2.10	0.04	2.01	0.05
prfB	peptide chain release factor 2	1.67	0.01	1.50	0.03

B. Unique to GMP

Locus tag	Annotated function	FC	p-val
Blon_0016	hypothetical protein Blon_0016	2.44	0.02
Blon_0017	hypothetical protein Blon_0017	-1.86	0.04
Blon_0040	transposase, mutator type	1.89	0.03
Blon_0043	extracellular solute-binding protein, family 1	1.60	0.05
Blon_0060	protein of unknown function DUF1212	1.67	0.03
Blon_0065	conserved hypothetical protein	2.00	0.00
Blon_0066	two component transcriptional regulator, LuxR family	1.78	0.01
Blon_0070	glycogen/starch/alpha-glucan phosphorylase	1.73	0.02
Blon_0086	protein of unknown function DUF6, transmembrane	1.54	0.02
Blon_0088	protein of unknown function DUF470	1.51	0.02
Blon_0130	major facilitator superfamily MFS_1	2.09	0.02
Blon_0131	ketol-acid reductoisomerase	1.62	0.01
Blon_0136	ketol-acid reductoisomerase	1.66	0.02
Blon_0137	alpha amylase, catalytic region	1.59	0.05
Blon_0139	4-alpha-glucanotransferase	1.98	0.02
Blon_0147	acyl-CoA reductase	1.64	0.02
Blon_0162	sortase family protein	1.66	0.02
Blon_0164	aspartate kinase	1.62	0.03
Blon_0184	putative transcriptional regulator	1.55	0.03
Blon_0185	major facilitator superfamily MFS_1	1.68	0.02
Blon_0192	hypothetical protein Blon_0192	2.68	0.01
Blon_0211	conserved hypothetical protein	1.54	0.05
Blon_0215	two component transcriptional regulator, LuxR family	1.57	0.03
Blon_0233	ribose-phosphate pyrophosphokinase	1.51	0.05
Blon_0250	MIP family channel protein	1.69	0.04
Blon_0268	glycoside hydrolase family 2, sugar binding	-1.54	0.03
Blon_0281	Inorganic diphosphatase	1.70	0.01
Blon_0283	LPXTG-motif cell wall anchor domain protein	1.86	0.02
Blon_0286	lactoylglutathione lyase (LGUL) family protein, diverged	1.69	0.05
Blon_0291	conserved hypothetical protein	2.84	0.00
Blon_0292	helix-turn-helix domain protein	2.24	0.03

Blon_0317	putative endoribonuclease L-PSP	1.74	0.02
Blon_0321	extracellular solute-binding protein, family 1	2.54	0.00
Blon_0335	putative transcriptional regulator, MerR family	2.22	0.00
Blon_0360	major facilitator superfamily MFS_1	2.18	0.03
Blon_0384	aminotransferase, class IV	1.51	0.04
Blon_0399	acetolactate synthase, large subunit, biosynthetic type	1.74	0.02
Blon_0418	ABC transporter related	1.70	0.02
Blon_0444	natural resistance-associated macrophage protein	1.80	0.01
Blon_0446	hypothetical protein Blon_0446	-1.66	0.01
Blon_0490	hypothetical protein Blon_0490	1.58	0.04
Blon_0492	hypothetical protein Blon_0492	1.50	0.02
Blon_0496	phosphoribosylglycinamide synthetase	1.68	0.01
Blon_0518	hypothetical protein Blon_0518	-1.59	0.01
Blon_0519	hypothetical protein Blon_0519	-1.57	0.01
Blon_0531	transcription factor WhiB	1.85	0.03
Blon_0540	lactaldehyde reductase	-1.80	0.01
Blon_0559	hypothetical protein Blon_0559	2.61	0.02
Blon_0578	inner-membrane translocator	1.64	0.05
Blon_0579	inner-membrane translocator	1.51	0.05
Blon_0581	ABC transporter related	1.66	0.03
Blon_0582	Sua5/YciO/YrdC/YwlC family protein	1.53	0.02
Blon_0606	nucleoside 2-deoxyribosyltransferase	2.21	0.00
Blon_0612	anaerobic ribonucleoside-triphosphate reductase	-1.60	0.03
Blon_0614	putative CoA-substrate-specific enzyme activase	-2.47	0.00
Blon_0638	Purine nucleosidase	-2.92	0.00
Blon_0639	major facilitator superfamily MFS_1	-2.51	0.00
Blon_0640	PfkB domain protein	-2.24	0.00
Blon_0646	glycosyl hydrolase, BNR repeat-containing protein	-1.92	0.03
Blon_0658	putative transposase	1.99	0.03
Blon_0667	Mandelate racemase/muconate lactonizing enzyme, N-terminal domain protein	-1.89	0.00
Blon_0703	metallophosphoesterase	1.93	0.04
Blon_0714	hypothetical protein Blon_0714	-1.70	0.01
Blon_0850	Peptidoglycan glycosyltransferase	-1.66	0.00
Blon_0874	regulatory protein, LacI	1.60	0.02
Blon_0876	hypothetical protein Blon_0876	-1.94	0.02
Blon_0885	binding-protein-dependent transport systems inner membrane component	-1.62	0.01
Blon_0890	FolC bifunctional protein	1.65	0.01
Blon_0902	initiation factor 3	1.69	0.02
Blon_0914	GTP-binding protein TypA	1.53	0.02
Blon_0915	major facilitator superfamily MFS_1	1.52	0.02
Blon_0918	Prephenate dehydratase	-1.60	0.01
Blon_0938	hypothetical protein Blon_0938	-1.66	0.02
Blon_0966	DNA gyrase/topoisomerase IV, subunit A	1.59	0.02
Blon_0980	transposase, mutator type	2.61	0.01
Blon_0986	hypothetical protein Blon_0986	2.61	0.01
Blon_0991	conserved hypothetical protein	1.53	0.03
Blon_0998	transposase for IS3509a	1.58	0.04
Blon_1007	pyridoxamine 5'-phosphate oxidase-related, FMN-binding	-1.67	0.01
Blon_1014	ComEC/Rec2-related protein	1.66	0.03
Blon_1019	O-sialoglycoprotein endopeptidase	1.56	0.02
Blon_1101	hypothetical protein Blon_1101	1.79	0.05

