JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Milk Oligosaccharide Variation in Sow Milk and Milk Oligosaccharide Fermentation in Piglet Intestine

Elisabetta Difilippo,[†] Feipeng Pan,[†] Madelon Logtenberg,[†] Rianne (H.A.M) Willems,[†] Saskia Braber,[‡] Johanna Fink-Gremmels,[‡] Henk Arie Schols,*[,†] and Harry Gruppen[†]

[†]Laboratory of Food Chemistry, Wageningen University, Wageningen, The Netherlands

[‡]Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences, Subdivision of Veterinary Pharmacology, Pharmacotherapy, and Toxicology, Utrecht University, Utrecht, The Netherlands

ABSTRACT: Porcine milk oligosaccharides (PMOs) were analyzed in six colostrum and two mature milk samples from Dutch Landrace sows. In total, 35 PMOs were recognized of which 13 were new for the PMO literature: neutral Hex*N*Ac-Hex, β4′-galactosyllactose, putative Gal*N*Ac(α/β1−3)Gal(β1−4)Glc, lacto-*N*-fucopentaose-II, lacto-*N*-tetraose, galactose substituted lacto-*N*-neohexaose, lacto-*N*-hexaose and difucosyl-lacto-*N*-hexaose, and acidic Neu5Ac(α2−6)Glc*N*Ac(β1−3)Gal(β1−4)Glc, sialyllacto-*N*-tetraose-a and -b, Neu5Ac$_2$−Hex$_3$, and sialyllacto-*N*-fucopentaose-II. PMOs were analyzed using capillary electrophoresis with laser-induced florescence detection or mass spectrometry and using liquid chromatography with mass spectrometry. Interindividual variation regarding PMO presence and concentration was observed between porcine milks. Within a limited sample set, a 43% decrease of the major PMOs was found during a 1 w lactation period. Interestingly, while some PMOs decreased, some other PMOs increased in concentration. PMOs were also monitored in fecal samples of suckling piglets. In feces of 1−2 d old piglets, few intact PMOs were found, indicating considerable PMO fermentation at early stage of life.

KEYWORDS: *pigs, mass analysis, variation, chromatography, cow, sugars, abundance*

INTRODUCTION

Milk is essential for newborns as it contains proteins, minerals, vitamins, lipids, lactose, and oligosaccharides.[1,2] Mammalian milk oligosaccharides (MMOs), being fermentable substrates for the intestinal microbiota, enhance the growth of bifidobacteria in the intestine.[3−5] Moreover, intestinal fermentation of MMOs leads to the production of short chain fatty acids (SCFAs) including butyric acid. The latter has been shown to be an important energy source for colonocytes and a possible inhibitor of inflammation and carcinogenesis in the intestine.[6] MMOs are also reported to protect the intestinal epithelium against pathogens.[2] Being soluble ligands for intestinal pathogens, MMOs avoid pathogen-attachment to the intestinal mucosa, thereby preventing infection.[2,4,5] In addition, 3′-sialyllactose, one of the most abundant acidic oligosaccharides in many mammal milks, has been suggested to decrease glycosyltransferase expression in epithelial cells. This down-regulation reduces the presence of sialic acid, fucose, and galactose at the epithelial cell surface, potentially inhibiting pathogen adhesion to the cells.[1,5,7,8]

Effects on the host immuno-system have been also suggested for MMOs. Both in vitro and ex vivo studies have demonstrated that MMOs decrease pathogen-associated inflammation.[9,10] Sialic acid levels in infant brains correlate with dietary sialic acids consumption.[11,12] It has been suggested that sialylated oligosaccharides increase learning speed and memory ability, as concluded from an in vivo piglet trial.[13,14] The development of the pig brain shows similarities with that of human infants, suggesting dietary sialic acid's contribution to the cognitive development of infants.[14] Because of the multiple beneficial functions of MMOs, there is increasing interest in the characterization of MMOs and in their fate in the intestinal tract.[2,4,5] MMO concentration varies among species and lactation period.[1,15,16] Commonly, colostrum contains the highest concentration of oligosaccharides, while mature milk contains a decreased concentration of MMOs.[1,15,16] In human milk, oligosaccharide concentration decreases from about 24 to 12 g/L, while in bovine milk, oligosaccharides concentration decreases from about 1 to 0.05 g/L, for colostrum and mature milk, respectively.[1,15,17] At the reducing end of the molecular structure of MMOs, either lactose or of *N*-actelyllactosamine is present. To these moieties, monosaccharides such as galactose, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, fucose, and sialic acids can be attached.[17] Addition of sialic acids to MMOs occurs via α2−3 or α2−6 linkages, while addition of fucose occurs via α1−2, α1−3, or α1−4 linkages, resulting in a wide variety of acidic and neutral oligosaccharides.[17] Moreover, sulfated and phosphorylated MMOs are reported to be present in domestic animal milks.[1,7,17] Bioactivity of MMOs is suggested to be closely related to their structure.[5] Oligosaccharide composition in milk varies due on diet, health, lactation stage, and genetic factors both in human and domestic animals.[2,5,17,19,20] Recently, 39 porcine milk oligosaccharides (PMOs) have been identified, of which 19 are neutral and 20 acidic.[7] Of the PMOs reported, 11 are also found in human colostrum. Differently from human milk oligosaccharides, PMOs present a quite lower proportion of fucosylated structures: 5% and 70% for porcine and human milk oligosaccharides, respectively.[1,7,15] Although PMOs were

Received: January 29, 2016
Revised: February 16, 2016
Accepted: February 16, 2016
Published: February 16, 2016

DOI: 10.1021/acs.jafc.6b00497
J. Agric. Food Chem. 2016, 64, 2087−2093

described[5,7,17] little is known about PMO variation per sow and per lactation period.

