Rapid, quantitative analysis of 3'- and 6'-sialyllactose in milk by flow-injection analysis–mass spectrometry: Screening of milks for naturally elevated sialyllactose concentration

Van Kelly,* Steve Davis,† Sarah Berry,† Janine Melis,‡ Richard Spelman,‡ Russell Snell,†§ Klaus Lehnert,†§ and David Palmer*¹

*Bender MassSpec, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

†ViaLacta Biosciences, PO Box 109-185, Newmarket, Auckland 1051, New Zealand

‡Livestock Improvement Corporation Limited, Private Bag 3016, Hamilton 3240, New Zealand

§School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

ABSTRACT

Non-protein-bound oligosaccharides are important bioactive components of cow milk, with potential human-health benefits such as stimulation of the growth of beneficial gut bacteria and defense against pathogens. In bovine milk, the majority of oligosaccharides are sialylated; 3'-sialyllactose (3'-*N*-acetylneuraminyld-lactose; 3'-SL) is the predominant sialylated oligosaccharide, followed by 6'-sialyllactose (6'-*N*-acetylneuraminyld-lactose; 6'-SL). Both 3'-SL and 6'-SL have antimicrobial activity. As bovine milk products such as infant formula can be an important component of the human diet, and the concentrations of 3'-SL and 6'-SL are lower in bovine milk compared with human milk, we aimed to identify cows that naturally produce higher concentrations of sialyllactose in their milk. Milk from such cows could be used to produce foods with an increased sialyllactose content, potentially providing increased health benefits. We speculated that cows overexpressing 3'-SL and 6'-SL would exist at low frequency in the population and, to allow their efficient identification, we developed a novel assay for 3'-SL and 6'-SL utilizing flow-injection analysis–mass spectrometry, which could be used for high-throughput analysis of milk samples. We then determined 3'-SL and 6'-SL concentrations in milk samples from 15,507 cows from Friesian, Jersey, and Friesian-Jersey crossbred animals. We found 329 cows with concentrations of 3'-SL or 6'-SL >2-fold higher than the mean, 26 cows with concentrations of 3'-SL or 6'-SL >3-fold higher than the mean, and 1 cow with concentrations of 3'-SL >4-fold higher than the mean. Although these outliers were observed across the 3 groups of cows, breed had a strong effect on mean 3'-SL and 6'-SL concentrations.

Key words: sialyllactose, mass spectrometry, milk

INTRODUCTION

Significant differences exist between the free oligosaccharides of human milk and those of farm animals. The concentration of oligosaccharides in human milk (8–14 g/L; Coppa et al., 1993; Martinez-Ferez et al., 2006) is much higher than that in caprine, bovine, or ovine milks, which contain 0.25 to 0.30, 0.03 to 0.06, and 0.02 to 0.04 g/L of oligosaccharide, respectively (Martinez-Ferez et al., 2006). Human milk appears to contain a more diverse array of oligosaccharide structures. Approximately 200 distinct oligosaccharides have been identified in human milk (Ninonuevo et al., 2006), whereas only about 40 distinct oligosaccharides have been identified in bovine milk (Tao et al., 2008). Approximately 70% of oligosaccharide species in human milk are fucosylated and approximately 20% are sialylated (Ninonuevo et al., 2006), whereas in bovine milk approximately 70% of the oligosaccharides are sialylated (Saito et al., 1987; Mariño et al., 2011) and only a few fucose-containing oligosaccharides have been described (Sundekilde et al., 2012). Goat milk also contains a large proportion of sialylated oligosaccharides (Martinez-Ferez et al., 2006) and there are conflicting reports about the presence of fucosylated oligosaccharides in goat milk (Nakamura and Urashima, 2004; Martinez-Ferez et al., 2006).

