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# Dynamic Temporal Variations in Bovine Lactoferrin Glycan Structures

Rivca L. Valk-Weeber,<sup>†</sup> Talitha Eshuis-de Ruiter,<sup>‡</sup> Lubbert Dijkhuizen,<sup>†,§</sup> and Sander S. van Leeuwen<sup>\*,†,||</sup>

<sup>†</sup>Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

<sup>‡</sup>Life Sciences, FrieslandCampina Research, Bronland 20, 6708 WH Wageningen, The Netherlands

## Supporting Information

**ABSTRACT:** It has been reported previously that glycosylation of bovine lactoferrin changes over time. A detailed structural overview of these changes over the whole course of lactation, including predry period milk, is lacking. In this study, a high-throughput analysis method was applied to the glycoprofile of lactoferrin isolated from colostrum, mature, and predry period mature milk, which was analyzed over two subsequent lactation cycles for 8 cows from diverse genetic backgrounds. In addition, comparisons are made with commercial bovine lactoferrin samples. During the first 72 h, dynamic changes in lactoferrin glycosylation occurred. Shifts in the oligomannose distribution and the number of sialylated and fucosylated glycans were observed. In some cows, we observed ( $\alpha$ 2,3)-linked sialic acid in the earliest colostrum samples. The glycoprofiles appeared stable from 1 month after delivery, as well as between cows. In addition, the glycosylation profiles of commercial lactoferrins isolated from pooled mature milk were stable over the year. Lactoferrin glycosylation in the predry period resembles colostrum lactoferrin. The variations in lactoferrin glycosylation profiles, lactoferrin concentrations, and other milk parameters provide detailed information that potentially assists in unraveling the functions and biosynthesis regulation of lactoferrin glycosylation.

**KEYWORDS:** lactoferrin, colostrum, milk, N-glycosylation, ( $\alpha$ 2,3)-sialic acid

## INTRODUCTION

Bovine milk is the most abundant milk produced worldwide, with many millions of tons produced annually.<sup>1</sup> In order to maintain milk production, the milk is produced in lactation cycles of approximately 12 to 13 months.<sup>2</sup> At the end of a lactation cycle, prior to the new delivery, milking is stopped (the dry period) to aid in the recovery of the mammary gland and to restore the constitution of the cow. Following delivery, maturation of the milk occurs over several periods, from colostrum to early, mid, and late lactation. The colostrum and early lactation period can be further divided into colostrum, transitional, and mature milk. However, the duration of these periods is not clearly defined. The colostrum period can be described as only consisting of the first milking, to up to 7 days *post parturition*.<sup>3</sup> In this work, the colostrum period is defined as the first 3 days (72 h) of lactation.

The composition of colostrum differs greatly from that of mature milk; it is an extra rich milk, produced only during the first days after parturition and contains higher concentrations of fat, immunoglobulins, and other beneficial proteins that support the newborn calf during the first few critical days. The most abundant saccharide in milk, lactose, is reduced in early colostrum and increases in concentration over time. In contrast, other components, such as fat, vitamin, and protein are present in higher concentrations in colostrum.<sup>3</sup> Colostrum provides passive immunity to the newborn via the highly increased concentrations of immunoglobulins.<sup>4</sup>

Similar to immunoglobulins, lactoferrin is a functional glycoprotein that is present in higher concentrations in

colostrum compared to mature milk. Lactoferrin is a 78 kDa protein that has a high iron-binding capacity and is found in several mammalian secretions.<sup>5</sup> Over the years, many important functions have been attributed to this protein.<sup>6,7</sup> The iron-binding capacity of lactoferrin has been linked to bactericidal effects by withholding iron from pathogens.<sup>8,9</sup> Lactoferrin also has potent antiviral<sup>10</sup> and antifungal properties,<sup>11</sup> thereby protecting the newborn from infections.

In addition, lactoferrin has been implicated as an anti-inflammatory protein and aids in the immune development of newborns, as extensively reviewed elsewhere.<sup>12,13</sup>

Lactoferrin is a glycoprotein, decorated with N-linked glycans in many mammals including humans and cows.<sup>14</sup> Human lactoferrin has three potential glycosites, Asn137, Asn478, and Asn623, of which one usually is not occupied. Mature human milk lactoferrin is decorated with sialylated diantennary glycans, with high levels of fucosylation.<sup>15</sup> Bovine lactoferrin has five potential glycosites, Asn281, Asn233, Asn368, Asn476, and Asn545, of which one (Asn281) only is glycosylated in 30% of cases.<sup>16,17</sup> The glycosylation profiles of human and bovine lactoferrins are very different. Bovine lactoferrin is mainly decorated with oligomannose-type glycans, in addition to low levels of hybrid and diantennary complex-type glycans. Moreover, bovine lactoferrin glycosyla-

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Table 1. Sample Parameters and Analysis Data of the Colostrum, Predry Period, and Mature Milk Samples<sup>a</sup>

Cow	1			2			3			4				
	Breed (%) <sup>b</sup>			FV-?-HF (62.5-25-12.5)			FV-HF-MRY (50-37.5-12.5)			HF-MRY (87.5-12.5)			SRB-HF-MRY (50-37.5-12.5)	
Lactation period / Sampling time <sup>c</sup>	Parity			2			7			2				
	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)		
Col	0-4 h	N/A	N/A	N/A	3.2	8.70	27.84	5.0	4.01	20.07	2.2	6.62	14.56	
	8-14 h	3.5	0.09	0.32	2.0	3.39	6.78	6.0	1.01	6.06	3.0	2.97	8.92	
	~24 h	1.1	0.11	0.12	3.0	1.80	5.40	7.2	0.52	3.72	3.5	1.97	6.89	
	~36 h	6.2	0.03	0.20	4.0	1.61	6.45	8.0	0.26	2.04	N.A.	N.A.	N.A.	
	~48 h	6.5	0.03	0.21	5.0	1.35	6.73	9.2	0.20	1.82	4.5	0.98	4.39	
	~60 h	6.2	0.04	0.24	4.8	0.91	4.36	8.0	0.19	1.52	4.5	0.67	3.02	
	~72 h	6.5	0.07	0.45	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	4.0	0.56	2.26	
Milk	30 d	11.0	0.03	0.28	7.5	0.19	1.41	12.0	0.16	1.97	7.5	0.11	0.80	
	60 d	11.0	0.02	0.26	8.0	0.21	1.68	16.5	0.10	1.62	7.6	0.08	0.63	
	90 d	11.0	0.06	0.67	9.5	0.22	2.04	15.0	0.19	2.90	8.5	0.11	0.95	
Dry	days	-13 d			-32 d			-15 d			N.A.			
Dry		4.1	0.16	0.64	1.8	0.58	1.04	1.0	3.14	3.14	N.A.	N.A.	N.A.	
Period 2	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)		
Col	0-4 h	5.0	0.46	2.32	N.A.	N.A.	N.A.	10.0	1.91	19.14	N.A.	N.A.	N.A.	
	8-14 h	2.5	0.40	1.01	3.5	1.07	3.74	6.5	0.63	4.10	N.A.	N.A.	N.A.	
	~36 h	N.A.	N.A.	N.A.	7.8	0.23	1.76	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	
	~60 h	8.5	0.14	1.16	10.0	0.11	1.09	10.0	0.28	2.80	N.A.	N.A.	N.A.	
	90 d	10.0	0.09	0.85	12.0	0.12	1.42	11.8	0.21	2.49	13.5	0.03	0.42	
Milk	30 d	10.0	0.07	0.70	14.8	0.08	1.12	12.7	0.17	2.11	3.6	0.05	0.17	
	90 d	10.0	0.07	0.70	14.8	0.08	1.12	12.7	0.17	2.11	3.6	0.05	0.17	
Cow	5			6			7			8				
	HF-MRY (50-50)			HF (100)			?			HF (100)				
Lactation period / Sampling time <sup>c</sup>	Parity			3			2			2				
	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)		
Col	0-4 h	3.7	1.56	5.76	4.5	1.69	7.59	4.5	2.68	12.06	10.0	0.48	4.76	
	8-14 h	4.2	0.51	2.14	4.0	0.46	1.84	4.0	0.86	3.42	6.5	0.15	0.99	
	~24 h	7.0	0.31	2.18	7.0	0.24	1.71	4.0	0.37	1.46	7.0	0.11	0.76	
	~36 h	7.5	0.21	1.55	9.0	0.15	1.34	6.0	0.21	1.23	7.5	0.10	0.74	
	~48 h	9.0	0.15	1.35	9.5	0.11	1.01	6.5	0.14	0.89	9.5	0.08	0.78	
	~60 h	11.0	0.14	1.53	N.A.	N.A.	N.A.	7.0	0.10	0.72	11.0	0.06	0.66	
	~72 h	N.A.	N.A.	N.A.	10.5	0.08	0.80	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	
Milk	30 d	13.0	0.06	0.79	15.0	0.07	1.05	10.0	0.03	0.30	15.5	0.04	0.62	
	60 d	12.5	0.07	0.88	16.4	0.05	0.84	10.0	0.05	0.54	14.0	0.08	1.12	
	90 d	15.0	0.10	1.49	15.5	0.09	1.44	9.0	0.03	0.29	14.2	0.08	1.12	
	90 d	15.0	0.10	1.49	15.5	0.09	1.44	9.0	0.03	0.29	14.2	0.08	1.12	
Dry	days	-16 d			19 d			-25 d			-31 d			
Dry		0.5	1.91	0.95	1.9	1.95	3.70	3.2	0.15	0.48	0.4	2.05	0.82	
Period 2	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)	Milk (L)	[LF] (mg/mL)	LF (g)		
Col	0-4 h	3.5	0.97	3.41	2.6	10.20	26.53	1.8	4.17	7.50	6.7	0.48	3.24	
	8-14 h	6.5	0.46	3.01	11.0	3.68	10.30	3.5	1.03	3.62	7.2	0.26	1.89	
	~36 h	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	
	~60 h	10.7	0.18	1.90	8.0	0.52	4.18	6.7	0.19	1.27	12.5	0.21	2.65	
	30 d	11.0	0.11	1.24	9.0	0.12	1.09	10.8	0.09	1.00	17.3	0.03	0.59	
	90 d	11.0	0.11	1.18	10.4	0.30	3.15	7.1	0.05	0.35	11.7	0.05	0.63	

