

## Natural Variability in Bovine Milk Oligosaccharides from Danish Jersey and Holstein-Friesian Breeds

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### **S** Supporting Information

**ABSTRACT:** Free oligosaccharides are key components of human milk and play multiple roles in the health of the neonate, by stimulating growth of selected beneficial bacteria in the gut, participating in development of the brain, and exerting antipathogenic activity. However, the concentration of oligosaccharides is low in mature bovine milk, normally used for infant formula, compared with both human colostrum and mature human milk. Characterization of bovine milk oligosaccharides in different breeds is crucial for the identification of viable sources for oligosaccharide purification. An improved source of oligosaccharides can lead to infant formula with improved oligosaccharide functionality. In the present study we have analyzed milk oligosaccharides by high-performance liquid chromatography chip quadrupole time-of-flight mass spectrometry and performed a detailed data analysis using both univariate and multivariate methods. Both statistical tools revealed several differences in oligosaccharide profiles between milk samples from the two Danish breeds, Jersey and Holstein-Friesians. Jersey milk contained higher relative amounts of both sialylated and the more complex neutral fucosylated oligosaccharides, while the Holstein-Friesian milk had higher abundance of smaller and simpler neutral oligosaccharides. The statistical analyses revealed that Jersey milk contains levels of fucosylated oligosaccharides significantly higher than that of Holstein-Friesian milk. Jersey milk also possesses oligosaccharides with a higher degree of complexity and functional residues (fucose and sialic acid), suggesting it may therefore offer advantages in term of a wider array of bioactivities.

**KEYWORDS:** oligosaccharides, dairy cow, sialyllactose, fucosylation, mass spectrometry

### **I** INTRODUCTION

Milk is a complex mixture of lipids, carbohydrates, proteins, and smaller metabolites and thus represents a key role in infant nourishment and development. Carbohydrates are one of the most predominant solid fractions of both human and bovine milk (4–7%),<sup>1</sup> and besides the main milk sugar, lactose, a number of more complex free oligosaccharides (OS) is also present in milk at lower abundance. Lactose is mainly used as an energy source for the neonate. However, the OS are indigestible by the enzymes in the gastrointestinal tract. Thus, they do not exist in milk for the purpose of nourishment. Comprehensive studies on human milk OS have elucidated a wide range of beneficial actions of milk OS on improving health. Oligosaccharides exhibit many specific biological functions, including stimulation of selected beneficial *Bifidobacteria* in *in vitro* studies,<sup>2–5</sup> participation in the innate immune system, preventing adhesion of pathogenic bacteria such as *Helicobacter pylori* and certain viruses.<sup>6–9</sup> Furthermore, sialic acid, a component of bovine milk OS, is essential for brain development and cognitive function.<sup>10</sup>

Oligosaccharides contain from 3 to 15 monosaccharides linked through a variety of glycosidic bonds. Most of the OS consist of a lactose core and additional hexoses, deoxyhexoses, *N*-acetylhexosamines, neuraminic acid, and the deoxyhexose fucose. Rhamnose, another deoxyhexose, is only found in plants. Two classes of OS have been identified in bovine milk: acidic OS-

containing sialic acid (in the form of *N*-acetyl neuraminic acid or *N*-acetyl glycolyl neuraminic acid) and neutral OS-containing *N*-acetylhexosamine.<sup>11</sup>

The highest concentration of oligosaccharides is found in early postparturition milk (colostrum). Human colostrum contains double the concentration of oligosaccharides that is found in mature milk (about 5–10 g/L), and more than 200 potential OS structures have been described, with over 75 fully elucidated and annotated structures.<sup>12–15</sup> Similarly, bovine colostrum contains a high concentration and structurally diversified OS (over 40 have been identified)<sup>11,16</sup> compared to mature bovine milk. Overall, the OS concentration and structural diversity is lower in milk of bovine origin compared to that in human milk. Previous work on characterization of OS in milk has mainly been performed on colostrum, and while this makes extraction and subsequent analyses easier due to the higher colostrum concentrations of OS, it does not reveal the OS profile of mature bovine milk. As infant formulas are manufactured predominantly with mature bovine milk components, investigations of OS in bovine milk other than colostrum are very important.<sup>17</sup>

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A complete understanding of milk and its bioactives, including OS, requires the development of toolsets to characterize these complex compounds. Many different analytical methods have been used in the characterization of the variety of human milk OS, including nuclear magnetic resonance (NMR), mass spectrometry, capillary electrophoresis, and various chromatographic techniques.<sup>18–22</sup> Despite technological advances, structural characterization of milk OS is a timely and demanding task because of the many isomeric forms for each structure as well as the fact that OS can be present at widely different abundances. Mass spectrometry with high resolving power and mass accuracy has proven to be a superior method to study OS in mammalian milks<sup>23</sup> and has generated high-quality and accurate data on bovine milk OS.<sup>11,16,24,25</sup> Multivariate data analysis methods have previously proven to be a valuable in data analysis of the data sets often generated by mass spectrometry and other analytical techniques. Recently, biochemical variability of bovine milk was analyzed using metabolomics, and the authors identified several differences in metabolites across milk of different origins,<sup>26</sup> which potentially could have impact on both nutritional and technological properties of the milk.<sup>27</sup> In 2009, Tao et al.<sup>24</sup> investigated the OS composition of milk from four Jersey cows and three Holstein-Friesian cows and did not find significant differences between the breeds; however, no larger systematic study has been conducted so far. Recently, a comprehensive analysis of the genes involved in OS metabolism by RNA sequencing and OS profiling did not find significant differences between milk from Jersey and Holstein-Friesian cows.<sup>28</sup> However, the data suggest a tendency toward increased amounts of OS with a polymerization larger than four in Jersey milk compared to that of Holstein-Friesian milk.<sup>28</sup> In the present study, we performed a complete characterization of OS in 20 mature milk samples from two bovine breeds (Jersey and Holstein-Friesian), widely applied for conventional dairy farming in Denmark. The use of high performance liquid chromatography chip/time of flight mass spectrometry (HPLC-Chip/TOF MS) allowed the characterization of the bovine milk glycome, including OS composition and abundance.