In bovine milk, 3'-sialyllactose (3'-*N*-acetylneuraminyld-lactose; **3'-SL**) is the predominant sialylated oligosaccharide; bovine milk contains much lower concentrations of its isomer 6'-sialyllactose (6'-*N*-acetylneuraminyld-lactose; **6'-SL**; Gopal and Gill, 2000; Martín-Sosa et al., 2003; Nakamura et al., 2003; McJarrow and van Amelsfort-Schoonbeek, 2004; Tao et al., 2009; Fong et al., 2011). Both 3'-SL and 6'-SL exhibit antimicrobial activities (see Newburg et al., 2005 for a review) and 3'-SL also has antiinflammatory activity (Bode et al., 2004). The levels of 3'-SL and 6'-SL in infant formula are lower than those in bovine milk (approximately 3 times less 3'-SL and 1.4 to 2.3

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¹Corresponding author: d.palmer@auckland.ac.nz

times less 6'-SL; Fong et al., 2011) from which it is derived, as well as than those in human milk (Martín-Sosa et al., 2003). In this study, we aimed to identify cows with considerably higher concentrations of 3'-SL, 6'-SL, or both, in their milk. Such cows could be used to produce ingredients for infant formula with much higher amounts of sialyllactose (SL).

Previous studies of SL in colostrum and mature milk have revealed variation in the SL concentrations among cows of the same breed and between breeds (Nakamura et al., 2003; McJarrow and van Amelsfort-Schoonbeek, 2004; Barile et al., 2010; Sundekilde et al., 2012). However, the number of animals in these studies was either low (≤ 10) or pooled herd samples were analyzed, and the observed range of variation in SL concentrations was low (≤ 2 -fold). We expected that cows producing much more than 2-fold higher concentrations of 3'-SL, 6'-SL, or both, would exist in the population at low frequency. We aimed to identify such phenotypic outliers by analyzing milk samples from thousands of cows, and for this a fast analytical method for 3'-SL and 6'-SL was required. Reported methods for the analysis of SL in milk are based on separation of oligosaccharides by HPLC after deproteination of the milk sample (Martín-Sosa et al., 2003; McJarrow and van Amelsfort-Schoonbeek, 2004; Barile et al., 2010; Fong et al., 2011; Sundekilde et al., 2012). Separation by HPLC enables resolution and analysis of a large number of oligosaccharides. However, the required analysis times are relatively long and their application to the analysis of SL in a large number of samples would be tedious. Therefore, we developed a rapid method for the simultaneous analysis of 3'-SL and 6'-SL in unfractionated samples based on flow-injection analysis-tandem mass spectrometry. The method is compatible with proteinaceous samples, which allows very rapid and simple sample processing. We applied this method to the analysis of milk samples from 15,507 individual cows from commercial dairy herds.

MATERIALS AND METHODS

Reagents

3'-Sialyllactose (A8681) and 6'-SL (A8556) were purchased from Sigma-Aldrich (St Louis, MO). Bronopol was purchased from Chemiplas NZ Ltd. (Auckland, New Zealand). Methanol and acetic acid were purchased from Merck (Palmerston North, New Zealand).

Milk Samples

Milk was collected from Friesian ($n = 5,253$), Jersey ($n = 3,400$), and Friesian-Jersey crossbred ($n = 6,854$) cows between mid-October and mid-December 2009

from 900 herds throughout the North Island of New Zealand. The majority of these cows calved in July or August 2009. Fifty herds contributed more than 14,300 of all samples, and the remainder of herds provided up to 5 samples each to complete the data set. The mean parity of the cows was 3.90 ± 0.02 (SEM) and the mean DIM was 77.8 ± 0.2 (SEM). Animals were milked twice daily and composite samples from the morning and afternoon milking from each animal were taken. Bronopol (10% wt/vol; 2-bromo-2-nitropropane-1,3 diol) was added as a preservative to 0.02% in the sample. The milk samples were taken as part of routine herd testing and an aliquot frozen at -80°C (approximately 24–48 h after sampling on-farm) until SL analysis was carried out.

Sample Preparation for SL Analysis

Milk samples were thawed, mixed, centrifuged ($1,000 \times g$, 5 min, room temperature) and then skimmed by pipetting from under the fat layer. Whey was prepared from skim milk by the addition of an equal volume of 2% acetic acid, mixing, and centrifugation ($3,200 \times g$, 20 min, 8°C) to sediment the caseins. The whey was aspirated and then serially diluted with 30% methanol:0.1% acetic acid to give a final dilution of 800-fold and analyzed by flow-injection analysis-mass spectrometry (FIA-MS).

