Molecular characterization of the level of sialic acids *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, and ketodeoxynonulosonic acid in porcine milk during lactation

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ABSTRACT

Sialic acids (Sia) are key monosaccharide constituents of sialylated glycoproteins (Sia-GP), human sialylated milk oligosaccharide (Sia-MOS), and gangliosides. Human milk sialylated glycoconjugates (Sia-GC) are bioactive compounds known to act as prebiotics and promote neurodevelopment, immune function, and gut maturation in newborns. Only limited data are available on the Sia content of porcine milk. The objective of this study was to quantitatively determine the total level of Sia *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and ketodeoxynonulosonic acid (KDN) in porcine milk and to compare these levels in gilt and sow milk during lactation. Milk from 8 gilts and 22 sows was collected at 3 stages of lactation (colostrum, transition, and mature milk). Standard and experimental samples were derivatized using 1,2-diamino-4,5-methylenedioxy-benzene and analyzed by ultra-high-performance liquid chromatography using a fluorescence detector. The following new findings are reported: (1) Gilt and sow milk contained significant levels of total Sia, with the highest concentration in colostrum (1,238.5 mg/L), followed by transition milk (778.3 mg/L) and mature milk (347.2 mg/L); (2) during lactation, the majority of Sia was conjugated to Sia-GP (41–46%), followed by Sia-MOS (31–42%) and a smaller proportion in gangliosides (12–28%); (3) Neu5Ac was the major form of Sia (93–96%), followed by Neu5Gc (3–6%) and then KDN (1–2%), irrespective of milk fraction or stage of lactation; (4) the concentration of Sia in Sia-GP and Sia-MOS showed a significant decline during lactation, but the level of ganglioside Sia remained relatively constant; (5) mature gilt milk contained a significantly higher concentration of Sia-GP than sow milk. The high concentration of total

Sia in porcine milk suggests that Sia-GC are important nutrients that contribute to the optimization of neurodevelopment, immune function, and growth and development in piglets. These findings provide an important rationale for the inclusion of Sia-GC in pig milk replacers to mimic porcine milk composition for the optimal growth and development of piglets.

Key words: sialic acids, glycoconjugate, lactation, sow and gilt milk, ultra-high-performance liquid chromatography

INTRODUCTION

The nutritional composition of milk is an important determinant of its value in supporting the growth and development of the neonate. Swine are one of the most important agricultural species and are recognized as an excellent model for monogastric nutritional studies, owing to similarities with humans in the physiology and anatomical structure of their digestive system (Baker, 2008; Patterson et al., 2008). Moreover, the developing brain structures and functions of piglets bear a resemblance to those of human infants (Pond et al., 2000). For these reasons, there has recently been an increased focus on the composition of porcine milk, although most analyses have concentrated primarily on protein, lipid, and carbohydrate (Simpson and Nicholas, 2002; Haselhorst et al., 2009; Tao et al., 2010) and paid little attention to the major sialylated glycoconjugate (**Sia-GC**) molecules, namely sialylated glycoprotein (**Sia-GP**), sialylated milk oligosaccharide (**Sia-MOS**), and gangliosides.

Sialic acid (**Sia**) is a member of the neuraminic acid family of 9-carbon acidic amino sugars that commonly occupy non-reducing termini of glycan chains. The carboxyl group at C1 confers a negative charge on Sia-GC expressed on the surface of mammalian cells. *N*-Acetylneuraminic acid (**Neu5Ac**), *N*-glycolylneuraminic acid (**Neu5Gc**), and the deacylated form, ketodeoxynonulosonic acid (**KDN**), are the 3 major forms of Sia most frequently found in nature (Davies and Varki, 2015).

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However, Neu5Ac and Neu5Gc are the 2 major Sia most commonly found in mammalian species; KDN is found more frequently in lower vertebrates, including many fish egg species (Iwasaki et al., 1990).

Humans cannot synthesize Neu5Gc due to a lack of the enzyme CMP-Neu5Ac hydroxylase, which catalyzes the conversion of Neu5Ac to Neu5Gc (Varki, 2010). There is no known alternate pathway in humans for the synthesis of Neu5Gc (Hedlund et al., 2007). However, very low levels of Neu5Gc are found in human tissues and are now established as coming from a diet rich in red meat and dairy products. Several studies have shown a positive correlation between red meat consumption and cancer and inflammatory diseases (Diaz et al., 2009; Samraj et al., 2014, 2015). More than 50 members of the Sia family have been reported to occur in nature (Kamerling et al., 2007). The cell membrane of all vertebrates, some invertebrates, and microorganisms—including viruses, pathogenic bacteria, and protozoa—contain Sia, which appears to be absent in plants (Corfield and Schauer, 1982; Kamerling et al., 2007).

