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**Profiling temporal changes in bovine milk lactoferrin glycosylation using lectin
microarrays**

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Abstract

The 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 oligosaccharide 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*.

Keywords:

Lactoferrin

Glycosylation profiling

High throughput

Lactation

Colostrum

Lectin microarray

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, & López-Fandiño, 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, Arevalo-Gallegos, & Rascon-Cruz, 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, Fukuwatari, & Shimamura, 1996) and iron-binding activities (Legrand, Mazurier, Colavizza, Montreuil, & Spik, 1990), as well as protecting the protein backbone from proteolysis (van Veen, Geerts, van Berkel, & Nuijens, 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, *et al.*, 2009) by day 5 post-partum (Abd El-Fattach, Abd Rabo, El-Dieb, & El-Kashef, 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, *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, Shinmura, & Fukunaga, 2000) but only 15% of bLF in mature milk (Kolodziejczyk, Tedeschi, Acquistapace, Bovetto, & Schmitt, 2011). As the glycosylation of bLF has been shown to play a role in its

bioactivity and affect its digestion profile (O’Riordan, *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, *et al.*, 2011), human LF (hLF) (Barboza, *et al.*, 2012) and bovine milk fat globule membrane (MFGM) (Wilson, Robinson, Donnet, Bovetto, Packer, & Karlsson, 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 purified 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 & Joshi, 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 $\rightarrow$ 4)-GlcNAc, LacdiNAc) (van Leeuwen, Schoemaker, Timmer, Kamerling, & Dijkhuizen, 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, Kacs Kovics, Breimer, Hammarström, & Holgersson, 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, Yang, Gong, & Chang, 2011; Zinger-Yosovich, Sudakevitz, Iluz, & Gilboa-Garber, 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, *et al.*, 2008; Froelich, *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, *et al.*, 2006). In brief, membrane adsorption cation exchange chromatography was performed using a Sartobind S SingleSep nano 1 mL capsule (36 cm²) 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 SourceTM 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. Commercially available bLF was also chromatographed for comparison. Isolated and

commercially available bLF were also analysed by SDS-PAGE (10% acrylamide gels) and stained with Coomassie Brilliant Blue and periodic acid-Schiff's reagent (Kilcoyne, *et al.*, 2009) to investigate sample purity (Fig. S1).

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, & Vogel, 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 KH₂PO₄, and appropriate mixture of 10 mM Na₂HPO₄ and NaH₂PO₄ 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, *et al.*, 2012; Gerlach *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 Ca²⁺ and Mg²⁺ ions (TBS; 20 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂) 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, *et al.*, 2012; Gerlach, *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, *et al.*, 2012; Gerlach *et al.*, 2014). 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, & Shimodaira, 2011) 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 °C for 1 h (Gallagher, Morris, & Dexter, 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 hydrolysate 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 °C for 4 h and analysed essentially as previously described (Kilcoyne, *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, *et al.*, 2012) while SP-Sepharose methods (Conesa, *et al.*, 2008) require an overnight incubation. Similar to Wei, Nishimura, & Yoshida (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, *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, *et al.*, 2006) and thus, the one-step purification technique described here may be suitable for up-scaling.

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 $\rightarrow$ 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, & Montreuil, 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, *et al.*, 2011; van Leeuwen, *et al.*, 2012), and Man₈GlcNAc₂ has been reported as the most abundant glycan structure present in mature bLF, accounting for 38% of the total glycan structures (Hua, *et al.*, 2011). The lectins PSA and Lch-B did not interact with any of the bLF samples, which implied 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*-acetylglucosamine (Gal-β-(1→4)-GlcNAc, LacNAc) and/or LacdiNAc motifs (van Leeuwen, *et al.*, 2012; Plate, *et al.*, 2006) (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 persistent 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, *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, *et al.*, 1991; Irazoqui, Vides, & Nore, 1999) but they may also have affinity for LacNAc motifs, which shares a similar structure of a Gal residue β-linked to an acetylated 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, *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 $\rightarrow$ 6)- and α -(2 $\rightarrow$ 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 $\rightarrow$ 6)-linked in agreement with structural analysis previously described for bLF (Spik, *et al.*, 1988).