3'-SL Standards

Standards of 3'-SL (0–3 mg/L) were prepared for FIA-MS in a sample of whey that was pooled from 100 randomly selected animals and diluted with 30% methanol:0.1% acetic acid. The final dilution of the whey was 800-fold.

FIA-MS

Analyses were carried out using an Agilent Technologies (Palo Alto, CA) SL ion-trap mass spectrometer fitted with an electrospray ionization interface. Solutions of 3'-SL and 6'-SL in 30% methanol:0.1% acetic acid were infused into the mass spectrometer at 12 $\mu\text{L}/\text{min}$ using a syringe pump to optimize the instrument and to collect MS and MS/MS spectra for these compounds. The optimized parameters were as follows: capillary exit -275 V, skimmer -50 V, octopole-1 -7.5 V, octopole-2 -0.5 V, and a trap drive of 35. The MS and MS/MS spectra of 3'-SL and 6'-SL were collected in negative ion mode, scanning 150 to 1,000 m/z .

The HPLC/MS system comprised an Agilent Technologies 1100 series isocratic pump (G1310A), fitted with a backpressure regulator (750 psi; Upchurch Sci-

entific, Oak Harbor, WA), and a thermostat-controlled well-plate sampler (G1367A; maintained at 6°C) with the mass spectrometer described above. The pump delivered 30% methanol:0.1% acetic acid at 0.1 mL/min, and 30 μ L of diluted whey samples and 3'-SL standards were injected during flow-injection analysis. The mass spectrometer was operated in negative ion MS/MS (pseudo-multiple reaction monitoring) mode, isolating the parent ions at 632.3 m/z , fragmenting for 40 ms at 1.3 V with SmartFrag off, and scanning the product ions from 280 to 580 m/z .

Calculation of 3'-SL and 6'-SL Concentrations from FIA-MS Data

1. The 572.2 m/z (a) and 290.0 m/z (b) product ion signals were summed over the flow-injection analysis duration by calculating extracted ion chromatograms using Agilent 6300 Series Ion Trap LC/MS data analysis software version 6.1 (Agilent Technologies).
2. The 572.2 m/z to 290.0 m/z ratio (c) was calculated from the product ion signals: $c = a/b$.
3. The relative proportion of 3'-SL (d) in the analyte was calculated from the 572.2:290.0 ratio (c) and interpolated from the calibration shown in Figure 2 using the best-fit equation: $d = -0.069769c^3 + 0.3942c^2 - 1.0216c + 1.039$.
4. The fractions of the 290.0 m/z signal derived from 3'-SL (g) and 6'-SL (h) were calculated according to the proportions of 3'-SL and 6'-SL in the analyte (from step 3) and the difference in intensity of the 290.0 m/z signals obtained at equimolar concentrations (3'-SL:6'-SL; 0.655:0.345. See Results for derivation of this ratio) derived from each compound. First,

$$\begin{aligned} \text{3'-SL component of the 290.0 } m/z \text{ signal (e)} = \\ d \times 0.655, \end{aligned}$$

$$\begin{aligned} \text{6'-SL component of the 290.0 } m/z \text{ signal (f)} = \\ (1 - d) \times 0.345. \end{aligned}$$

Then, the values are normalized so that they sum to 1:

$$\text{3'-SL fraction of the 290.0 } m/z \text{ signal (g)} = e/(e + f),$$

$$\text{6'-SL fraction of the 290.0 } m/z \text{ signal (h)} = f/(e + f).$$

5. The 290.0 m/z signal (b) was then divided into 2 components corresponding to 3'-SL and 6'-SL:

$$\text{3'-SL 290.0 } m/z \text{ signal (i)} = g \times b,$$

$$\text{6'-SL 290.0 } m/z \text{ signal (j)} = h \times b.$$

6. The absolute concentration of 3'-SL was calculated by comparing the 3'-SL 290.0 m/z signal (i) to the 290.0 m/z signals of the 3'-SL standard curve (Figure 3), correcting for dilution of the sample (800-fold), and correcting for the 290 m/z signal in the standard curve arising from endogenous SL in the whey diluent. The concentration of 6'-SL was calculated by comparing the 6'-SL 290.0 m/z signal (j) to the 290.0 m/z signals of the 3'-SL standard curve and correcting for the higher 290.0 m/z signal from 3'-SL (3'-SL:6'-SL; 0.655:0.345), dilution of the sample (800-fold), and correcting for the 290 m/z signal in the standard curve arising from endogenous SL in the whey diluent.