In this study, the oligosaccharide content in porcine colostrum and mature milk in Dutch Landrace sows was investigated, with attention to PMO interindividual variation and changes in PMO abundance during 1 w of sow lactation. The fate of PMOs in the intestine of piglets after 1 day and 1 w of nursing was also investigated. PMOs were characterized and quantitated using a combination of capillary electrophoresis with fluorescence or using mass spectrometric detection and liquid chromatography with mass spectrometric detection.

MATERIALS AND METHODS

Oligosaccharide Standards and Capillary Electrophoresis Analysis Kit. Xylose, glucose, galactose, maltotriose, 3′- and 6′-sialyllactose, 3′- and 6′-sialyl-*N*-acetyllactosamine, and lacto-*N*-fucopentaose-V were purchased from Sigma-Aldrich (St. Louis, MO). Lacto-*N*-tetraose, β3′-, β4′-, and β6′-galactosyllactose were from Carbosynth (Compton, UK). Lactose-*N*-neotetraose, 2′- and 3′-fucosyllactose, lacto-*N*-neohexaose, lacto-*N*-hexaose, lacto-*N*-fucopentaose-I,-II, and- III, lacto-*N*-difucosylhexaose, lacto-*N*-fucohexaose-III, and sialyllacto-*N*-tetraose-a, -b, and -c were purchased from Dextra Laboratories (Reading, UK). Labeling of oligosaccharides was performed using the Carbohydrate Labeling & Analysis Kit (Beckman Coulter, Fullerton, CA). All other chemicals were of analytical grade. Millipore water (Millipore, Darmstadt, Germany) was used throughout the text as water.

Colostrums and Fecal Samples. Eight milk samples from Dutch-Landrace sows were collected within 0.5−2 d and at 1 w postpartum, and frozen (−20 °C) until use. Milk samples collected within 0.5−2 d postpartum are referred to as colostrum, while milk samples collected at 1 w postpartum are referred to as mature milk. Two colostrum samples (7 and 8) were donated by Animal Nutrition Group (Wageningen University) (Table 1), while six samples, of which 4 were colostrum and 2 were mature milk samples, matching the corresponding colostrum samples, were obtained from Proefaccommodatie de Tolakker (Utrecht University, Utrecht, The Netherlands). For oligomer identification purpose, samples 1−8 were mixed to ensure sufficient amounts for oligosaccharide purification and characterization. Individual fecal samples were collected from three piglets, 1−2 d and 1 w old (Proefaccommodatie de Tolakker), within 24 h after porcine milk ingestion. Fecal samples were stored at −80 °C until use. An overview of matching porcine colostrum, mature milk samples, and piglet fecal samples is displayed in Table 1.

Table 1. Overview of the Sows, Porcine Milks, and Piglet Fecal Samples Used in This Study[a]

sow code	time of sample collection	milk containing PMOs	fecal samples from 3 piglets fed from the corresponding sow milk		
1	1 day	1M	f1-a	f1-b	f1-c
	1 week	1M#	f1-d	f1-e	f1-f
2	1 day	2M	–	–	–
	2 days	–	f2-a	f2-b	f2-c
	1 week	–	f2-d	f2-e	f2-f
3	0.5 day	3M	–	–	–
	2 days		f3-a	f3-b	f3-c
	1 week	3M#	f3-d	f3-e	f3-f
4	2 days	4M	–	–	–
5	0.5 day	5M	–	–	–
6	1 day	–	f6-a	f6-b	f6-c
	2 days	6M	–	–	–
	1 week	–	f6-d	f6-e	f6-f

[a]# = Mature milk. − = sample not present

Extraction, Purification and Fractionation of PMOs. *Milk Samples.* For qualitative analysis of PMOs present in porcine colostrum, the colostrum samples available (5 mL) were pooled, and the carbohydrates were extracted as reported previously.[1] After carbohydrate extraction, solutions were freeze-dried. In order to remove lactose from the extracted carbohydrates, size exclusion chromatography (SEC) was used.

SEC was used to obtain pools of PMOs with different degrees of polymerization (DP). In total, extracted carbohydrates (200 mg) were fractionated on three Superdex 30 Hiload 26/600 preparative grade columns (GE Healthcare, Pittsburgh, PA) connected in series, using an AKTA Purifier (GE Healthcare) as previously reported.[1] In total, 4 SEC pools were obtained: SEC pools 1 and 2 contained mainly acidic PMOs, while SEC pools 3 and 4 contained mainly neutral PMOs. After freeze-drying, the resulting pools were re-solubilized in 1 mL of water, and the solutions were used for PMO characterization. Part of the SEC pools was recombined, in order to have a representative PMO mixture with reduced lactose content (lactose-free PMOs). Another part of the SEC pools, as well as lactose-free PMOs, was labeled with 9-aminopyrene-1,4,6-trisulfonate (APTS) and subsequently analyzed by CE-LIF and CE-MS^n. A third part of each SEC pool and lactose-free PMOs was analyzed using HILIC-MS^n without prior labeling. Oligosaccharide standards and a human milk oligosaccharide mixture as characterized in a previous study were used as reference for the characterization of PMOs.[18]

For quantitative analysis, SPE was used. The carbohydrates extracted from individual colostrum and mature milk samples (1 mL of 1 mg/mL solution) were loaded onto a graphitized carbon cartridge (150 mg bed weight, 4 mL tube size) (Grace, Deerfield, IL), previously activated by a MeCN/water solution (80/20, v/v) (1.5 mL) with trifluoroacetic acid (0.1% (v/v)) followed by a water wash (1.5 mL).[1] Lactose removal and oligosaccharide elution were performed as reported previously.[1] The fractions obtained containing PMOs were dried overnight under a stream of nitrogen and afterward solubilized in 0.5 mL of water.