Blon_1106	hypothetical protein Blon_1106	1.90	0.04
Blon_1114	hypothetical protein Blon_1114	1.51	0.04
Blon_1115	hypothetical protein Blon_1115	1.72	0.05
Blon_1176	hypothetical protein Blon_1176	2.07	0.03
Blon_1177	SNF2-related protein	1.98	0.03
Blon_1180	hypothetical protein Blon_1180	1.97	0.02
Blon_1181	hypothetical protein Blon_1181	1.99	0.02
Blon_1182	LigA	2.04	0.03
Blon_1185	leucine rich repeat variant	2.09	0.02
Blon_1186	hypothetical protein Blon_1186	1.86	0.02
Blon_1188	hypothetical protein Blon_1188	2.83	0.01
Blon_1193	hypothetical protein Blon_1193	1.88	0.04
Blon_1201	hypothetical protein Blon_1201	1.95	0.01
Blon_1202	hypothetical protein Blon_1202	2.04	0.03
Blon_1203	hypothetical protein Blon_1203	1.75	0.01
Blon_1207	hypothetical protein Blon_1207	1.90	0.05
Blon_1208	hypothetical protein Blon_1208	1.97	0.04
Blon_1210	hypothetical protein Blon_1210	1.79	0.04
Blon_1211	hypothetical protein Blon_1211	2.27	0.02
Blon_1212	hypothetical protein Blon_1212	2.20	0.04
Blon_1213	hypothetical protein Blon_1213	2.22	0.01
Blon_1214	transcriptional regulator, XRE family	1.65	0.03
Blon_1217	hypothetical protein Blon_1217	2.73	0.01
Blon_1231	hypothetical protein Blon_1231	1.68	0.02
Blon_1232	conserved hypothetical protein	1.65	0.01
Blon_1245	hypothetical protein Blon_1245	2.09	0.03
Blon_1246	CHAP domain containing protein	1.83	0.03
Blon_1247	hypothetical protein Blon_1247	2.02	0.04
Blon_1248	hypothetical protein Blon_1248	1.93	0.02
Blon_1249	DNA polymerase III, beta subunit	1.79	0.03
Blon_1250	hypothetical protein Blon_1250	1.79	0.01
Blon_1252	hypothetical protein Blon_1252	2.29	0.03
Blon_1254	hypothetical protein Blon_1254	1.72	0.02
Blon_1255	hypothetical protein Blon_1255	1.96	0.02
Blon_1258	cell divisionFtsK/SpoIIIE	2.17	0.04
Blon_1262	hypothetical protein Blon_1262	1.74	0.05
Blon_1267	hypothetical protein Blon_1267	2.12	0.02
Blon_1268	hypothetical protein Blon_1268	2.24	0.02
Blon_1269	transcription factor WhiB	1.92	0.02
Blon_1270	hypothetical protein Blon_1270	1.99	0.02
Blon_1271	hypothetical protein Blon_1271	2.23	0.01
Blon_1273	hypothetical protein Blon_1273	1.56	0.05
Blon_1288	metallophosphoesterase	1.56	0.02
Blon_1294	hypothetical protein Blon_1294	1.62	0.03
Blon_1296	hypothetical protein Blon_1296	1.57	0.03
Blon_1297	hypothetical protein Blon_1297	2.29	0.01
Blon_1309	hypothetical protein Blon_1309	2.13	0.01
Blon_1335	hypothetical protein Blon_1335	1.75	0.01
Blon_1342	hypothetical protein Blon_1342	1.65	0.03
Blon_1373	apurinic endonuclease Apn1	1.65	0.01
Blon_1376	VanZ family protein	1.91	0.02
Blon_1421	putative transcriptional regulator, MerR family	1.65	0.02

Blon_1426	hypothetical protein Blon_1426	1.87	0.02
Blon_1468	MazG nucleotide pyrophosphohydrolase	1.71	0.03
Blon_1469	hypothetical protein Blon_1469	1.84	0.01
Blon_1480	extracellularosolute-binding protein, family 5	1.87	0.04
Blon_1498	hypothetical protein Blon_1498	1.73	0.03
Blon_1501	hypothetical protein Blon_1501	1.51	0.04
Blon_1503	hypothetical protein Blon_1503	1.53	0.04
Blon_1505	single-strand binding protein	1.62	0.03
Blon_1506	hypothetical protein Blon_1506	1.64	0.02
Blon_1507	hypothetical protein Blon_1507	1.51	0.05
Blon_1546	HNH endonuclease	3.09	0.01
Blon_1547	hypothetical protein Blon_1547	2.27	0.00
Blon_1548	hypothetical protein Blon_1548	3.08	0.00
Blon_1549	hypothetical protein Blon_1549	2.50	0.01
Blon_1550	hypothetical protein Blon_1550	3.50	0.01
Blon_1551	hypothetical protein Blon_1551	2.96	0.00
Blon_1552	hypothetical protein Blon_1552	2.76	0.01
Blon_1553	hypothetical protein Blon_1553	2.88	0.01
Blon_1554	hypothetical protein Blon_1554	3.23	0.00
Blon_1555	hypothetical protein Blon_1555	4.38	0.00
Blon_1556	hypothetical protein Blon_1556	4.82	0.00
Blon_1557	hypothetical protein Blon_1557	4.95	0.00
Blon_1558	hypothetical protein Blon_1558	2.99	0.01
Blon_1559	hypothetical protein Blon_1559	2.17	0.02
Blon_1560	hypothetical protein Blon_1560	2.21	0.04
Blon_1561	hypothetical protein Blon_1561	2.95	0.01
Blon_1562	hypothetical protein Blon_1562	3.47	0.00
Blon_1563	Fibronectin, type III domain protein	2.25	0.03
Blon_1569	hypothetical protein Blon_1569	2.70	0.04
Blon_1570	hypothetical protein Blon_1570	2.74	0.03
Blon_1571	glycoside hydrolase, family 25	3.51	0.01
Blon_1609	hypothetical protein Blon_1609	1.63	0.03
Blon_1624	hypothetical protein Blon_1624	1.96	0.00
Blon_1625	hypothetical protein Blon_1625	2.55	0.00
Blon_1640	DEAD/DEAH box helicase domain protein	1.71	0.01
Blon_1694	protein of unknown function DUF214	1.64	0.04
Blon_1697	Phosphomethylpyrimidine kinase type-1	1.60	0.01
Blon_1703	O-methyltransferase, family 3	1.61	0.01
Blon_1714	pyruvate formate-lyase activating enzyme	-2.20	0.00
Blon_1715	formate acetyltransferase	-2.32	0.00
Blon_1722	Fructose-6-phosphate phosphoketolase	-1.56	0.01
Blon_1738	hypothetical protein Blon_1738	1.65	0.02
Blon_1739	phage integrase family protein	2.15	0.01
Blon_1742	protein of unknown function DUF34	-1.54	0.03
Blon_1745	Pyruvate kinase	-1.58	0.01
Blon_1755	Methenyltetrahydrofolate cyclohydrolase	1.58	0.03
Blon_1770	transcription factor WhiB	1.76	0.00
Blon_1825	Fibronectin, type III domain protein	2.72	0.02
Blon_1828	hypothetical protein Blon_1568	2.73	0.05
Blon_1831	glycoside hydrolase, family 25	3.46	0.01
Blon_1832	holin	1.82	0.05
Blon_1842	protein of unknown function UPF0182	-1.64	0.01