<sup>a</sup>Colostrum (col), predry period (dry), and milk samples were collected from two subsequent lactation cycles of 8 cows of the Rietveldhoeve Farm, Aduard, Groningen. <sup>b</sup>Abbreviations used for the genetic background of the cows: Swedish red (SRB), HF, Meuse-Rhine-Yssel (MRY), Fleckvieh (FV), and unknown (?). <sup>c</sup>Sampling times, lactoferrin concentrations, total amounts of lactoferrin per milking, and parity at the time of the first lactation are shown. Time is specified in hours (h) or days (d), respectively. The samples taken in the predry period are shown as negative days until the parturition. Milk sample volumes are shown in liters (L). N.A.: sample not available. Approximate sampling times are shown; for exact sampling times see Table S2.

tion contains LacDiNAc motifs, N-glycolyl neuraminic acid, and the Gal-( $\alpha$ 1,3)-Gal epitope ( $\alpha$ -Gal) epitope, which do not occur in the human milk lactoferrin protein.<sup>18</sup>

Not only protein concentrations differ in colostrum versus mature milk, but also the glycosylation profiles of the glycoproteins present vary. For example, the glycans expressed

on bovine immunoglobulins are known to undergo changes during the early lactation period.<sup>19</sup> Similar changes have been reported for human lactoferrin<sup>20</sup> and bovine lactoferrin.<sup>21</sup>

While information is available about the changes occurring in total levels of sialic acid, fucose, and general glycan complexity of bovine lactoferrin, information on the exact glycan structures present on bovine colostrum lactoferrin is currently lacking. In addition, no information is available on the glycan structures of bovine lactoferrin in the predry period milk. In this paper we describe the exact structural modifications that take place in these glycans during early and end-period lactation. Lactoferrin in mature milk from Holstein-Friesian (HF) cows has been studied most. In this study, lactoferrin was isolated from 8 cows from diverse genetic backgrounds, as well as from commercial sources, to investigate the heterogeneity of mature lactoferrin glycosylation. Lactoferrin concentrations and other milk parameters were also determined. The observed (changes in) lactoferrin glycosylation profiles over time provide novel information and may provide new insights into the functionality and regulation of lactoferrin and its glycans.

## MATERIALS AND METHODS

**Materials.** Bovine lactoferrin (purity 95.7%) was provided by FrieslandCampina Domo (Amersfoort, the Netherlands). In addition, 10 commercial lactoferrin samples from a single production plant in the Netherlands were provided by FrieslandCampina. Peptide-N-glycosidase F (PNGase F, *Flavobacterium meningosepticum*) was obtained from New England Biolabs (Ipswich, UK). Jack bean  $\alpha$ -mannosidase (75 U/mL in 3.0 M  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 mM zinc acetate, pH 7.5) was purchased from Sigma-Aldrich Chemie N.V. (Zwijndrecht, the Netherlands). Green coffee bean  $\alpha$ -galactosidase (25 U/mL 100 mM sodium phosphate pH 6.5, containing 0.25 mg/mL bovine serum albumin), bovine testis  $\beta$ -galactosidase (5 U/mL in 20 mM sodium citrate phosphate, 150 mM NaCl, pH 4.0), *Streptococcus pneumoniae*  $\alpha$ -sialidase, with strong preference for  $\alpha(2,3)$  linkages (4 U/mL 20 mM Tris-HCl, 25 mM NaCl, pH 7.5), and *Arthrobacter ureafaciens*  $\alpha$ -sialidase (5 U/mL in 20 mM Tris HCl pH 7.5, containing 25 mM NaCl) were obtained from ProZyme (Ballerup, Denmark).

**Milk.** Colostrum and milk samples were collected from 8 cows from a local organic farm (Rietveldhoeve farm, Aduard, Groningen, the Netherlands), for over 2 subsequent lactation cycles. In the first cycle, colostrum was collected directly after calving, and then at approximately 12, 24, 36, 48, and 60 h postpartum (Table 1). Milk samples were collected at 1, 2, and 3 months after calving and then a final sample just before the start of the dry period. In the subsequent cycle, colostrum was collected directly after calving, 12 and 60 h postpartum and milk at 1 and 3 months. At these time points, the complete milk samples were collected from each individual cow and their total milk volume was registered. The complete milk sample was homogenized and an aliquot of ~40 mL was directly stored at  $-20\text{ }^\circ\text{C}$  until the sample was transported to the analysis facility. After transport, the samples were stored at  $-80\text{ }^\circ\text{C}$  until analysis.

**Whey Preparation.** Frozen colostrum and milk were thawed in a water bath at  $37\text{ }^\circ\text{C}$  and homogenized. An aliquot was defatted by centrifugation at 2000g for 30 min at  $4\text{ }^\circ\text{C}$ . The defatted milk/colostrum was collected and caseins were removed by ultracentrifugation at 100 000g for 1 h at  $4\text{ }^\circ\text{C}$  using a Sorvall RCM120GX microcentrifuge (Thermo Fisher Scientific, Waltham, MA). To allow the precipitation of the caseins in early colostrum, the samples collected at day 1 (0 and 12 h) were diluted 1:1 with Milli-Q water prior to ultracentrifugation. The supernatant whey fraction was filtered over a  $0.22\text{ }\mu\text{m}$  syringe filter 25 mm GD/X cellulose acetate (Whatman, GE Healthcare, UK).

**Lactoferrin Isolation.** Lactoferrin was captured by cation exchange on SP-Sepharose Fast Flow (GE Healthcare Bio-Sciences

AB, Uppsala, Sweden) as described,<sup>22</sup> with modifications to allow for a small scale batch protocol. Optimal NaCl concentrations used during the binding and washing steps, to eliminate interfering proteins, were determined as 250 mM for binding, 400 mM for washing, and 1.2 M for elution (Supporting Information, Figure S13).