The aim of the present study was to systematically investigate OS profiles of two common dairy breeds by advanced MS analyses and to couple this information with multivariate data analysis techniques. Indeed, the analyses revealed novel differences in the OS profiles of Holstein-Friesian milk and Jersey milk samples.

## MATERIALS AND METHODS

**Samples and Sample Collection.** Milk samples were collected as part of the Danish/Swedish Milk Genomics Initiative. In total 892 milk samples (morning milking) were collected from a total of 40 nonorganic herds across Jutland, Denmark. All cows were housed in loose housing systems, fed with total mixed ration according to standard practice in Denmark during their indoor period, and milked twice a day. Immediately after milking, milk samples were placed on ice for transport to the laboratory. Once at the laboratory, the milk samples were aliquoted, skimmed, and frozen at  $-80\text{ }^{\circ}\text{C}$ . The cows included were selected from a scheme setup to maximize the genetic variability, while still matching the cows within the first-third parity and midlactation period. Ten milk samples (days since calving: 138–232) from 6 herds of Jersey cows and 10 milk samples from 5 herds of Holstein-Friesian cows were selected on the basis of a prescreening of milk samples by both  $^1\text{H}$  NMR spectroscopy and by assessment of milk fat, protein, and lactose concentrations. Prescreening was performed in order to select samples with a broad range of features. Detailed information on days, milk parity, and concentrations of protein, fat, and lactose are given in Table 1. Samples included in this study were Jersey 0402, 0406, 0502, 0854,

**Table 1. List of Samples Included in the Present Study (HF, Holstein-Friesian)**

cow ID	breed <sup>a</sup>	parity <sup>b</sup>	days <sup>c</sup>	fat <sup>d</sup>	protein <sup>d</sup>	lactose <sup>d</sup>
0402	Jersey	1	174	4.82	3.97	4.74
0406	Jersey	1	161	4.6	3.74	4.89
0502	Jersey	1	168	5.88	4.23	4.6
0854	Jersey	1	196	5.38	4.19	4.76
1119	Jersey	1	180	5.75	4.12	4.67
1149	Jersey	3	219	5.43	4	4.59
1230	Jersey	2	188	7.71	4.23	4.51
1231	Jersey	2	184	3.31	4.66	4.81
1410	Jersey	3	198	5.94	3.97	4.51
1416	Jersey	1	232	5.83	3.85	4.67
4401	HF	2	157	4.93	4.31	4.79
4403	HF	1	138	4.1	3.53	4.84
4406	HF	1	164	4.01	3.43	4.83
4419	HF	1	144	3.67	3.08	4.92
4529	HF	1	135	3.74	3.27	4.78
4937	HF	2	173	4.77	3.2	4.66
5007	HF	3	193	4.37	3.42	4.75
5015	HF	3	184	2	3.32	4.11
5152	HF	1	179	3.49	3.35	4.91
5166	HF	3	183	4.62	3.31	4.81

<sup>a</sup>Bovine breeds: Danish Jersey and Danish Holstein-Friesians. <sup>b</sup>Parity number. <sup>c</sup>Number of days since calving. <sup>d</sup>Fat, protein, and lactose concentrations measured by Milkoscan (in percent).

1119, 1149, 1230, 1231, 1410, and 1416, and Holstein-Friesian 4401, 4403, 4406, 4419, 4529, 4937, 5007, 5015, 5152, and 5166. Each milk sample was analyzed for concentration of milk fat, protein, casein, and lactose by Milkoscan FT 2 (Foss Electric, Hillerød, Denmark) and for somatic cell count using Fossomatic 5000 (Foss Electric) at Eurofins Laboratory (Holstebro, Denmark). Milk samples included in the study had somatic cell counts below  $5 \times 10^5$  cells/mL.

**Oligosaccharide Isolation and Purification.** Skimmed milk samples (0.5 mL) were diluted with an equal volume of nanopure water and centrifuged at 4000g for 30 min at  $4\text{ }^{\circ}\text{C}$  to remove remaining milk lipids. Four volumes of a chloroform/methanol solution (2:1 v/v) were subsequently added and centrifuged (same conditions as above) to remove some of the protein. The upper layer containing OS was carefully transferred to a separate vial, and 2 volumes of pure ethanol were added. After overnight precipitation at  $4\text{ }^{\circ}\text{C}$ , the mixture was centrifuged (same conditions as above) to sediment the denatured proteins. The carbohydrate-rich supernatant was recovered and dried in vacuum. The dried OS were resuspended in 1 mL of nanopure water and subjected to solid-phase extraction to remove residual contaminants and to concentrate the OS. Residual peptides were removed using C8 columns in solid-phase extraction (DSC-C8 Discovery, 3 mL tube capacity, 500 mg bed weight, Supelco, Bellefonte, PA). The C8 sorbent was conditioned with three cartridge volumes of pure acetonitrile, followed by three cartridge volumes of nanopure water. The carbohydrate-rich solutions were slowly loaded onto the cartridges, and the eluate was collected in three consecutive cycles. An additional 1 mL of nanopure water was applied to the cartridge to ensure the collection of residual OS trapped in the cartridge. The OS-rich solution was further purified by solid-phase extraction using conditioned graphitized carbon cartridges G28 (150 mg of carbon, 4 mL tube capacity, Alltech, Deerfield, IL) to remove salts, monosaccharides, and lactose. Each cartridge was conditioned with three cartridge volumes of nanopure water, 0.05% (v/v) trifluoroacetic acid (TFA) in 80% acetonitrile in water (v/v), and three cartridge volumes of nanopure water before sample loading. The carbohydrate-rich solutions were applied to the cartridges; the salts were removed by washing with three cartridge volumes of nanopure water at a flow rate of 1 mL/min. Purified OS were eluted from the cartridges in two steps, first using 6 mL of 10% and 20% acetonitrile and then 6 mL of 40% acetonitrile/TFA 0.1% in

nanopure water (v/v) and dried in vacuum prior to mass spectrometry analysis.