Blon_1886	cobalt transport protein	1.86	0.01
Blon_1887	ABC transporter related	1.68	0.02
Blon_1895	Auxin Efflux Carrier	1.73	0.02
Blon_1905	Beta-glucosidase	-1.58	0.02
Blon_1913	transcriptional regulator, TetR family	1.62	0.04
Blon_1917	Camphor resistance CrcB protein	1.92	0.02
Blon_1919	hypothetical protein Blon_1919	1.67	0.02
Blon_1969	ribosomal protein L36	-1.56	0.01
Blon_1984	hypothetical protein Blon_1984	-2.37	0.01
Blon_1986	ABC transporter related	-1.65	0.03
Blon_1994	pyridoxine biosynthesis protein	1.52	0.03
Blon_1999	conserved hypothetical protein	1.53	0.05
Blon_2054	hypothetical protein Blon_2054	-1.68	0.00
Blon_2063	galactose-1-phosphate uridylyltransferase	-1.63	0.02
Blon_2072	narrowly conserved hypothetical protein	-1.51	0.03
Blon_2085	protein of unknown function DUF1526	-1.58	0.01
Blon_2090	hypothetical protein Blon_2090	1.66	0.02
Blon_2094	IS3 family transposase	1.75	0.03
Blon_2098	IS3 family transposase	2.07	0.01
Blon_2102	transposase, mutator type	1.91	0.04
Blon_2129	conserved hypothetical conserved transmembrane protein in the DedA family	-1.76	0.01
Blon_2143	OsmC family protein	1.55	0.02
Blon_2145	NLP/P60 protein	1.56	0.02
Blon_2171	UDP-glucose 4-epimerase	-1.53	0.02
Blon_2174	hypothetical protein Blon_2174	-2.34	0.00
Blon_2175	binding-protein-dependent transport systems inner membrane component	-1.75	0.03
Blon_2176	binding-protein-dependent transport systems inner membrane component	-1.99	0.02
Blon_2252	4Fe-4S ferredoxin, iron-sulfur binding domain protein	1.64	0.02
Blon_2258	conserved hypothetical protein	1.65	0.02
Blon_2259	GCN5-related N-acetyltransferase	1.65	0.03
Blon_2262	SAF domain protein	1.74	0.01
Blon_2278	hypothetical protein Blon_2278	1.72	0.02
Blon_2288	biotin--acetyl-CoA-carboxylase ligase	1.61	0.01
Blon_2307	major facilitator superfamily MFS_1	-1.55	0.03
Blon_2309	Mandelate racemase/muconate lactonizing enzyme, C-terminal domain protein	-1.80	0.04
Blon_2311	protein of unknown function DUF214	1.63	0.02
Blon_2312	ABC transporter related	2.06	0.00
Blon_2322	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	-2.37	0.00
Blon_2323	dihydroorotate dehydrogenase	1.70	0.01
Blon_2351	extracellular solute-binding protein, family 1	-1.52	0.02
Blon_2356	Haloacid dehalogenase domain protein hydrolase	1.59	0.03
Blon_2370	glycerophosphoryl diester phosphodiesterase	1.58	0.02
Blon_2371	Glutamate--tRNA ligase	2.20	0.01
Blon_2372	ATPase AAA-2 domain protein	1.98	0.01
Blon_2373	GCN5-related N-acetyltransferase	1.76	0.03
Blon_2379	binding-protein-dependent transport systems inner membrane component	-1.61	0.03
Blon_2380	extracellular solute-binding protein, family 1	-1.65	0.03
Blon_2415	regulatory protein, LacI	2.02	0.01

Blon_2419	extracellular solute-binding protein, family 5	1.58	0.04
Blon_2420	oligopeptide/dipeptide ABC transporter, ATPase subunit	2.45	0.01
Blon_2421	binding-protein-dependent transport systems inner membrane component	2.68	0.01
Blon_2422	binding-protein-dependent transport systems inner membrane component	1.96	0.01
Blon_2423	Bleomycin hydrolase	3.02	0.00
Blon_2440	conserved hypothetical protein	1.50	0.03
Blon_2498	ribonuclease P protein component	1.62	0.02
dnaK	chaperone protein DnaK	1.74	0.01
groES	chaperonin Cpn10	1.52	0.02
gyrB	DNA gyrase, B subunit	1.59	0.02
ispF	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	1.81	0.01
leuD	3-isopropylmalate dehydratase, small subunit	1.76	0.01
mraW	S-adenosyl-methyltransferase MraW	-1.54	0.01
nagB	glucosamine-6-phosphate isomerase	-1.67	0.04
rplS	ribosomal protein L19	1.98	0.00
rpmA	ribosomal protein L27	1.56	0.03
rpmE	ribosomal protein L31	1.53	0.04
rpmG	ribosomal protein L33	1.53	0.03
rpmJ	ribosomal protein L36	1.59	0.02
uvrC	excinuclease ABC, C subunit	1.52	0.02