Fecal Samples. Fecal samples (±100 mg) were defrosted, and the slurries were diluted in water (2 mL). The suspensions were rotated head over tail overnight (4 °C), and afterward, they were centrifuged (15 min, 15000*g*, 4 °C).[18,19] The supernatants were collected and filtered through a 0.22 μm cellulose acetate membrane as microbial fecal sample cleanup (GE Healthcare).[18] Fecal enzyme inactivation was performed by boiling the solutions (5 min at 100 °C), and samples were purified using SPE as reported elsewhere.[18,19] The fractions obtained from fecal samples were dried overnight under a stream of nitrogen and afterward solubilized in 0.5 mL of water.

Capillary Electrophoresis with Laser-Induced Fluorescent Detection (CE-LIF). The PMOs obtained, either after SEC or SPE, were labeled with APTS and analyzed by CE-LIF as reported elsewhere.[20] CE-LIF peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA). In the CE-LIF technique, quantitation of oligosaccharides is obtained by the use of an internal standard. The internal standard chosen was xylose, as reported previously.[1,20] In CE-LIF, one APTS molecule labels the reducing end of each oligosaccharide molecule, generating linear correlation between peak area and oligosaccharide mole concentration, independently from the precise structure of the specific oligosaccharide investigated.[1,20] The oligosaccharide mole concentrations are therefore converted into the corresponding milligrams through the oligosaccharide molecular weight.[1,20] After manual peak integration, the peak areas were converted into the corresponding PMO concentrations (g/L).

Capillary Electrophoresis with Mass Spectrometric Detection (CE-MS^n). APTS-labeled SEC PMO pools were analyzed by CE on a PA 800 plus system (Beckman Coulter) coupled to an ion-trap mass spectrometer (LTQ Velos Pro ion trap MS, Thermo Scientific, Waltham, MA). The CE-MS^n analysis was performed as previously reported.[20]

Hydrophilic Interaction Liquid Chromatography with Mass Spectrometric Detection (HILIC-MS^n). In order to analyze PMOs present in minor abundances in the milk samples, nonlabeled SEC PMO pools were analyzed by HILIC-MS^n as previously described,

DOI: 10.1021/acs.jafc.6b00497
J. Agric. Food Chem. 2016, 64, 2087−2093

Table 2. Overview of Porcine Milk Oligosaccharides Found in This Study

Milk Oligosaccharides in Porcine Milk				
no.	proposed structure	molecular weight	ref. PMOs[a]	ref. HMOs[a]
	Neutral			
1	**Hex*N* Ac-Hex**	383.37		
2	2′-FL	488.44	7	21
3	3′-FL	488.44	7	21
4	*β*3′-GL	504.44	7,16	21
5	***β*4′-GL**	504.44	16	21
6	*β*6′-GL	504.44	7	21
7	*Gal(α 1-3)Gal(β1-4)Glc*	504.44	7	
8	3′ -FLN	529.52	7	
9	**GaI*N* Ac(*α*/*β*1-3)Gal(*β*1-4)Glc**	545.53		
10	**LNT**	707.63		*21, 23*
11	LNnT	707.63	*7, 16*	*21, 23*
12	*Novo-LNP-I*	869.83	*7, 16*	
13	**LNFP- II**	853.77		*21, 23*
14	LNFP- III/V	853.77	7	*21, 23*
15	**Gal-LNnH**	1235.12		
16	**LNH**	1072.96		*21, 23*
17	LNnH	1072.96	*7, 16*	21
18	Gal(*α*1-3)+Gal(*β*1-4)Glc*N* Ac(*β*1-6)[Gal(*β*1-3)]Gal(*β*1-4)Glc	1032.01	7	
19	**LNDFH**	1365.27		21
	Sialylated			
20	3′-SL	633.55	*7, 16*	21
21	6′-SL	633.55	*7, 16*	21
22	3′-SLN	674.64	*7, 16*	
23	6′-SLN	674.64	*7, 16*	
24	Neu5Ac(*α*2-3)Gal(*β*1-3)Gal(*β*1-4)Glc	795.71	7	
25	Neu5Ac(*α*2-6)GlcNAc(*β*1-6)Gal(*β*1-4)Glc	836.80	7	
26	**Neu5Ac(*α* 2-6)GlcNAc(*β*1-3)Gal(*β*1-4)Glc**	836.80		
27	**LSTa**	998.88		21
28	**LSTb**	998.88		21
29	LSTc	998.88	16	21
30	**Neu5Ac-Neu5Ac-Hex-Hex-Hex**	1087.02		
31	*S-novo-LNP-I*	1161.10	*7, 16*	
32	**S-LNFP-II**	1145.04		
33	S-LNH[b]	1364.23	*7, 16*	21
34	FS-LNH[b]	1510.38	16	21
	Phosphorylated			
35	**P+lactose**	422.29		

[a]ref = reference. HMOs = human milk oligosaccharides. PMOs = porcine milk oligosaccharides. [b]PMO isomer not further specified; structure names in italic = putative isomeric PMO structures. Structures in bold: PMOs novel for the porcine milk literature.[7,16]

with some modification.[1] The mobile phases used were A = water + 1% (v/v) acetonitrile (MeCN), B = MeCN, and C = 200 mM ammonium formate buffer (pH 4.5). A flow rate of 300 μL/min was used. Mobile phases were eluted according to the following profile: 0−1 min isocratic 85% B; 1−31 min from 85 to 60% B; 31−35 min from 60 to 40% B; 35−36 min isocratic 40% B; 36−36.1 min from 40 to 85% B; and 36.1−45 min isocratic 85% B. The mobile phase C was kept constant at 5% during the entire gradient. The autosampler and column oven were kept at 15 and 35 °C, respectively. Mass-spectrometric data settings were ion transfer tube temperature of 350 °C and 3.5 kV source voltage, capillary temperature of 350 °C, and source heater temperature of 225 °C. MS data in negative ion mode were collected over a range of m/z of 300−2000. The mass spectrometer was tuned using maltotriose (0.3 mg/mL) in 70:30 (v/v) MeCN/water containing 5% (v/v) 200 mM ammonium formate buffer (pH 4.5).