An aliquot of 100  $\mu\text{L}$  SP-Sepharose was added to 3 mL of filtered whey. NaCl was added to a final concentration of 250 mM and the samples were gently rotated overnight at  $4\text{ }^\circ\text{C}$ . The Sepharose was precipitated by centrifugation at 400g for 10 min. The supernatant was carefully removed and discarded. The Sepharose was resuspended in 200  $\mu\text{L}$  of 250 mM NaCl in 20 mM phosphate buffer at pH 7.5 and transferred to a 96 well filter plate (0.45  $\mu\text{m}$  GHP, Pall). The solvent was removed by a 96 well plate vacuum manifold at 5–10 In. Hg (Supelco, Bellefonte, PA, USA).

The Sepharose was washed 2 times by the addition of 400  $\mu\text{L}$  250 mM NaCl in 20 mM phosphate buffer at pH 7.5 and mixing at 1000 rpm for 5 min. The solvent was removed by vacuum. Additional washing was performed with 400  $\mu\text{L}$  400 mM NaCl in 20 mM phosphate buffer at pH 7.5. Elution of lactoferrin was performed by the addition of  $2 \times 400\text{ }\mu\text{L}$  of 1.2 M NaCl in 20 mM phosphate buffer at pH 7.5.

The lactoferrin was desalted and concentrated with centrifugal molecular weight cutoff filters (30 kDa Amicon Ultra, Merck Millipore, Tullagreen, Cork, IRL). Lactoferrin concentration was determined by absorption measurements at 280 nm ( $\epsilon$  1 %, 12.7) against a calibration curve of 0.1–10 mg/mL reference lactoferrin (corrected for 95.7% purity, FrieslandCampina) using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

**Lactoferrin Concentration Determination.** The concentration of lactoferrin in the milk samples was quantified by the bovine lactoferrin ELISA quantitation set (E10-126, Bethyl Laboratories, Montgomery, TX, USA). Manufacturer's protocol was strictly followed with the addition of the Roche ELISA Blocking reagent as described below. ELISA plates (Costar, #3590, Sigma) were coated with the capture antibody followed by a blocking step using a blocking agent for ELISA (#11112589001, Roche Diagnostics, Mannheim, Germany). Milk and colostrum samples were diluted within the calibration curve range of 7.8–500 ng/mL using a blocking agent for ELISA. As a last step, horseradish peroxidase-labelled detection antibody was diluted in the ELISA blocking reagent. 3,3',5,5'-Tetramethylbenzidine substrate solution (Thermo Fisher Scientific, Waltham, MA) was added to the wells. After 15 min color development was stopped using 1 M HCl (Sigma). Absorbance was measured at 450 nm using a Bio-Rad iMark ELISA plate reader (Bio-Rad Laboratories, USA). Data were analyzed using a four-parameter logistic (4-PL) curve-fit.

**PNGase F Digestion.** An exact amount of 25–50  $\mu\text{g}$  of lactoferrin was lyophilized and redissolved at a concentration of 2.5 mg/mL in 100 mM phosphate buffer at pH 7.5, containing 1% of sodium dodecyl sulfate (SDS), 1%  $\beta$ -mercaptoethanol, and 3 ng of maltohexaose per  $\mu\text{g}$  of lactoferrin.

The lactoferrin was denatured by heating at  $85\text{ }^\circ\text{C}$  for 5 min in a MyCycler Thermal Cycler (Bio-Rad Laboratories, USA) to ensure homogeneous heating. NP-40 (NP-40 substitute, Sigma) was added to a final concentration of 1% and the samples were mixed before addition of PNGase F (50 U/experiment). Incubation was performed in a MyCycler Thermal Cycler (Bio-Rad Laboratories, USA) kept at a constant  $37\text{ }^\circ\text{C}$  overnight.

**Glycan Labeling (2-AA).** Isolated glycans were labeled with anthranilic acid (2-AA, Sigma). PNGase F digests were diluted 1:1 with labeling solution 0.35 M 2-AA and 1 M sodium cyanoborohydride (Sigma) in dimethylsulfoxide (DMSO, Sigma): glacial acetic acid (7:3, v/v) and incubated in a thermal cycler kept at a constant  $65\text{ }^\circ\text{C}$  for 2 h. The labeled samples were purified by 96 well microcrystalline cellulose SPE.<sup>23</sup>

**Glycan Labeling (2-AB).** For mass spectrometry (MS) identification, the samples were labeled with 2-aminobenzamide (2-AB, Sigma). Aliquots of 0.5 mg of lactoferrin, isolated from mature milk or colostrum, were digested with PNGase F under the conditions described earlier. PNGase F digests were diluted 1:1 with labeling

solution 0.35 M 2-AA and 1 M 2-picoline-borane (Sigma) in DMSO (Sigma): glacial acetic acid (7:3, v/v). Incubations were performed for 2 h at 65 °C in a heating block. The labeled samples were purified by 96 well microcrystalline cellulose SPE.<sup>23</sup>

**HPLC Analysis.** Labeled glycans (2-AB and 2-AA) were separated on an Acquity UPLC Glycan BEH Amide column (2.1 mm × 100 mm, 1.7 μm, Waters, Etten-Leur, the Netherlands), using a UltiMate 3000 SD HPLC system (Thermo Fisher Scientific, Waltham, MA) equipped with a Jasco FP-920 fluorescence detector ( $\lambda_{\text{ex}}$  330 nm,  $\lambda_{\text{em}}$  420 nm, Jasco Inc, Easton, MD). An injection volume of 3 μL was used.

Ternary gradients were run using Milli-Q water, acetonitrile, and 250 mM formic acid adjusted to pH 3.0 (with ammonia) at a flow rate of 0.5 mL/min. A Milli-Q gradient from 25 to 35% (total concentration) was used for 45 min. For improved separation during the exoglycosidase assays, a longer gradient was used (22–40% Milli-Q for 67.5 min). A constant 20% formic acid solution at pH 3.0 was maintained throughout the run. The remaining percentage of the solvent composition comprised acetonitrile. After completion of the gradient, the final gradient conditions were maintained for 9 min and the column reconditioned back to initial conditions for 13 min. Chromatograms were scaled to the protein amounts, based on the internal maltohexaose peak.

**Mass Spectrometry Analysis.** MS analysis was performed in a positive ion mode, using a maXis plus QTOF mass spectrometer (Bruker, Billerica, MA, USA). The ion source was set with the following settings, capillary voltage 3000 V, nebulizer pressure 5 bar, dry gas 5 L/min, and a dry temperature of 200 °C. Ion transfer settings were funnel 1 RF, 400 V<sub>pp</sub>, isCID 0 eV, and multipole RF 400 V<sub>pp</sub>. Collision cell settings were collision energy 10 eV, collision RF 3000 V<sub>pp</sub>, transfer time 100 μs, and a pre pulse storage of 30 μs. Spectra were collected in single MS mode, with an *m/z* range from 1000 to 3000 and a rolling average rate of 2 times 2 Hz. Chromatography was performed by an Acquity I-Class UPLC system (Waters, Milford, MA, USA). The above described ternary gradient was modified to a binary solvent system, with the final solvent conditions and gradient slope identical to a regular high-performance liquid chromatography (HPLC) setup. Glycans were identified by their (derivatized) monoisotopic molecular mass, using the GlycoMod tool<sup>24</sup> (<https://web.expasy.org/glycomod/>) and a 0.2 Da mass tolerance.

## RESULTS

Colostrum and milk samples were collected over 2 lactation cycles of 8 cows from diverse genetic backgrounds and subjected to detailed analysis of various parameters (Table 1).

**Lactoferrin Concentrations.** The concentrations of lactoferrin in the milk and colostrum samples were determined by ELISA (Table 1). Lactoferrin concentration was highest in early colostrum and decreased rapidly over the first 72 h. Between cows, large differences were observed, with concentrations ranging from approximately 100 μg/mL to 10 mg/mL in early colostrum. In some cows there was also significant variation in lactoferrin production between lactation cycles. For example, cow 2 produced higher concentrations in the first lactation cycle (8.66 mg/mL) than in the second (1.07 mg/mL), although there is an 8 h difference in the sampling time. In contrast, cow 6 produced more lactoferrin in the second lactation cycle; 10.20 mg/mL versus 1.69 mg/mL in the first lactation cycle. In other cows (e.g., 5 and 7), the lactoferrin concentrations in the first and second lactation cycles were very comparable. No clear correlation between the lactoferrin concentration and parity was observed.