Neural cell membranes in mammals contain ~20-fold higher levels of Sia than other cell membranes, and this is indicative of the key role that Sia glycans play in neural structure and function (Schauer, 1982). In humans, Sia is also detectable in milk, plasma, and urine (ten Bruggencate et al., 2014). It is also found in other body fluids—including saliva, gastric juice, and tears—in the form of Sia-GP or as terminal sugars of oligosaccharide chains of mucins (Wang and Brand-Miller, 2003). Human milk is a rich source of Sia, with concentrations ranging from 1,277 to 1,558 mg/L in colostrum (d 1 to 5), 911 to 1,070 mg/L in transition milk (d 7 to 17), and 525 to 600 mg/L in mature milk (d 28 to 45) (Carlson, 1985; Wang et al., 2001; Martín-Sosa et al., 2004). In human milk, Sia-MOS account for about 75% of the total Sia (Nakano et al., 2001). However, in bovine milk, the concentration of Sia is about 771 to 1,700 mg/L in colostrum collected immediately after parturition, and then rapidly declines to 339 to 520 mg/L at 24 h, 150 to 165 mg/L in transition milk (d 3 to 7), and 143 to 150 mg/L in mature milk (mo 3) (Morrissey, 1973; Puente et al., 1992; Nakamura et al., 2003). Infants cannot digest Sia-MOS; Sia-MOS remain largely intact until they reach the large intestine, where they can be used by beneficial intestinal bacteria, contributing to the prebiotic activities of milk (Tao et al., 2008). As well, Sia-MOS are involved in mediating a variety of important physiological cell-cell interactions (Schauer, 2009), mediating immune functions (Eiwegger et al., 2010; Zenhom et al., 2011; Böhm et al., 2012; Cabral et

al., 2013; Comstock et al., 2014), and preventing bacterial and viral infections (Gopal and Gill, 2000; Coppa et al., 2004; Martinez-Ferez et al., 2006; Boehm and Stahl, 2007; LoCascio et al., 2007). In piglets, such pathogens include enterotoxigenic *Escherichia coli* (Malykh et al., 2003) and influenza, corona, rota, and rubula viruses (Vallejo et al., 2000). Pretreatment of target cells with sialidases can block infections with sialidase-sensitive rotaviruses in both calves and piglets (Isa et al., 2006). Sialidase hydrolyzes glycosidic linkages of terminal Sia residues in Sia-MOS, and the released Sia competes for rotavirus binding sites on epithelial surfaces to prevent their adhesion. The high concentration of Sia-MOS in sow milk may confer protection for the neonate (Tao et al., 2010).

In human milk, Sia-MOS also prevent the binding of rotavirus (Newburg et al., 2005), rheovirus (Pacitti and Gentsch, 1987; Barton et al., 2003), *Helicobacter pylori* (Yang et al., 2008), and some *E. coli* strains associated with neonatal meningitis and cholera toxin (Idota et al., 1995; Noguchi et al., 2010). All of these factors are associated with infant diarrhea (Wang, 2009). These microorganisms and toxins can also influence the growth of *Bifidobacteria* spp. in the intestinal tract of neonates (Rueda et al., 1998). In a recent study, Yang et al. (2014) showed that the sialylated milk glycoprotein lactoferrin upregulates the gene expression of brain-derived neurotrophic factor, ubiquitin carboxy-terminal hydrolase L1, and polysialic acid; decreases the colon microbiota of *E. coli*; reduces early weaning diarrhea; and improves gut maturation in piglets. The Sia-MOS also play an important functional role in neural development and neuroprotection (Nakano et al., 2001; Wang, 2003). Eight-arm radial maze learning studies showed that the Sia-GC glycomacropeptide could improve learning and memory in postnatal piglets (Wang et al., 2007). In spite of these recent advances, however, information is lacking on the quantitative levels and distribution of Sia-GC in gilt and sow milk. Accordingly, the objective of this study was to determine the developmental changes in the concentration of Neu5Ac, Neu5Gc, and KDN in Sia-GP, Sia-MOS, and gangliosides in the colostrum, transition, and mature milk of gilt and sow. We used ultra-high-performance liquid chromatography (UHPLC)-fluorescence, the most rapid and sensitive method for Sia detection and quantification. This comprehensive analysis of Sia in gilt and sow milk indicates a broad range of Sia distribution and concentration during lactation and serves as a guide for piglet feeding, including pig milk replacer development to optimize the growth and development of piglets.