RESULTS AND DISCUSSION

Characterization of Porcine Milk Oligosaccharides. In order to elucidate the structure of porcine milk oligosaccharides (PMOs), colostrum samples from different sows were collected and pooled. As shown elsewhere, fractionation by size exclusion chromatography of mammalian milk oligosaccharides (MMOs) facilitates their characterization by increasing the signal-to-noise ratio during the analysis.[1,7,16] For most of the PMOs, full structural characterization was possible by comparing elution times of PMOs with those of available standards using CE-LIF and HILIC-MS^n, and by comparing mass spectrometric fragmentation data in HILIC-MS^n with those described elsewhere.[1] All PMOs found are listed in Table 2, where the new PMO structures are highlighted in bold. In total, 35 PMOs were annotated of which 19 were neutral, 15 were sialylated, and 1 was phosphorylated. Comparing the outcomes of this study with previous literature on porcine milk,[7,16] 13 of the 35 PMOs were new: neutral Hex*N*Ac-Hex, *β*4′-galactosyllactose,

DOI: 10.1021/acs.jafc.6b00497
J. Agric. Food Chem. 2016, 64, 2087−2093

putative Gal*N*Ac(α/β1-3)Gal(β1-4)Glc, lacto-*N*-fucopentaose-II, lacto-*N*-tetraose, galactose linked to lacto-*N*-neohexaose, lacto-*N*-hexaose and difucosyl-lacto-*N*-hexaose, and acidic Neu5Ac(α2-6)Glc*N*Ac(β1-3)Gal(β1-4)Glc, sialyllacto-*N*-tetraose-a and -b, $NeuAc_2$-Hex_3, and sialyllacto-*N*-fucopentaose-II. For 3 PMOs (numbers 7, 12, and 31) (Table 2), only partial characterization was possible. PMO number 7 is a trisaccharide, as concluded from mass spectrometry. Comparing this finding with literature reporting on trisaccharides in cow, goat, sheep, and horse milk,[7] the trisaccharide in porcine milk was assigned putatively to Gal(α1-3)Gal(β1-4)Glc. PMO numbers 12 and 31 were assigned putatively to novo-lacto-*N*-pentaose-I (novo-LNP-I) and sialyl-novo-LNP-I as literature on domestic animal milks report solely the presence of a neutral pentameric structure: novo-LNP-I.[7,16,17] Proportionally, PMOs comprise almost twice as many sialylated structures as human milk oligosaccharides (HMOs), 43% and 20%, respectively. In colostrum, sialylated PMOs are comparable to those in other MMOs, being 60−90, 57, 51, and 20−52% of all MMOs reported for cow, goat, sheep, and horse colostrum, respectively.[1,7] The opposite trend is noticed for fucosylated structures, which covered 23% of all PMO structures and about 70% of HMOs.[21] Fucosylated PMOs are numbers 2, 3, 8, 13, 14, 19, 32 in Table 2.[16] Regarding the number of structures, PMOs showed a higher proportion of fucosylated structures when compared with other domestic animal MMOs, being fucosylated for 8, 8, 7, and 5% for cow, sheep, goat, and horse colostrum, respectively.[7] Nineteen of the 35 PMOs showed structural overlap with structures present in human milk, as indicated in Table 2. Eight PMOs carried the so-called bifidogenic factor (Gal(β1-3)Glc*N*Ac) and, therefore, were considered potential prebiotics (PMO numbers 10, 13, 14, 16, 19, 27, 28, and 32) (Table 2).[21] One molecule of lactose carrying a phosphorylated group was observed (PMO number 35) (Table 2). This phosphorylated oligosaccharide has been reported to be present in ovine, caprine, and equine milk, representing only 1−2% of the total milk oligosaccharides structures.[7,17,22] This is the first time that it has been identified in porcine milk.[7,16,17]

PMO Pattern for Different Sow Colostrum Samples. CE-LIF profiles of PMOs present in the individual porcine milks were used in order to compare PMO pattern in colostrum and mature milk samples. The CE-LIF has been recognized as a suitable method for oligosaccharide quantitation due to its good peak resolution and separation. Therefore, CE-LIF was chosen for the quantitation of PMOs in the individual porcine colostrum samples.[20] In the CE-LIF profile of one porcine milk (3M) (Figure 1), 17 peaks were annotated by comparing migration times of available oligosaccharides standards, of characterized PMO-SEC pools (current study), and of fully identified HMO samples from previous studies within the same CE-LIF analysis. HMOs were characterized and reported in a previous study.[23] In addition, CE-MS^n data were compared with those of HMOs reported previously.[1,18] The most abundant neutral PMO present was the putative Gal(α1-3)Gal(β1-4)Glc. The α1−3 linkage has been reported to be present between galactose moieties in oligosaccharides from caprine colostrum as well.[24] The most abundant acidic PMO was 3′-sialyllactose (3′-SL), similar to that in horse and cow colostrum.[1,22,25] Three main PMO peaks, migrating at 4.76, 5.59, and 5.76 min (Figure 1), could not be characterized. From their migration times, it can be hypothesized that they are a dimer and two tetramers as present in equine and human milk, respectively.[1,26] Using the same annotation as in Figure 1, peaks were identified in Figure 2 for the individual porcine colostrum and mature milk samples. The main PMOs present in the CE-LIF profiles were selected for quantitative analysis. The characterized PMOs and their corresponding concentrations in the samples are listed in Table 3. Comparison of the CE profiles reveals that 9 of the 11 PMOs quantitated were present in all milk samples, although they highly varied in concentration among the samples. Lacto-*N*-neohexaose (LNnH) and sialyllacto-*N*-hexaose (S-LNH, isomer not further specified) were absent in some of the colostrum samples (1M, 4M, and 6M) and in mature milk samples (1M# and 3 M#). Among the colostrum samples, the total PMO concentrations ranged from 7.38 to 29.35 g/L. The corresponding values for HMOs, EMOs, and bovine milk oligosaccharides (BMOs) present in the corresponding colostrums are about 24, 2.8, and 1 g/L, respectively. Hence, it can be noticed that in this respect porcine colostrum resembles human colostrum the most.[1,27] Acidic PMOs represented the most abundant structures, accounting for 77% of the total amount of PMOs quantitated. This finding corresponds with the previous data in which 82% peak abundance was reported for acidic PMOs.[16]