In mature milk, lactoferrin concentrations were lower with a much smaller bandwidth, ranging from 0.03 to 0.30 mg/mL. In all cases, the lactoferrin concentrations measured in the predry period milk were significantly higher than in mature milk of the

same cow. In some cases (cows 5, 6, and 8), the concentration of lactoferrin found in the predry period milk (1.91, 1.95, and 2.05 mg/mL, respectively) was higher than the concentration found in the first collected colostrum sample (1.56, 1.69, and 0.48 mg/mL, respectively). For cows 5 and 8 the predry period lactoferrin concentrations were also higher than in the first colostrum samples of the second lactation cycle.

The lactoferrin concentrations found in this study are in line with earlier reports on colostrum, predry period, and mature milk.<sup>25–27</sup> Taken together, these data show that the levels of lactoferrin production varies between cows and lactation cycles. The range of lactoferrin concentrations in these bovine colostrum samples had a bandwidth of a factor 100 (from 0.1 to 10 mg/mL).

Milk parameters (cell count, urea, protein, fat, and lactose concentrations) were obtained from the Rietveldhoeve farm administration selecting the quality control measurements of the last milk sample preparturition and the first milk sample postparturition (Table S1). Pearson correlations (two-tailed, 95% confidence intervals) were calculated by GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA) on the postparturition data and the lactoferrin concentrations of the 1 month milk samples (closest time point to analysis date) (Table 1). No significant correlation was found between the protein, fat, urea, and lactose concentrations and the lactoferrin concentrations in the milk. A significant ( $P < 0.05$ ) correlation was found between the cell counts and lactoferrin concentrations (Table 2).

**Table 2. Pearson Correlations between Milk Lactoferrin Concentrations and Other Milk Parameters Listed<sup>a</sup>**

	Pearson <i>r</i>	R squared	<i>P</i> value (two-tailed)
protein	−0.10	0.01	0.76 (ns)
fat	−0.46	0.21	0.14 (ns)
cell count	0.59	0.35	0.04 (*)
lactose	−0.35	0.12	0.26 (ns)
urea	0.08	0.01	0.80 (ns)

<sup>a</sup>Two tailed *P*-values are annotated as significant (\*) or not significant (ns).

**Lactoferrin Production Levels.** In mature milk, the total amount of lactoferrin produced per milk sample was relatively constant (Table 1). In colostrum however, marked increases in the total lactoferrin occurred compared to mature milk. For most cows, highest production was observed in the first colostrum sample, with rapid decreases over the 3 day colostrum period. In cow 1 during the first lactation cycle (parity 1), the increase in lactoferrin production in the first collected sample (14 h) was minimal. The second lactation cycle of the same cow (cow 1, parity 2) displayed a more marked increase, as observed for the other cows. For a number of cows (2–5), an increased production was maintained throughout the 72 h colostrum period. In others (6–8), the increased production was limited to the first day of colostrum, after which total lactoferrin produced was similar to that in mature milk samples. While the milk volume collected during the dry period was much lower than that in mature milk, the total amount of lactoferrin secreted into the milk remained similar (Table 1).

**Glycan Structure Analysis.** Glycans isolated from lactoferrin were either labelled with 2-AA for superior sensitivity in fluorescent detection, or with 2-AB for detection

Table 3. Overview of the Lactoferrin N-Glycan Structures Detected by MS Analysis<sup>a</sup>

No.	Theoretical mass (Free end Monoisotopic)	Found m/z (+2-AB)	Theoretical m/z (+2-AB)	Structure image	No.	Theoretical mass (Free end Monoisotopic)	Found m/z (+2-AB)	Theoretical m/z (+2-AB)	Structure image
1	1234.4334	1355.5080*	1355.5095		22	1890.6610	1006.3633#	1006.3722	
2	1275.4600	1396.5364*	1396.5361		23	1915.6927	1018.8880#	1018.8881	
3	1316.4865	1437.5609*	1437.5626		24	1931.6876	1026.8853#	1026.8855	
4	1396.4863	1517.5582*	1517.5624		25	1931.6876	1026.8862#	1026.8855	
5	1437.5128	1558.5857*	1558.5889		26	1972.7141	1047.4003#	1047.3988	
6	1478.5393	1599.6110*	1599.6154		27	1972.7141	1047.3991#	1047.3988	
7	1558.5391	1679.6112*	1679.6152		28	2013.7407	1067.9070#	1067.9121	
8	1599.5656	1720.6370*	1720.6417		29	2036.7189	1079.4029#,!	1079.4012	
9	1607.5819	1728.6534*	1928.658		30	2077.7455	1099.9121#	1099.9145	
10	1640.5922	1761.6625*	1761.6683		31	2093.7404	1107.9074#	1107.9119	
11	1640.5922	1761.6625*	1761.6683		32	2118.7720	1120.4267#	1120.4277	
12	1681.6187	1802.6841*	1802.6948		33	2118.7720	1120.4146#	1120.4277	
13	1720.5919	1841.6627*	1841.6680		34	2134.7670	1128.4210#	1128.4252	
14	1722.6453	1843.7175*	1843.7214		35	2159.7986	1140.9405#	1140.9410	
15	1728.6082	1849.6775*	1849.6843		36	2175.7935	1148.9398#	1148.9385	
16	1769.6348	1890.7058*	1890.7109		37	2222.7830	1172.4455#,!	1172.4332	
17	1786.6501	1907.7255*	1907.7262		38	2238.7779	1180.4284#	1180.4307	
18	1802.645	1923.7049	1923.7211		39	2263.8096	1192.9475#,!	1192.9465	
19	1827.6766	1948.7575*	1948.7527		40	2368.8409	1245.4533#,!	1245.4622	
20	1868.7032	1989.7676*	1989.7793		41	2409.8675	1265.9679#,!	1265.9755	
21	1882.6447	2003.7167*	2003.7208						

<sup>a</sup>Glycans were labeled with 2-AB prior to analysis. Structures are marked with \* when detected as  $[M + H]^+$  or with # when detected as  $[M + 2H]^{2+}$ . Doubly sialylated structures (marked with !) were found as ( $\alpha 2,3/\alpha 2,6$ ) and ( $\alpha 2,6/\alpha 2,6$ ) isomers.

with improved sensitivity in the positive-ion mode during MS analysis. While glycans labeled with 2-AB have a higher retention in the chromatography setup used, the overall chromatographic patterns were the same (Figure S14). The structures obtained by the 2-AB labeled profile could therefore directly be appointed in the 2-AA labeled profiles. During MS, the glycans were detected as both singly charged ions  $[M + H]^+$  and doubly charged ions  $[M + 2H]^{2+}$  (Table 3). Neutral glycans presented the highest intensities for the singly charged ion species, while the doubly charged ions were more intense for the sialylated structures. Predicted structures obtained by GlycoMod were verified by comparing with known structures from the literature, as well as by exoglycosidase assays (Figures

S2 and S3).<sup>18,21,24</sup> The most abundant structures were of the oligomannose type (Man-5 to Man-9) (Table 4, Figure 1).