Table 1. Number, breed, age, and parity of pigs used in this study

No. of pigs	Breed	Age (d)	Parity		No. of animals devoid of mature milk sample
			>1	1	
4	Belgian Landrace	461–697	3	1	0
10	Large White	352–1,552	9	1	1
8	Landrace	351–1,263	5	3	1
8	Duroc	346–939	5	3	3 (1) ¹

¹The number of gilts is in parentheses.

MATERIALS AND METHODS

Animals

The pigs (*Sus scrofa*, Belgian Landrace, Large White, Landrace, and Duroc) including gilts (young female pigs that have not farrowed yet; $n = 8$) and sows (mature female pigs that have bred at least once; $n = 22$) were included in this study. All pigs were obtained from the commercial farrowing shed at the Pig Improvement Company facility (Grong Grong, NSW, Australia), as was the source of all milk samples reported in this study. The study protocol was approved by the Animal Care and Ethics Committee of Charles Sturt University, Wagga Wagga, NSW, Australia (13/103).

The gilts and sows were healthy, aged 1 to 4 yr, and had uncomplicated perinatal histories (Table 1). The gestational period of litters from both gilts and sows was 112 to 121 d. Pigs were fed a commercial gestation diet (2.4 kg/d) from mating to the end of gestation, and then a lactation diet (5.8 kg/d) over the next 21 d until weaning. The nutritional composition of the commercial pig feed is shown in Table 2. All animals had access to water ad libitum and were housed under standard commercial production conditions.

Collection of Milk Samples

Milk samples were collected from gilts and sows in sterile plastic vials by manual expression during the same time each morning (0900–1200 h). Colostrum was collected from each gilt and sow on d 1, transition milk at d 3, and mature milk at d 15 of lactation. Complete data were available for all 3 time points in 25 pigs, including 18 sows and 7 gilts: mature milk samples were not collected from 4 sows and 1 gilt because they were culled before 15 d of lactation due to poor milk production. Unlike dairy cows, the porcine mammary gland does not have cisternae in which to store milk. Milk collection can only be carried out after inducing the milk ejection reflex through release of oxytocin induced by the suckling stimulus of piglets for at least 1 min (Fraser, 1980; Hartmann and Holmes, 1989). Sows nurse

piglets approximately 20 times per day, and the milk flow lasts for about 10 to 20 s (Fraser, 1980; Hartmann and Holmes, 1989). In this study, 2 to 5 mL of milk was collected from each gilt and sow after the milk letdown was induced by the suckling stimulus from piglets during the 10- to 20-s window of opportunity. Samples were immediately placed on ice after collection and stored at -20°C until analyzed.

Preparation of Milk Samples

Milk components were fractionated into Sia-GP, Sia-MOS, and gangliosides using a slight modification of the method of Wessel and Flügge (1984). Briefly, 1 mL of milk was centrifuged at $13,000 \times g$ at 4°C for 30 min, and the upper cream layer was removed using a small spatula. A mixture of methanol, chloroform and water

Table 2. Nutritional composition (% unless otherwise noted) of commercial pig feed

Nutrient	Lactating sow	Dry sow
Pig digestible energy (MJ/kg)	14.05	13.022
Pig ME (Mcal/kg)	3.225	2.989
Moisture	9.187	9.608
CP	17.938	14.3
Lysine	1.12	0.819
Methionine	0.32	0.264
Methionine + cystine	0.706	0.601
Threonine	0.709	0.553
Isoleucine	0.78	0.586
Leucine	1.273	0.954
Tryptophan	0.22	0.167
Arginine	1.185	0.924
Histidine	0.453	0.354
Valine	0.862	0.71
Available lysine	0.936	0.651
Crude fat	5.487	5.5
Crude fiber	4.787	6.285
Ash	6.006	6.846
Calcium	0.89	1.149
Available phosphorus	0.4	0.4
Total phosphorus	0.829	0.909
Salt	0.342	0.387
Sodium	0.127	0.16
Chloride	0.213	0.235
Choline (mg/kg)	1,909.948	1,598.472