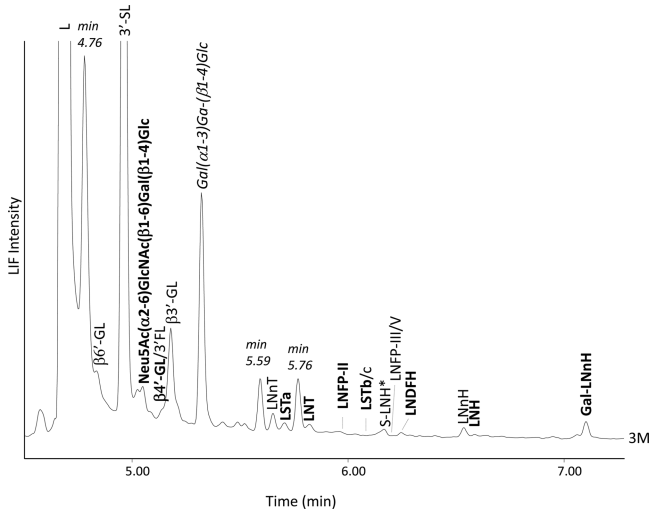

Figure 1. CE-LIF electropherogram of PMOs in sow colostrum (3M). * = PMO isomer not further specified; structures in italic = putative isomeric PMOs structure. Structures in bold: PMOs novel for the porcine milk literature.[7,16,17] L = lactose, β3′-, 4′-, 6′-GL = β3′-, 4′-, 6′-galactosyllactose, 3′-FL = 3′-fucosyllactose, LN(n)T = lacto-*N*-(neo)tetraose, LNFP-II, III.

The relatively high contribution of acidic structures in colostrum has been also shown for BMOs, for which the abundance of acidic and neutral structures is 70−91 and 9−30%, respectively.[7,28] In all sow colostrum samples, the most abundant PMOs was the acidic 3′-SL, with a concentration ranging from 5.03 to 20.98 g/L, representing 68−71% of the quantitated PMOs. 3′-SL is also the most abundant oligosaccharide in cow colostrum (about 49% of the total colostral BMOs), while it is a minor component in human colostrum.[7,15,26]

PMO Concentration during Lactation. Having only two porcine mature milks available matching the corresponding colostrum samples, a first quick inventory regarding PMO composition of colostrum and mature milk was made (1M-1M# and 3M-3M#, for colostrum and mature milk, respectively) (Table 3). Overall, the PMO concentrations decreased from

DOI: 10.1021/acs.jafc.6b00497
J. Agric. Food Chem. 2016, 64, 2087−2093

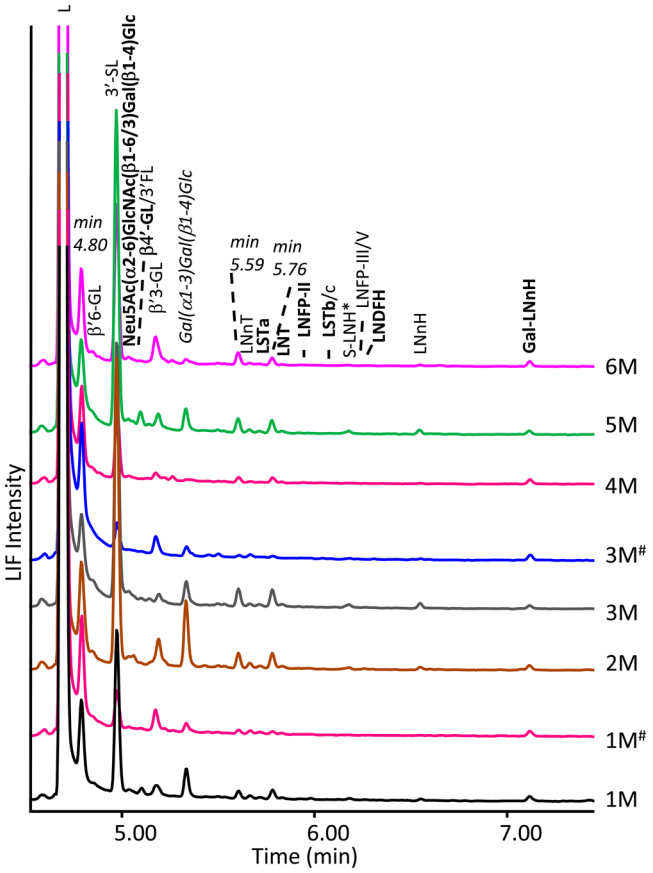

Figure 2. CE electropherograms of PMOs from six porcine milk samples. 1−6 M = sow colostrum, 1 and 3 M# = sow mature milks. * = PMO isomer not further specified; structures in italic = putative isomeric PMOs structure. Structures in bold: PMOs novel for the porcine milk literature.[7,16,17] L= lactose; β3′-, 4′-, and 6′-GL = β3′-, 4′-, and 6′-galactosyllactose; 3′-FL = 3′-fucosyllactose; LN(n)T = lacto-*N*-(neo)tetraose; LNFP-II, III, and V = lacto-*N*-fucopentaose-II, III, and V; LSTa, b, and c = sialyl-lacto-*N*-tetraose-a, -b, and -c; LNDFH = lacto-*N*-difucohexaose; S-LNH = sialyllacto-*N*-hexaose; LN(n)H = lacto-*N*-(neo)hexaose; and Gal-LNnH = galactose-sialyllacto-*N*-neohexaose.