In addition, hybrid and diantennary structures were found. Diantennary structures were decorated with galactose, or *N*-acetylglucosamine, or in rare cases with an  $\alpha$ -Gal motif. Single and doubly sialylated structures were present, decorated with Neu5Ac. Minor decorations with Neu5Gc were also observed. Contrary to previous studies, we observed ( $\alpha 2-3$ )-linked Neu5Ac epitopes in some samples. In addition, a small number of fucosylated biantennary structures were detected. No tri- or tetra-antennary structures were found. The structures found in this study are consistent with those in earlier reports.<sup>18</sup>

**Lactoferrin Glycoprofiles in Mature Milk Versus Early Colostrum.** The lactoferrin glycoprofiles were dominated by

**Table 4. Total and Relative Percentages of the Oligomannose-Type Glycans of Lactoferrin in Colostrum<sup>a</sup>**

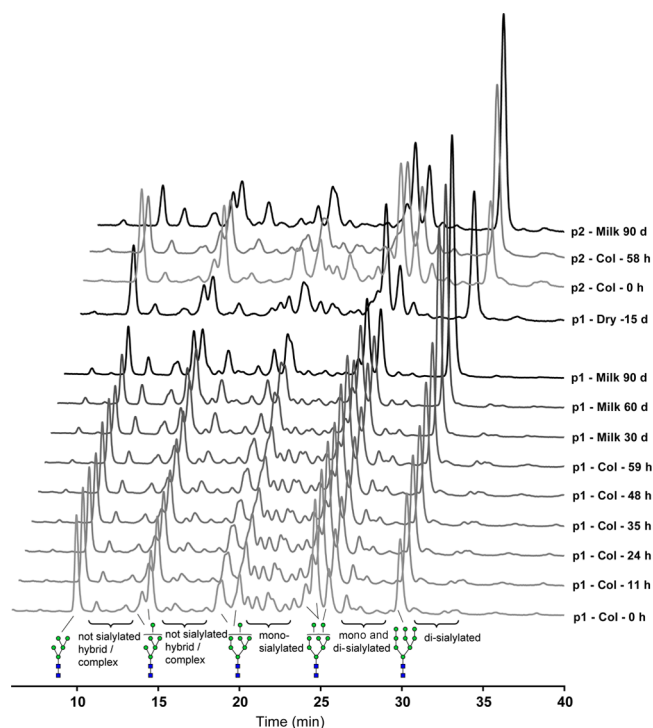
	colostrum ~0 h	colostrum ~72 h	dry	mature
Man-5	14.3 ± 2.2	9.0 ± 2.2	10.5 ± 1.6	5.6 ± 0.6
Man-6	17.4 ± 4.5	14.7 ± 1.4	14.8 ± 2.4	16.7 ± 0.9
Man-7	17.4 ± 2.5	15.5 ± 1.6	14.0 ± 0.7	14.5 ± 1.0
Man-8	33.5 ± 3.6	31.9 ± 3.6	35.1 ± 3.1	26.2 ± 1.5
Man-9	17.4 ± 5.0	28.9 ± 5.9	25.6 ± 3.0	37.2 ± 2.4
Man-5 to 9 (% of total) <sup>b</sup>	80.3 ± 2.6	82.5 ± 2.7	78.4 ± 2.7	84.0 ± 2.6

<sup>a</sup>Relative percentages based on the total area of all Man-5 to Man-9 isomer peaks. Number of samples: colostrum ( $n = 15$ ), predry period ( $n = 7$ ), and mature milk ( $n = 16$ ). <sup>b</sup>Man-5 to Man-9 total percentages were calculated against the total area of the chromatogram.

the oligomannose glycans Man-5 to Man-9. Also, hybrid structures were observed where the first antenna was expanded with a GlcNAc, followed by either a galactose (LacNAc) or a GalNAc (LacdiNAc). Complex-type glycan structures were also identified, consisting of diantennary structures of varying compositions. No tri- or tetra-antennary structures can be found in this study. With the exception of the oligomannose-type glycans, the structures were capped with sialic acid, either Neu5Ac or Neu5Gc.

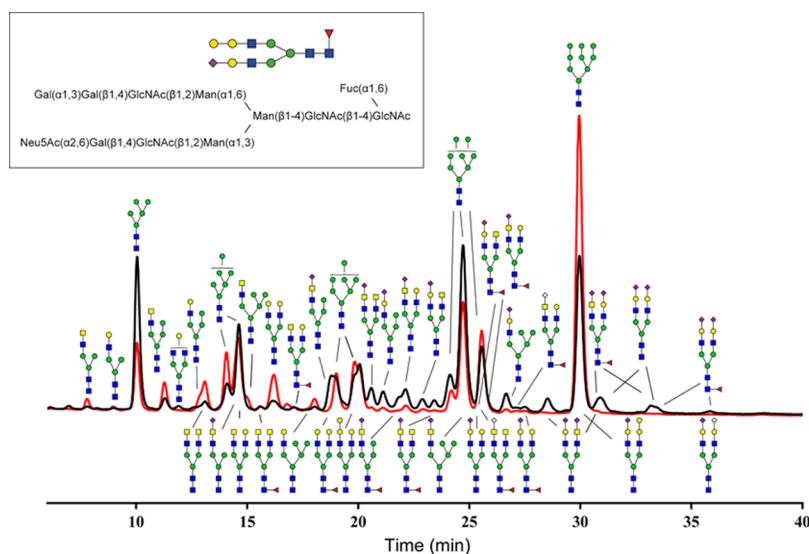
The oligomannose glycans were readily identified by their elution at regular intervals (Figure 2), with the smaller glycans eluting earlier than the larger ones. Man-5 and Man-9 occurred in single configurations, while isoforms of Man-6, Man-7, and Man-8 were present, with different configurations of the outer mannoses on the glycan antennae. These isoforms were resolved by chromatography, resulting in multiple peaks. When interpreting the chromatograms of these oligomannoses, care should be taken that the isoforms are added together when estimating the total amount of Man-6 to Man-8 (Supporting Information, Figure S1).

When comparing the colostrum lactoferrin glycoprofiles, several changes became apparent (Figure 2). The largest observable shift occurred in the oligomannose structures. The



**Figure 2.** Lactoferrin glycoprofiles from colostrum (Col), milk (Milk), and predry (Dry) period milk over two subsequent lactation cycles of cow 3 [period 1 (p1) and period 2 (p2)]. Time notation in hours (h) and days (d). The glycoprofiles of the other cows are shown in Figures S5–S11.

obtained chromatograms were integrated to gain insight into the exact distribution of these glycans in colostrum, mature, and predry period milk samples. In addition, the percentage of oligomannose-type glycans against the total chromatogram area was calculated (Table 4). In all samples, from early colostrum to mature milk, the percentage of oligomannose glycans versus the total glycan pool was approximately 80%. In early colostrum, Man-8 was the most abundant glycan, more than 30% of the total oligomannose pool. Man-5, 6, 7, and 9



**Figure 1.** Overlay of the lactoferrin glycoprofiles of colostrum (black line) versus mature milk (red line) of cow 4. Identified structures are annotated. A list of the corresponding glycan masses can be found in Table 3.

were present at nearly equal percentages. Compared to mature milk, Man-5 was present in significantly higher quantities in early colostrum. In contrast, the largest oligomannose structure, Man-9, was present in a much decreased concentration in early colostrum. Both these glycans showed a more than twofold change in concentration over the sampling period (Table 4). No large difference was observed in the total concentration of Man-6 and 7, although shifts occurred in the isomer distribution.

In addition to the shifts in the oligomannose pattern, the degree of sialylation also showed clear temporal changes. In early colostrum, sialylated hybrid and complex-type biantennary structures were observed. The concentration of sialylated structures rapidly decreased over the colostrum period, while the concentration of the unsialylated counterparts increased later in lactation. To quantify these changes, two glycans were selected, based on their separation from the oligomannose peaks, making them suitable for integration (Table 3, nos. 5, 14, 15, and 28). The sialylated glycan percentages were highest in colostrum and predry period milk, and the ratio shifted to the nonsialylated form in mature milk (Table 5). In the predry

**Table 5. Peak Area Percentages (against the Total Chromatogram) of Two Selected Glycan Structures<sup>a</sup>**

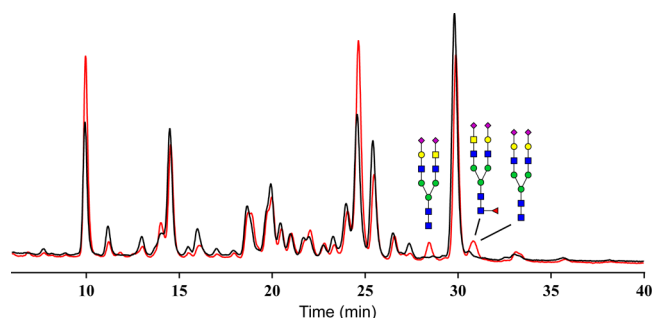
	colostrum ~0 h	colostrum ~72 h	predry	mature
not sialylated (5,14)	1.1 ± 0.3	2.0 ± 0.5	1.8 ± 0.5	2.8 ± 0.3
sialylated (15,28)	3.8 ± 0.6	2.6 ± 0.6	2.1 ± 0.8	1.2 ± 0.6
total	4.9 ± 0.6	4.6 ± 0.6	3.9 ± 0.5	3.9 ± 0.6

<sup>a</sup>Selected glycan structures: sialylated (Table 3, no. 15 and 28) and without sialylation (Table 3, no 5 and 14) in colostrum ( $n = 15$ ), predry period ( $n = 7$ ), and mature milk ( $n = 16$ ).

period a reversal of the ratio was observed, making it similar to colostrum. While the ratio shifted, the total amount of the sialylated and nonsialylated forms remained stable.