(4:2:3, 9 mL) was added to the skim milk, and the sample was vortexed before centrifugation at $4,000 \times g$ at 4°C for 15 min. The upper aqueous methanol phase containing free Sia-MOS was collected in a separate tube with a glass pipette. The bottom chloroform layer containing gangliosides was collected and added to the tube containing the cream layer. The protein pellet was carefully collected. To examine if free Sia-MOS and gangliosides were separated well using the current method, we qualitatively determined the concentrations of gangliosides GM3 and GD3 in the upper aqueous methanol layer, cream layer, and bottom chloroform layer using the published validated method of liquid chromatography–high-resolution mass spectrometry (Fong et al., 2009). We confirmed that there were no gangliosides GM3 and GD3 in the upper methanol layer from any of the milks from the 3 time points. In the cream and bottom chloroform layer, however, GM3 and GD3 were detected in milk samples from all 3 time points or in the mature milk only (manuscript in preparation).

The Sia-MOS were analyzed after hydrolysis of the upper aqueous layer with an equal volume of 0.1 *M* trifluoroacetic acid at 80°C for 30 min. Chloroform was evaporated from the gangliosides layer in a vacuum apparatus (Gallenkamp, London, UK) at 20°C with 8,000 Pa pressure for about 4 h, then mixed with 1 mL of 0.1 *M* trifluoroacetic acid and hydrolyzed at 80°C for 30 min. To determine the level of Sia-GP, the pellet was resuspended in 2 mL of 0.05 *M* sulfuric acid and heated for 2 h for 80°C to release the Sia. The optimal condition for releasing Sia from Sia-GC was predetermined using a variety of acids at different time intervals to determine the maximal release of Sia with minimum degradation (Wang et al. 2001). After acid hydrolysis, all samples were filtered through a 0.22 μm Millipore membrane (Millex, Merck Millipore, Germany). Then, 1 g of commercial pig feed as a dried pellet was weighed, homogenized, and added to 2 mL of 0.05 *M* H₂SO₄ and hydrolyzed at 80°C for 1 h. After cooling to room temperature, the samples were filtered through a 0.22- μm Millipore membrane filter. A schematic diagram describing how the milk samples were prepared is provided in Figure 1.

Method for Derivatizing Neu5Ac, Neu5Gc, and KDN

The 1,2-diamino-4,5-methylenedioxybenzene (DMB) reagent to introduce the fluorescent label for chromatographic detections was prepared according to the method of Hara et al. (1989) as follows: 7 mM DMB in 1.4 *M* acetic acid containing 0.75 *M* 2-mercaptoethanol and 18 mM sodium hydrosulfite. The experimental samples and standards of Neu5Ac, Neu5Gc, and KDN (Sigma Aldrich, St. Louis, MO) were filtered through

a 0.22- μm Millipore membrane filter, and 50 μL of the DMB reagent was mixed with 50 μL of the filtered sample or standard (Sigma Aldrich) in the insert in a brown high-performance liquid chromatography vial (Agilent Technologies, Santa Clara, CA) to protect the samples from light. All samples were mixed by vortex and heated at 80°C for 50 min before being cooled on ice for 5 min. Immediately after the DMB derivatization, samples were quantitatively analyzed for Sia concentration by UHPLC as described below.

Quantitative Determination of Neu5Ac, Neu5Gc, and KDN by UHPLC

The DMB-derivatized standards and experimental Sia samples were analyzed using an Agilent 1290 Infinity system (Agilent Technologies, Germany) with a 1260 Infinity Fluorescence Detector (Agilent Technologies, Germany). A Zorbax SB-Aq Rapid Resolution column (3.5 μm , 4.6 $\times$ 50 mm, Agilent Technologies, Santa Clara, CA) was held at 30°C, and the auto sampler temperature was held constant at 4°C. The DMB-derivatized Sia samples were eluted from the column using a mixture of water, methanol, and acetic acid (75:25:0.05 vol/vol/vol) at a flow rate of 1.5 mL/min for 3.5 min. Columns were washed with a mixture of methanol, water, and acetic acid (80:20:0.05 vol/vol/vol) for 1.4 min before being re-equilibrated for 4 min (with a total run time of 9 min). Fluorescence was detected at 448 nm emissions and 373 nm excitation using the 1260 Infinity FLD detector (Agilent Technologies, Germany). All samples were analyzed in duplicate, and the injection volume was 10 μL . The quantitative level of Sia was determined by comparison with a standard curve prepared using commercial standards of Neu5Ac, Neu5Gc, and KDN. The final concentration of all Sia was expressed as milligrams of Sia per liter of milk.