11.85 to 12.19 g/L in the porcine colostrum to 6.82−6.98 g/L in the mature milk samples (Table 3). This reduction corresponds with data for BMOs and HMOs reporting a decrease of about 56% of milk oligosaccharides within the initial 14 d of lactation.[17,26] In those studies, no information on the decrease of individual oligosaccharides was reported.[15,29] In the current study, levels of individual PMOs highly varied depending on the milk analyzed. Nevertheless, the abundance of some PMOs showed a comparable trend in concentration from colostrum to mature milk samples: putative Gal(α1-3)Gal(β1-4)Glc, 3′-SL, and S-LNH (isomer not further specified) decreased in concentration, while β3′-GL, LNnT, and SLTa increased in concentration. Overall, in porcine milk, proportion of sialylated PMOs decreased from 77% to 49% of the total PMOs, for colostrum and mature milk samples, respectively, being in accordance with previous literature.[16] For sialylated BMOs also a decrease of about 30% has been observed.[15,30] The proportion of neutral PMOs increased from 23% to 58% of the total PMOs, for colostrum and mature milk samples, respectively. Neutral BMOs have been shown to increase about 40% in concentration during the first 6 d postpartum.[15]

Fermentation of PMOs. In order to investigate PMO fermentation, fecal samples from piglets were collected after 1−2 d and after 1 w of nursing. Oligosaccharides present in fecal samples were analyzed by CE-LIF and HILIC-MS^n, and their presence was correlated with those of the corresponding milk samples. In Figure 3, CE profiles of PMOs from fecal samples of three piglets per sow (f-1$_{a-c}$ and f-6$_{a-c}$) fed for 1 day on the corresponding sow colostrum (1 M and 6M) are shown. In the CE profiles of oligosaccharides of fecal samples, hardly any PMOs, as present in the milk, could be traced back. Very minor amounts of 3′-SL, LSTa, LNT, LNnT, and LNFP-II might be present in some fecal samples. Next to these minor amounts of known PMOs, several oligosaccharides with DP3 and DP4, as suggested from their CE peak migration times (min 5−6) (Figure 3), were observed. Using mass spectrometry, however, no specific structures could be assigned to these oligosaccharides. Since piglets were exclusively fed on colostrum, it can be hypothesized that the unidentified oligosaccharides were

Table 3. Presence and Concentration of Main PMOs in Colostrum and Mature Milk (#) of Seven Sows[a]

	PMO Concentration in Colostrum and Mature Milk (g/L)							
	1M	1M#	2M	3M	3M#	4M	5M	6M
				Neutral				
β6′-GL	1.09	0.94	0.97	0.64	1.23	0.73	0.83	0.69
β3′-GL	0.69	1.20	1.68	0.19	1.42	0.34	0.59	1.98
Gal(α 1-3)Gal(β 1-4)Glc	1.04	0.47	3.61	0.73	0.57	0.07	1.11	0.23
LNnT	0.13	0.22	0.34	0.07	0.31	0.15	0.21	0.09
LNT	0.06	0.07	0.14	0.07	0.03	0.07	0.08	0.05
LNnH	–	–	0.28	0.30	–	–	0.43	0.10
GAL-LNnH	0.45	0.72	0.54	0.19	0.79	0.84	0.50	0.67
				Acidic				
3′-SL	7.60	3.05	20.98	9.48	1.86	5.03	17.93	10.67
Neu5Ac(α 2-6)GlcN Ac(β 1-6)Gal(β 1-4)Glc	0.59	0.10	0.24	0.13	0.48	0.02	1.05	0.29
SLTa	0.08	0.20	0.25	0.10	0.12	0.12	0.16	0.10
S-LNH*	0.13	–	0.32	0.29	–	–	0.40	–
Total PMOs	12.01	6.98	29.35	12.19	6.82	7.38	23.28	14.87

[a]*PMO isomer not further specified; structure names in italic = putative isomeric PMO structure; structures in bold = PMOs novel for the porcine milk literature.[7,16] – = not present.

