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Comparison of bovine milk oligosaccharides in native North European cattle breeds

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Abstract

Milk oligosaccharides are of high interest due to their bioactive properties. This study is the first to characterise milk oligosaccharides from native North European cattle breeds, as represented by 80 milk samples collected from eight native breeds originated from Norway (Norwegian Doela cattle and Norwegian Telemark cattle), Sweden (Swedish Mountain cattle), Denmark (Danish Red anno 1970), Iceland (Icelandic cattle), Lithuania (native Lithuanian Black and White) and Finland (Western Finncattle and Eastern Finncattle). Using high-performance liquid-chromatography chip/quadrupole time-of-flight mass-spectrometry, 18 unique monosaccharide compositions and a multitude of isomers were identified. No N-glycolylneuraminic acid was identified among these breeds. Western Finncattle milk was most abundant in neutral, acidic and fucosylated oligosaccharides. Further, Eastern Finncattle milk was significantly higher in acidic oligosaccharides and Icelandic cattle milk significantly higher in fucosylated oligosaccharides, compared to the mean. This study highlights specific native breeds of particular interest for future exploitation of milk oligosaccharides and breeding strategies.

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Declaration of interest: none

1. Introduction

Human milk is the ideal source of nutrition for neonates as it consists of a complete source of macronutrients and provides numerous biological activities (Andreas, Kampmann, & Le-Doare, 2015; Ballard & Morrow, 2013). These activities originate from multiple constituents in milk, including oligosaccharides (OS). OS are indigestible by human metabolising enzymes, but play key health-promoting roles, such as acting as prebiotics in the gut (Bode, 2012; Cheng, Akkerman, Kong, Walvoort, & de Vos, 2020), modulating the immune system (He, Liu, Leone, & Newburg, 2014), and preventing pathogen adhesion to the intestinal epithelium (Kong et al., 2019; Moran, Gupta, & Joshi, 2011). Further, sialic acid-containing OS have shown to be important contributors to brain function and cognitive development in animals (Obelitz-Ryom et al., 2019; Oliveros et al., 2018). In bovine milk many OS structures resemble those present in human milk (Aldredge et al., 2013; Fischer-Tlustos et al., 2020; Tao et al., 2008). Bovine milk OS have in several studies been recognized as potentially bioactive food components of interest in industrial applications, such as infant formula, nutraceutical products, and functional and therapeutic foods. The OS from bovine milk may hold the potential to alleviate numerous prevalent disorders in both infants and adults, including metabolic and inflammatory conditions. Several animal studies have demonstrated that consumption of bovine milk OS may decrease intestinal permeability, prevent development of diet-induced obesity and improve inflammatory conditions in the gut (Boudry et al., 2017; Cani et al., 2008; Hamilton et al., 2017). Purified sialic acid-containing OS have moreover shown to promote, in animal models of infant undernutrition, organ growth and improvements in metabolic regulation (Charbonneau et al., 2016). The OS in bovine milk are highly sialylated, with nearly 70% of the structures in colostrum and ~50% of the structures in mature milk containing sialic acid (Tao et al., 2008; Tao, DePeters, German, Grimm, & Lebrilla, 2009).

To date, analytical studies on milk OS structures and compositions have mainly focused on human milk and milk from common dairy breeds (Holstein and Jersey) (Robinson et al., 2019), while no studies have, until now, profiled the OS distribution in milk from native Nordic cattle breeds. The native Nordic cattle breeds are today a result of low effective population size. As an example, the number of registered cows within the Finnish breeds, Western Finncattle and Eastern Finncattle, was of approximately 500,000 in 1950, this number declined to a few thousands today (Hiemstra, Haas, Mäki-Tanila, & Gandini, 2010). The same dramatic decline in population size was reported for Danish Red anno 1970 representing 70% of the production cattle in Denmark in 1950, and today with a breeding population size of approximately 170 (Poulsen et al., 2016; Szekeres, Schönherz, Nielsen, & Guldbrandtsen, 2016). Further, the approximate population sizes of Norwegian Doela cattle, Norwegian Telemark cattle, native Lithuanian Black and White cattle, Icelandic cattle and Swedish Mountain cattle are today of 250, 380, 1290, 80,000 and 500 respectively (Gautason, Schönherz, Sahana, & Guldbrandtsen, 2019; Sæther, Holene, Fjellstad, & Rasmussen, 2019; Šveistienė & Razmaitė, 2013; Växa-Sweden, 2020). Several studies have characterised compositional differences between two of the most commonly used breeds for dairy milk production: Holstein and Jersey. A few of these studies have examined OS variations among breeds (Robinson et al., 2019; Sundekilde et al., 2012; Tao et al., 2009).

The most recent findings show that Jersey milk contained higher relative abundance of most OS structures with a higher degree of complexity, containing more fucose and sialic acid residues (Robinson et al., 2019; Sundekilde et al., 2012).

Molecular characterisations suggest the native dairy breeds to be genetically different from common production breeds, which may have lost some unique traits due to intensive selective breeding (Kantanen et al., 2000). Further, studies have shown that the native Nordic cattle breeds possess a great phenotypic diversity (Kantanen et al., 2000; Lien et al., 1999; Tapio et al., 2006). These indigenous breeds display genetic distinctiveness due to genetic drift, inbreeding and isolation. A previous study by Lien et al. (1999) has shown, that certain allelic frequencies (e.g., the B allele of κ-casein, *CSN3*), related to good manufacturing properties for cheese (Aaltonen & Antila, 1987; Marziali & Ng-Kwai-Hang, 1986; Schaar, Hansson, & Pettersson, 1985), were higher in native Nordic breeds compared with common production breeds (Holstein and Jersey). On the other hand, the β-casein genetic variant F has a strikingly high frequency in Danish native breeds (Danish Red anno 1970 and Jutland cattle) compared with common breeds (Danish Holstein and Jersey), and seems to be associated with poor milk coagulation properties (Poulsen et al., 2016).

Given these differences between native and common cattle breeds, there is substantial interest to determine whether these differences among breeds also manifest in the variation of the valuable bovine milk OS. The present study comprises the first characterisation of OS in milk from native Nordic cattle breeds, to reveal whether such breeds hold unique OS distribution and variation. The study was conducted using high performance liquid-chromatography chip/quadrupole time-of-flight mass spectrometry (HPLC-Chip/Q-TOF MS) and thereby creating comprehensive libraries for each breed based on tandem MS, as well as a relative quantification of all OS identified.

2. Material and methods

2.1. Milk samples and sample collection

Milk samples were collected from the following eight native Nordic and Baltic cattle breeds: Norwegian Doela cattle, Norwegian Telemark cattle, Danish Red anno 1970, native Lithuanian Black and White cattle, Swedish Mountain cattle, Icelandic cattle, Eastern Finncattle and Western Finncattle (Fig. 1). Representative milk samples of 50–100 mL each were collected from a single morning milking from 10 individual cows within each breed (n = 80). Within each breed, all cows were reared in the same herd and no two breeds were reared together in the same herd (10 herds in total). All cows had been routinely milked for commercial production. The cows were on average within the third parity (range: 1 to 9 calvings), and with an average stage of lactation of 133 days (range: 21 to 558 days of lactation) (Table 1). Immediately after collection, all samples were frozen and transported to Aarhus University (Tjele, Denmark). Here all samples were skimmed (2500 × *g*, 30 min, 4 °C) and frozen at −80°C. The samples were shipped on dry ice to the University of California, Davis. Milk samples within each breed were pooled (n = 8) for the MS/MS analysis to establish comprehensive OS libraries and individual milk samples (n = 80) were analysed by MS using the newly assembled libraries. Additionally, a full fat (3.5%) commercial milk sample (most likely representing milk from American Holstein) was

included in this study as a bulk milk control for comparison with the native Nordic breeds milk OS composition. This sample was skimmed (2500 × *g*, 30 min, 4 °C), followed by oligosaccharide extraction and analysis in quadruplicate.