RESULTS

Development of an Assay for 3'-SL and 6'-SL

3'-Sialyllactose and 6'-SL are isomers that are observed by mass spectrometry at the same m/z ratio, both yielding pseudomolecular ions at 632.3 m/z in negative ion mode (Figure 1). However, these compounds can be distinguished using tandem MS (MS/MS) techniques. In MS/MS, 3'-SL yields a major ion at 290.0 m/z and minor ions at 470.2 and 572.2 m/z , whereas 6'-SL yields major ions at 290.0, 470.2, and 572.2 m/z (Figure 1). As these product ions are present in distinct ratios, we developed a method for simultaneously assaying 3'-SL and 6'-SL based on utilizing these ratios to decipher the relative proportion of 3'-SL and 6'-SL present in the analyte. Once the relative proportions of 3'-SL and 6'-SL have been determined, these compounds can be quantified by attributing a proportion of a common product ion signal to each compound followed by comparison to a standard.

The first step in developing the method was to define the relationship between product ion ratios and the proportion of 3'-SL to 6'-SL in the analyte. Mixtures of 3'-SL and 6'-SL in various proportions at a total SL concentration of 20 μ M were analyzed by FIA-MS. Analysis of the data revealed that plotting the product ion signal at 572.2 m/z divided by the product ion signal at 290.0 m/z (hereafter termed 572/290) against the proportion of 3'-SL in the analyte gave a reproducible nonlinear relationship (Figure 2). This calibration curve was then used to calculate the relative proportions of 3'-SL and 6'-SL in an analyte from the observed 572/290. Flow-injection analysis-MS was also

Figure 1. Analysis of 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL) by mass spectrometry (MS). Solutions of 3'-sialyllactose (A) and 6'-sialyllactose (B), both 50 μ M, were infused into the mass spectrometer and MS spectra (upper panels) were collected in negative ion mode. The ion observed at 632.3 m/z from each compound was fragmented by collision-induced dissociation and tandem MS (MS/MS) spectra (lower panels) were collected.

performed on mixtures of 3'-SL and 6'-SL at total SL concentrations of 5 and 50 μ M. The data (not shown) were similar to those obtained at 20 μ M, indicating that the observed relationship is not concentration dependent. Utilization of the product ion at 470.2 m/z , which in the MS/MS spectrum of 3'-SL is less intense than 572.2 m/z , proved to be impractical because the variability among replicate analyses was too high.

Figure 2. Analysis of mixtures of 3'- and 6'-sialyllactose by mass spectrometry; effect of the 3'-SL to 6'-SL ratio on the 572.2 m/z to 290.0 m/z ratio. Samples of 3'-sialyllactose mixed with 6'-sialyllactose in different ratios to give 20 μ M total sialyllactose were analyzed by flow injection analysis-mass spectrometry. Signals at 572.2 and 290.0 m/z were extracted from the tandem MS (MS/MS) data. Data are shown as the means of triplicate measurements with error bars representing 1 SD.

For quantitation, we used the product ion at 290.0 m/z , which is a major ion in the MS/MS spectra of both 3'-SL and 6'-SL (Figure 1). Analysis of 3'-SL and separately of 6'-SL at the same concentration by FIA-MS revealed that 3'-SL yielded a 1.9 times more intense signal at 290.0 m/z than 6'-SL [the mean 290 m/z signal for 3'-SL was 600,221 arbitrary units ($n = 20$) and the mean 290 m/z signal for 6'-SL was 316,361 arbitrary units ($n = 20$)]. Therefore, in an equimolar mixture, 65.5% of the 290.0 m/z signal is derived from 3'-SL and 34.5% is derived from 6'-SL. This ratio, 0.655:0.345, combined with the relative proportion of 3'-SL and 6'-SL present in the analyte, as calculated from 572/290, was used to split the 290 m/z signal into the components derived from 3'-SL and 6'-SL. The 2 components of the 290 m/z signal were then compared with SL standards to determine the absolute concentrations of 3'-SL and 6'-SL in the analyte.