DOI: 10.1021/acs.jafc.6b00497
J. Agric. Food Chem. 2016, 64, 2087−2093

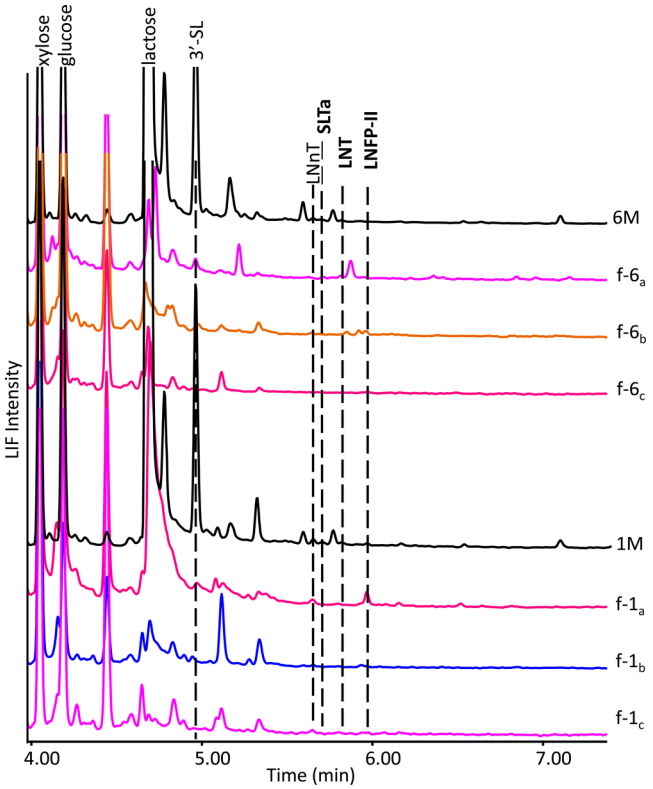

Figure 3. CE electropherograms of fecal PMOs (f-1_{a-c} and f-6_{a-c}) from 3 piglets fed 1 day on the corresponding sow colostrum (1M and 6M). Structures in bold: oligosaccharides novel for the porcine milk literature.[7,16,17] 3′-SL = 3′-sialyllactose, LN(n)T = lacto-*N*-(neo)-tetraose, LSTa, = sialyl-lacto-*N*-tetraose-a, and LNFP-II = lacto-*N*-fucopentaose-II.

metabolic products derived from PMOs or released from endogenous glycoproteins.[18,31] Already at the first day of life, piglet feces lack intact PMOs, indicating quite some intestinal fermentation. Differently, infant feces have been described to present intact dietary HMOs, even after 2 months of life.[23] As reported previously, within 12 h after piglet birth, bacteria already colonize the colon of piglets (10^9 −10^{10} bacteria/g colonic content), and within 2 d, piglet microbiota could already be established.[32,33] A decrease of about 41% in CE-LIF peak area was found for oligosaccharides present in feces of 1 w old piglets (data not shown) compared with 1 day old piglets. By HILIC-MS^n, only one other dimer with a Hex-NAcHex structure, in addition to 3′-SL, was recognized (data not shown) in fecal samples from 1 w old piglets.

In conclusion, 35 PMOs were recognized in porcine colostrum, of which 13 structures were found for the first time.[7,16,17] Eleven major PMOs were quantitated individually analyzed showing high interindividual PMO variation, both regarding their presence and abundance in porcine milk samples. Although only estimated for two matching samples, the abundance of PMOs in colostrum and mature milk was analyzed and correlated. An overall decrease in abundance of about 43% was found among the major PMOs during the first week of lactation, confirming the trend already found for cow's milk.[15] However, while the concentration of acidic PMOs decreased, that of neutral PMOs increased. Already during the first days of life, fecal samples of piglets contain only a few intact PMOs, which are not present anymore in fecal samples of 1 w old piglets. Fecal oligosaccharides different from PMOs were recognized, indicating PMO intestinal fermentation products.

AUTHOR INFORMATION

Corresponding Author

*Phone: +31 317 482239. Fax: +31 317 484893. E-mail: henk.schols@wur.nl.

Funding

This project is jointly financed by the European Union, European Regional Development Fund, and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta, the Municipality of Groningen, the Provinces of Groningen, Fryslân and Drenthe, the Dutch Carbohydrate Competence Center (CCC WP25; www.cccresearch.nl), Danone Nutricia Research, and FrieslandCampina.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to thank Alexandra Ferreira da Silva Ginja for the valuable technical assistance and Edwin Bakx (Laboratory of Food Chemistry, Wageningen University) for the fruitful discussions on the interpretation of the mass spectra.

ABBREVIATIONS USED

DSL,disialyllactose; DS-LNnT,disialyllacto-*N*-neotetraose; Fuc,fucose; 2′-FL,2′-fucosyllactose; 3′-FL,3′-fucosyllactose; Gal,D-galactose; GalNAc,*N*-acetylgalactosamine; *β*3′-GL,*β*3′-galactosyllactose; *β*4′-GL,*β*4′-galactosyllactose; *β*6′-GL,*β*6′-galactosyllactose; Glc,D-glucose; Gal-LNnH,galactose-sialyllacto-*N*-neohexaose; GlcNAc,*N*-acetylglucosamine; Hex,hexaose; HexNAc,*N*-acetyl-hexosamine; L,lactose; LNDFH,lacto-*N*-difucohexaose; LNFP-II, -III, -V,lacto-*N*-fucopentaose-II, -III, -V; LNH,lacto-*N*-hexaose; LN(n)T,lacto-*N*-(neo)tetraose; LNnP-I,lacto-*N*-novo-pentaose I; LST-a, -b, -c,sialyl-lacto-*N*-tetraose-a, -b, -c; SL,3′-sialyllactose; 6′-SL,6′-sialyllactose; 3′-SLN,3′-sialyl-*N*-acetyllactosamine; 6′-SLN,6′-sialyl-*N*-acetyllactosamine; S-LNH,sialyllacto-*N*-hexaose; LN(n)H,lacto-*N*-(neo)-hexaose

REFERENCES

(1) Difilippo, E.; Willems, H. A.; Vendrig, J. C.; Fink-Gremmels, J.; Gruppen, H.; Schols, H. A. Comparison of milk oligosaccharides pattern in colostrum of different horse breeds. *J. Agric. Food Chem.* **2015**, *63*, 4805−14.

(2) German, J. B.; Dillard, C. J.; Ward, R. E. Bioactive components in milk. *Curr. Opin. Clin. Nutr. Metab. Care* **2002**, *5*, 653−8.

(3) Gyorgy, P.; Norris, R. F.; Rose, C. S. Bifidus factor. I. A variant of *Lactobacillus bifidus* requiring a special growth factor. *Arch. Biochem. Biophys.* **1954**, *48*, 193−201.

(4) Gopal, P. K.; Gill, H. S. Oligosaccharides and glycoconjugates in bovine milk and colostrum. *Br. J. Nutr.* **2000**, *84* (Suppl 1), S69−74.

(5) Bode, L. Recent advances on structure, metabolism, and function of human milk oligosaccharides. *J. Nutr.* **2006**, *136*, 2127−30.