C. Unique to GMP-P			
Locus tag	**Annotated function**	**FC**	**p-val**
Blon_0019	metallophosphoesterase	1.74	0.01
Blon_0030	protein of unknown function DUF21	1.80	0.04
Blon_0053	extracellular solute-binding protein, family 5	1.60	0.03
Blon_0054	binding-protein-dependent transport systems inner membrane component	1.54	0.03
Blon_0057	conserved hypothetical protein	1.51	0.05
Blon_0067	hypothetical protein Blon_0067	1.61	0.02
Blon_0092	DNA-cytosine methyltransferase	1.51	0.04
Blon_0095	heat shock protein Hsp20	1.51	0.05
Blon_0126	Camphor resistance CrcB protein	1.56	0.01
Blon_0145	Xanthine/uracil/vitamin C permease	1.56	0.05
Blon_0167	conserved hypothetical protein	1.77	0.03
Blon_0179	conserved hypothetical protein	1.57	0.02
Blon_0190	conserved hypothetical phage AbiD protein	1.64	0.03
Blon_0194	putative transcriptional regulator	1.59	0.02
Blon_0195	conserved hypothetical membrane protein with unknown function	1.91	0.03
Blon_0208	6-phosphogluconolactonase	1.55	0.02
Blon_0232	alanine racemase domain protein	1.56	0.05
Blon_0248	Alpha-L-fucosidase	1.80	0.05
Blon_0256	hypothetical protein Blon_0256	1.58	0.04
Blon_0293	hypothetical protein Blon_0293	1.65	0.04
Blon_0294	hypothetical protein Blon_0294	1.55	0.04
Blon_0323	binding-protein-dependent transport systems inner membrane component	1.86	0.05
Blon_0330	methylated-DNA--protein-cysteine methyltransferase	1.68	0.04
Blon_0334	RelB antitoxin	1.73	0.01
Blon_0385	para-aminobenzoate synthase, subunit I	1.65	0.05

Blon_0431	sugar transporter	1.58	0.01
Blon_0443	narrowly conserved hypothetical protein	1.50	0.03
Blon_0467	protein of unknown function DUF214	1.71	0.04
Blon_0473	hypothetical protein Blon_0473	1.53	0.03
Blon_0474	ABC transporter related	1.54	0.05
Blon_0487	hypothetical protein Blon_0487	2.07	0.04
Blon_0500	hypothetical protein Blon_0500	1.55	0.02
Blon_0514	ABC transporter related	2.02	0.04
Blon_0516	regulatory protein, LacI	1.51	0.03
Blon_0528	hypothetical protein Blon_0528	1.54	0.04
Blon_0532	hypothetical protein Blon_0532	1.77	0.03
Blon_0534	hypothetical protein Blon_0534	1.52	0.03
Blon_0535	hypothetical protein Blon_0535	2.33	0.04
Blon_0547	tRNA(Ile)-lysidine synthetase	1.53	0.01
Blon_0576	modification methylase, HemK family	1.51	0.03
Blon_0607	AMP-dependent synthetase and ligase	1.51	0.03
Blon_0616	transposase, IS605 OrfB family	1.67	0.05
Blon_0621	Glucan 1,3-beta-glucosidase	1.54	0.02
Blon_0624	transcriptional regulator, TetR family	1.53	0.03
Blon_0626	nucleoside-diphosphate-sugar epimerase and GAF domain protein	1.55	0.01
Blon_0671	transposase IS3/IS911 family protein	1.71	0.05
Blon_0672	Integrase, catalytic region	1.62	0.05
Blon_0684	conserved hypothetical protein	1.57	0.02
Blon_0695	protein of unknown function DUF909	1.71	0.03
Blon_0708	hypothetical protein Blon_0708	1.78	0.05
Blon_0713	conserved hypothetical secreted protein	1.61	0.03
Blon_0717	major facilitator superfamily MFS_1	1.78	0.00
Blon_0728	conserved hypothetical protein	1.56	0.03
Blon_0734	ABC transporter related	1.64	0.03
Blon_0754	inner-membrane translocator	1.55	0.01
Blon_0763	Integrase, catalytic region	1.81	0.04
Blon_0806	ribonuclease BN	1.56	0.01
Blon_0821	carbohydrate kinase, FGGY	1.60	0.02
Blon_0935	protein of unknown function DUF262	1.55	0.04
Blon_0977	Integrase, catalytic region	1.51	0.04
Blon_0987	hypothetical protein Blon_0987	1.70	0.03
Blon_0988	major facilitator superfamily MFS_1	2.07	0.02
Blon_0993	hypothetical protein Blon_0993	1.59	0.01
Blon_1000	bacteriocin ABC transporter, permease protein subunit, putative	1.51	0.03
Blon_1029	Methylase of polypeptide chain release factors-like protein	1.65	0.02
Blon_1030	ABC transporter related	1.58	0.03
Blon_1034	conserved hypothetical protein	1.58	0.01
Blon_1072	narrowly conserved protein with unknown function	1.52	0.03
Blon_1073	conserved hypothetical protein	2.35	0.02
Blon_1099	hypothetical protein Blon_1099	1.77	0.04
Blon_1112	hypothetical protein Blon_1112	1.62	0.03
Blon_1123	hypothetical protein Blon_1123	1.75	0.04
Blon_1130	hypothetical protein Blon_1130	1.92	0.05
Blon_1136	hypothetical protein Blon_1136	1.95	0.02
Blon_1139	hypothetical protein Blon_1139	2.42	0.04
Blon_1168	transposase for IS3509a	1.62	0.05