The lectins AAL, LTA and UEA-I have preferential binding affinities for α -(1 $\rightarrow$ 6)-, α -(1 $\rightarrow$ 3)- and α -(1 $\rightarrow$ 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 $\rightarrow$ 6)-linkage only in all structures, in agreement with previous structural analysis (van Leeuwen, *et al.*, 2012; Spik, *et al.*, 1988). 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 $\rightarrow$ 3)-Gal (Teneberg, Alsén, Ångström, Winter, & Goldstein, 2003). The Gal- α -(1 $\rightarrow$ 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, *et al.*, 2012). The Gal- α -(1 $\rightarrow$ 3)-Gal epitope has been linked to allergen-related immune responses in humans (Commings, *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 pmoles/ μ g bLF in colostrum to 88.3 ± 18.5 pmoles/ μ g 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, *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, *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, *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, *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 $\rightarrow$ 3)-Gal epitope. The lectins VRA and EEA, which indicated the presence of Gal- α -(1 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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, & 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.

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, *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, *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.

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, Garcia-Pardo, & Hueso, 2001). Sialic acid is important for stabilisation of LF due to its calcium binding ability, which affects the antibacterial activity of bLF (Rossi, *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, & Nakajima, 1994) and therefore, it may contribute to the prebiotic effect observed for bLF (Rahman, Kim, Ito, Kumura, & Shimazaki, 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, *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, *et al.*, 2008), such as ingested bovine milk. The absorption of dietary Neu5Gc may elicit an immune response in humans (Padler-Karavani, *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, & Shaw, 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, *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 $\rightarrow$ 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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Figure legends

Figure 1 BLF Isolation Elution profile of whey applied to a Sartobind S SingleSep nano 1mL 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 bovine lactoferrin. **Insert** RP-HPLC profile of pooled C1-C3 fractions following dialysis to ascertain purity of isolated bLF.

Figure 2 Lectin profiles Lectin microarray profiles of bovine lactoferrin isolated from three cows from different timepoints in lactation **A** colostrum (day 1) **B** transitional milk (day 6) **C** mature milk (day 90). Cow A: black. Cow B: white. Cow C: grey.

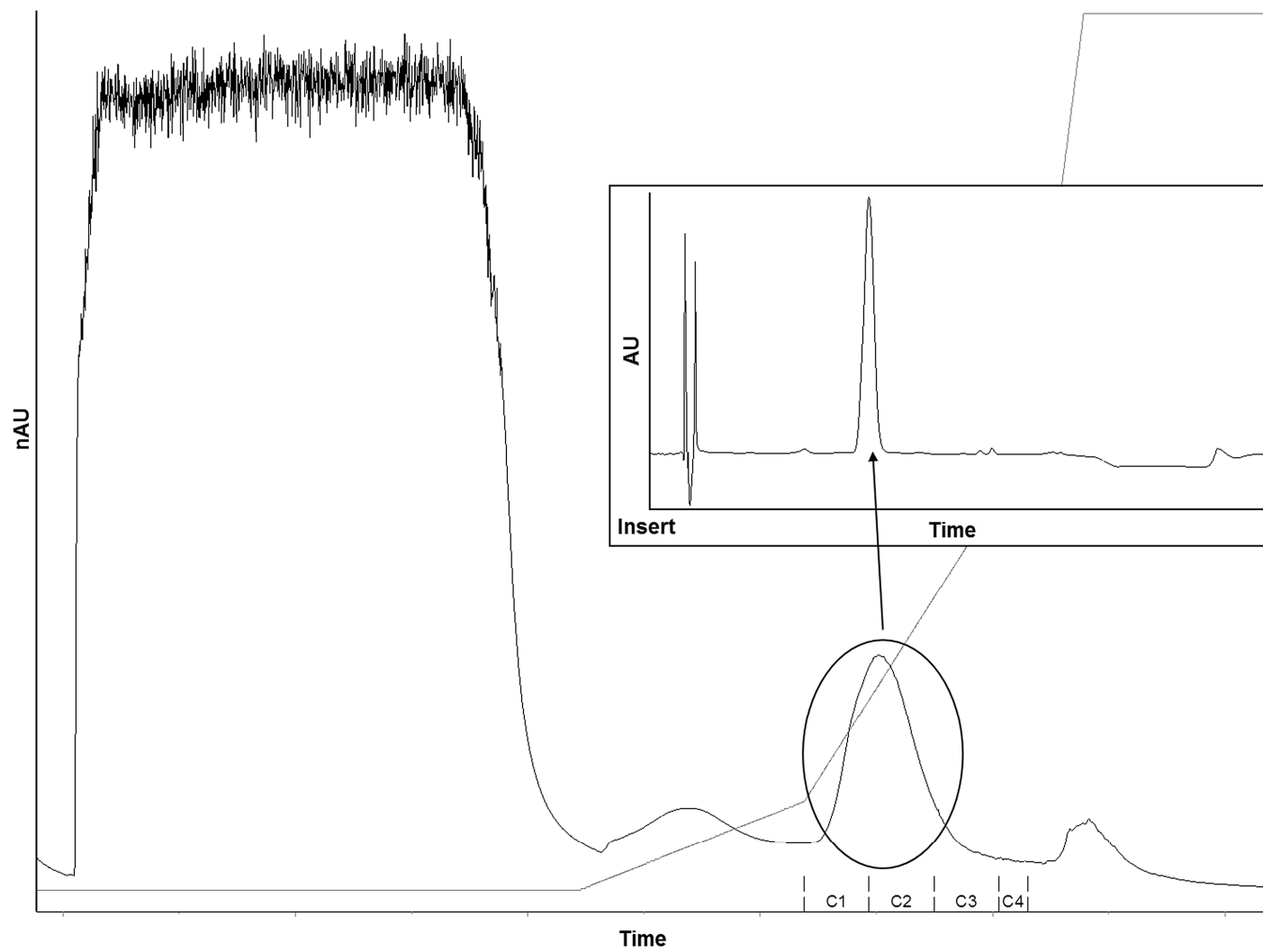
Figure 3 Cluster analysis Cluster dendrogram of lectin binding profiles for bovine lactoferrin samples from three cows (A-C) from day 1 to day 90 of lactation. Clusters with a P value ≥ 0.95 were selected. **A** Timepoints from mature milk cluster closely together.