2.2. Oligosaccharide extraction and purification

Thawed skim milk samples were vortexed and 200 μL were mixed with four volumes of 2:1 (v/v) chloroform: methanol. The samples were subsequently centrifuged (4000 × *g*, 30 min, 4 °C) to remove protein and residual milk fat from the samples. Two hundred and fifty microlitres of the upper methanol layer containing the OS fraction was collected and mixed with two volumes of −30 °C pure ethanol. Further denaturation and precipitation of proteins were allowed for 1 h at −30 °C followed by centrifugation at the same conditions as above. The supernatant was dried by centrifugal evaporation at 30 °C (Genevac MiVac Quattro concentrator, Genevac Ltd., Ipswich, UK). The dried OS isolate was reconstituted in 150 μL 18.2 MΩ-cm (Milli-Q) water (EMD Millipore, Billerica, MA, USA) and shaken until complete dissolution. Removal of residual peptides and purification of OS was achieved by microplate C18 solid-phase extraction (SPE, Glygen Corp., Columbia, MD, U.S.A.) conditioned with 3 × 100 μL 100% acetonitrile (ACN), followed by 3 × 100 μL Milli-Q water. After sample loading, the wells were washed with 600 μL Milli-Q water. The eluate that was collected from the sample loading and washing steps was loaded to graphitic carbon SPE microplate (Glygen Corp., Columbia, MD, USA) to remove monosaccharides and residual salts using the conditions as described previously (Parc et al., 2017).

The purified OS fraction collected after graphitic carbon SPE was dried by centrifugal evaporation, then resuspended in 150 μL Milli-Q water and shaken (ThermoMixer C, Eppendorf, Hamburg, Germany) for 1 h to complete rehydration. Samples for the MS/MS analysis were based on pooled samples of equal amounts of milk from the ten individual cows within each breed, whereas individual milk samples were analysed by MS.

2.3. Analysis of oligosaccharides by Nano-LC-Chip/Quadropole TOF MS and development of oligosaccharide libraries

Prior to MS analysis, the concentrated OS stock was diluted 50 times with Milli-Q water and the internal standard, xylosyl-cellobiose (Megazyme, Chicago, IL, USA), was added in a final concentration of 0.5 mg L^{-1}. The samples were analysed using an Agilent 6520 Accurate-Mass quadrupole time-of-flight (Q-TOF) liquid chromatography-mass spectrometry (LC/MS) system, equipped with a microfluidic Chip Cube interface (Agilent Technologies, Santa Clara, CA, USA). Liquid chromatographic solvents consisted of 3% ACN with 0.1% formic acid in water (A), and 89.9% ACN with 0.1% formic acid in water (B) as described by Tao et al. (2009). Samples were injected in aliquots of 2 μL and loaded into the LC system with solvent A at a flow rate of 4 μL min^{-1} by the capillary pump. The gradient used for separation was as follows: 0–2.5 min, 0% B; 2.5–20.0 min, 0–16% B; 20.0–30.0 min, 16–44% B; 30.0–35.0 min, 44–100% B; 35.0–45.0 min, 100% B; followed by 0% B for 20 min to equilibrate the column, at a flow rate of 0.3 μL min^{-1}. Inrun calibration of the equipment was achieved by infusion of calibrant ions with *m/z* 922.009798 and 1221.990637. Data was collected in positive acquisition mode with scan ranges of 450 to 2500 *m/z* (MS) and 100 to 2500 *m/z* (MS/MS).

Comprehensive OS libraries for each native Nordic breed (n = 8) were constructed and validated by tandem MS based on the fragmentation profiles (Supplementary Material Fig. S2). Only OS that were structurally confirmed by tandem MS were added to the libraries. Eight libraries were combined into a final library. The relative abundance of OS within each sample (n = 80) was measured by integration of area under the curve (AUC) using Batch Targeted Feature Extractor from MassHunter Profinder software version B.08.00 (Agilent Technologies). The software extracted chromatograms with a mass tolerance of 20 ppm, a retention time window of ± 2 min, a required glycan isotopic model with charge states of 1–2, and minimum absolute height of 1000 counts. All integrated peaks were checked individually, and any incorrect peak integration from the automatic assignments were reintegrated manually. The relative abundance was reported as the AUC of OS peaks relative to AUC of the internal standard.

2.4. Uni- and multivariate statistical data analysis

Wilcoxon-Mann-Whitney test was used to compare relative abundances of the OS within each breed to the mean of all breeds including the commercial milk sample. This test was chosen due to a non-normal distribution of OS abundances. All univariate statistical tests were performed in R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). A principal component analysis (PCA) was performed in SIMCA version 16 (Sartorius Stedim Biotech, Umeå, Sweden) using 50 OS isoforms as x-variables.

3. Results

3.1. Variation in oligosaccharide profiles among breeds

A total of 50 OS peaks, including isomers and anomers, with 18 unique monosaccharide compositions were identified in milk from all the native Nordic breeds. A combined library was assembled (shown in Table 2), as well as a complete list of isomers and anomers in Supplementary Table S3. Ten OS were neutral non-fucosylated (neutral) containing only hexose (Hex) and N-acetylhexosamine (HexNAc), six were acidic containing Hex, HexNAc and N-acetylneuraminic acid (sialic acid; NeuAc) and two were neutral fucosylated (fucosylated) containing Hex, HexNAc and fucose (Fuc), ranging from small trisaccharides to a polymerisation degree as high as ten monosaccharides. None of the OS compositions contained the monosaccharide N-glycolylneuraminic acid (NeuGc).

Representative base peak chromatograms (BPC) for each breed from the HPLC-Chip/Q-TOF MS analysis are shown in Fig. 2, illustrating major differences in the overall OS profiles. The internal standard, xylosyl-cellobiose, included in all chromatograms, is highlighted in red colour. Surprisingly, neutral OS with composition 2 Hex 1 HexNAc was highly variable in abundance among the analysed breeds, displaying the highest abundance in Western Finncattle, Norwegian Doela cattle, Eastern Finncattle and Swedish Mountain cattle (Fig. 2A–D), and lowest in Danish Red anno 1970 (Fig. 2F) and Norwegian Telemark cattle (Fig. 2G). Less variable in abundance was the major acidic OS 3’-sialyllactose (3’-SL, 2 Hex 1 NeuAc). This OS was most abundant in milk from Western Finncattle, and lowest in abundance in milk from native Lithuanian Black and White cattle, Danish Red anno 1970 and Norwegian Telemark cattle as well as in the commercial milk sample. Further, various

peaks of minor OS were observed, including simple structures such as 3 Hex and 4 Hex, as well as the more complex fucosylated OS.