Blon_1191	hypothetical protein Blon_1191	1.79	0.04
Blon_1192	hypothetical protein Blon_1192	2.05	0.05
Blon_1209	hypothetical protein Blon_1209	1.68	0.05
Blon_1216	hypothetical protein Blon_1216	1.70	0.03
Blon_1222	hypothetical protein Blon_1222	1.59	0.04
Blon_1235	hypothetical protein Blon_1235	1.73	0.02
Blon_1243	hypothetical protein Blon_1243	2.38	0.01
Blon_1266	preprotein translocase, SecG subunit	2.01	0.05
Blon_1280	peptidase C60, sortase A and B	1.97	0.02
Blon_1284	conserved hypothetical protein	1.67	0.05
Blon_1287	hypothetical protein Blon_1287	1.71	0.04
Blon_1298	hypothetical protein Blon_1298	1.76	0.04
Blon_1314	hypothetical protein Blon_1314	1.61	0.05
Blon_1319	hypothetical protein Blon_1319	1.61	0.05
Blon_1325	hypothetical protein Blon_1325	1.51	0.02
Blon_1330	hypothetical protein Blon_1330	1.70	0.04
Blon_1331	hypothetical protein Blon_1331	1.65	0.04
Blon_1333	hypothetical protein Blon_1333	1.84	0.03
Blon_1346	DNA methylase N-4/N-6 domain protein	2.01	0.05
Blon_1386	major facilitator superfamily MFS_1	1.67	0.01
Blon_1396	ABC transporter related	1.77	0.05
Blon_1446	dihydroorotate dehydrogenase family protein	1.53	0.02
Blon_1526	DNA-cytosine methyltransferase	1.60	0.03
Blon_1610	cytidine deaminase	1.50	0.04
Blon_1633	two component transcriptional regulator, LuxR family	1.60	0.01
Blon_1647	family M20D unassigned peptidase	1.68	0.01
Blon_1650	drug resistance transporter, EmrB/QacA subfamily	1.72	0.03
Blon_1657	hypothetical protein Blon_1657	1.55	0.02
Blon_1659	hypothetical protein Blon_1659	1.64	0.03
Blon_1663	ABC transporter related	1.53	0.03
Blon_1675	major facilitator superfamily MFS_1	1.82	0.03
Blon_1676	hypothetical protein Blon_1676	1.53	0.05
Blon_1692	integral membrane sensor signal transduction histidine kinase	1.58	0.03
Blon_1734	hypothetical protein Blon_1734	2.03	0.03
Blon_1748	hypothetical protein Blon_1748	1.61	0.03
Blon_1750	bacteriocin, lactococcin 972 family	1.56	0.05
Blon_1763	integral membrane sensor signal transduction histidine kinase	1.59	0.02
Blon_1764	putative prophage repressor	1.63	0.02
Blon_1772	cell divisionFtsK/SpoIIIE	1.57	0.03
Blon_1815	phage structural protein	2.33	0.04
Blon_1852	hypothetical protein Blon_1852	1.68	0.02
Blon_1908	histidine kinase	1.56	0.03
Blon_1929	IstB domain protein ATP-binding protein	1.70	0.02
Blon_1936	glycosyl transferase, group 1	1.52	0.02
Blon_1937	aminotransferase, class I and II	1.62	0.02
Blon_1967	ribosomal protein S14	1.62	0.03
Blon_1971	putative high-affinity zinc ABC transporter	1.71	0.05
Blon_1976	hypothetical protein Blon_1976	1.78	0.04
Blon_1988	transcriptional regulator, RpiR family	1.97	0.05
Blon_1992	putative transcriptional regulator, GntR family	1.55	0.02
Blon_1993	SNO glutamine amidotransferase	1.55	0.03
Blon_2000	quorum-sensing autoinducer 2 (AI-2), LuxS	1.50	0.03

Blon_2004	cystathionine beta-synthase	1.64	0.03
Blon_2017	pseudouridine synthase	1.53	0.02
Blon_2057	periplasmic binding protein/LacI transcriptional regulator	1.56	0.03
Blon_2069	biotin/lipoate A/B protein ligase	1.58	0.04
Blon_2087	proline symporter	1.56	0.03
Blon_2148	putative phosphoserine aminotransferase	1.59	0.02
Blon_2190	ribonuclease H	1.54	0.05
Blon_2272	ABC transporter related	1.52	0.04
Blon_2273	ABC transporter related	1.61	0.04
Blon_2274	hypothetical protein Blon_2274	1.76	0.01
Blon_2275	hypothetical protein Blon_2275	1.67	0.04
Blon_2281	hypothetical protein Blon_2281	1.61	0.01
Blon_2285	Propionyl-CoA carboxylase	1.52	0.01
Blon_2291	regulatory protein, IclR	1.78	0.01
Blon_2292	regulatory protein, IclR	2.03	0.02
Blon_2304	Auxin Efflux Carrier	1.61	0.03
Blon_2416	Beta-galactosidase	1.91	0.03
Blon_2428	conserved hypothetical protein	1.70	0.03
Blon_2429	conserved hypothetical protein	1.51	0.05
Blon_2456	binding-protein-dependent transport systems inner membrane component	1.62	0.04
Blon_2472	major facilitator superfamily MFS_1	1.57	0.02
dnaG	DNA primase	1.55	0.01
lacY	oligosaccharide/H+ symporter, major facilitator superfamily (MFS)	1.91	0.04
recO	DNA repair protein RecO	1.50	0.02