MS analysis consistently showed double peaks for the doubly sialylated diantennary glycans. This indicated the presence of not only the commonly described ( $\alpha$ 2,6)-linked sialic acid, but also of ( $\alpha$ 2,3)-linked sialic acid. The ( $\alpha$ 2,6) and ( $\alpha$ 2,3) isomers were separated by hydrophilic interaction liquid chromatography, with structures containing ( $\alpha$ 2,3)-linked sialic acid eluting earlier than their ( $\alpha$ 2,6)-linked counterparts.<sup>28</sup> We confirmed the presence of ( $\alpha$ 2,3) sialic acid in an exoglycosidase digestion with ( $\alpha$ 2,3) specific sialidase (Figure S2). Structures containing ( $\alpha$ 2,3) were most prominent in the earliest colostrum sample of cow 4 (Figure 3), with lesser levels in cows 1–2. Only trace amounts were detected in colostrum of cows 5 and 7, while for cows 6 and 8 these structures were very low or absent and were not visible past the first colostrum sample (Supporting Information, Figures S5–S11). The ( $\alpha$ 2,3) sialylated structures were not detected in the mature milk samples of all 8 cows. Structures capped with Neu5Gc instead of Neu5Ac were rare, with the higher quantity detected in colostrum and decreasing to trace levels in mature milk.

In early colostrum samples, fucosylated diantennary complex-type structures were present, and the majority of which was also capped with one or two sialic acids. Based on the decreasing peak area of identified fucosylated structures observed in the chromatograms, the amount of fucosylated structures was highest in early colostrum and decreased over



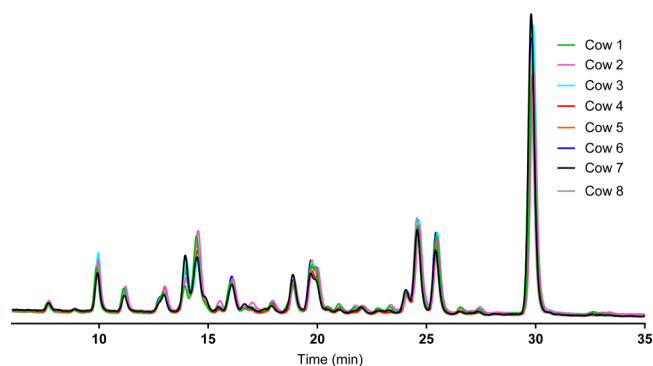
**Figure 3.** Comparison of the lactoferrin glycosylation profiles in the first colostrum sample of cows 4 (SRB–HF–MRY/50–37.5–12.5%; red line) and 8 (HF/100%; black line). Doubly sialylated glycans with both ( $\alpha$ 2,3)- and ( $\alpha$ 2,6)-linked Neu5Ac are annotated in the HPLC chromatogram.

time, in a similar way as the sialylated structures. Only a very limited number of fucosylated glycan structures was observed in mature milk.

The overall glycosylation shifts in oligomannoses, sialylated and fucosylated structures occurred very rapidly in some cows (4, 7, and 8), stabilizing after 72 h into a profile that resembled that of mature milk. In other cows (2, 3, and 5), the shifts were more gradual with the glycosylation profile not maturing during this relatively short time period. In all cases however, the mature profile was observed at the 1 month time point.

The glycoprofiles of lactoferrin obtained from milk samples at the end of lactation (start of the dry period) resembled those of the colostrum samples. Man-5 increased in concentration, while the Man-9 glycan decreased in concentration when compared to the 3 months milk samples. In addition, more sialylated glycans were found in this predry period milk. In order to investigate if the number of days until delivery influenced the glycoprofile, a visual comparison was made between the predry period milk obtained  $14 \pm 5$  days prior to delivery (cows 1, 3, 5, and 6) and  $30 \pm 5$  days (cows 2, 7, and 8). In this dataset, no clear relation between the number of days prior to delivery and the alteration of the glycoprofile could be established (Figure S12).

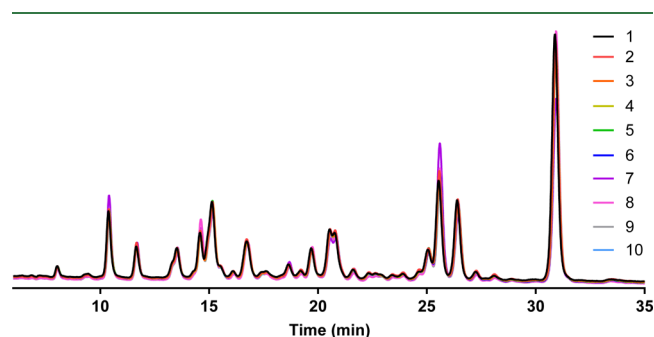
**Comparison of the Glycosylation Profiles of Lactoferrin in Mature Milk and Lactoferrin from Commercial Sources.** The lactoferrin glycoprofiles of mature milk from 8 different cows of diverse genetic breeds (Table 1) were very similar (Figure 4). This suggests an evolutionarily conserved



**Figure 4.** Glycoprofiles of lactoferrin in mature milk collected at 3 months postparturition, from 8 cows of diverse genetic backgrounds (Table 1).



strong regulation of lactoferrin glycosylation. It is highly likely that the glycosylation pattern of lactoferrin is important for its functionality. In addition, we obtained 10 commercial lactoferrin samples collected over a year from a single production plant. Again, the glycoprofiles were strikingly similar, with the exception of sample 7, which showed an oligomannose profile corresponding to not fully matured milk (Figure 5). Although the samples were not dated, based on the glycoprofile, this sample may have been produced in the fall season, as this is peak calving season in the Netherlands.<sup>29</sup>



**Figure 5.** Glycoprofiles of 10 commercial lactoferrin samples. Over a year, samples were periodically collected from a single production plant. Each sample was analyzed in triplicate. The results of the triplicate analysis were averaged prior to plotting the data.

## DISCUSSION

Isolation of lactoferrin from milk is a relatively simple procedure, for which several well described methods exist.<sup>22,30–32</sup> Capturing lactoferrin with cation-exchange resins is a very robust and reliable way of isolating this protein. Other glycoproteins also adsorb to cation-exchange resins, but lactoferrin binds most strongly, allowing elimination of such contaminating proteins in washing steps.<sup>33</sup> While commonly applied, manual extraction of single samples either in batch, or column mode is unsuitable for processing of a larger number of samples. The isolation of lactoferrin can be significantly sped up by downscaling and optimizing the existing techniques and adopting high-throughput options, such as the use of a 96 well plate vacuum manifold. With the protocol described here, approximately 50 samples can be analyzed in just a few days (Figure S4). The limiting factor now lies in the chromatographic analysis, which can take up to 1 h per sample depending on the required separation and detail needed for interpretation of the data. When using fluorescent labeling and detection, the analysis can be performed with very low amounts of lactoferrin and allows isomeric glycan separation.

Using this optimized protocol, lactoferrin was isolated from a total of 116 individual milk samples, from 8 cows with varying genetic backgrounds, over two subsequent lactation cycles. In addition, 10 commercial lactoferrins from a single production plant were analyzed to investigate the heterogeneity of their glycosylation profiles. The data generated from these samples provided an in-depth view into the glycosylation of lactoferrin in early colostrum, mature milk, and predry period milk.