Statistical Analyses

The concentration of Sia in the Sia-GP, Sia-MOS, and gangliosides during the 3 time periods of lactation was analyzed using a 2-factor repeated-measures ANOVA model with a Greenhouse-Geisser adjustment for asphericity. To investigate the different time trends for gilt and sow milk, the interaction between the dependent variable and the grouping factor was determined. The overall statistical comparison of Sia levels between gilt and sow milk was obtained from repeated measures of analyses of the variance models and compared at individual time points using *t*-tests. All analyses were completed using SPSS Statistics version 20 for Windows (SPSS Inc., Chicago, IL). Values were considered significant at *P* = 0.05.

Figure 1. Schematic diagram depicting the experimental methods used to isolate, fractionate, and hydrolyze the milk sample for quantitative analyses by ultra-high-performance liquid chromatography.

RESULTS AND DISCUSSION

Quantitative Determination of Total Sia in Porcine Milk

The total concentrations of Sia in porcine colostrum, transition, and mature milk during lactation are shown in Figure 2A. The mean total Sia concentration was

1,238.5 mg/L in colostrum, 778.3 mg/L in transition milk, and 347.2 mg/L in mature milk. The overall difference in the total level of Sia at the different stages of lactation was statistically significant ($P < 0.001$).

Our quantitative analyses of the total concentration of Sia as a function of the developmental stage of lactation showed a significant decline over the course of lactation, with total Sia in colostrum being about

Figure 2. (A) Developmental changes in the concentration of total sialic acid (Sia) expressed in sialylated glycoprotein (Sia-GP), sialylated milk oligosaccharide (Sia-MOS), and gangliosides in colostrum, transition milk, and mature milk. *Significant difference ($P < 0.001$) between mature milk and colostrum; †significant difference ($P < 0.001$) between mature milk and transition milk ($P < 0.001$). Values are mean $\pm$ SEM. (B) Proportions of total Sia in Sia-GP, Sia-MOS, and gangliosides in porcine colostrum, transition milk, and mature milk. Values are mean of all samples analyzed (colostrum $n = 30$, transition milk $n = 30$, mature milk $n = 25$).

Table 3. Total and individual concentrations of sialic acid (Sia) in glycoproteins (Sia-GP), milk oligosaccharides (Sia-MOS), and gangliosides of porcine milk during lactation (mg/mL of milk)¹

Milk type and Sia	<i>N</i> -Acetylneuraminic acid (Neu5Ac)	<i>N</i> -Glycolylneuraminic acid (Neu5Gc)	Ketodeoxynonulosonic acid (KDN)	Total
Sia-GP				
Colostrum (n = 30)	538.88 ± 23.3	25.54 ± 1.0	9.93 ± 0.71	574.52 ± 23.9
Transition milk (n = 30)	304.858 ± 14.8	14.62 ± 0.8	2.66 ± 0.3	322.07 ± 15.2
Mature milk (n = 25)	138.64 ± 9.44	8.52 ± 0.4	1.12 ± 0.29	148.29 ± 9.5
<i>P</i> -value	<0.001	<0.001	<0.001	<0.001
Sia-MOS				
Colostrum (n = 30)	497.09 ± 23.3	11.23 ± 1.0	6.66 ± 0.7	514.99 ± 23.9
Transition milk (n = 30)	235.45 ± 14.8	6.77 ± 0.8	3.24 ± 0.2	241.00 ± 15.2
Mature milk (n = 25)	122.67 ± 9.4	3.30 ± 0.4	2.89 ± 0.2	128.87 ± 9.5
<i>P</i> -value	<0.001	<0.001	<0.001	<0.001
Gangliosides				
Colostrum (n = 30)	142.21 ± 23.3	4.51 ± 1.0	2.25 ± 0.7	148.98 ± 23.9
Transition milk (n = 30)	206.48 ± 14.8	5.77 ± 0.8	2.25 ± 0.2	214.24 ± 15.2
Mature milk (n = 25)	67.093 ± 9.4	2.27 ± 0.4	0.66 ± 0.2	70.04 ± 9.5
<i>P</i> -value	<0.001	<0.001	<0.001	<0.001

¹Values are means ± SE.