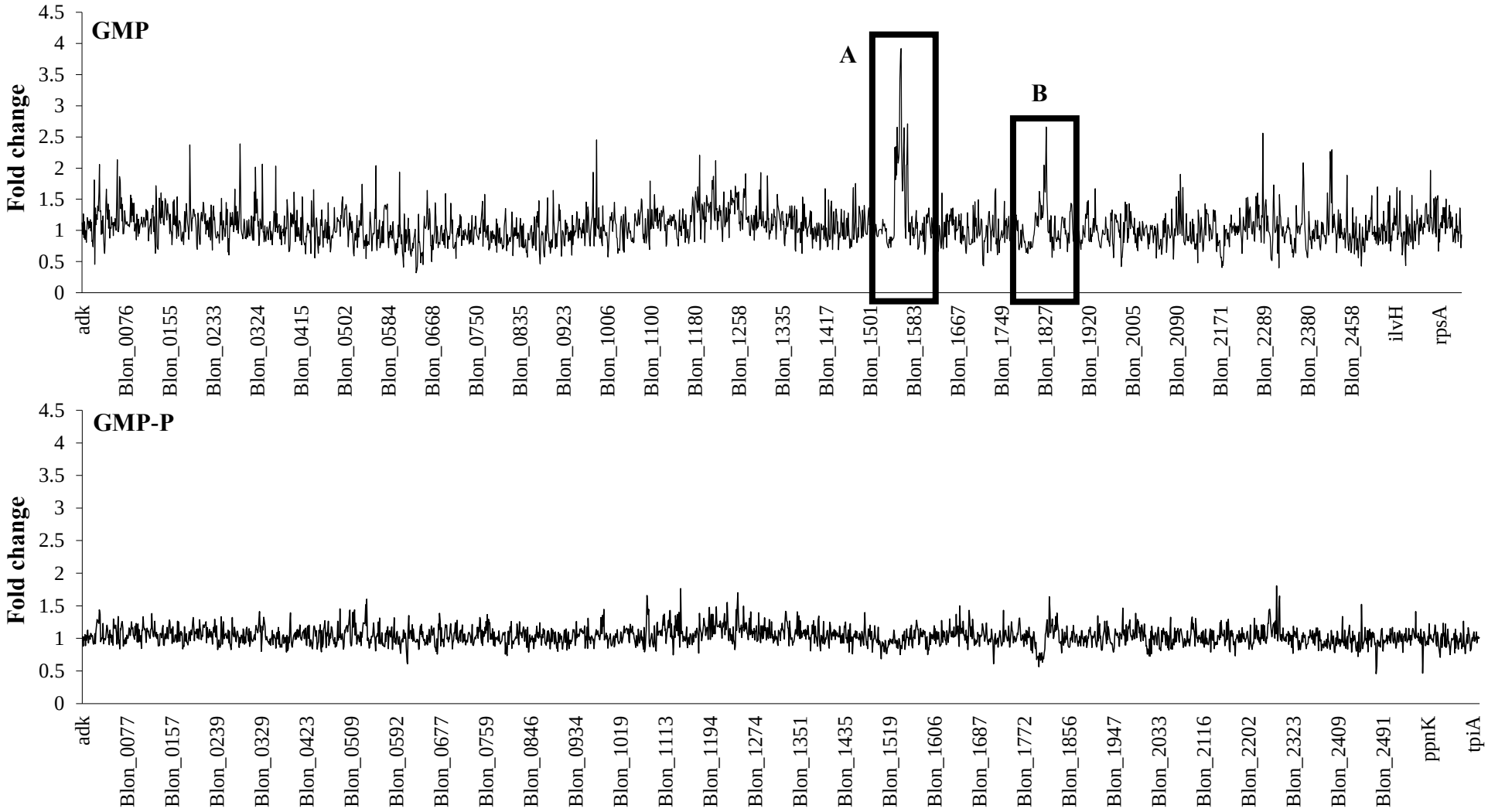

Figure S2 Whole genome plot of differential gene expression following GMP or GMP-P treatment. Fold change reported on y-axis. Boxes indicate up-regulated clusters of genes containing glycoside hydrolases, family 25, and fibronectin type III domain proteins.

Table S3 Percentage sequence identity between the amino acid sequences of the five identified fibronectin type III domain proteins from bifidobacteriuim, as analysed by BLASTX.

	BLON_1563	BLON_1825	BIFADO_01393	BIFADO_01092	BIFBRE_01730
BLON_1563	100	61	52	50	51
BLON_1825	61	100	54	49	53
BIFADO_01393	52	54	100	65	81
BIFADO_01092	50	49	65	100	64
BIFBRE_01730	51	53	81	64	100

Table S4 Summary of fold changes following growth in the presence of GMP and GMP-P, as determined by microarray and qPCR analysis. '*' indicates values which were determined insignificant due to their high p-value.

	GMP		**GMP-P**	
Locus tag and annotated function	**Array**	**qPCR**	**Array**	**qPCR**
Blon_0139 4-alpha-glucanotransferase	1.98	4.73	*	-1.43
Blon_0646 glycosyl hydrolase, BNR repeat-containing protein	-1.92	-6.45	*	-1.53
Blon_1555 hypothetical protein	4.38	4.78	*	1.00
Blon_1562 hypothetical protein	3.47	5.71	*	1.03
Blon_1563 Fibronectin, type III domain protein	2.25	6.51	*	1.00
Blon_1571 glycoside hydrolase, family 25	3.51	4.66	*	-1.04
Blon_1831 glycoside hydrolase, family 25	3.51	4.66	*	-1.04
Blon_1825 Fibronectin, type III domain protein	2.72	6.08	*	-1.08
Blon_2421 binding-protein-dependent transport systems inner membrane component	2.68	2.63	*	-1.06
Blon_2423 Bleomycin hydrolase	3.02	3.05	*	1.41

Overall summary and general discussion

Overall summary and general discussion

Mammalian milk is a remarkably complex matrix of macronutrients and bioactive molecules, including hormones, antimicrobials, oligosaccharides, immunoglobulins, glycoproteins and glycolipids (Pacheco, Barile et al. 2015). The composition of human milk is dynamic and varies according to lactation stages, feedings, and between mothers, with the most significant differences in compositions observed between mature milk and colostrum (Chatterton, Nguyen et al. 2013). Likewise, the nutritional composition and production volume of bovine milk are influenced by breed, season, lactation stage, milking frequency, health, environment and diet (Linn 1988, Auldist, Walsh et al. 1998, Kelly, Reid et al. 1998, Litwinczuk, Krol et al. 2011, Nóbrega and Langoni 2011). In bovine milk, the fat composition is particularly susceptible to change based on these parameters and the impact of diet, breed and organic farming practices on the fatty acid composition has been previously described (Sutton 1989, Stanton, Lawless et al. 1997, Lawless, Stanton et al. 1999, Ellis, Innocent et al. 2006).