The glycosylation of lactoferrin from mature milk had been well studied,<sup>14,15,18,34</sup> but limited data existed on the glycosylation of lactoferrin from colostrum.<sup>19,21</sup> Previously, Takimori et al. studied the *N*-glycome of bovine milk glycoproteins during lactation. In their study samples were

taken weekly for a duration of 4 weeks after parturition. The oligomannose-type glycans were quantified, which originate predominantly from lactoferrin. Marked differences were observed in the oligomannose balance, between the first sample and the one-week sample, with minor changes occurring after 1 week.<sup>19</sup>

A more detailed analysis of early stage temporal changes in lactoferrin glycosylation in colostrum has been reported previously.<sup>21</sup> This study determined glycan composition using a lectin microarray which provided insight into the types of glycans present, and the shifts occurring over a 10 day period postparturition. Based on the results, they hypothesized that changes occurred in the oligomannose levels of lactoferrin. In addition, shifts in the sialylation and fucosylation were reported. Our study reproduces data of these earlier reports, but also reports the exact oligomannose balance, in depth visual structural information, together with detailed information about the sialic acid linkage types present. In addition, we report the glycoprofile of the predry period milk. Lastly, the lactoferrin concentration and its total production in all samples was determined; information that may allow further interpretation of the implications of the glycoprofile differences observed.

The glycosylation of lactoferrin in colostrum dynamically changed in time, involving modifications to the oligomannose glycans (1), or to the hybrid and complex-type glycans (2). The glycoprofile of lactoferrin is dominated by oligomannose-type glycans, rendering changes in this group most evident. In early colostrum, higher amounts of Man-5 and Man-8 were detected. Over a short 72 h period, the profile underwent a rapid shift, with Man-5 and Man-8 decreasing and Man-9 becoming the dominant oligomannose of lactoferrin in mature milk. Earlier, Man-8 was reported to be the major oligomannose of mature milk lactoferrin.<sup>18,35</sup> This discrepancy may be because of differences in the methods of quantification and sample processing used. The fluorescence detection used in our study results in a more reliable quantification than MS. The analysis of lactoferrin glycans from a large number of individually isolated milk samples from separate sources clearly shows that Man-9 is dominant in mature milk lactoferrin.

In mature milk, the lactoferrin oligomannose pattern is very stable, yielding similar results in the 8 cows of mixed genetic background (Figure 4), as well as in 10 different commercial lactoferrin samples collected over a year (Figure 5).

We observed a large diversity of, and variation in, the lactoferrin hybrid and complex-type glycans over the measured period. The abundance of sialylated structures is one of the most prominent variations observed in the colostrum versus mature glycoprofile. Mono and doubly sialylated structures were readily observed in the earliest colostrum sample, decreasing rapidly in quantity over the 72 h colostrum period, down to very low levels in mature milk. The main sialic acid observed was Neu5Ac, whereas Neu5Gc was detected in low amounts only, as reported previously.<sup>18</sup>

In previous studies, no ( $\alpha$ 2,3)-linked sialic acid was detected.<sup>18,21</sup> However, in this study ( $\alpha$ 2,3)-linked Neu5Ac was detected on doubly sialylated diantennary glycans and confirmed by exoglycosidase assay (Figure S2). This ( $\alpha$ 2,3)-linked sialic acid was observed with the highest intensity in the early colostrum samples of cow 4 (SRB–HF–MRY/50:37.5:12.5). It was also observed in other cows with a mixed genetic background (cow 1, FV–unknown–HF/62.5–25–12.5) (cow 2, FV–HF–MRY/50–37.5–12.5). Trace

amounts were detected in Meuse-Rhine-Yssel (MRY)–HF mixed breeds (cow 3, HF–MRY/87.5–12.5) (cow 5, HF–MRY/50–50) and in one cow with unknown genetic background (cow 7). The amount of ( $\alpha$ 2,3)-linked sialic acid was lowest in the pure HF breeds (6 and 8). In mature milk, ( $\alpha$ 2,3)-linked sialic acid was not detected. While the number of cows was limited in this study, there may be a genetic factor that contributes to the presence of ( $\alpha$ 2,3)-linked sialic acid on lactoferrin glycans in the colostrum phase. In previous studies, lactoferrin from pure HF cows and/or mature milk-derived lactoferrin, was used for the determination of glycans, which may explain the absence of ( $\alpha$ 2,3)-linked sialic acid in these reports. In future studies, colostrum from MRY, Fleckvieh (FV), Swedish Red (SRB), and HF pure breeds will be investigated in more detail to give insight into this specific type of sialylation on bovine whey proteins over the course of lactation.

The  $\alpha$ -Gal epitope was detected in trace amounts, on diantennary structures with and without sialylation (Table 3, Figure S3). In contrast, the GalNAc-( $\beta$ 1,4)-GlcNAc (LacdiNac) epitope is highly abundant in bovine glycosylation, reflected by the large number of structures containing this epitope detected in this study. Similar to the overall higher sialylation early on in lactation, the sialylated  $\alpha$ -Gal or LacdiNac structures were detected more in colostrum samples, while the nonsialylated structure is more prevalent in mature milk. Also, more fucosylated structures were observed early in lactation, compared to mature milk.

The maturation of the lactoferrin glycoprofile was very rapid, completing within 72 h in some cows (1 and 8) and in all cows within the first month of lactation. In mature milk, no further modifications were observed in the first three months which were analyzed in this study.

The altered colostrum glycan profiles (compared to the stable mature milk profiles) may suggest that they play a functional role during the first few days after parturition. Interestingly, in this study we found that shortly before the dry period between lactation cycles, the lactoferrin glycoprofile started to revert to a structural composition resembling that of late colostrum. The modification of the glycoprofile in predry period milk may reflect a preparation for colostrum production for the expected newborn calf. Alternatively, it may be the result of a changed regulation of transcription of glycosyltransferase genes, based on metabolic adaptations during the recovery period. Finally, the lower milk production during the predry period stage may influence processing time of glycans in the Golgi and thereby influence the glycoprofile. In this study, we did not observe a correlation between the number of days predelivery and either the glycoprofile, or the lactoferrin concentration in the milk. This suggests that the glycosylation of lactoferrin during the predry period is not regulated in anticipation of the newborn calf, but the number of samples in our study was too limited to draw solid conclusions. A more in-depth analysis of end-stage lactation and predry period milk therefore is an interesting target for future study.

Milk yield in cows is optimal when a short interruption is made in the lactation between subsequent deliveries (Kuhn, Hutchison, and Norman, 2005). During this dry period, the mammary cells can recover and prepare for the subsequent lactation cycle (Capuco, Akers, and Smith, 2010). In addition to improving the overall milk yield, this period is also beneficial for the cow's health, minimizing the risk of mastitis (Odensten, Berglund, Waller, and Holtenius, 2010). The cows are dried off

for an average of 7 weeks, although a shorter or longer period can be chosen by the farmer (Bertulat, Fischer-Tenhagen, and Heuwieser, 2015). Shorter periods of 30 days or less, will result in a lower milk yield during early lactation, but improves the energy balance of the cow during this critical stage (van Knegsel, Rummelink, Jorjong, Fievez, and Kemp, 2014). In this study, the cows were dried off for this shorter period.

The concentration of lactoferrin was constant during the mature phase of the lactation and increased after cessation of normal milking.<sup>36</sup> We also observed higher concentrations of lactoferrin in the milk collected just before the dry period compared with the mature milk phase (Table 1). This may suggest that the production rate of lactoferrin is higher in the predry and colostrum periods. Based on the calculated total amount of lactoferrin produced, a higher production rate of lactoferrin in colostrum was confirmed. The production rate of lactoferrin in the predry period appeared to be similar to that in mature milk (Table 1). Nevertheless, in the predry period milk the lactoferrin glycosylation resembles that of colostrum. The observed changes in glycosylation may be the result of increased expression or activity of the glycosidases and transferases involved in protein glycosylation. An increased expression of these transferase genes was observed in goat colostrum versus mature milk phases.<sup>37</sup> In the same study, nucleotide sugar transporter genes were also found to be upregulated in the colostrum period. Similar studies on the expression of these enzymes in bovine colostrum are very limited. In one study, an increased expression of these transferase and glycosidase genes was observed in late lactation milk (day 250) compared to transitional milk (day 15).<sup>38</sup> The changes in lactoferrin glycosylation in colostrum and predry period milk observed in our work thus may be based on an increased activity of the protein glycosylation pathway. This remains to be studied further.