The summed relative abundances of all OS detected within each breed are shown in Fig. 3A. The highest relative abundance of total OS was seen in milk from Western Finncattle, with significantly higher levels compared to the mean of all breeds ($P = 0.005$). Total OS in milk from Western Finncattle was approximately double as high as levels in milk from the commercial milk sample. Milk from the Danish Red anno 1970 and the Norwegian Telemark cattle displayed the lowest relative abundance of total OS, of where Norwegian Telemark cattle milk was significantly lower than the mean of all samples ($P = 0.005$).

The relative abundances of neutral, acidic and fucosylated OS are shown in Fig. 3B–D. The distributions of neutral and acidic OS followed similar patterns among breeds. The Western Finncattle milk was significantly higher than the mean in neutral OS ($P = 0.009$) and acidic OS ($P = 0.005$), and Eastern Finncattle milk was significantly higher in acidic OS ($P = 0.027$). Lower relative abundance compared to the mean was seen in Norwegian Telemark cattle milk for neutral ($P = 0.015$) and acidic ($P = 0.032$) OS. The distribution of fucosylated OS (Fig. 3D) revealed a different pattern among breeds compared to neutral and acidic OS. Major differences in fucosylated OS relative to neutral and acidic types were seen for Swedish Mountain cattle milk and Danish Red anno 1970 milk, which both had relative abundances of fucosylated OS significantly lower than the mean of all samples ($P = 0.0002$ and $P = 0.027$, respectively). Further, milk from both Icelandic cattle and Western Finncattle had relative abundances of fucosylated OS significantly higher than the mean ($P = 0.049$ and $P = 0.028$, respectively).

3.2. Clustering of breeds based on distinct oligosaccharides

An unsupervised PCA model was applied to explore any clustering behaviour of the native Nordic breeds. Fig. 4A displays a scores plot of the first two principal components (PC), explaining 34% and 22% of the variation, respectively. Most of the samples were clustered close to the centre of the plot, where some samples from each breed were further distributed towards quadrants II and IV, mainly. Mainly samples of Icelandic cattle and Norwegian Doela cattle were represented in all four quadrants. Clustering of the eight breeds revealed interesting correlations with the OS detected (Fig. 4B). Notably the fucosylated OS, 3 Hex 1 HexNAc 1 Fuc, was located in the negative area of PC1 (quadrant I), opposite to most other OS. The major fucosylated OS, 3 Hex 6 HexNAc 1 Fuc, was located in quadrant IV, dominating in samples of Western Finncattle, Icelandic cattle, native Lithuanian Black and White cattle and Norwegian Doela cattle. Neutral OS isomers of 4 Hex were found in the positive area of PC2, dominating in Danish Red anno 1970, Icelandic cattle and Swedish Mountain cattle milk samples. The highest variation within breeds were observed for Western Finncattle, Norwegian Doela cattle and Icelandic cattle and lowest variation within Danish Red anno 1970, Norwegian Telemark cattle and native Lithuanian Black and White.

The study showed a significant variation ($P \leq 0.05$) in the abundance of several distinct OS among breeds. Relative abundances of six OS (2 Hex 1 HexNAc, 4 Hex, 3’-SL, 6’-SL, 3 Hex 6 HexNAc 1 Fuc and 3 Hex 1 HexNAc 1 Fuc) are shown in Fig. 5A–F. Relative abundances of the remaining OS identified in the study are shown in Supplementary

Material Fig. S1. Relative abundances reported are based on the sum of all isomers/anomers identified with the given composition. The major neutral OS detected was 2 Hex 1 HexNAc (Fig. 5A), less abundant was the neutral OS 4 Hex (Fig. 5B). Very low levels of 2 Hex 1 HexNAc were detected in milk from Danish Red anno 1970 and Norwegian Telemark cattle, significantly lower than the mean. In contrast, Danish Red anno 1970 milk was significantly higher than the mean in 4 Hex ($P = 0.043$). The abundance of 2 Hex 1 HexNAc was significantly above the mean in Western Finncattle ($P = 0.004$, Fig. 5A) and slightly above the mean in the commercial milk sample. The major acidic OS detected was 3'-SL (Fig. 5C), revealing an almost similar distribution among breeds as the overall acidic OS distribution. The most abundant fucosylated OS detected was 3 Hex 6 HexNAc 1 Fuc (Fig. 5E), which was significantly higher than the mean in milk from Western Finncattle, Norwegian Doela cattle and Icelandic cattle ($P = 0.004$, $P = 0.02$ and $P = 0.04$, respectively). Less abundant was the more simple fucosylated OS 3 Hex 1 HexNAc 1 Fuc (Fig. 5F), with significantly higher relative abundance in samples of Norwegian Telemark cattle ($P < 0.001$) compared with the mean.

4. Discussion

4.1. Oligosaccharide identification

Based on the specific OS libraries the study was able to identify a total of 50 OS, including isomers and anomers, with 18 unique compositions. The use of tandem MS to analyse pooled samples from each breed revealed a very comprehensive verification of the OS identified. Besides providing a high accuracy, the method allows identification of isomers and anomers eluting at distinct RT, but with the same monosaccharide composition. Overall, more OS structures have been identified in bovine milk previously, though most of these structures were found in colostrum or early lactation milk and were only present in low abundances or not present in mature milk (Fischer-Tlustos et al., 2020; Tao et al., 2009). Since the present study is based on mature milk (range: 21 to 558 days of lactation), these differences may explain why less compositions were identified in the study. None of the OS compositions contained the monosaccharide NeuGc, which is most commonly found in colostrum and early lactation milk (Tao et al., 2009). Similarly, Robinson et al. (2019) identified no OS containing NeuGc in mid-lactation milk, while Sundekilde et al. (2012) identified three OS structures containing NeuGc in Holstein milk with an average lactation stage of 165 days. The absence of NeuGc in mid-lactation milk may thus be a uni4que feature of the native breeds, as the samples are at a comparable lactation stage (average lactation of 133 days), though it may be present in colostrum of these breeds as also shown previously in mature milk and colostrum from Holstein cows (Tao et al., 2009). Absence of NeuGc in mid-lactation milk may be a benefit, since NeuGc has been associated with negative health outcomes, by possibly promoting inflammation and cancer progression (Samraj et al., 2015). Further, no OS in human milk contains NeuGc (Ninonuevo et al., 2006; Wu, Grimm, German, & Lebrilla, 2011). The absent expression of NeuGc in mature milk from the native Nordic breeds may thus be a beneficial trait in order of being ideal for manufacturing of infant formula.

The samples used in this study were frozen prior to OS extraction and analysis. Although the effect of freezing on the stability and solubility of milk OS has not been extensively studied, milk OS extraction steps performed at −80 °C have previously been conducted without detriment to OS recovery: a study by Totten et al. (2014) showed no differences when OS profiles were monitored by HPLC-Chip/Q-TOF MS with and without protein precipitation via freezing in ethanol.