**Matrix-Assisted Laser Desorption/Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry.** Matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance mass spectrometry (MALDI-FTICR MS) with tandem capabilities was used to screen OS composition in the milk samples as described in Barile et al.<sup>29</sup> A MALDI-FTICR MS instrument (IonSpec Corporation Pro, Lake Forest, CA) equipped with a 7.0 T actively shielded superconducting magnet and an external MALDI source capable of hexapole ion accumulation and fitted with a pulsed Nd:YAG laser (355 nm) was used. External accumulation of ions produced by a variable number of MALDI laser pulses (typically from 18 to 27) was used to obtain optimum total ion intensity for each sample analyzed. For MALDI screening, 0.5  $\mu\text{L}$  of a solution containing purified OS was spotted on a polished stainless steel target followed by 0.25  $\mu\text{L}$  of 0.01 M NaCl (for positive mode) and 0.5  $\mu\text{L}$  of 0.4 M 2,5-dihydroxybenzoic acid as a matrix. The spots were then allowed to dry under vacuum prior to mass spectrometric analysis. Internal calibration with OS from other food matrixes was performed to obtain accurate and precise mass-to-charge information.<sup>29</sup> Oligosaccharide composition was elucidated by collision-induced dissociation within the ICR cell.

**HPLC-Chip/TOF MS.** The dried OS samples were reconstituted in 100  $\mu\text{L}$  of nanopure water and analyzed using the 6200 Series HPLC-Chip/TOF MS instrument (Agilent Technologies, Santa Clara, CA) according to the method reported.<sup>16,22</sup> Oligosaccharide separation was achieved by using binary gradient A, 3% acetonitrile in 0.1% formic acid solution, and B, 90% acetonitrile in 0.1% formic acid solution. The column was initially equilibrated and eluted with a flow rate of 0.3  $\mu\text{L}/\text{min}$  for the nanopump and 4  $\mu\text{L}/\text{min}$  for the capillary pump. The 65-min gradient was as follows: 2.5–20.0 min, 0–16% B; 20.0–30.0 min, 16–44% B; 30.0–35.0 min, B increased to 100% and held at 100% B for 10 min, and finally, 0% B for 20 min to equilibrate the separation column inside the chip before the next sample injection. Internal calibration was performed using internal reference masses constantly infused during the run (ESI-TOF Tuning Mix G1969–85000, Agilent Technologies). Each sample was run in duplicate. The mass lists obtained from the HPLC-Chip/TOF MS were deconvoluted using the Molecular Feature Extractor from MassHunter Qualitative Analysis software version B.03.01 (Agilent Technologies), and OS compositions were predicted using an in-house program, Glycan Finder, written in Igor Pro version 5.04B (WaveMetrics Inc., Portland, OR). Oligosaccharide compositions were determined for masses with a mass error  $\leq 5$  ppm. OS composition was determined based on computational tables and known reproducibility of retention times of OS created from previous OS analyses, as well as determination by MALDI-FTICR MS/MS.<sup>11,16,22,24,30</sup> Furthermore, a few samples were analyzed using a 6200 Series HPLC-Chip/qTOF MS instrument (Agilent Technologies) capable of doing MS/MS analyses. Relative quantification of each OS was in some instances performed based on its intensity (counts per second, cps) and more accurately by integration of the area under the chromatogram (AUC) of extracted ion chromatogram (EIC) of selected OS peaks. Furthermore, the molecular feature function in Mass Hunter Qualitative Analysis (Agilent Technologies) was used to generate intensity data for the multivariate data analysis.

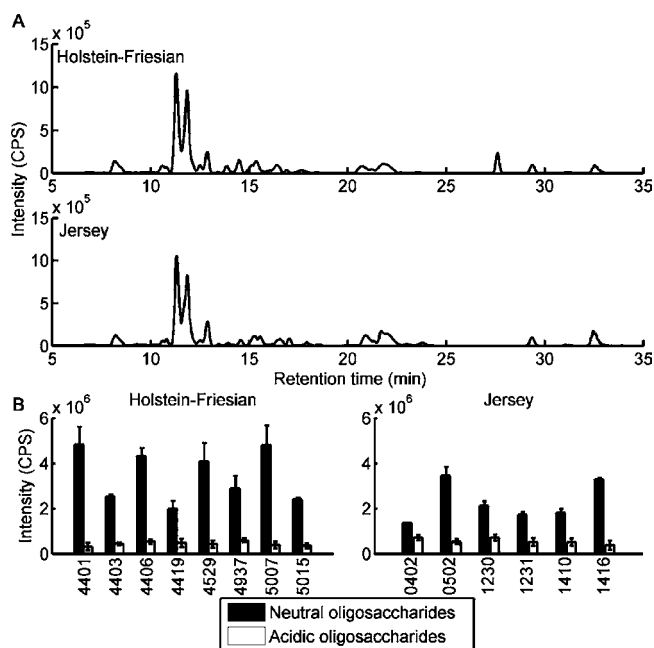
**Multivariate Data and Statistical Analyses.** Multivariate data analysis was applied to examine systematic variation in a data matrix in order to identify underlying variables that contribute to differences between the milk samples. Principal component analysis (PCA) was used to provide a linear transformation of the original variables (OS intensity data) measured in samples into a substantially reduced set of uncorrelated variables, the principal components (PC).<sup>16,31</sup> PCA was performed using SIMCA-P+ 12.0.1.0 (Umetrics AB, Sweden). PCA was applied to the data extracted using Molecular Feature in MassHunter Qualitative Analysis software version B.03.01 (Agilent Technologies) to explore any clustering behavior of the samples. Prior to PCA, the data was centered and scaled to unit variance to ensure each OS was given equal opportunity to influence the model. PCA was also applied to the base peak chromatograms (BPC) of the 10 Jersey milk samples and 10 Holstein-Friesian samples measured, resulting in 761 variables in the