The standard curve used for quantitation was constructed by diluting 3'-SL into 800-fold-diluted whey (the dilution at which whey samples were assayed) pooled from 100 randomly selected samples. The response of the FIA-MS assay was linear up to the highest 3'-SL concentration tested, 3.0 mg/L (Figure 3), which is equivalent, allowing for dilution, to approximately 23 to 57 times the concentration of 3'-SL in mature

milk (Martín-Sosa et al., 2003; McJarow and van Amelsfort-Schoonbeek, 2004). Standards of 6'-SL produced similar results (not shown) but were not required as 6'-SL could be quantified using the 3'-SL standard by allowing for the difference in the 290 m/z signal between 6'-SL and 3'-SL (3'-SL:6'-SL; 0.655:0.345).

The precision of the assay was assessed by replicate measurements. A single milk sample was divided into 96 aliquots, which were independently processed and assayed. The coefficients of variation observed for 3'-SL and 6'-SL were 5.2 and 8.5%, respectively (Figure 4). The precision of the assay could be improved by increasing the volume of sample injected or decreasing the HPLC flow rate during FIA-MS, but at the expense of increased analysis time. The FIA-MS method as described required 2.7 min per sample and for our purposes this was a good balance between sample throughput and measurement precision.

The FIA-MS assay was very robust. The sample analyzed in this method is diluted whey, a very complex sample that contains protein components that can foul hardware. However, the HPLC/MS system delivered consistent signals over extended periods. For example, standard curves measured approximately 18 h apart, with analysis of 384 whey samples in between, corresponded very well (Figure 3). Neither the HPLC nor

Figure 3. 3'-Sialyllactose standard curve and standard curve reproducibility. Samples of 3'-sialyllactose diluted in whey were analyzed by flow-injection analysis-mass spectrometry. The signals at 290.0 m/z were extracted from the tandem MS (MS/MS) data and used to construct the standard curve. The standard curve was determined twice: immediately before (■) and immediately after (▲) the analysis of 384 samples of whey over a total analysis time of approximately 18 h.

Figure 4. Assessment of the reproducibility of the 3'- and 6'-sialyllactose assay. Ninety-six aliquots of a sample of skim milk were processed independently and 3'-sialyllactose and 6'-sialyllactose were assayed by flow injection analysis-mass spectrometry.

the MS required any significant cleaning after such analyses.

Measurement of 3'-SL and 6'-SL Concentrations in Milk

The concentrations of 3'-SL and 6'-SL were determined in milk samples from 15,507 individual cows from Friesian, Jersey, and Friesian-Jersey crossbred herds (Figure 5). Combining data from the 3 breeds, we found 329 cows with greater than twice the mean concentrations of 3'-SL or 6'-SL (Figure 5B). One cow had >4-fold higher than the mean concentration of 3'-SL (i.e., > 695 μM).

We followed up sampling on the 10 highest SL concentrations during the same milking season. The mean concentrations in the original samples were 515 and 59 μM for 3'- and 6'-SL, respectively. These means were maintained in 2 replicate samplings in later lactation, demonstrating that the observed high concentrations were stable features of these cows.

Analysis of the data by breed (Figure 6) revealed a strong effect of breed on 3'-SL concentrations ($P < 0.001$ by ANOVA). Further, Jerseys had the highest 3'-SL level and this was significantly greater than the concentration found in crossbred milks, which in turn were greater than the average concentration of 3'-SL in Friesian milk. Similar ranking of breeds was found for 6'-SL, although numerical differences were much smaller.