4.2. Inter-breed differences related to oligosaccharide profiles

To our knowledge, this is the first study to investigate OS variations in native Nordic cattle breeds, with the approach of exploring existing variations in milk OS among local native breeds representing a high phenotypic, geographical and phylogenetic diversity (Kantanen et al., 2000). Most native breeds have, to some extent, suffered a demographic bottleneck and/or inbreeding resulting in loss of heterozygosity and thereby potential loss of important genetic polymorphisms (Kantanen et al., 2000). However, as the genetic influence on milk OS is generally high (Poulsen, Robinson, Barile, Larsen, & Buitenhuis, 2019), the interesting variations in the relative abundances of OS observed among the native Nordic cattle breeds included may reflect the phylogenetic variation of these breeds. Molecular characterisations of 35 Northern European breeds suggested three distinct phylogenetic clusters of these breeds into Black and White type, Baltic Red and Nordic cattle (Tapio et al., 2006), where the Western Finncattle, Eastern Finncattle, Swedish Mountain cattle, Icelandic cattle, Norwegian Doela cattle and Norwegian Telemark cattle cluster into the Nordic type, Danish Red anno 1970 into the Baltic Red cluster and native Lithuanian Black and White cattle into the Black and White type cattle (Tapio et al., 2006). The genetic groups suggested by Tapio et al. (2006) were largely confirmed by Kantanen et al. (2000), apart from Norwegian Telemark cattle, which they found to cluster with Danish Red anno 1970 into a group containing Southern Scandinavian breeds, and the Norwegian Doela cattle was not really placed into any group in this study (Kantanen et al., 2000).

The overall relative abundances of all milk OS detected within each breed revealed significantly higher levels of OS in milk from Western Finncattle compared with the mean of all samples, which makes this breed particularly interesting (Fig. 3A). Generally, milk from Norwegian Doela cattle, Eastern Finncattle, Swedish Mountain cattle and Icelandic cattle, all belonging to the Nordic group, were also high in OS, suggesting that a higher total OS abundance may be connected to polymorphisms present in these breeds. The distribution of fucosylated OS differed from the distribution of acidic and neutral OS, suggesting that the underlying synthesis pathways for fucosylated OS may be different from those synthesising acidic and neutral OS.

In a recent genome-wide association study (Poulsen et al., 2019) on Danish Holstein and Danish Jersey, promising candidate genes encoding specific glycosyltransferases (e.g. β3-N-acetylglucosaminyltransferases) were identified as affecting the biosynthesis of specific neutral (including fucosylated OS) and acidic bovine milk OS and polymorphism in these genomic regions may also affect the OS pattern observed here among the Nordic breeds. The OS in the commercial milk was approaching the mean of neutral and acidic OS, but was lower than the mean value for fucosylated OS, demonstrating that milk from the native

breeds analysed generally contains higher levels of fucosylated OS. More specifically, milk from three breeds, Western Finncattle, Norwegian Doela cattle and Icelandic cattle, possessed OS containing a high degree of functional residues (fucose and sialic acid). They were significantly higher in abundance of the complex fucosylated OS 3 Hex 6 HexNAc 1 Fuc compared with the mean of all breeds, and they were among the five breeds, Western Finncattle, Norwegian Doela cattle, Eastern Finncattle, Swedish Mountain cattle and Icelandic cattle, with the highest relative abundances of the major acidic OS 3'-SL, among the native breeds. Inclusion of these breeds in future breeding strategies, towards milk with higher bioactivities, should thus be considered. Earlier findings document comparable breed specific differences, in this case between Holstein and Jersey, in which Holstein milk was shown to have overall higher levels of neutral OS, though lower levels of fucosylated OS, as compared with Jersey milk (Sundekilde et al., 2012).

The OS profiles permitted an overall clustering of samples from each breed, as illustrated by multivariate data analysis including the variation observed for the identified 50 isomers/anomers (Fig. 4). For some of the breeds, the ten individual cows within a breed were clustered closely together, demonstrating a low intra-breed variation in OS. Given the low sample population per breed, high genetic relationship among sampled cows must be considered as a factor. Norwegian Telemark cattle, Danish Red anno 1970 and native Lithuanian Black and White cattle displayed notable low intra-breed variation, while the remaining breeds displayed higher intra-breed variations. However, the clustering of samples from each breed was to some extent overlapping with other breeds, which suggests that despite large phylogenetic diversity, OS profiles among cows from different breeds may still be quite similar and that the breeds as such do not produce highly unique OS profiles, but rather subtle differences in OS abundance. The Icelandic cattle milk seemed to display high variation clustering into all four quadrants. This may demonstrate that, despite isolation on Iceland since the settlement of humans in the ninth century (Sigurdsson & Jonmundsson, 2011), the relatively large effective population size may have preserved the within-population genetic variation. Further, previous studies have suggested a relatively high level of genetic diversity within the Icelandic cattle, using molecular markers (Ásbjarnardóttir, Kristjánsson, Jónsson, & Hallsson, 2010).

Clustering of the breeds revealed interesting correlations with the OS identified (Fig. 4B). The two fucosylated OS (3 Hex 1 HexNAc 1 Fuc and 3 Hex 6 HexNAc 1 Fuc) were located in almost opposite corners of the PCA plot, which is also observed in the relative abundances of these OS among breeds (Fig. 5E,F). This may reflect origins from different synthesis pathways, despite of their compositional similarities. It is possible that 3 Hex 1 HexNAc 1 Fuc serves as a precursor of 3 Hex 6 HexNAc 1 Fuc, which would also explain the low levels of the precursor in samples with high relative abundance of 3 Hex 6 HexNAc 1 Fuc. Alternatively, 3 Hex 1 HexNAc 1 Fuc may be a degradation product from the action of carbohydrate-degrading enzymes on 3 Hex 6 HexNAc 1 Fuc. Further, 4 Hex seems to have a major effect on the clustering of breeds along PC2, and there was a very strong positive association among the 13 identified 4 Hex isomers/anomers. This OS composition was not detected in the commercial milk sample, though it has been detected in common cattle breeds (Holstein and Jersey), previously (Aldredge et al., 2013). The 4 Hex composition was further relatively low in Western Finncattle and Norwegian Doela cattle

milk, which in contrast were high in 2 Hex 1 HexNAc, opposite to the trends observed for Danish Red anno 1970 and Norwegian Telemark cattle milk. These findings may possibly reflect that mechanisms of the synthesis pathways of these neutral OS are reversely regulated.

4.3. Variation in milk oligosaccharides among native breeds and common dairy cattle

Relative abundances of OS in the commercial milk sample were in most cases close to the mean of all samples, or lower. However, the abundance of the major neutral OS, 2 Hex 1 HexNAc, was relatively high in commercial milk, and generally varied a lot among the native breeds. This can be observed in the representative chromatograms, where consistently very low abundances in Danish Red anno 1970 and Norwegian Telemark cattle are striking and may suggest that these breeds are unable to synthesize these isomers (Fig. 2, Fig. 5A). 2 Hex 1 HexNAc has previously been reported to be a major OS in the common dairy breeds, Holstein and Jersey (Aldredge et al., 2013). As the data here reported are based on small sample populations and each breed is representing only one herd, a close genetic relationship among sampled cows could also affect the results. Furthermore, it should be noted that parameters such as variations in lactation stage, parity, feed and farm management may as well influence the OS output within and among breeds included in the study, but to elucidate this a more comprehensive study would be needed.