region 5–25 min. Prior to this analysis, the BPCs were aligned using Correlation Optimized Warping.<sup>32</sup> The data was centered and Pareto scaled prior to PCA, also used to explore any clustering behavior of the samples. Pareto scaling is often used for continuous variables.<sup>33</sup> Statistical significance was evaluated by using paired Student's *t* test and one-way analysis of variance (ANOVA) where applicable using the Statistics Toolbox in MATLAB 7.9 (MathWorks Inc., Natick, MA).

## RESULTS AND DISCUSSION

### Oligosaccharide Identification by Mass Spectrometry.

In total, 52 individual OS masses were observed, with 29 being present ostensibly in all the samples and confirmed by Tandem MS/MS. Representative BPCs from the HPLC-Chip/TOF analyses of the Holstein-Friesian and Jersey milk OS profiles are presented in Figure 1A. Overall, Holstein-Friesian milk



**Figure 1.** (A) Representative base BPC of both Holstein-Friesian and Jersey milk oligosaccharides (Cow ID: Holstein-Friesian: 4529, Jersey: 0502). (B) Neutral and acidic oligosaccharide abundances in 14 samples measured in duplicates (values: mean  $\pm$  SD). Samples are colored according to neutral OS (black) and acidic OS (white).

samples display higher intensity (measured as counts-per-second) than that of Jersey milk samples, which suggests a higher level of OS in milk of the Holstein-Friesian cows.

MALDI FT ICR mass spectrometry with tandem (MS/MS) capabilities and HPLC-Chip/qTOF MS/MS were used to confirm the actual monomeric composition predicted by the software and to assign some structures of each OS identified by the HPLC-Chip/TOF analyses. In total the composition of 29 OS was confirmed by tandem analysis (Tables 2 and 3). The remaining 23 OS were not present in all samples and if present, their concentration was too low to obtain reliable MS/MS events. Further MS/MS analyses and enzymatic assays are needed in order to unambiguously characterize these OS and assign structures. Tandem MS spectra of selected OS are presented in the Supporting Information.

**Analysis of Neutral and Acidic Oligosaccharides.** A recent study investigated the methods for absolute quantification of OS from milk samples and identified a possible cocrystallizing effect of lactose and OS by ethanol precipitation.<sup>34</sup> In the present

Table 2. List of the Detected Neutral Oligosaccharides (unreduced neutral mass) from Holstein-Friesian Sample 5015<sup>a,b</sup>

OS ID	neutral mass		delta (ppm)	oligosaccharides			RT	abundance
	expt <sup>c</sup>	cal		Hex	HexNAc	fucose		
1	504.169	504.169	0.860	3			8.97	6732887
1	504.169	504.169	0.066	3			11.01	4115087
1	504.169	504.169	0.496	3			12.42	12120882
1	504.168	504.169	1.289	3			14.23	3773572
2	545.195	545.196	0.472	2	1		12.13	19055487
5	666.220	666.222	2.176	4			17.10	194208
8	707.248	707.248	1.202	3	1		11.97	73384
8	707.247	707.248	1.485	3	1		13.82	314853
8	707.247	707.248	1.980	3	1		15.03	1657912
8	707.247	707.248	2.074	3	1		17.82	163224
9	748.275	748.275	0.401	2	2		10.55	6288
9	748.274	748.275	2.005	2	2		14.25	458787
9	748.272	748.275	4.277	2	2		15.83	91097
14	869.299	869.301	2.531	4	1		11.23	32225
14	869.301	869.301	0.690	4	1		18.12	3630953
15	910.327	910.328	1.153	3	2		13.37	55158
15	910.327	910.328	1.062	3	2		15.93	2373331
18	990.327	990.327	0.353	6			11.29	69719
18	990.325	990.327	2.070	6			14.43	97367
21	1072.382	1072.381	1.119	4	2		14.73	11419
21	1072.381	1072.381	0.280	4	2		18.52	658142
22	1113.408	1113.407	0.539	3	3		17.24	228764
23	1152.377	1152.380	2.430	7			9.54	4468
23	1152.375	1152.380	4.512	7			15.76	33025
26	1234.430	1234.433	2.592	5	2		17.34	5933
28	1275.460	1275.460	0.157	4	3		18.42	12918
32	n/a <sup>d</sup>	1396.486	n/a	6	2		n/a	n/a
36	1462.541	1462.545	2.256	3	4	1	17.53	2326
37	n/a <sup>d</sup>	1478.539	n/a	4	4		n/a	n/a
38	n/a <sup>d</sup>	1519.566	n/a	3	5		n/a	n/a
42	1624.597	1624.597	0.246	4	4	1	16.12	16985
43	1640.596	1640.592	2.499	5	4		13.63	6752
45	1681.617	1681.619	0.951	4	5		16.00	8257
46	1722.650	1722.645	2.583	3	6		15.38	52068
47	1786.650	1786.650	0.168	5	4	1	16.49	52239
49	1827.676	1827.677	0.547	4	5	1	17.57	33073
52	1868.703	1868.703	0.241	3	6	1	16.94	126084