Multiple regression analysis revealed that fitting a model including breed, parity, and DIM explained 29% of the variation in 3'-SL and 5% of the variation

Figure 5. Identification of milk samples with high concentrations of 3'- and 6'-sialyllactose. 3'-Sialyllactose and 6'-sialyllactose concentrations were determined in 15,507 milk samples collected between mid-October and mid-December from individual Friesian, Jersey, and Friesian-Jersey crossbred cows by flow injection analysis-mass spectrometry (A). The numbers of milk samples identified with greater than twice the mean concentrations of 3'-sialyllactose or 6'-sialyllactose are indicated (B).

in 6'-SL concentrations in milk. All parameters had a significant ($P < 0.001$) effect on both 3'-SL and 6'-SL. For 3'-SL, breed, parity, and DIM accounted for 16, 10, and 3% of the variation, respectively. For 6'-SL, breed, parity, and DIM explained 2.5, 1.5, and 0.5% of the variation, respectively. The effect of parity was most apparent in the first 2 lactations, where both 3'-SL and 6'-SL concentrations in milk were 5 to 10% lower (relative to the mean) in the first 2 lactations, with no apparent trend thereafter. The concentration of 3'-SL increased as lactation progressed, with a linear increase in milk concentration of 0.43 μ M/d ($P < 0.001$), where the sampling range was up to 160 DIM. For 6'-SL in milk, a small decrease of 0.0001 μ M/d was observed with DIM ($P < 0.001$).

Figure 6. Box plots of 3'- and 6'-sialyllactose concentrations in milk from Friesian, Friesian-Jersey crossbred, and Jersey cows. The distributions of 3'-sialyllactose concentrations (A) in Friesian ($n = 5,253$, mean = 147, SD = 37), Friesian-Jersey crossbred ($n = 6,854$, mean = 174, SD = 51), and Jersey ($n = 3,400$, mean = 214, SD = 64) cows and 6'-sialyllactose concentrations (B) in Friesian ($n = 5,253$, mean = 33, SD = 10), Friesian-Jersey cross bred ($n = 6,854$, mean = 35, SD = 10), and Jersey ($n = 3,400$, mean = 38, SD = 12) cows are shown. * $P < 0.001$.

DISCUSSION

We have developed an assay for 3'-SL and 6'-SL that does not require separation of these compounds from each other or from other constituents of whey. This allowed a rapid analysis to be implemented, utilizing

FIA-MS with a very simple sample preparation, essentially just the preparation of whey from skim milk. In contrast, previously reported methods for the analysis of SL in milk (Martín-Sosa et al., 2003; McJarrow and van Amelsfort-Schoonbeek, 2004; Barile et al., 2010; Fong et al., 2011; Sundekilde et al., 2012) require deproteinization of the sample and chromatographic separation of the oligosaccharides. The FIA-MS assay delivered reasonably low coefficients of variation for 3'-SL and 6'-SL, which were appropriate to detect several-fold differences among animals. In spite of the relatively crude sample analyzed, instrument performance was consistent over long periods of analysis time, which was essential for our high-throughput application. It is important to note that implementing this method with different fragmentation parameters or on a different type of instrument would likely alter the ratio of signals for 3'-SL and 6'-SL at 290 m/z and that the relationship between the 572/290 ratio and the proportions of 3'-SL and 6'-SL in the sample may be slightly different. Therefore, it would be essential to check these parameters with standards and adjust the calculations accordingly.

Although our focus was on SL, the FIA-MS method could be expanded to assay additional bovine milk oligosaccharides for which standards are commercially available: 3'-sialyllactosamine, 6'-sialyllactosamine, and disialyllactose, although the presence of 3'-sialyllactosamine in bovine milk is disputed (Martín-Sosa et al., 2003; Fong et al., 2011). Protein components such as β -lactoglobulin and α -lactalbumin can also be assayed in the same diluted whey sample used for SL analysis by modifying the FIA-MS to full-scan MS in positive ion mode [H. Ward (ViaLactia Biosciences, Auckland, New Zealand), S. Davis, S. Berry, K. Lehnert, D. Palmer, R. Spelman, V. Kelly, K. Henty (School of Biological Sciences, University of Auckland, Auckland, New Zealand), and R. Snell, unpublished data]. More generally, the approach of using product ion ratios to deconvolute signals from mixtures of isomers could be applied to any compound in which the isomers do not yield unique major ions in tandem MS.