One major trait selected for in common dairy breeds is milk yield. In that context, previous studies have shown that higher relative abundances of OS were found in milk from beef cattle (i.e., breeds that were not bred for milk yield) compared with common dairy breeds (Sischo, Short, Geissler, Bunyatratchata, & Barile, 2017), possibly suggesting that breeding for higher milk yield may result in lower OS content. Studies by Sundekilde et al. (2012) and Robinson et al. (2019) found acidic and fucosylated OS being more abundant in Jersey milk, compared with Holstein milk. Although both breeds are commercial breeds, Holstein has been through a more intensive breeding for higher milk yields compared with Jersey (Prendiville, Pierce, & Buckley, 2009). However, these studies did not document OS abundance as being dependent on milk yield. Milk from the two Finnish breeds, Eastern Finncattle and Western Finncattle, of the present study, revealed significantly higher levels of acidic OS compared to the mean of all samples, whereas the commercial milk sample, in comparison, was just below the mean (Fig. 3C). Three native breeds, Western Finncattle, Norwegian Doela cattle and Icelandic cattle, had a significantly higher abundance of the milk OS, 3 Hex 6 HexNAc 1 Fuc, compared with the mean, while the commercial milk sample again was just below the mean (Fig. 5E). As the yields of Western Finncattle, Norwegian Doela cattle and Icelandic cattle are ~2720–6780 kg $year^{-1}$ (Lilja, Soini, & Mäki-Tanila, 2009; Sæther et al., 2019), compared with Holstein with a yield of ~10,000 kg $year^{-1}$ (Januś, Borkowska, Wilgos, & Czaplicka, 2013; Kristensen et al., 2015), this could suggest a tendency towards more acidic and fucosylated OS with lower yield. More samples are though required to confirm this association.

Fischer-Tlustos et al. (2020) showed that the concentration of certain OS decreased markedly from colostrum to seven days in lactation, illustrating the importance of excluding samples collected less than one week in lactation when comparing OS in mature milk. In the

present study Norwegian Telemark cattle represented the lowest lactation stage with an average of 48±32 days, while native Lithuanian Black and White represented the highest of 228±131 days (Table 1). Thus, the difference in lactation stage among these breeds may have influenced the OS abundances presented in this study. However, we believe that this effect is unlikely to be significant for two reasons: first, several studies have shown that the most substantial temporal changes in OS abundance occur during the first 1–2 weeks of lactation, with much more subtle and gradual changes occurring afterward (Tao et al., 2009; Wickramasinghe et al., 2011); second, it was observed that Norwegian Telemark cattle, which represent the lowest average day of lactation, had the lowest relative abundance of total OS, and Western Finncattle, which represent relatively high average day of lactation (189±115 days) had the highest. Therefore, variation related to lactation stage is unlikely to cause the differences observed among the Nordic breeds.

It is well known that bovine milk contains higher abundances of 3'-SL compared with 6'-SL over lactation (Wickramasinghe et al., 2011). In contrast, mature human milk contains higher amounts of 6'-SL compared with 3'-SL (Ferreira et al., 2020; Tonon, Miranda, Abrao, de Morais, & Morais, 2019; Wang & Brand-Miller, 2003). In the present study, the commercial milk sample and most of the native breeds contained approximately 10 times higher relative abundance of 3'-SL compared to 6'-SL. In native Lithuanian Black and White cattle milk the ratio between 3'-SL/6'-SL was 15.2 and in Danish Red anno 1970 milk only 6.6, indicating that Danish Red anno 1970 samples more closely resemble human milk in relation to this specific trait compared with the other Nordic breeds included in the study.

5. Conclusions

Several differences in composition and abundance of OS among eight native Nordic dairy breeds have been elucidated in the present study for the first time. Multivariate data analysis revealed interesting inter-breed OS clustering. In particular, the two fucosylated OS with composition 3 Hex 6 HexNAc 1 Fuc and 3 Hex 1 HexNAc 1 Fuc, as well as 4 Hex isomers were important for the variation observed in the data. The distribution of fucosylated OS among native breeds was highly different from the distribution of neutral and acidic OS, demonstrating that other regulation mechanisms or other synthesis pathways may regulate the synthesis of fucosylated OS. Three breeds had rather unique OS pattern (Western Finncattle, Norwegian Doela cattle and Icelandic cattle), in terms of their significantly higher relative abundance of the complex fucosylated OS with composition 3 Hex 6 HexNAc 1 Fuc. Furthermore, milk from Western Finncattle was notable for being substantially more abundant in most OS identified in the study. This characterization reveals new knowledge on the milk glycome in native dairy breeds and highlights breeds of particular interest for commercial use, conservation purposes and possibly for future selective breeding strategies for potential bioactive food components.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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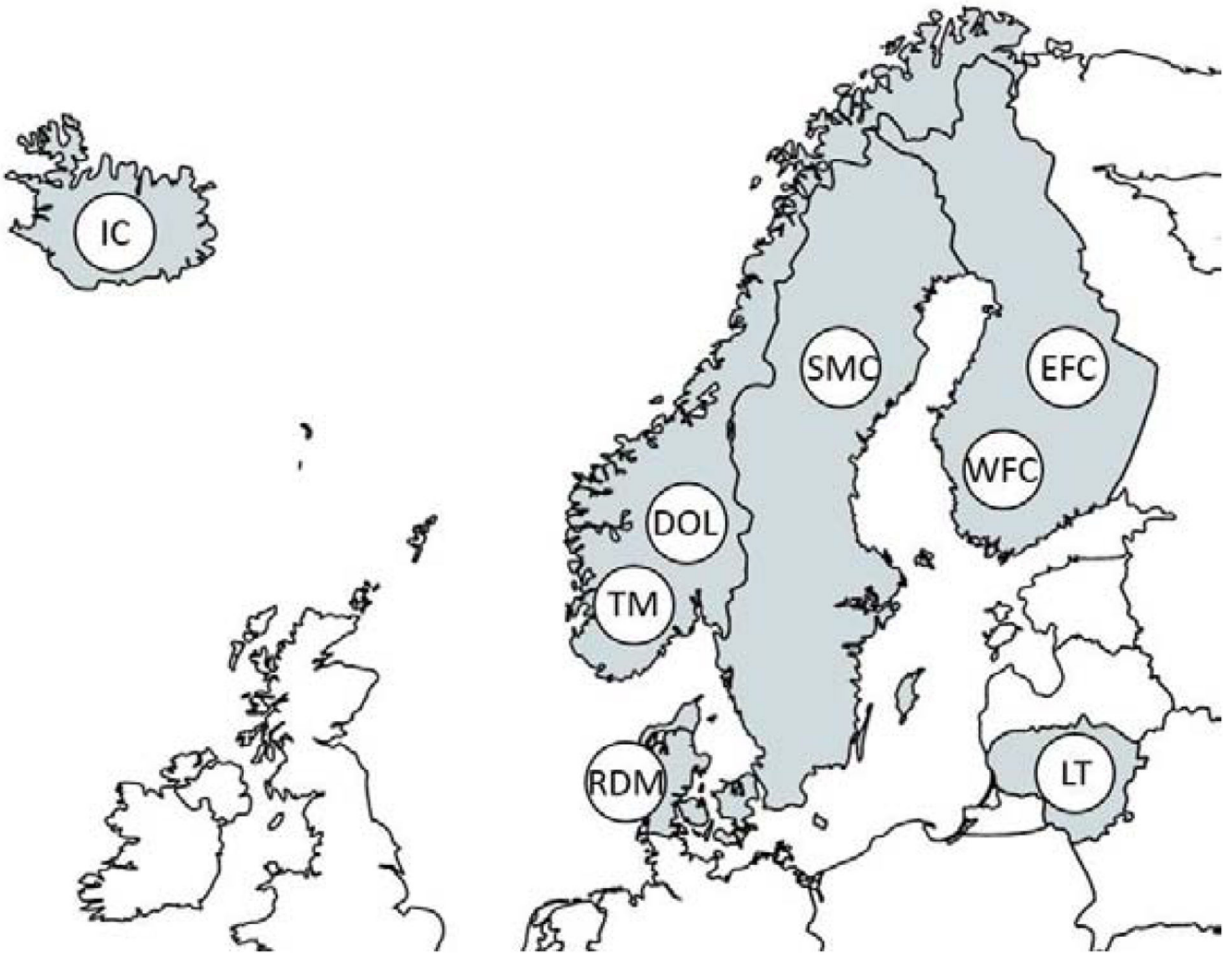

Fig. 1.