Glycoproteins in bovine milk are a group of proteins of industrial interest due to their presence in the waste streams of cheese (whey) and butter (serum) processing in relatively high concentrations and their greater ease of isolation compared to bovine milk oligosaccharides. In Chapter 1 of this thesis, the structural and biological importance of the attached glycans on bovine milk proteins was reviewed along with the influence of breed, lactation stage and daily milk production of the animal on glycoprotein concentration (Cheng, Wang et al. 2008, Krol, Litwinczuk et al. 2010) and glycan structures (Wilson, Robinson et al. 2008, Takimori, Shimaoka et al. 2011). The experimental work to support this thesis aimed to further build on this repository of information and expand our knowledge on bovine milk protein glycosylation.

Milk protein glycosylation is driven *in vivo* by glycosyltransferases and glycosidases expressed in the mammary gland, which cleave terminal saccharide residues from glycans. Chapter 2 describes the monitoring of the activity of the major glycosidases in bovine milk over the first 90 days post-partum to investigate the biochemical mechanisms behind milk glycan variation over lactation. The highest glycosidase activity was present in colostrum, and decreased through transitional milk production to minimal but constant activity in mature milk. *N*-acetyl-beta-D-glucosaminidase, alpha-L-fucosidase,

alpha-galactosidase and *N*-acetyl-neuraminidase appeared to be the most biologically relevant glycosidases in bovine milk. Analysis of the presence of glycosidases in whole milk, skimmed milk and whey suggested that glycosidases were in the majority present in the whey fraction of milk but a loss of activity was observed for a number of enzymes upon removal of the fat phase, *N*-acetylneuraminidase being the most biological relevant of these. Further investigation should be undertaken to confirm presence of this enzyme in the fat phase and how this impacts the glycosylation of the MFGM, in particular the terminal Neu5Ac content.

The saccharide substrates of the detected enzymes are the terminal residues of bovine milk oligosaccharides and glycoproteins. The elevated presence of these glycosidases in early lactation aligns to the increased level of their target substrates in bovine colostrum oligosaccharides (Nakamura, Kawase et al. 2003, Tao 2009), LF (O'Riordan, Gerlach et al. 2014), GMP (Fiat, Chevan et al. 1988) and MFGM (Ross, Gerlach et al. 2016). To confirm that the glycosidases profiled in this work hydrolyse bovine milk glycan structures, bovine milk should be inoculated with the individual purified glycosidases and changes in the free and bound saccharide chains monitored. Downstream separation of oligosaccharides and glycoconjugates would provide insight as to if glycosidases preferentially digest terminal saccharides on free oligosaccharides versus those bound to lipid or protein backbones, due to the lack of steric hindrance of the active enzyme site to the glycan structures.

In this work, it was hypothesised that the elevated enzymatic activity in colostrum may play a role in the digestion of bovine milk glycans in the infant mammal, prior to colonisation of the lower GI tract by the gut microbiota. However, questions still remain as to what section of the calf GIT the glycosidases would be active, if they would survive transit through the harsh acidic environment of the stomach to reach the lower GIT or if they would be active earlier in the digestion process. How would proteases in the bovine digestive system affect glycosidase survival through the GIT? It would also be interesting to profile glycosidase expression of the bovine intestinal flora to identify overlaps and functionalities in common between both milk and microbiota sources.

Overall summary and general discussion

Chapter 3 of this described the changes in the specific glycoprofile of bovine LF over lactation using of lectin microarrays. This technology proved effective as a high-throughput method for accurately monitoring changes LF's terminal glycosylation structures, as verified by monosaccharide HPLC analysis on the samples. This technology has also recently been employed to monitor glycosylation changes on the bovine MFGM over lactation (Ross, Gerlach et al. 2016). The MFGM study verified the ability of the lectin microarrays to profile complex glycoprotein mixtures and this method could therefore be used to investigate the glycan profiles of dairy streams such as cheese whey and buttermilk as potential sources of bioactive components. This method could be used to further expand our knowledge on the impact of environmental and dietary factors on protein glycosylation through feeding and housing studies and any correlation between these external factors and the content of biologically relevant glycan structures. Lectin microarrays offer the potential to monitor the stability of milk glycans in an industrial setting during processing and the effect of parameters such as heat and holding times on the saccharides to minimise degradation, for example, during industrial scale drying processes

A distinct shift in the glycosylation between colostrum and mature milk LF was observed in this study. In the initial days post-partum, a high Neu5Ac content and a diverse glycoprofile was present on lactoferrin. As lactation stage progressed, a decrease in the heterogeneity of the glycans was observed, with a predominance of high mannose structures in mature milk. As our knowledge of the molecular structure of LF grows, it would be interesting to look at how the variation in protein glycosylation affects the tertiary and quaternary structure of the protein and its biological roles. As there is a higher content of acidic monosaccharides in colostrum, could there be weak attraction/repulsion forces with the charged amino and carboxyl groups present on the amino acid components influencing protein folding, which would be absent in the more neutral monosaccharide dominant mature milk profile? This aspect of LF will have an impact on the presentation of the glycans *in vivo* and their availability to interact with mammalian and bacterial cell receptors.

If there is variation in the protein folding over lactation, it would be interesting to explore how this influences the digestion of the protein *in vivo*. LF

glycosylation has previously been shown to impact the susceptibility of the protein backbone to enzyme degradation due to steric hindrance (van Veen, Geerts et al. 2004). The use of a model system which replicates the acidity and enzyme profile of the bovine GIT would allow the study of any differences in the peptide profiles which would be generated from colostrum and mature milk LF *in vivo*. These peptides could then be evaluated for differences in biological activity as they would be more representative of the true structures which would be present in the GIT to deliver biological benefits.