In order to interpret the implications of the modifications to the glycoprofile in early lactation and predry period milk, the specific function of the carbohydrate structures has to be understood. However, the specific function of glycan structures with different composition is still relatively unknown. For example, while LacdiNac epitopes are highly abundant on bovine lactoferrin, little is known about the function of this epitope. In humans, this epitope is expressed on the hormone lutropine, where it is involved in the clearance of the protein.<sup>39</sup> It is also found on glycodefin, a protein with high immunosuppressive properties.<sup>40</sup> The relation of this LacdiNac epitope with immune modulation is also supported by the expression on highly antigenic parasitic worms, where the glycans are suggested to be involved in modulating the host immune system.<sup>41</sup> In bovines, the LacdiNac epitope is involved in the differentiation of mammary epithelial cells.<sup>42</sup>

The  $\alpha$ -Gal epitope is absent in certain primates, including humans, because of a defect in the  $\alpha$ -1,3-galactosyltransferase gene.<sup>43</sup> The presence of this epitope on proteins may trigger transplant rejection<sup>44</sup> and red meat allergy<sup>45</sup> in humans. While the  $\alpha$ -Gal epitope is relatively abundant on other bovine glycoproteins such as thyroglobulin,<sup>46</sup> only trace amounts were detected on lactoferrin.

Changes in bovine immunoglobulin G (IgG) glycosylation did not influence its interaction with FcRn protein.<sup>19</sup> In contrast, modifications in the oligomannose profile of lactoferrin had different effects on reporter cell lines.<sup>47</sup> These experiments were performed with lactoferrin with glycoprofile modifications, either with a trimmed oligomannose, or with a

desialylated glycoprofile. While these modifications are more extreme than is observed in the colostrum glycosylation shift of lactoferrin, these experiments give an insight into the potential function of the oligomannose profile and sialylation of early colostrum. Lactoferrin with a trimmed oligomannose profile demonstrated a stronger stimulatory effect on Toll-like receptor 4 (TLR4). This specific pattern recognition receptor is known for having a strong link with the innate immune system, yielding a proinflammatory response on activation.<sup>48</sup> In contrast, desialylated lactoferrin demonstrated a lower response compared to the native profile. The observation of a higher sialylation early on in lactation and the higher occurrence of shorter oligomannoses on colostrum lactoferrin therefore seems to complement each other to potentially elicit a stronger TLR4-mediated response.

Lactoferrin has a very high bacteriostatic function, and the high concentration in the predry period milk has been directly linked to a high bacterial resistance of the mammary gland.<sup>49</sup> The altered glycoprofile of the lactoferrin in the predry period milk could further enhance the immune properties and aid in the protection of the mammary glands in this critical rest period. This hypothesis is supported by the TLR4-mediated response reported.<sup>47</sup>

In addition to TLR-mediated properties of the glycans of lactoferrin, they also act as important decoys for microorganisms. Certain pathogens, such as *Clostridium difficile*, secrete toxins that bind to the  $\alpha$ -Gal epitopes.<sup>50</sup> Neu5Gc is present in most mammalian species, with the exception of humans, because of a mutation in the CMP-Neu5Ac hydroxylase gene. While the presence of the foreign Neu5Gc on cow milk proteins is generally considered as unfavorable for human consumption, it does provide additional functionalities for the newborn calf. Certain bacterial toxins, such as the SubAB toxin produced by Shiga toxigenic *Escherichia coli*, bind with a strong specificity to Neu5Gc.<sup>51</sup> Viruses also have affinity to sialic acid structures, varying in strength depending on the linkage type ( $\alpha$ 2,3) or ( $\alpha$ 2,6);<sup>52</sup> *E. coli* species express FimH, a lectin that is used to attach to mannose-expressing host cells.<sup>53</sup> This lectin is also found in certain *Salmonella* species.<sup>54</sup> It has a strong binding affinity to Man-5 to Man-9, the glycans that are predominantly expressed on bovine lactoferrin. Certain pathogenic bacteria adhere to fucosylated glycans,<sup>55</sup> suggesting that higher fucosylated lactoferrin acts as a potential decoy as well.<sup>56</sup>

Sialic acid residues are also of nutritional value to the newborn calf. The majority of the sialic acid found in bovine milk is protein bound, therefore the glycoproteins are the major source of sialic acid for the newborn calf.<sup>57</sup> It can be released from the glycoproteins by bacterial neuraminidases and taken up by the intestinal tract.<sup>58,59</sup> In a study on piglets, dietary sialic acid has been proven to be important for neural development.<sup>60</sup>

Rossi et al. demonstrated that the sialic acid present on lactoferrin assists in the binding of calcium by lactoferrin.<sup>61</sup> By acting as a calcium chelator, lactoferrin enhances the lipopolysaccharide release from Gram negative bacteria, which as a result damages their cell wall.<sup>62</sup>

Sialic acid also aids in iron binding to the lactoferrin structure.<sup>63</sup> The iron-containing holo-lactoferrin is more stable and resistant to bacterial digestion than the iron-free apo-lactoferrin, thereby increasing its longevity in the milk. The bacteriostatic properties of lactoferrin have also been strongly

linked to its iron-binding capacities,<sup>8,9</sup> which thus also may be increased by the sialylation status of lactoferrin.

While the specific function of core fucose on lactoferrin is currently unknown, this motif is known to highly influence receptor docking of IgG.<sup>64</sup> Core fucosylated glycans present in milk are known to have a positive effect on the growth and colonization of certain bacterial species in the infant gut. For example, growth of *Bifidobacterium* and *Lactobacillus* strains are selectively promoted by specific glycans. Furthermore, the metabolites produced by these bacterial strains stimulate B-cell activation, thereby supporting the immune system.<sup>65</sup> Fucose can be released from the glycoproteins by microbial  $\alpha$ -fucosidases expressed by *Bacteroides thetaiotaomicron*.<sup>66</sup> This released fucose can then be utilized by the host, as well as by other microorganisms.<sup>67</sup>

While a large amount of data is now available on the glycosylation of lactoferrin in different lactation stages, many questions are still unanswered. While the last sample prior to the dry period displays a modified glycoprofile, the initiation of these changes potentially occurs earlier already and warrants further study of the end stage lactation. Currently, most of the research toward lactoferrin has been performed on HF cows. Glycosylation studies toward other breeds, such as the SRB and FV, potentially will yield new insights, such as the higher prevalence of the ( $\alpha$ 2,3) sialic acid that was detected in this study. Lactoferrin isolated from colostrum offers a unique glycoprofile with different functional characteristics from mature lactoferrin. In vitro and in vivo studies with colostrum lactoferrin may provide new insights into the specific functionality of these glycans. Such studies toward the effects of modifications in glycan composition on their functional properties are underway, first of all necessitating efficient protocols for the isolation of larger amounts of lactoferrin glycans.<sup>47,68</sup> A comparison of lactoferrin and its isolated glycans from mature milk and colostrum therefore is an interesting target for future studies.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.9b06762>.

Sample collection times of the milk and colostrum samples; parturition dates, offspring gender of both periods; milk parameters, determined during the control of the regular milking, collected pre- and postparturition; isomer elution pattern of the oligomannose glycans; exoglycosidase assay procedure and chromatograms; sample preparation schedule of the lactoferrin analysis procedure; lactoferrin glycoprofiles of colostrum milk and predry period milk of cow 1, 2, 4, 5, 6, 7, and 8; chromatogram overlays of predry period milk; SDS-polyacrylamide gel electrophoresis of lactoferrin isolation by cation exchange resin; and chromatogram comparison of 2-AB versus 2-AA labeled glycans (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*E-mail: [s.s.van.leeuwen@umcg.nl](mailto:s.s.van.leeuwen@umcg.nl)

### ORCID

Rivca L. Valk-Weeber: 0000-0001-7186-6045

Lubbert Dijkhuizen: 0000-0003-2312-7162

Sander S. van Leeuwen: 0000-0003-0017-2841

## Present Addresses

<sup>§</sup>Carbexplore Research BV, Zernikepark 12, 9747 AN Groningen.

<sup>||</sup>Laboratory Medicine, University Medical Center Groningen (UMCG), Hanzplein 1, 9713 GZ, Groningen, The Netherlands.

## Notes

The authors declare no competing financial interest.

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