

Research Article

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

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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.

Sialic acid is a generic name given to various kinds of neuraminic acid derivatives which together constitute a large family. Sialic acids (SA) play a significant role in biological processes since they are prominent molecules on the cell surface (the so-called glycocalyx), not only in position but also in function, and they can be considered as cytoprotectors. However, in this position, they are often attacked and destroyed by pathogens, especially viruses, and they can also be misused by malignant cells (Schauer and Kamerling, 2018). Neu5Ac and Neu5Gc are two of the most abundant derivatives and Neu5Gc is produced from Neu5Ac through enzymatic hydroxylation catalysed by the activity of CMP-NeuAc hydroxylase. In humans, Neu5Gc cannot be produced due to the inactivation of the hydroxylase coding gene (Hayakawa *et al.*, 2001). Since humans frequently consume Neu5Gc (mainly with red meat), they incorporate a small portion of this SA into their tissues (Tangvoranuntakul *et al.*, 2003). Neu5Gc is recognized as a xeno-antigen and causes the production of anti-Neu5Gc antibodies which, in turn, are reported to have implications for human health and disease. However, contradictory results on Neu5Gc effects on human health are present and well documented, for instance in Soullidou and Padler-Karavani (2020). Some papers report the Neu5Ac and Neu5Gc concentrations in the organ or skeletal muscle of animal species (Jahan *et al.*, 2021) and milk and milk-based products (Spichtig *et al.*, 2010). However, the analysed dairy products are from the supermarket or bulk tank and to our knowledge quantification studies of these SA in individual cow milk samples of different breeds are scarce. Because SA are key components of milk with beneficial effects on human health in general whilst harmful consequences for Neu5Gc are suspected, we wanted to quantify them in various situations. We addressed the following hypotheses: (a) Does the stage of lactation affect the production of Neu5Ac and Neu5Gc? (b) Do Neu5Ac and Neu5Gc concentrations differ between cow breeds? (c) Can two distinct raising systems affect the amount of Neu5Ac and Neu5Gc in milk? (d) Does phenotypic variation exist within cow breeds?

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 silage, 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

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

SA	Sampling time (days)		Breeds			
	60	120	HO	SM×HO	SM	POD
Neu5Ac	54.37 ^A ± 10.49	117.28 ^B ± 11.05	101.58 ^A ± 4.82	84.76 ^B ± 4.4	72.12 ^B ± 4.21	84.83 ^B ± 9.1
Neu5Gc	1.78 ^A ± 0.43	3.92 ^B ± 0.45	2.99 ^A ± 0.2	2.85 ^A ± 0.18	2.86 ^A ± 0.17	2.69 ^B ± 0.37

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 $P < 0.001$.

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

SA	Breed	Sampling time (days)		
		60	120	P60>120
Neu5Ac	HO	63.81 ± 12.18	139.35 ^A ± 12.25	0.000506
Neu5Ac	SM×HO	54.37 ± 11.94	115.14 ^B ± 12.40	n.s.
Neu5Ac	SM	41.58 ± 12.15	102.66 ^B ± 11.31	n.s.
Neu5Ac	POD	57.72 ± 13.48	111.95 ^B ± 15	n.s.
Neu5Gc	HO	1.68 ± 0.5	4.29 ^A ± 0.5	0.000170
Neu5Gc	SM×HO	1.79 ± 0.5	3.9 ^{AB} ± 0.5	0.034164
Neu5Gc	SM	1.7 ± 0.5	4.02 ^A ± 0.46	0.000625
Neu5Gc	POD	1.93 ± 0.55	3.46 ^B ± 0.61	n.s.

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 \times 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 crossbreeds 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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