Determination of $N$-acetylneuraminic and $N$-glycolylneuraminic acids in unprocessed milk of four cattle breeds

Alessandra Crisà, Cinzia Marchitelli, Sebastiana Failla and Michela Contò

Abstract

This research communication reports concentrations of two sialic acids (SA), $N$-acetylneuraminic (Neu5Ac) and $N$-glycolylneuraminic (Neu5Gc), in fresh milk from different cow breeds throughout lactation. According to published studies, the two SA types found in animal-derived products have diverse and conflicting effects on human health, but SA content is not routinely analysed in individual milk cows samples. We measured the content of Neu5Ac and Neu5Gc in milk from Holstein Friesian (HO), Simmental (SM), Simmental × Holstein crossbred (SM×HO), and Podolica (POD) cows at 60 and 120 d following calving. HO, SM and SM×HO were reared in an intensive production while POD were raised in an extensive system. Results showed that total Neu5Ac was overall thirty times more abundant than Neu5Gc, and their concentrations were higher at 120 d than at 60 d ($P < 0.001$). Neu5Gc values were greater in HO, SM, and SM×HO than in POD ($P < 0.001$), while HO had a higher Neu5Ac value than the other three breeds ($P < 0.001$). These findings shed light on the differences in SA content among cow breeds and lay the groundwork for future research to select animals that produce milk with desirable characteristics for human health.

Material and methods

Animals and management

The study was carried out in Holstein Friesian (HO), Simmental (SM), Simmental (sire) × Holstein (dam) crossbred (SM×HO) and Podolica (POD) breeds. HO, SM and SM×HO were reared in the CREA experimental farm located in Central Italy. Animals were kept under the same management and feeding conditions with a total mixed ration (alfalfa, Medicago sativa italicus L.) hay, polyphyta hay, sorghum slilage, barley, corn, triticale, soybean) in an intensive system. The POD cows were reared in the South of Italy in an extensive system based on grazing, with differences in forage essences and chemical composition depending on
the spring and summer sampling. Individual milk samples of 30 animals from each breed were collected from milking morning at 60 and 120 d of lactation (total of 240 samples) and stored frozen at −20 °C until analysis.

Analytical procedures

The concentration of total SA as Neu5Ac and Neu5Gc was determined using the Spichtig et al. (2010) procedure with some modifications. For the release of SA from glycoconjugates (glycoprotein, glycolipids, and lipopolysaccharides), acidic hydrolysis was used, followed by reversed-phase high-performance liquid chromatography (HPLC) after derivatization of the SA using 1,2-diamino-4,5-methylenedioxybenzene (DMB) (protocol in online Supplementary file).

Statistical analysis

The statistical analysis of the SA content was carried out by the STATISTICA© 12.0 package (StatSoft Inc., Tulsa, OK, USA) using a mixed model that included the breed, and the sampling time (days 60 and days 120), and their interaction as fixed effects. The statistical significance of the difference in the level of SA and least-squares means were determined using Tukey’s test with a probability level of P < 0.05.

Results and discussion

The average Neu5Ac and Neu5Gc level in all 240 milk samples tested was 88.21 μg/ml and 2.95 μg/ml, respectively. Between Neu5Ac and Neu5Gc, we found a statistically significant Pearson’s correlation coefficient (r = 0.63; P < 0.001). Considering the possible effect of milk production on Neu5Ac and Neu5Gc concentrations we found a statistically positive weak correlation of r = 0.1540; P = 0.019 and r = 0.2065; P = 0.002, respectively.

Our results showed that Neu5Ac represented 97% of the total SA, whereas Neu5Gc represented the remaining 3% in agreement with findings previously reported by Albrecht et al. (2014) in bovine HO colostrum. In an examination of bovine colostrum oligosaccharides, Tao et al. (2008) discovered that those carrying Neu5Gc made up a very tiny fraction of the total milk oligosaccharides. Considering that the bulk are sialylated (70%), only about 5% included Neu5Gc. Even when colostrum or late lactation are taken into account, the Neu5Ac to Neu5Gc ratio remains identical. Furthermore, Tao et al. (2009) observed just a trace quantity of Neu5Gc in Holstein milk collected at day 120 of lactation.

In our study, we were interested in whether there was any variation in the content of SA during the course of lactation and we found that both Neu5Ac and Neu5Gc concentrations were higher at day 120 than at day 60 (P < 0.001) (Table 1). Martin et al. (2001) did not report any difference in Neu5Ac levels in milk samples from four Spanish-Brown cows obtained in mature (3rd month) and late-lactation (10th month) (26.1 and 26.9 mg/l respectively), whilst Puente et al. (1992) found that Neu5Ac concentration in milk increased more rapidly from 90 to 180 d in 6 Spanish-Brown cattle, similar to our own observation.