<sup>a</sup>Corresponding data on a Jersey sample is available in Table S1. <sup>b</sup>Abbreviations: expt, experimental, cal, calibrated, RT, retention time (minutes); Hex, hexose; HexNAc, N-acetylhexosamine. <sup>c</sup>expt: experimental mass values have been averaged. <sup>d</sup>Oligosaccharide is not detected in sample 5015, but it is present in other milk samples.

study, OS were extracted from milk samples as previously established,<sup>11</sup> and care was taken in order to maximize reproducibility of the extraction procedure as described.<sup>35,36</sup> The present study compares relative abundances of individual OS of different milk samples, and the composition of the identified OS are presented in Tables 2 and 3 and Tables S1 and S2 in the Supporting Information. The tables present lists of the neutral and acidic OS studied in this work, along with their accurate mass, monomeric composition, unique retention times, and abundances. Several OS were found to elute at different retention times (RT), indicating different positional isomers, e.g., OS ID 1, consisting of three hexoses, which elute at four different RT corresponding to four different isomers. Additional isomers were found for several other OS including OS ID 3, 7–9, 14, 15, and 21. Figure 1A displays the representative BPC of Holstein and Jersey milk OS (Holstein cow 4529, Jersey cow 0502). The neutral OS HexNAc-lactose (OS ID: 2; Table 2), eluting at 12 min, was observed to be the most abundant OS in both breeds.

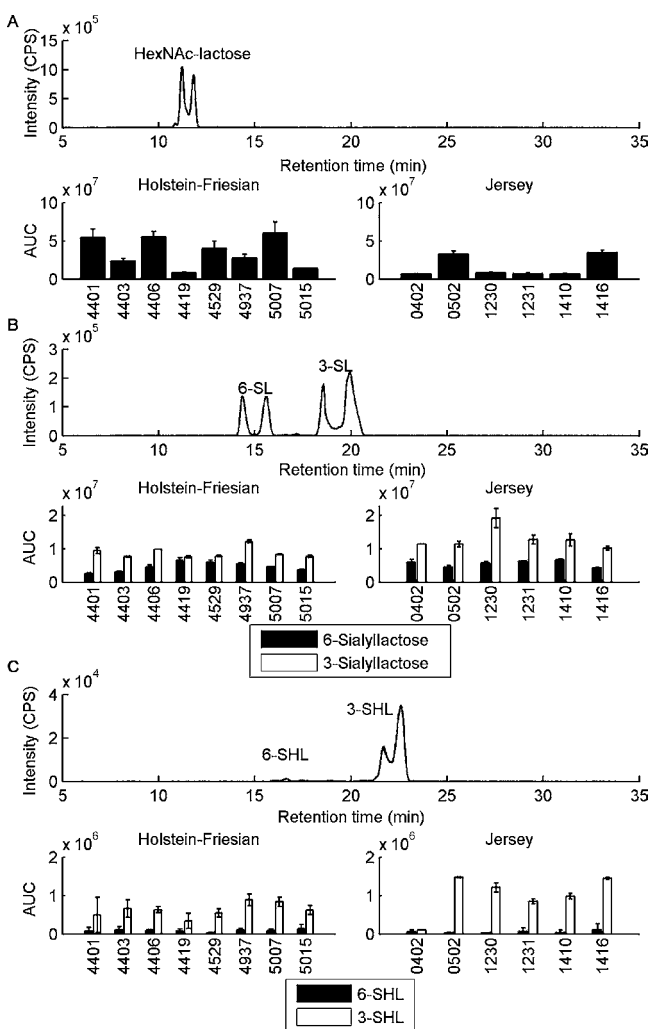
Figure 1B shows the distribution of neutral versus acidic OS species in the milk samples studied measured as peak height intensities (counts per second, CPS) of all neutral and acidic OS species pooled together. Neutral OS species are significantly higher than acidic OS species (paired Student's *t* test;  $p < 0.05$ ) which is in agreement with previous findings.<sup>24</sup> The intensity data was analyzed by ANOVA, and although not significant, there is a tendency toward Jersey milk containing higher levels of acidic OS ( $p = 0.066$ ) and there is a trend toward higher OS intensities in Holstein-Friesian milk ( $p = 0.057$ ).

The extracted ion chromatogram and abundance (represented as AUC) of the three most abundant OS found in the milk samples (Figure 2) revealed that the neutral HexNAc-lactose was more abundant in milk from Holstein-Friesian than in Jersey milk, thus indicating a breed-specific trend toward lower levels of this neutral OS in Jersey milk than in Holstein-Friesian milk (Figure 2A). Interestingly, OS ID 2 has previously been reported to be present at low levels in both colostrum and midlactation

Table 3. List of the Detected Acidic Oligosaccharides (unreduced neutral mass) from Holstein-Friesian sample 5015<sup>a,b</sup>

OS ID	neutral mass			oligosaccharides				RT	abundance
	expt <sup>c</sup>	cal	delta (ppm)	Hex	HexNAc	NeuAc	NeuGc		
3	633.210	633.212	1.842	2		1		15.88	3858014
3	633.210	633.212	2.579	2		1		21.79	8045793
4	n/a <sup>d</sup>	649.206	n/a	2			1	n/a	n/a
6	674.237	674.238	1.928	1	1	1		16.59	232755
7	690.232	690.233	1.449	1	1		1	7.24	3902
7	690.232	690.233	1.449	1	1		1	16.29	10914
10	795.264	795.264	0.671	3		1		16.96	67956
10	795.262	795.264	3.018	3		1		22.85	480621
12	836.288	836.291	3.229	2	1	1		15.92	69422
19	n/a <sup>d</sup>	998.344	n/a	3	1	1		n/a	n/a
24	1160.396	1160.397	0.625	4	1	1		25.35	108672
25	1201.419	1201.423	3.662	3	2	1		24.79	6558
39	1525.526	1525.529	1.901	5	2	1		24.69	5465

<sup>a</sup>Corresponding data on a Jersey sample is available in Table S2. <sup>b</sup>Abbreviations: expt, experimental; cal, calibrated; RT, retention time (minutes); Hex, hexose; HexNAc, N-acetylhexosamine. <sup>c</sup>expt: experimental mass values have been averaged. <sup>d</sup>Oligosaccharide is not detected in sample 5015, but it is present in other milk samples.