1.5-fold higher than in transition milk and about 4-fold higher than in mature milk. This decline may reflect a time-dependent reduction in the biosynthetic capacity to synthesize Sia-GC and an increased dilution of Sia or both, due to an increase in milk production during the course of lactation. Spinka et al. (1997) reported that the milk output per teat increased from 18.0 ± 1.1 g/h on d 1 to 21.5 ± 1.0 g/h on d 2 and 24.5 ± 1.3 g/h on d 3. Similar results were also reported for bovine milk (Nakamura et al., 2003) and for human and bovine milk (Martín-Sosa et al., 2003). Human infants and piglets early in life may not have the biosynthetic capacity to meet the demand for Sia as a required precursor for the synthesis of brain Sia glycans, including gangliosides and sialylated glycoproteins: milk, particularly colostrum and transition milk, is the only source of Sia for newborns. Another reason for this decline in milk Sia during lactation may be piglets' lower requirements for Sia from milk because the rate-limiting bifunctional enzyme UDP-GlcNAc-2-epimerase, which is responsible for Sia synthesis, becomes more active as growth progresses (Wang, 2009).

Verstegen et al. (1998) reported that the transition from colostrum to mature milk was associated with a substantial decline in total solids from 25 to 18% and proteins from 15 to 5%, but observed an increase in lactose concentration from 3.4 to 5.3% and fat from 6 to 8% in sow milk. The overall decrease in TS content of porcine milk, particularly protein, may in part explain the decline in Sia concentration in both gilt and sow milk during lactation. Further, Useh et al. (2008) have suggested that the higher level of total Sia in colostrum compared with mature milk may relate to more com-

plex interactions between Sia-GC associated with the development of mucosal immunity to combat enteric pathogens.

Concentration of Sia in Sia-GP, Sia-MOS, and Gangliosides in Colostrum, Transition Milk, and Mature Milk

The total levels of Sia in Sia-GP, Sia-MOS, and gangliosides in porcine milk at the 3 different stages of lactation are shown in Table 3. The highest level of Sia was in Sia-GP (41 to 46%), followed by Sia-MOS (31 to 42%) and gangliosides (12 to 28%), irrespective of stage of lactation (Figure 2B). In colostrum, the level of Sia in both Sia-GP and Sia-MOS was about 3.5-fold higher than in gangliosides. In transition milk, however, the total level of Sia in Sia-MOS and gangliosides was about 25 and 33% lower than in Sia-GP. In mature milk, total Sia in gangliosides was about 2 times lower than that of Sia-GP and Sia-MOS (128.87 mg/L). The overall differences in the level of total Sia in the Sia-GP, Sia-MOS, and gangliosides in colostrum, transition, and mature milk were statistically significant ($P < 0.001$).

The concentration of total Sia in Sia-GP, Sia-MOS, and gangliosides of porcine milk decreased significantly regardless of whether all data available were included in the analysis (as shown in Table 3) or whether the 25 pigs with complete data for all time points were analyzed separately ($P < 0.05$, Figure 3). By the 15th day of lactation, only ~26% of the initial concentration of Sia in the Sia-GP and Sia-MOS remained. The total concentration of Sia in the Sia-MOS decreased by

53% in the progression from colostrum to transition milk and by 46.7% from transition milk to mature milk. During this period, the level of Sia-GP decreased by 43.9% from colostrum to transition milk and by 53.9% from transition milk to mature milk. For the gangliosides, however, the total Sia concentration increased by 43.8% from colostrum to transition milk and then decreased by 67.3% from transition milk to mature milk (Table 3).

In contrast to porcine milk, the highest level of Sia in human milk has been found in Sia-MOS (73%) followed by Sia-GP (27%), but ganglioside forms <0.5% of the total Sia (Wang et al., 2001). This difference may relate to the higher concentrations of Sia-GP in porcine milk (16.65% in colostrum and 5.83% in mature milk) than in human milk (0.8–0.9% in mature milk) (Jenness, 1979; Csapo et al., 1996). Interestingly, although the

Sia concentration in Sia-GP and Sia-MOS decreased during lactation, the level in gangliosides remained the highest in transition milk. This may relate to higher fat content in porcine milk between 48 and 72 h postpartum (Kehoe et al., 2007). Csapo et al. (1996) reported that porcine colostrum contained 5.3% fat, increased to 13.1% fat at 48 to 72 h, and then decreased to 6.5% fat in mature milk.