We observed that the milk SA content was influenced by the cow breed. HO had higher Neu5Ac values than SM × HO, SM and POD (P < 0.001), whilst Neu5Gc values were greater in HO, SM × HO and SM than in POD (P < 0.001) (Table 1). When

### Table 1. Effect of breeds and sampling times on sialic acid (SA) content

<table>
<thead>
<tr>
<th>SA</th>
<th>Sampling time (days)</th>
<th>Breeds</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
<td>120</td>
<td>HO</td>
<td>SM×HO</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>54.37 ± 10.49</td>
<td>117.28 ± 11.05</td>
<td>101.58 ± 4.82</td>
<td>84.76 ± 4.4</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>1.78 ± 0.43</td>
<td>3.92 ± 0.45</td>
<td>2.99 ± 0.2</td>
<td>2.85 ± 0.18</td>
</tr>
</tbody>
</table>

SA, sialic acids; SA concentration is reported as μg/ml; se, standard error; HO, Holstein:Friesian; SM, Simmental; SM×HO, cross bred; POD, Podolica. Values are expressed as least-square means ± se. Uppercase superscripts within rows indicate significantly different LS means at P < 0.001.

### Table 2. Effect of interactions of breed and sampling time on sialic acid (SA) content

<table>
<thead>
<tr>
<th>SA</th>
<th>Breed</th>
<th>Sampling time (days)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
<td>120</td>
<td>HO</td>
<td>SM×HO</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>63.81 ± 12.18</td>
<td>139.35 ± 12.25</td>
<td>0.00506</td>
<td></td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>54.37 ± 11.94</td>
<td>115.14 ± 12.40</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>41.58 ± 12.15</td>
<td>102.66 ± 11.31</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>57.72 ± 13.48</td>
<td>111.95 ± 15</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>1.68 ± 0.5</td>
<td>4.29 ± 0.5</td>
<td>0.000170</td>
<td></td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>1.79 ± 0.5</td>
<td>3.94 ± 0.5</td>
<td>0.03464</td>
<td></td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>1.7 ± 0.5</td>
<td>4.02 ± 0.46</td>
<td>0.000625</td>
<td></td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>1.93 ± 0.55</td>
<td>3.46 ± 0.61</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

SA, sialic acids; SA concentration is reported as μg/ml; se, standard error; n.s., not significant; HO, Holstein:Friesian; SM, Simmental; SM×HO, cross bred; POD, Podolica. Values are expressed as least square means ± se. Uppercase superscripts within columns indicate significantly different LS means at P < 0.001. P-values of the differences between sampling times are reported in the last column.
the breed and sampling period interaction effect was taken into account, there was a statistical difference in SA content between breeds only at day 120; Neu5Ac content in HO was higher than the other breeds (P < 0.001), whilst Neu5Gc content in HO and SM was higher than POD (P < 0.001) (Table 2). The breed, management method, nutritional and genetic factors could all potentially influence the variance in SA content in the enrolled cattle breeds. HO represents the most common breed with the highest milk production, whilst SM and POD are dual-purpose breeds selected for milk and meat production and the SM × HO crossbreed tries to combine the best traits of both parents. In the CREA herd, with animals under the same management system, HO showed the highest Neu5Ac and Neu5Gc content and the crossbred showed intermediate concentration relative to the purebreds. The level of Neu5Ac in crossbreds was comparable to that of POD. In a nutritional trial on HO cows at around days 100 after calving, Asakuma et al. (2010) reported that grazing animals fed fresh forage produced milk with higher Neu5Ac concentration than those kept inside and fed grass silage. When comparing milk samples collected from dairy and non-dairy breed cows in the period of 70 to 120 d postpartum, Goto et al. (2010) found no changes in Neu5Ac concentration.

In conclusion, we observed that POD had lower Neu5Ac and Neu5Gc levels than the other breeds, which is likely related to the fact that it is a local breed raised in an extensive system and fed on pasture. In comparison to previous research, we used a large number of cows and were able to demonstrate phenotypic diversity within breeds for SA (online Supplementary Figures S1 and S2). Genomic and transcriptomic studies are ongoing to uncover genetic signals associated with the phenotype results which can be used to improve breeding for better compositional properties of the milk.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000620.

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References


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