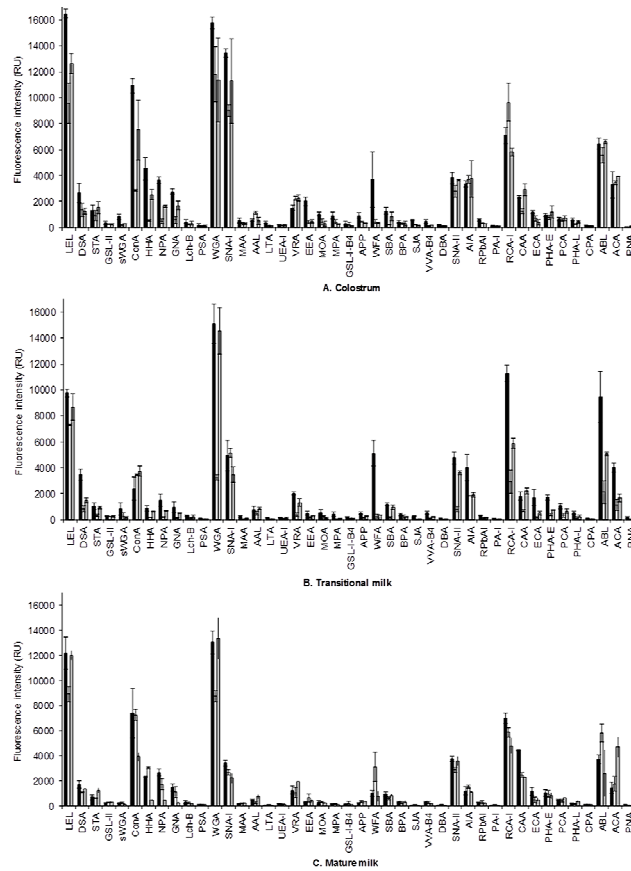
Figure 4 Sialic acid Percentage abundance of Neu5Ac and Neu5Gc (pmoles monosaccharide per μg bovine lactoferrin) from the milk of three cows over the first three months of lactation.