We were unable to detect free Sia in the Sia-GP, Sia-MOS, or ganglioside fractions of porcine milk. In human milk, however, <3% Sia, or about 43 and 21 mg/L were found as free Sia in colostrum and mature milk, respectively (Wang et al., 2001). It is possible that minor differences in methodology in the preparation of milk and its analyses between the 2 studies may explain this difference. No Sia was detected in the commercial pig feed used in the present study.

Figure 3. Developmental changes in the concentration of total sialic acid (Sia), *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and ketodeoxynonulosonic acid (KDN) in sialylated glycoprotein (Sia-GP), sialylated milk oligosaccharide (Sia-MOS), and gangliosides in porcine milk during the course of lactation ($n = 25$). Only sows and gilts whose data were complete for all 3 time points are included: (A) total Sia, (B) total Neu5Ac, (C) total Neu5Gc, and (D) total KDN. *Significant difference ($P < 0.005$) between mature milk and colostrum; †significant difference ($P < 0.001$) between mature milk and transition milk ($P < 0.001$). Values are mean $\pm$ SEM.

Table 4. Concentration of sialic acids (Sia) in glycoproteins (Sia-GP), milk oligosaccharides (Sia-MOS), and gangliosides of colostrum, transition milk, and mature milk in gilts and sows during lactation (mg/mL of milk)

Sialic acid	Colostrum (n = 30)		Transition milk (n = 30)		Mature milk (n = 25)	
	Gilt (n = 8)	Sow (n = 22)	Gilt (n = 8)	Sow (n = 22)	Gilt (n = 7)	Sow (n = 18)
Sia-GP	561.62 ± 61.2	579.13 ± 31	304 ± 19.6	328.59 ± 18.6	200.42 ± 26.4*	128.1 ± 11.6
Sia-MOS	548.75 ± 62.9	502.72 ± 30.4	228.75 ± 25	246.72 ± 17.2	128.71 ± 13.4	128.83 ± 5.9
Gangliosides	161.62 ± 32.3	144.4 ± 15.6	213.62 ± 32.5	214.36 ± 20.3	71 ± 11.2	69.66 ± 5.9
Total Sia	1,272 ± 96.4	1,226.27 ± 58.1	746.75 ± 36.1	789.68 ± 29.9	400.28 ± 20.7*	326 ± 5

¹Values are means ± SE.*Significant difference ($P < 0.05$) compared with sow milk.

Quantitative Determination of Neu5Ac, Neu5Gc, and KDN in Sia-GP, Sia-MOS, and Gangliosides in Porcine Milk

The total levels of Neu5Ac, Neu5Gc, and KDN in Sia-GP, Sia-MOS, and gangliosides in porcine milk at the 3 different stages of lactation are shown in Table 3. Most of the total Sia (95–96%) from either gilt or sow milk was Neu5Ac, irrespective of the stage of lactation. Neu5Gc comprised 3 to 4% of Sia, but only 1 to 2% was present as KDN. The overall difference between the 3 forms of Sia was statistically significant in the Sia-MOS, Sia-GP, and gangliosides during the course of lactation ($P < 0.001$).

The total concentration of Neu5Ac, Neu5Gc, and KDN in porcine milk of Sia-GP, Sia-MOS, and gangliosides was highest in colostrum, and decreased significantly as lactation progressed. The overall difference in the level of Neu5Ac, Neu5Gc, and KDN in the Sia-GP, Sia-MOS, and gangliosides between the 3 stages of lactation was significant ($P < 0.001$, Table 3). We observed no significant difference, however, in the concentration of Neu5Ac and KDN in gangliosides between colostrum and transition milk, or in the concentration of KDN in the Sia-GP and Sia-MOS between transition and mature milk.

Unlike porcine milk, human milk Sia consists almost exclusively of Neu5Ac (Puente and Hueso, 1993; Newburg and Neubauer, 1995), because of an exon deletion/frame shift mutation in the human *CMAH* gene. This mutation results in a loss of the ability of humans to synthesize Neu5Gc (Varki 2010). In marked contrast, bovine milk contains 5 to 35% Neu5Gc, depending on the stage of lactation (Puente and Hueso, 1993). Thus, the concentration and forms of Sia in milk is species-specific. Relatively little information is available on the level of KDN in either human or mammalian milk. Further studies will be required to elucidate the biological role of KDN in porcine milk. To our knowledge, these findings provide the first comprehensive analysis

of concentrations of Neu5Ac, Neu5Gc, and KDN in gilt and sow milk.