Geographic origin of the breeds included in the study. Western Finncattle (WFC), Norwegian Doela cattle (DOL), Eastern Finncattle (EFC), Swedish Mountain cattle (SMC), Icelandic cattle (IC), native Lithuanian Black and White cattle (LT), Danish Red anno 1970 (RDM) and Norwegian Telemark cattle (TM).

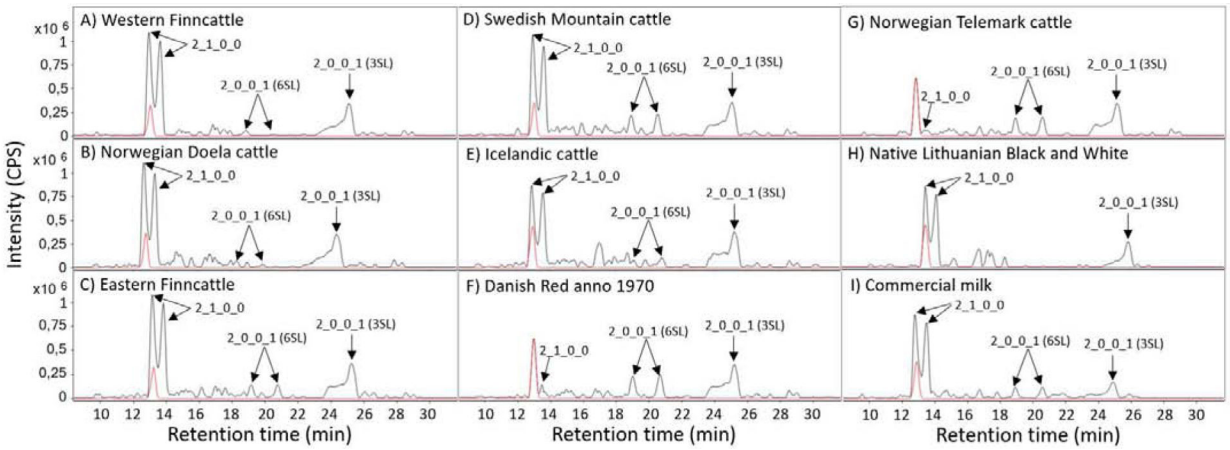

Fig. 2.

Representative base peak chromatograms (BPC), from HPLC-Chip/Q-TOF MS, of each of the eight native cattle breeds (A) Western Finncattle, (B) Norwegian Doela cattle, (C) Eastern Finncattle, (D) Swedish Mountain cattle, (E) Icelandic cattle, (F) Danish Red anno 1970, (G) Norwegian Telemark cattle, (H) native Lithuanian Black and White and (I) commercial milk sample. Internal standard is shown in red. The animals selected were within lactation stage 99–194 days. The OS are described by their monosaccharide compositions, denoted as the number of each monosaccharide type present in the following order Hex_HexNAc_Fuc_NeuAc, where: Hex, hexose; HexNAc, N-acetylhexosamine; Fuc, fucose; NeuAc, N-acetylneuraminic acid (sialic acid).

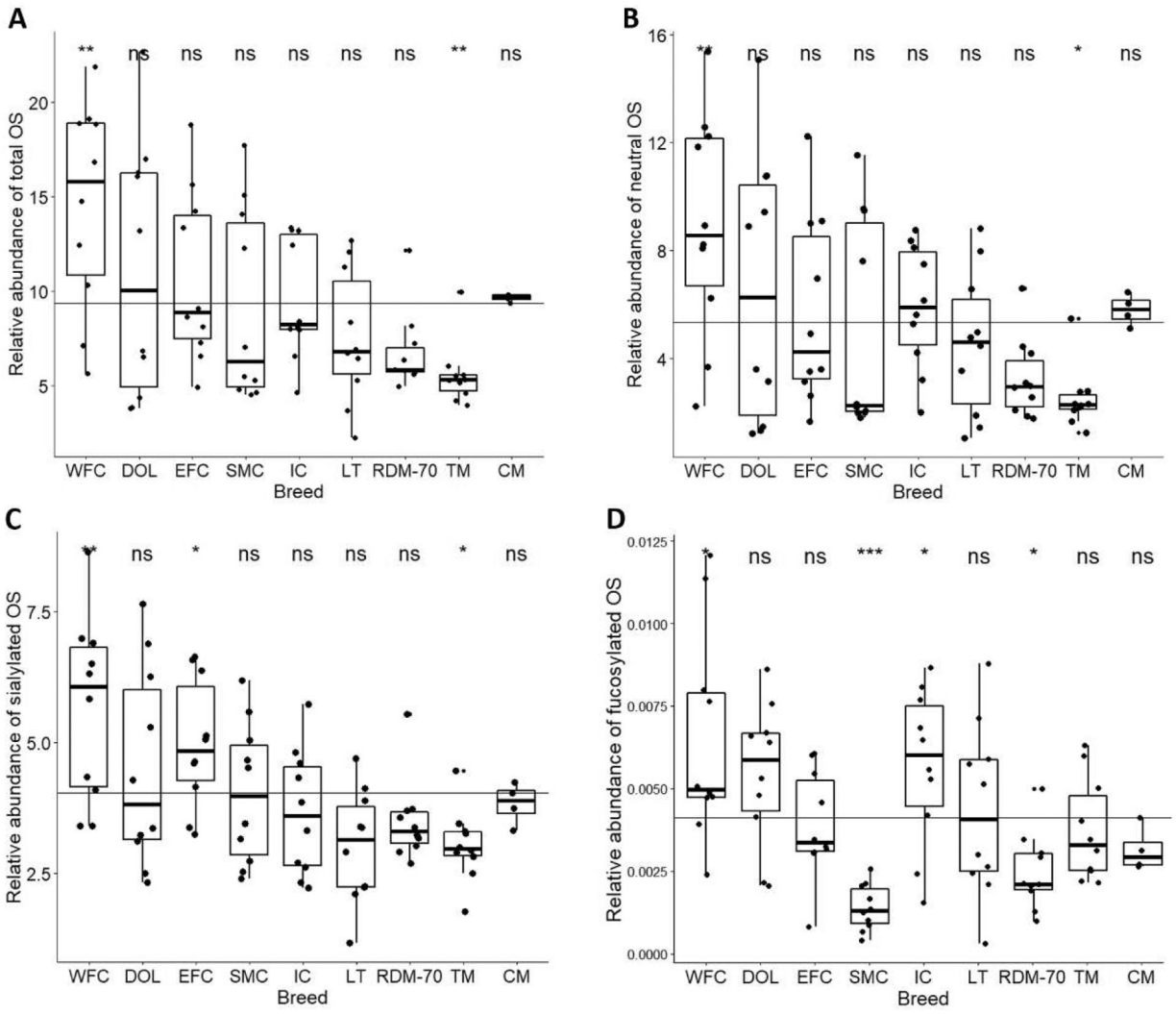

Fig. 3.