It was hypothesised that the change in glycosylation profile of LF may be linked to the changing protection required by the calf from milk, its primary food and nutrient source. This hypothesis was further explored when the bacterial binding of LF from colostrum and mature milk was compared in chapter 4. The binding of a panel of pathogenic bacteria to LF from the various lactation timepoints was increased for mannose rich mature milk. This suggested that mature LF may offer a non-specific defence against bacterial invasion in the developing infant. Colostrum LF had a reduced bacterial binding activity, suggesting the glycovariants may offer different functionality *in vivo*. The elevated Neu5Ac content in colostrum LF suggests the proteins role in early lactation may be to support the establishment of the gut microbiota which ultimately protects the neonate from microbial or the protein elicates it's antibacterial activity in a manner not reliant on direct binding to the bacterial cell. There may be less of a biological driver for antimicrobial activity from LF in the intial days after birth as the immunoglobulin concentration in milk during the first days postpartum is very high (Marnila and Korhonen 2002). Further anti-infective *in vivo* studies are required to expand on this hypothesis and evaluate the functionality of colostrum and mature milk LF in the more complex gut environment. It will also be important to understand the difference in the *in vivo* digestion profiles of colostrum and mature LF in the bovine GIT and if the generated peptides also demonstrate bacterial binding.

Chapter 5 of this work focused on GMP, a peptide cleaved from kappa casein during the cheese making process, which has anti-microbial and prebiotic activities. In this study, a growth enhancing effect was observed for *B. infantis* when cultured in 2 mg/ml GMP. This prebiotic effect was lost upon sodium

metaperiodate treatment of the peptide. In order to validate if these results translate into an increase in bifidobacterial growth *in vivo*, feeding studies should be undertaken with varying GMP concentrations to identify the optimum dose rate of the glycopeptide which results in a growth promotion of beneficial bacteria in the lower GIT. If an increase in the bifidobacterial numbers are not evident, it will also be important to look at the adhesion of these bacteria *in vivo* as well as profile of the microbiota present to determine if there is an increase in other beneficial bacteria strains or a reduction in the persistence of infectious bacteria.

In contrast to the majority of other milk glycoproteins, there is information already available on the survival of GMP during transit through the human GIT. Intact CMP, released from milk kappa casein in the stomach, has been detected in the plasma of humans at physiologically significant concentrations (Chabance, Marteau et al. 1998, Thoma-Worringer, Sorensen et al. 2006) and glycosylation and sialylation have been shown to reduce digestion of the glycopeptide by brush border membrane endopeptidases, allowing it to be absorbed into the blood (Doi, Kawaguchi et al. 1979, Addeo, Martin et al. 1984, Boutrou, Jardin et al. 2008).

A gene expression microarray provided further insights into the molecular basis for the prebiotic effect of bovine milk GMP on *B. infantis*, with a number of genes associated with carbohydrate metabolism upregulated in the presence of GMP. Incubation studies with *B. infantis* and GMP should be conducted to investigate if the bacteria digest the peptide backbone or oligosaccharide structures of the glycoprotein. Expression and purification of the glycoside hydrolases upregulated in the bacteria cultured in GMP fortified growth media would provide added information on these enzymes and their specificity.

This study on the effect of GMP on the probiotic *B. infantis* strain supports information currently available highlighting this peptide as a potential ingredient for fortified foods and infant formula. The peptide glycosylation, and in particular the high Neu5Ac content, is critical to the beneficial biological activities associated with GMP. When the potential sources of this peptide for industrial isolation are considered, cheese whey is the most obvious dairy stream to focus on due to its wide availability in the Irish and European dairy industry. However, cheese production involves the use of live bacterial cultures which express a variety of enzymes which hydrolyse milk proteins and sugars. The impact of this

fermentation process on the glycosylation and sialic acid content of GMP will have to be investigated and the potential to optimise and standardise this process to ensure reproducible glycosylation on GMP after each production.

Genetic variation has an impact on milk protein composition, especially bovine milk kappa-casein (κ-CN) composition, and a recent study detailed the impact of genetic variation on the presence and glycosylation of κ-CN (Bonfatti, Chiarot et al. 2014). Unfortunately, no such studies exist to date for any other bovine milk glycoproteins. The potential of somatic cells as a more readily available source of transcriptomic information offers the opportunity to explore in greater detail the genes involved in the synthesis and post-translational modifications of bovine milk glycoproteins.

Gene expression in somatic cells excreted in bovine milk have been shown to be a good representation of the cell transcriptomes in the mammary gland and offer a more non-invasive method of profiling gene transcription in the bovine mammary gland (Boutinaud and Jammes 2002, Canovas, Rincon et al. 2010). Using this approach, Wickramasinghe et al. (2011) detected 92 genes involved in milk oligosaccharide metabolism expressed in bovine milk somatic cells, including genes encoding for glycotransferases, glycosidases and sugar transporters. Further studies of this type, with a more narrow focus on milk from the first days post-partum, would provide a greater understanding of the genetic regulation behind the changes in bovine milk oligosaccharide structures, protein glycosylation and glycosidase expression described during this period. This study may also provide insight on how to further select for the *in vivo* expression of genes responsible for favourable modifications, such as sialylation, which have positive biological impacts. Questions to be further explored include; What are the genetic mechanisms in place to regulate protein expression and glycosylation?; What is the impact of factors such as lactation stage, breed, diet and infection on this gene regulation?; Can these parameters be optimised to increase the content of biologically relevant glycoproteins in milk, delivering enhanced benefits to the calf and increasing the commercial value of bovine milk as a source of bioactive components for the infant formula and nutraceutical industries?

As infant formulae are typically based on bovine milk-derived ingredients, with a focus on components which deliver novel functionalities, as the food

industry desires nutritionally enriched and beneficial products, and as dairy farmers in Ireland and worldwide are under increasing pressure to deliver consistant milk quality, insight into the impact of feeding, housing and milking practices which can harness further value from milk would be universally advantageous. Overall, the work described in this thesis has highlighted the potential of Irish milk as a source of nutritionally and bioactively beneficial components which could have product application across infant formulae, functional and fortified foods and immune supplements.

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