The mean concentrations of 3'-SL and 6'-SL measured in this study correspond well with reported concentrations of these compounds in milk. However, very large variation exists in the reported concentrations of 3'-SL and 6'-SL in mature milk, from 47 to 188 μM and from 10 to 139 μM , respectively (concentrations in these reports have been converted to μM for comparison purposes; Martín-Sosa et al., 2003; Nakamura et al., 2003; Fong et al., 2011). The reasons for these differences were discussed by Fong et al. (2011) and were attributed to loss of SL during sample preparation, the effect of interfering compounds co-eluting in some HPLC

methods, variability resulting from sampling only a few animals, as well as biological variation due to breed and feed. Our data fit most closely to the concentrations of 3'-SL and 6'-SL and their ratio reported by Goto et al. (2010) for mature milk from the Holstein breed, of 131 μM for 3'-SL and 35 μM for 6'-SL.

Differences in 3'-SL and 6'-SL were significantly greater in Jerseys than in Friesians, although generally within-breed variation was similar to among-breed variation. The mean concentrations of 3'-SL and 6'-SL in the Friesian-Jersey crossbreeds were intermediate between those of Friesian and Jersey. Our data from Friesian and Jersey are similar to those reported for mature milk from Danish Jersey and Holstein-Friesian breeds (Sundekilde et al., 2012), where 3'-SL was significantly higher in Jersey and 6'-SL was not significantly different between these breeds. A study of SL in early milk from New Zealand Friesian and Jersey breeds (McJarrow and van Amelsfort-Schoonbeek, 2004) revealed a significantly higher concentration of 6'-SL in Friesian in the first milking compared with Jersey. However, by the fifth milking, Jersey had a higher concentration of 6'-SL, consistent with our observations in mature milk. In comparing breeds to identify richer sources of 3'-SL and 6'-SL, it is interesting that there is no significant difference between the concentrations of these compounds in the mature milk of Holstein and 4 nondairy breeds (Goto et al., 2010).

The fact that 3'-SL concentration appeared to be slightly lower in cows in their first 2 lactations is hard to explain. The effect size was very small (5–10%) but occurred in all breeds and was independent of sampling date. The difference may be related to the higher metabolic demands in heifers through concurrent growth and lactation affecting glucose supply to the mammary gland. However, such an explanation is speculative. Overall, the effect of parity on variation in both 6'-SL and 3'-SL was very small.

Stage of lactation had a significant effect on 3'-SL concentrations, in particular. Again, this effect was seen in all breeds, but the effect size was very small, accounting for <3% of the overall variance when breed and parity were accounted for in the statistical model. McJarrow and van Amelsfort-Schoonbeek (2004) also reported minor effects of lactation stage on 3'-SL and 6'-SL concentrations in milk. It should be noted that most of the sampling for this study was undertaken through peak lactation, and identification of outliers was done initially on a within-herd basis, minimizing the effect of any temporal variation.

More substantial than differences between breeds or the effects of stage of lactation or parity was the detection of phenotypic outlier cows in all breeds with much higher concentrations of 3'-SL or 6'-SL. Across

the 3 breeds, we observed 329 cows with more than twice the mean concentrations of 3'-SL or 6'-SL, 26 cows with more than 3 times the mean concentrations of 3'-SL or 6'-SL, and 1 cow with more than 4 times the mean 3'-SL concentration (Figure 6). The outliers were observed in all 3 breeds investigated. Importantly, a subset of the outliers had higher concentrations of both 3'-SL and 6'-SL; 38 cows had more than twice the mean concentration of both 3'-SL and 6'-SL, and 2 cows had more than 3 times the mean concentrations of both 3'-SL and 6'-SL. As 3'-SL and 6'-SL are produced by different enzymes (different gene products), this observation would be expected if a sufficiently large population was sampled.

In a parallel study, we used a genome-wide association approach to identify genetic polymorphisms associated with the observed phenotypic variation in milk SL content [S. Davis, S. Berry, K. Lehnert, D. Palmer, R. Spelman, V. Kelly, K. Henty (School of Biological Sciences, University of Auckland, Auckland, New Zealand), and R. Snell, unpublished data], confirming a genetic basis for the outlier phenotypes.

CONCLUSIONS

We have developed a novel high-throughput method for determining 3'-SL and 6'-SL concentrations in milk. We applied this method to a large set of samples and observed several substantial phenotypic outliers expressing higher concentrations of these bioactive oligosaccharides. Given the variation observed in the present study, it would be interesting to sample a much larger population to search for more extreme outliers.

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