Developmental Changes and Quantitative Comparison Between Concentrations of Neu5Ac, Neu5Gc, and KDN in Porcine Milk

Table 4 compares the total levels of the Sia in Sia-MOS, Sia-GP, and gangliosides in colostrum, transition, and mature milk from gilts and sows. Colostrum and mature milk from gilts contained 3.6 and 18.5% higher levels of total Sia than sow milk at the same stages of lactation: however, we observed no such difference in transition milk. For mature milk, the difference in the concentration of total Sia between gilts and sows was statistically significant ($P < 0.05$). In both gilt and sow milk, Sia-GP were again the major source of Sia, followed by Sia-MOS, and gangliosides. The concentration of total Sia in Sia-GP and Sia-MOS gradually decreased in both gilt and sow milk during the course of lactation. This was not the pattern, however, for the gangliosides, because the highest concentration was found in transition milk, followed by colostrum and mature milk. The difference in Sia-GP between gilt and sow milk was statistically significant for mature milk ($P < 0.05$), at which time gilt milk contained almost one-third higher concentrations of Sia than sow milk (Table 4).

The concentration of total Neu5Ac, Neu5Gc, and KDN in gilt and sow milk during lactation is summarized in Figure 4. The predominant form of Sia was Neu5Ac, followed by Neu5Gc and KDN in both gilt and sow milk throughout the course of lactation. Colostrum and mature milk from gilts contained significantly lower concentrations of KDN and higher concentrations of Neu5Ac than sow milk ($P < 0.05$). The developmentally related changes in concentration of Neu5Ac, Neu5Gc, and KDN in gilt and sow milk with time were also consistent, irrespective of whether all data were included in the analyses (Figure 4) or whether the 18 sows and 7 gilts with a complete data for all time

Figure 4. Comparison of total levels of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and ketodeoxynonulosonic acid (KDN) in colostrum, transition milk, and mature milk in gilts ($n = 8$) and sows ($n = 22$) during lactation. *Significant difference ($P < 0.05$) between sow and gilt milk. Values are mean $\pm$ SEM. Mature milk, $n = 7$ for the gilt and $n = 18$ for the sow.

points were analyzed separately (Supplementary Figure S1; <http://dx.doi.org/10.3168/jds.2016-11187>). We also observed no significant difference in concentration of total Sia, Neu5Ac, Neu5Gc, and KDN in the milk of different breeds of sows or gilts.

In pig production, gilts are more likely to deliver low-birth-weight piglets than sows (Quesnel et al., 2008). This may partially explain the reason for the higher concentration of Sia-GC in mature milk from gilts compared with sows, to support the growth and development of their offspring. In pig production facilities, gilts are included in the breeding system as soon as they attain puberty, even though they have not attained a mature body weight. Thus, they must be able to partition nutrients between those required for lactation and those needed for their own growth and development. In managing this process, providing higher levels of Sia to the gilt's litter may help compensate for deficiencies in milk volume and total nutrient availability. Another reason might be the lower milk production by the gilts, so that the overall concentration of Sia is relatively higher in their milk. In this way, the gilts could provide the same amount of Sia to the piglets. Further studies are required to test this hypothesis.

Porcine milk is a rich source of Sia, with the predominant form being Neu5Ac (comprising more than 95% of total Sia), followed by Neu5Gc (which formed less than 4% of total Sia). The lowest proportion of Sia was

KDN, which provided less than 1% of total Sia, regardless of source (sows or gilts) or duration of lactation. The distribution of Neu5Ac, Neu5Gc, and KDN in Sia-GP, Sia-MOS, and ganglioside in sow and gilt milk was consistent throughout lactation. At the same stage of lactation, we observed a ~ 2.5 -fold difference in total Sia concentration in 8 gilts and 22 sows, making Sia one of the most variable components in porcine milk reported to date. The structure of Sia-MOS from pig and human are significantly different (Tao et al., 2010), but the variation in the total concentration of Sia reported for porcine milk reflected similar variations found for human colostrum, transition, and mature milk, providing further evidence that the piglet's digestion system and nutritional requirements are very similar to that of the human infant.

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