Relative abundance of (A) total, (B) neutral non-fucosylated, (C) sialylated and (D) neutral fucosylated oligosaccharides detected in milk of Western Finncattle (WFC), Norwegian Doela cattle (DOL), Eastern Finncattle (EFC), Swedish Mountain cattle (SMC), Icelandic cattle (IC), native Lithuanian Black and White cattle (LT), Danish Red anno 1970 (RDM-70), Norwegian Telemark cattle (TM) and commercial milk (CM). The boxes highlights the interquartile range (IQR) of the 25th to 75th percentile. The horizontal line in the boxes indicates the median and the overall horizontal line indicates the mean of all samples: ns = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ based on a Wilcoxon-Mann Whitney test. Exact *P*-values are provided as Supplementary material Table S1.

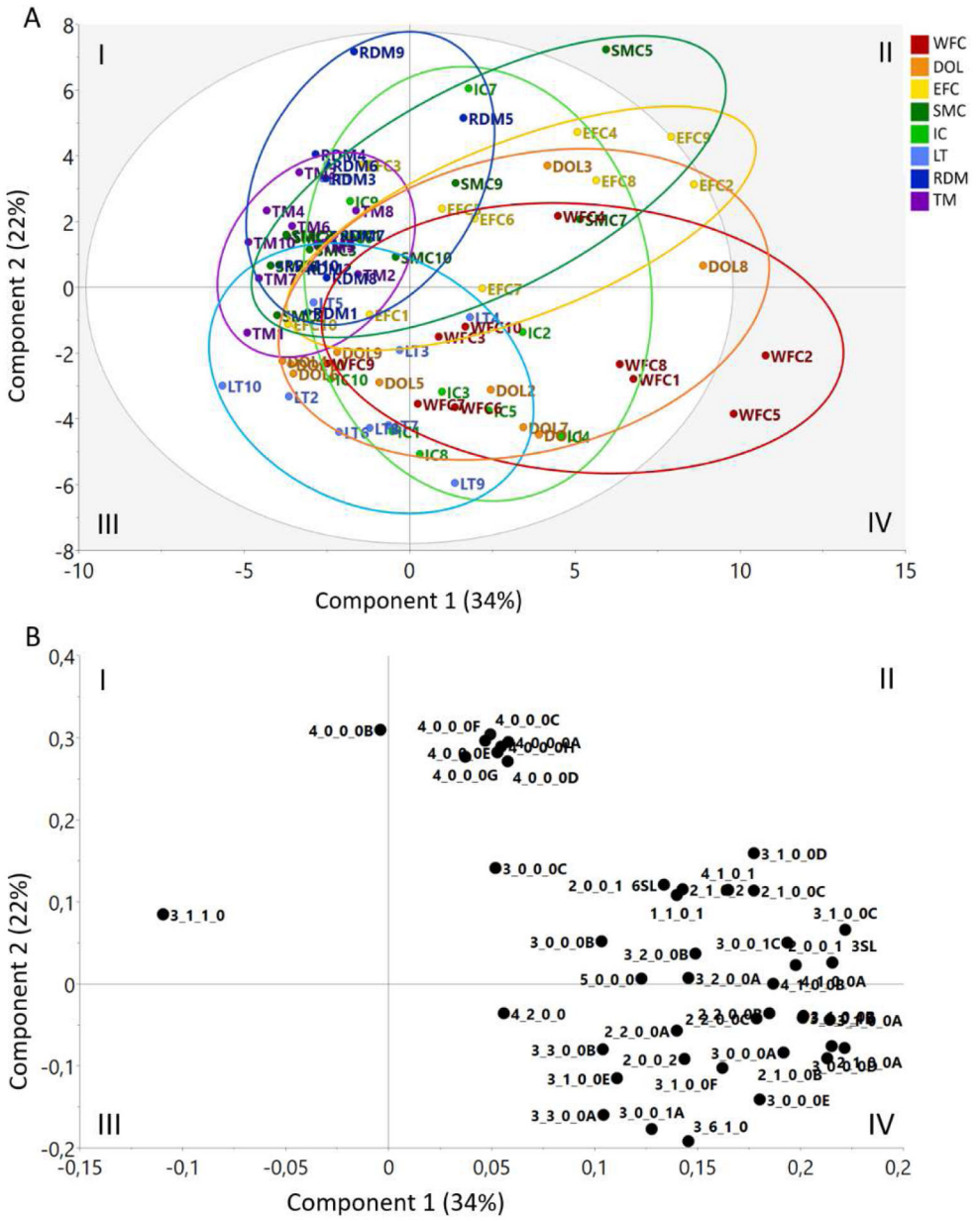

Fig. 4.

Principal component analysis (PCA) displaying the two first principal components: (A) scores plot of ten samples within each of the eight breeds; (B) loading plot of integrated areas of oligosaccharide isomers/anomers from EIC. Data have been scaled to unit variance prior to analysis. The oligosaccharides are described by their monosaccharide compositions, denoted as the number of each monosaccharide present in the following order Hex_HexNAc_Fuc_NeuAc, where: Hex, hexose; HexNAc, N-acetylhexosamine; Fuc, fucose; NeuAc, N-acetylneuraminic acid (sialic acid). Western Finncattle (WFC1–10), Norwegian Doela cattle (DOL1–10), Eastern Finncattle (EFC1–10), Swedish Mountain cattle (SMC1–10), Icelandic cattle (IC1–10), native Lithuanian Black and White cattle

(LT1–10), Danish Red anno 1970 (RDM1–10) and Norwegian Telemark cattle (TM1–10). Isomers/anomers are denoted A–H.