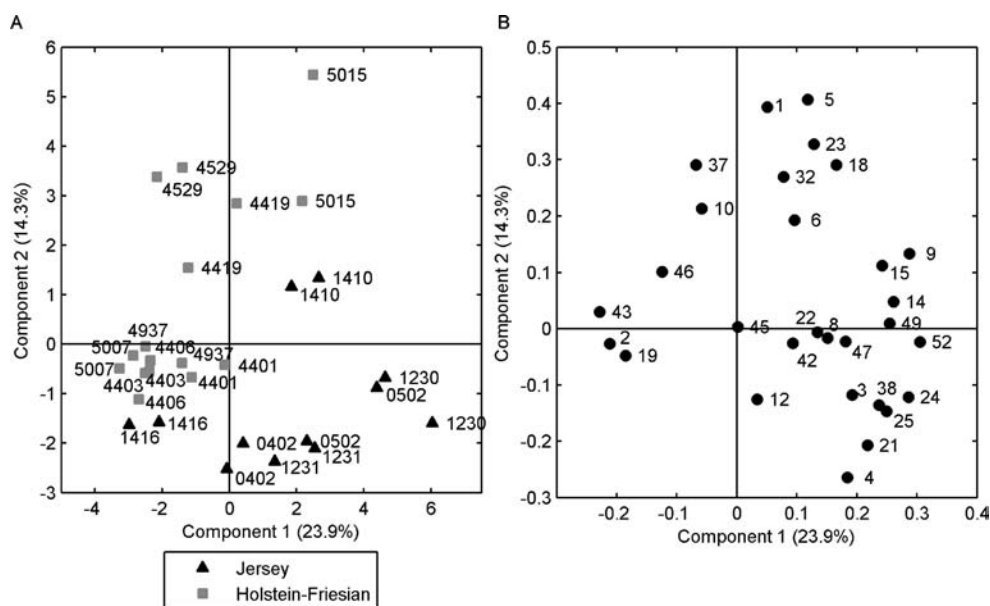


**Figure 2.** Extracted ion chromatograms and calculated AUC in 14 samples measured in duplicate (mean  $\pm$  SD) (A) HexNAc-lactose. (B) The two isomers of sialyllactose (3-SL and 6-SL). (C) Sialyl-hexosyl-lactose.

bovine milk samples.<sup>24</sup> In contrast, the most abundant acidic OS, the acidic isomer  $\alpha$ 2-3 of sialyllactose (3-SL; ID: 3; Table 3) is

present at higher levels in Jersey milk than in Holstein-Friesian milk (Figure 2B). It is well established that 3-SL is one of the most abundant OS in bovine milk, while the other isomer, 6-sialyllactose (6-SL), is less abundant.<sup>17,37</sup> In the present study, it is shown that in Jersey milk, the 3-SL isomer is significantly (ANOVA;  $p = 0.008$ ) higher in abundance than in milk from Holstein-Friesian cows, while the amount of the 6-SL isomer is not significantly different in the two breeds (Figure 2B). Jersey and Holstein-Friesian colostrum have previously been examined for differences in sialyl OS indicating higher amounts of 3-SL in Jersey milk.<sup>38</sup> Interestingly, McJarow and colleagues found significantly higher amounts of 6-SL in Holstein-Friesian colostrum samples compared to that in Jersey samples.<sup>38</sup> However, our data suggest that in mature milk, there is no difference between abundances of 6-SL between breeds (Figure 2B). Another highly abundant sialylated OS is the sialyl-hexosyl-lactose (SHL, ID: 10, Table 3), which contains one additional hexose compared to sialyllactose. As with sialyllactose, two isomers are found in all milk samples, although the  $\alpha$ 2-6-sialyl-hexosyl-lactose (6-SHL) is at very low abundance compared to  $\alpha$ 2-3-sialyl-hexosyl-lactose (3-SHL; Figure 2C). In contrast to the majority of OS, which decrease as the milk matures, the level of 3-SHL has previously been shown to increase in mature milk compared to colostrum.<sup>24</sup> Statistical analyses show a trend toward a higher proportion of the 3-SHL isomer in Jersey samples (ANOVA;  $p = 0.068$ ), while the 6-SHL isomer is significantly higher in Holstein-Friesian milk (ANOVA;  $p = 0.049$ ; Figure 2C). Sample 0402 deviates from the other samples within the breed, as both isomers are in very low abundance. However, the sample has a normal level of  $\alpha$ 2-3-sialyllactose (compared to the other samples); consequently, the missing SHL isomers from this individual cow cannot be caused by lack of activity of the enzyme required for the production of this specific linkage ( $\beta$ -galactoside- $\alpha$ 2,3-sialyltransferase), and the reason for the absence of SHL isomers in the milk from this animal requires further investigation.