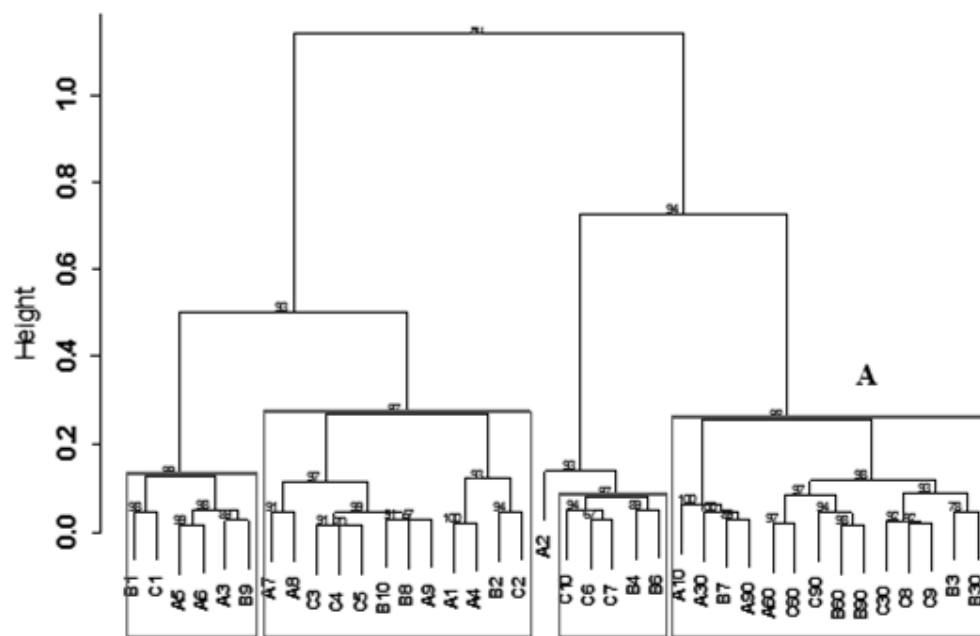
Table 1 Average concentration ($\pm$ standard deviation) of monosaccharides (pmoles per μg bLF) collected from three cows over the first three months of lactation, based on three separate analyses of bLF isolated from each animal (n=9).

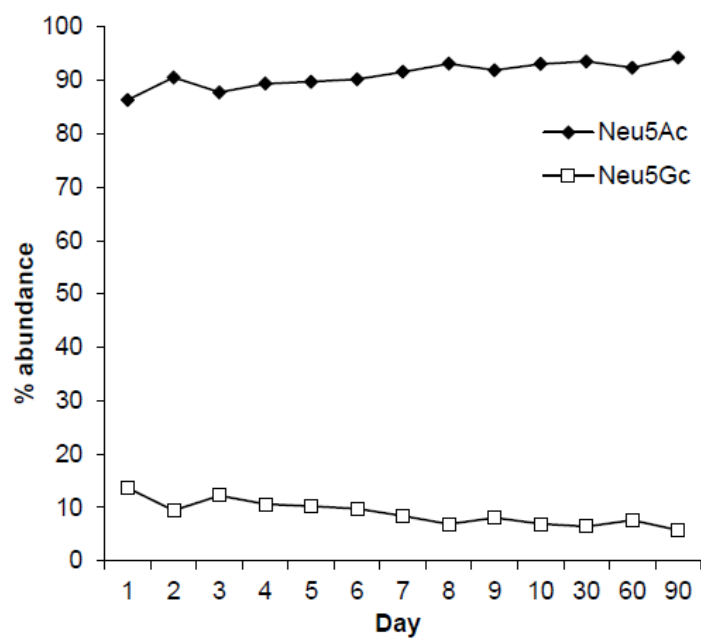
Day	GlcN	Man	GalN	Gal	Neu5Ac	Neu5Gc	Fuc
1	98.4 $\pm$ 30.1	45.8 $\pm$ 17.7	44.8 $\pm$ 9.6	26.6 $\pm$ 13.2	87.7 $\pm$ 16.8	13.9 $\pm$ 1.0	4.2 $\pm$ 2.4
2	95.5 $\pm$ 18.2	48.1 $\pm$ 6.4	22.1 $\pm$ 9.7	23.2 $\pm$ 12.7	67.3 $\pm$ 13.5	7.0 $\pm$ 1.1	2.8 $\pm$ 1.9
3	78.1 $\pm$ 2.2	50.3 $\pm$ 44.8	23.0 $\pm$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

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O’Riordan *et al* 2013 Highlights

- LF was isolated from bovine milk over lactation by cation exchange chromatography.
- Lectin binding and monosaccharide profiles of bLF varied over lactation.
- Sialylation (Neu5Ac and Neu5Gc) was quantified, most abundant in early lactation.
- The presence of high mannose type glycans increased as lactation progressed.
- Lectin arrays as a platform to monitor glycosylation changes of bLF.