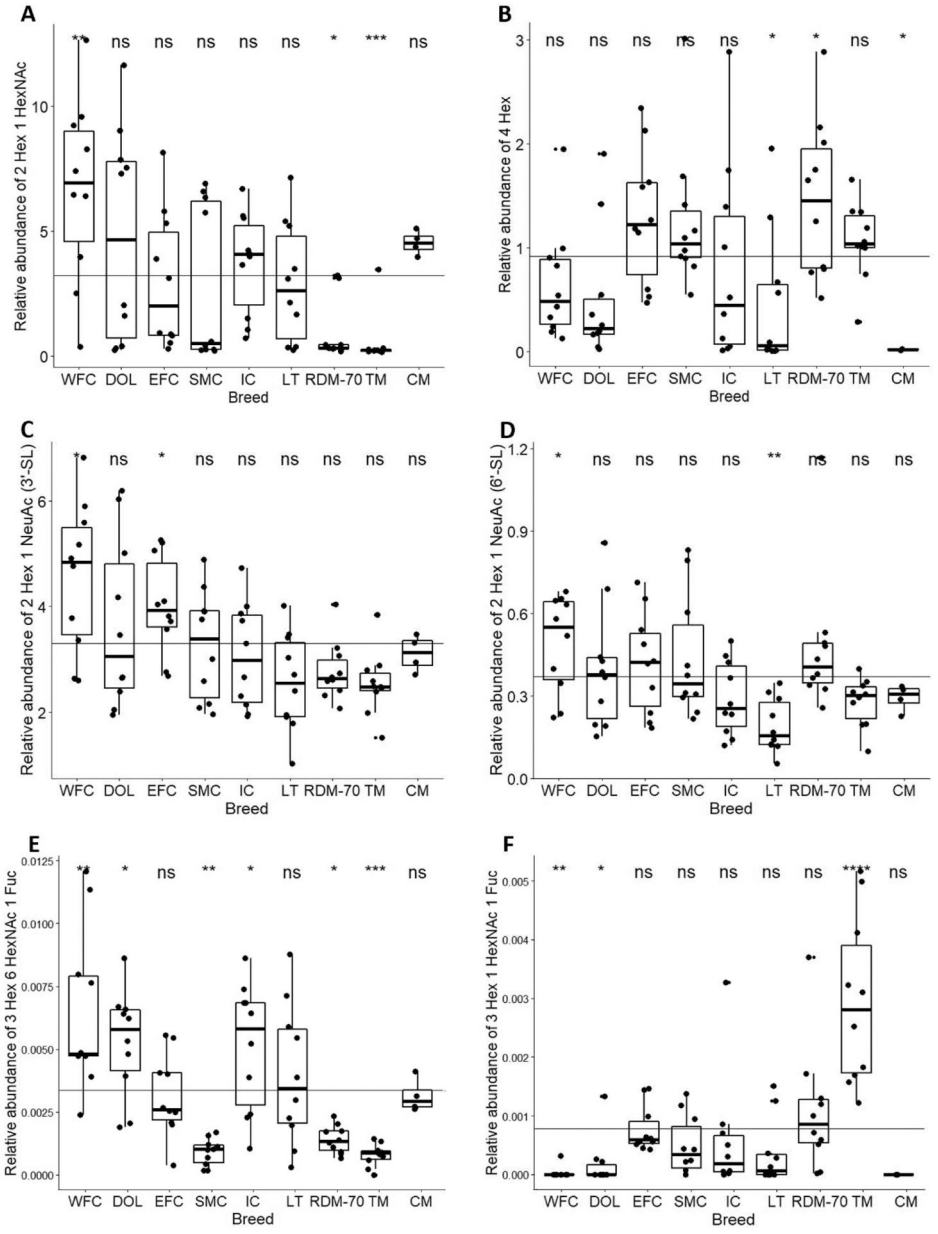

Fig. 5.

Relative abundance of the neutral oligosaccharides (A) 2 Hex 1 HexNAc and (B) 4 Hex, the sialylated oligosaccharides (C) 3'-SL and (D) 6'-SL, as well as the fucosylated oligosaccharides (E) 3 Hex 6 HexNAc 1 Fuc and (F) 3 Hex 1 HexNAc 1 Fuc, in milk of Western Finncattle (WFC), Norwegian Doela cattle (DOL), Eastern Finncattle (EFC), Swedish Mountain cattle (SMC), Icelandic cattle (IC), native Lithuania Black and White cattle (LT), Danish Red anno 1970 (RDM-70), Norwegian Telemark cattle (TM) and commercial milk (CM). The horizontal line in the boxes indicates the median and the horizontal line in the plot indicates the mean of all samples; ns = $P > 0.05$; * = $P < 0.05$; **

= $P < 0.01$; *** = $P < 0.001$ based on a Wilcoxon-Mann Whitney test. Exact *P*-values are provided as Supplementary material Table S1.

Table 1

Average lactation stage and parity of samples included in the study.[a]

Breed	Cow ID	Lactation stage	Parity
Western Finncattle	WFC	188.5 ± 114.7	2.6 ± 2.0
Norwegian Doela cattle	DOL	88.7 ± 33.3	3.1 ± 1.7
Eastern Finncattle	EFC	195.5 ± 52.7	4.1 ± 2.8
Swedish Mountain cattle	SMC	109 ± 58.5	3.7 ± 1.8
Icelandic cattle	IC	95.6 ± 55.2	2.5 ± 1.8
Native Lithuanian Black and White	LT	228 ± 131.4	2.8 ± 0.8
Danish Red anno 1970	RDM-70	109 ± 62.5	1.9 ± 0.9
Norwegian Telemark cattle	TM	48.4 ± 32.3	4.3 ± 2.3

[a]Values are the mean ± standard deviation; ten samples were taken for each breed. Lactation stage is the average number of days in milk; parity is the average parity

Table 2

Library of the 18 unique oligosaccharide (OS) compositions identified in all breeds.[a]

OS ID	Exp. mass	RT	Oligosaccharide composition				Relative abundance
			Hex	HexNAc	Fuc	NeuAc	
1	545.2025	9.6–13.7	2	1	0	0	3.2 ± 2.0
2	748.2818	15.9–19.5	2	2	0	0	0.018 ± 0.0083
3	504.1761	9.8–16.9	3	0	0	0	0.74 ± 0.26
4	707.2553	13.2–20.4	3	1	0	0	0.21 ± 0.079
5	853.314	16.5–16.6	3	1	1	0	0.0008 ± 0.0008
6	910.3351	18.2–19.5	3	2	0	0	0.035 ± 0.022
7	1113.417	20.1–21.5	3	3	0	0	0.033 ± 0.015
8	1868.716	21.6–21.9	3	6	1	0	0.0034 ± 0.0022
9	666.2286	11.8–24.7	4	0	0	0	0.94 ± 0.45
10	869.3079	19.9–21.7	4	1	0	0	0.13 ± 0.063
11	1072.391	22.6–23.0	4	2	0	0	0.041 ± 0.018
12	828.283	28.9–29.0	5	0	0	0	0.0014 ± 0.00066
13	674.2451	18.4–19.0	1	1	0	1	0.0105 ± 0.0043
14 (6’SL)	633.2188	17.0–19.0	2	0	0	1	0.37 ± 0.15
14 (3’SL)	633.2188	23.3–25.0	2	0	0	1	3.3 ± 1.2
15	924.314	25.3–26.3	2	0	0	2	0.011 ± 0.0088
16	1127.395	31.6	2	1	0	2	0.0057 ± 0.0038
17	795.2714	24.5–26.8	3	0	0	1	0.303 ± 0.14
18	1160.407	28.3–28.7	4	1	0	1	0.025 ± 0.017

[a]Multiple isomers exists for each composition but due to the lack of commercial pure standards, these were not included separately. An exception is the more abundant 3’SL and 6’SL (OS ID 14), which are isomers of the same monosaccharide composition, but are listed separately since their commercial standards (and hence retention times) can be easily obtained. Abbreviations are: Exp. mass, neutral experimental mass values (averaged); RT, retention time; Hex, hexose; HexNAc, N-acetylhexosamine; Fuc, fucose; NeuAc, N-acetylneuraminic acid. Relative abundance is the sum of relative abundances of the isomers/anomers taken as averaged values from all eight breeds, including standard deviations.