**Interbreed Differences in Oligosaccharides.** Principal component analysis was performed on MS chromatographic data extracted using the molecular feature extraction of the 29 confirmed OS present in all samples (Figure 3). All isomer differences were eliminated by grouping the intensity corresponding to the same mass value. The PCA model successfully



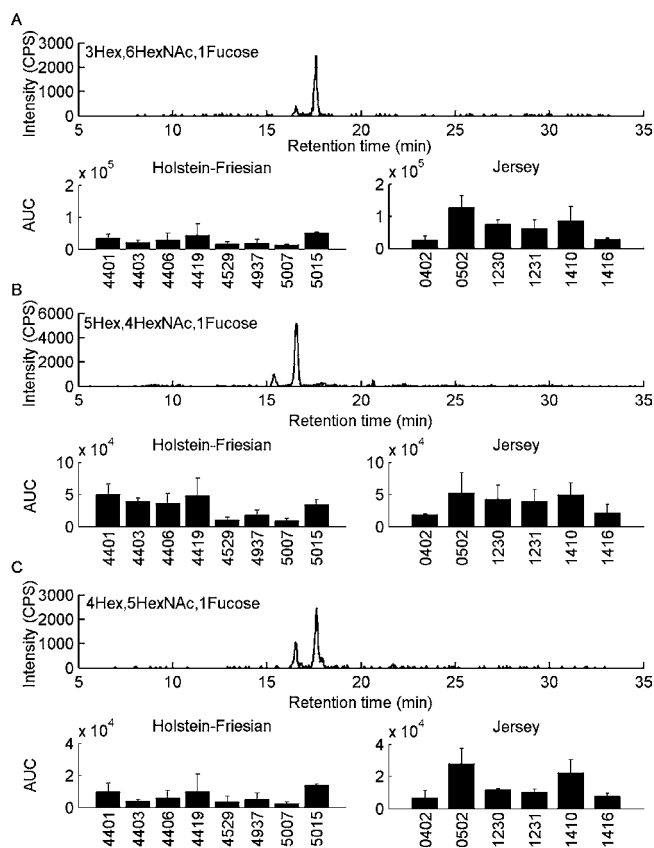
**Figure 3.** (A) PCA score scatter plot of PC1 and PC2 showing targeted analysis of 29 oligosaccharides EIC being integrated by Molecular Feature using MassHunter software (Agilent). (B) Corresponding loading plot. Oligosaccharide IDs refer to Tables 2 and 3.

clustered the milk samples according to the breed using exclusively the first two principal components that explained, respectively, 23.9% and 14.3% of the variance (Figure 3A). The clustering into breeds indicates that there are differences in the OS profile between the breeds. Jersey samples 1410 and 1416 are most similar to the Holstein-Friesian samples, indicated by the proximity of the samples to the Holstein-Friesian cluster. The corresponding loading scatter plot in Figure 3B shows the OS that are responsible for this particular clustering within the two breeds; while the OS present in the top left part of the scatter plot are relatively higher in abundance in Holstein-Friesian milk samples than in Jersey milk samples the opposite is true for the OS present in the bottom right part of the scatter plot, which are relatively higher in abundance in Jersey milk (Figure 3B). The most abundant neutral OS hexosyl-lactose (OS ID: 1) and HexNAc-lactose (OS ID: 2) are relatively higher in milk from Holstein-Friesian milk compared to Jersey milk as seen in Figure 3. Furthermore, we found several differences within the Holstein-Friesian samples. The OS hexosyl-lactose is relatively higher in samples 5015, 4529, and 4419, while HexNAc-lactose HexNAc-lactose is more abundant in 4401, 4403, 4406, 4937, and 5007 (Figure 3AB). The overall higher abundance of neutral OS in Holstein-Friesian milk compared to that in Jersey milk confirms the trend observed in Figure 1B where a univariate approach was applied. Neutral human milk OS with a degree of polymerization of less than seven been shown to be preferentially consumed *in vitro* by some beneficial *Bifidobacteria* strains.<sup>4</sup> Bovine milk contains similar neutral OS, such as the OS with composition 3Hex and 1HexNAc (OS ID: 8) and 4Hex and 2HexNAc (OS ID: 21), and intriguingly one isomer of the latter, eluting at RT 18.52 min, was found to be in higher abundance in Jersey milk than in Holstein-Friesian milk as indicated by the loading plot (Figure 3B). Because this particular OS was previously shown to have a selective prebiotic effect *in vitro*,<sup>4</sup> these findings suggest that milk from the two breeds may have a different impact on the health benefits of milk consumption such as growth stimulation of *Bifidobacteria*. Noticeably, this is also reflected in the PCA analysis (OS ID: 3, 6, 10, 12, 24, and 25), which reveals that two sialylated OS are more abundant in Holstein-Friesian milk

[sialyl-hexosyl-lactose (OS ID: 10) and sialyllactosamine (OS ID: 6)] while four other sialylated OS are more abundant in Jersey milk (OS ID: 3, 12, 24, and 25). Both the univariate and the multivariate data analyses indicate that Jersey cows appear to produce higher levels of the more complex sialylated OS in the milk.

Fucosylated OS are the dominant OS species in human milk,<sup>22</sup> while they have been found in bovine colostrum in only one study,<sup>39</sup> and the occurrence of the fucosylated species is still not readily found in mature bovine milk, with the exception of cheese whey.<sup>11,40</sup> Recently, fucosylated OS species with a polymerization less than four have been identified enzymatically in bovine milk.<sup>41</sup> Furthermore, in a study of the genes involved in OS metabolism, Wickramasinghe et al. identified the genes in the bovine genome responsible for the *de novo* synthesis of fucosylated OS.<sup>28</sup> Additionally, the same authors also identified expression of genes encoding fucosidases, which could explain why fucosylated OS species have not been found in bovine milk previously.<sup>28</sup> It is therefore intriguing that we, in the present study, were able to characterize four fucosylated OS with a polymerization larger than eight, and interestingly, these OS were found to be present in higher abundance in Jersey milk than in Holstein-Friesian milk (OS ID: 42, 47, 49, and 52; Figure 3). The tandem MS/MS spectra obtained by MALDI FT-ICR of some of the fucosylated species are shown in the Supporting Information. The extracted ion chromatograms of the three most abundant and larger fucosylated OS are shown in Figure 4, with 3Hex, 6HexNAc, 1Fucose (OS ID: 52) being the most intense, and the levels of this OS being significantly higher in Jersey samples (ANOVA;  $p = 0.017$ ; Figure 4A). Figure 4B shows another fucosylated OS (5Hex, 4HexNAc, 1Fucose; ID: 47) that is present in comparable levels in the two breeds, while the OS shown in Figure 4C is again significantly higher in abundance in milk from Jersey cows (4Hex, 5HexNAc, 1Fucose; ID: 49; ANOVA;  $p = 0.049$ ).

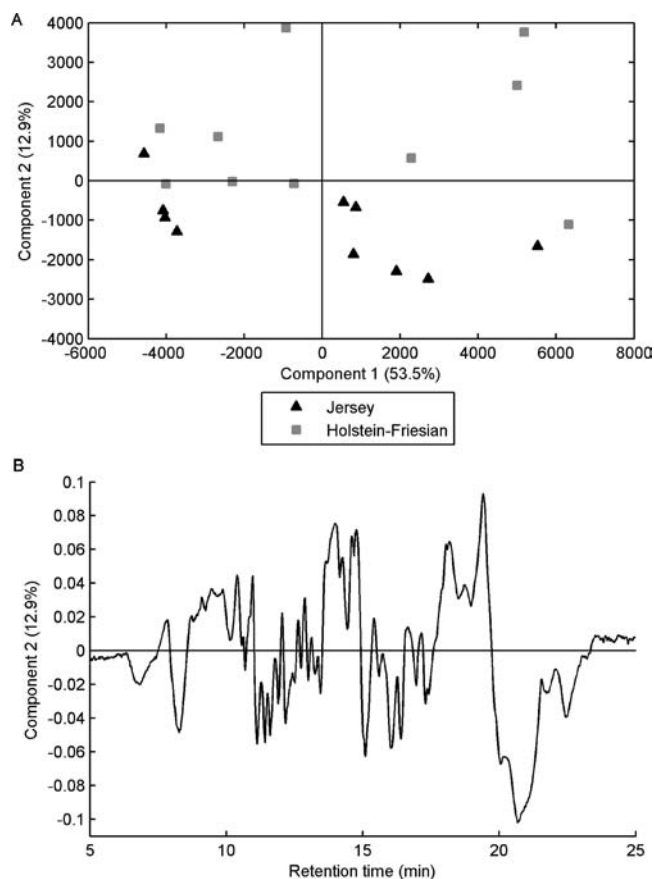
**Multivariate Data Analysis of Base Peak Chromatograms.** In addition to examining the data extracted by the molecular feature extraction of the 29 selected OS masses in MassHunter software, we decided to analyze the data untargeted



**Figure 4.** Extracted ion chromatogram and calculated AUC in 14 samples measured in duplicates (mean  $\pm$  SD). (A) 3Hex, 6HexNAc. (B) 5Hex, 4HexNAc, 1Fucose. (C) 4Hex, 5HexNAc, 1Fucose.

and exploratively by multivariate data analysis of the BPC. Base peak chromatograms of the LC-MS analysis of the milk samples (10 Jersey and 10 Holstein-Friesian) were extracted and aligned. Score scatter plots obtained from PCA on the mean-centered, Pareto-scaled BPC data demonstrated similar groupings to the clustering obtained with the PCA carried out on data extracted by using the molecular feature extraction of selected OS masses present in all samples (Figure 5). Figure 5A shows a score scatter plot of the first and second principal components explaining 53.5% and 12.9% of the variance, respectively. The classification into breeds can almost exclusively be attributed to the second PC, and the corresponding loading plot of the second PC is displayed as a line plot, which resembles the original BPC (Figure 5B). As can be seen from Figure 5A, the Holstein-Friesian milk samples are clustered in quadrants I and II, while the Jersey milk samples are clustered in quadrants III and IV. Thus, a positive peak in Figure 5B indicates that this feature is more abundant in Holstein-Friesian milk samples compared with Jersey samples, and negative peaks are more abundant in Jersey milk samples. The main peaks responsible for the grouping of samples into breeds are galactosyl-lactose (RT: 13.97 min),  $\alpha$ 2-6-sialyllactose (RT: 15.07 min), and  $\alpha$ 2-3-sialyllactose (RT: 19–20 min). As expected when using the BPC, the most abundant OS are influencing the grouping into breeds, while the lesser abundant OS are not visible.

In conclusion, the OS profiles of the two bovine breeds often used in the production of dairy milk were analyzed for differences in OS composition and abundance using a combination of accurate mass spectrometry and multivariate data analyses. On the basis of the data analyses, we have established a number of



**Figure 5.** (A) PCA score scatter plot of PC1 and PC2 showing untargeted analysis of 20 different milk samples purified for oligosaccharides. Base peak chromatograms were extracted and 761 continuous variables were used. (B) Corresponding loading line plot.

differences between the OS profile in Danish Jersey and Holstein-Friesian breeds. Jersey milk contains higher levels of sialylated and also complex neutral fucosylated OS, while Holstein-Friesian milk contains higher levels of the less complex neutral OS. Additionally, Jersey milk samples contained relatively higher levels of the neutral OS which are known to be preferentially utilized by selected beneficial *Bidifobacteria*.<sup>4</sup> Thus, results of the present study point toward Jersey milk as having a higher amount of fucosylated and sialylated OS species than that in Holstein-Friesian milk, which may be important with respect to certain health-promoting benefits for consumers. These findings are novel, and further studies including additional animals would be valuable to corroborate the present findings.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Figure S1: MALDI FTICR tandem MS showing fragmentation patterns of two different oligosaccharides. Tables S1 and S2: showing OS data of a Jersey sample corresponding to Tables 2 and 3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## Notes

The authors declare no competing financial interest.

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