(6) Hamer, H. M.; Jonkers, D.; Venema, K.; Vanhoutvin, S.; Troost, F. J.; Brummer, R. J. Review article: the role of butyrate on colonic function. *Aliment. Pharmacol. Ther.* **2008**, *27*, 104−19.

(7) Albrecht, S.; Lane, J. A.; Marino, K.; Al Busadah, K. A.; Carrington, S. D.; Hickey, R. M.; Rudd, P. M. A comparative study of free oligosaccharides in the milk of domestic animals. *Br. J. Nutr.* **2014**, *111*, 1313−28.

(8) Angeloni, S.; Ridet, J. L.; Kusy, N.; Gao, H.; Crevoisier, F.; Guinchard, S.; Kochhar, S.; Sigrist, H.; Sprenger, N. Glycoprofiling

DOI: 10.1021/acs.jafc.6b00497
J. Agric. Food Chem. 2016, 64, 2087−2093

with micro-arrays of glycoconjugates and lectins. *Glycobiology* **2005**, *15*, 31−41.
(9) He, Y.; Liu, S.; Kling, D. E.; Leone, S.; Lawlor, N. T.; Huang, Y.; Feinberg, S. B.; Hill, D. R.; Newburg, D. S. The human milk oligosaccharide 2′-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation. *Gut* **2016**, *65*, 33−46.
(10) He, Y.; Liu, S.; Leone, S.; Newburg, D. S. Human colostrum oligosaccharides modulate major immunologic pathways of immature human intestine. *Mucosal Immunol.* **2014**, *7*, 1326−39.
(11) Wang, B.; McVeagh, P.; Petocz, P.; Brand-Miller, J. Brain ganglioside and glycoprotein sialic acid in breastfed compared with formula-fed infants. *Am. J. Clin. Nutr.* **2003**, *78*, 1024−9.
(12) Wang, B.; Miller, J. B.; McNeil, Y.; McVeagh, P. Sialic acid concentration of brain gangliosides: variation among eight mammalian species. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **1998**, *119*, 435−9.
(13) Wang, B.; Yu, B.; Karim, M.; Hu, H.; Sun, Y.; McGreevy, P.; Petocz, P.; Held, S.; Brand-Miller, J. Dietary sialic acid supplementation improves learning and memory in piglets. *Am. J. Clin. Nutr.* **2007**, *85*, 561−9.
(14) Pond, W. G.; Boleman, S. L.; Fiorotto, M. L.; Ho, H.; Knabe, D. A.; Mersmann, H. J.; Savell, J. W.; Su, D. R. Perinatal ontogeny of brain growth in the domestic pig. *Proc. Soc. Exp. Biol. Med.* **2000**, *223*, 102−8.
(15) Tao, N.; DePeters, E. J.; German, J. B.; Grimm, R.; Lebrilla, C. B. Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. *J. Dairy Sci.* **2009**, *92*, 2991−3001.
(16) Tao, N.; Ochonicky, K. L.; German, J. B.; Donovan, S. M.; Lebrilla, C. B. Structural determination and daily variations of porcine milk oligosaccharides. *J. Agric. Food Chem.* **2010**, *58*, 4653−9.
(17) Urashima, T.; Saito, T.; Nakamura, T.; Messer, M. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate J.* **2001**, *18*, 357−71.
(18) Albrecht, S.; Schols, H. A.; van den Heuvel, E. G. H. M.; Voragen, A. G. J.; Gruppen, H. Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk. *Carbohydr. Res.* **2011**, *346*, 2540−50.
(19) Moro, G. E.; Stahl, B.; Fanaro, S.; Jelinek, J.; Boehm, G.; Coppa, G. V. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. *Acta Paediatr.* **2005**, *94*, 27−30.
(20) Albrecht, S.; Schols, H. A.; Klarenbeek, B.; Voragen, A. G.; Gruppen, H. Introducing capillary electrophoresis with laser-induced fluorescence (CE-LIF) as a potential analysis and quantitation tool for galactooligosaccharides extracted from complex food matrices. *J. Agric. Food Chem.* **2010**, *58*, 2787−94.
(21) Urashima, T.; Asakuma, S.; Messer, M. Milk Oligosaccharides. In *Comprehensive Glycoscience. From Chemistry to System Biology*; Kamerling, J. P., Boons, G. J., Lee, Y. C., Suzuki, A., Taniguchi, N., Voragen, A. G. J., Eds.; Elsevier: Oxford, UK. 2007; Vol. *4*, pp 695−722.
(22) Parkkinen, J.; Finne, J. Isolation of sialyl oligosaccharides and sialyl oligosaccharide phosphates from bovine colostrum and human urine. *Methods Enzymol.* **1987**, *138*, 289−300.
(23) Albrecht, S.; Schols, H. A.; van den Heuvel, E. G. H. M.; Voragen, A. G. J.; Gruppen, H. CE-LIF-MS^n profiling of oligosaccharides in human milk and feces of breast-fed babies. *Electrophoresis* **2010**, *31*, 1264−73.
(24) Kiskini, A.; Difilippo, E. Oligosaccharides in goat milk: structure, health effects and isolation. *Cell Mol. Biol. (Noisy-le-grand)* **2013**, *59*, 25−30.
(25) Fong, B.; Ma, K.; McJarrow, P. Quantification of bovine milk oligosaccharides using liquid chromatography−selected reaction monitoring−mass spectrometry. *J. Agric. Food Chem.* **2011**, *59*, 9788−95.
(26) Albrecht, S. Gastrointestinal-Active Oligosaccharides from Human Milk and Functional Foods. Ph.D. Thesis, Wageningen University, Wageningen, The Netherlands. 2011.
(27) Bode, L. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* **2012**, *22*, 1147−62.
(28) ten Bruggencate, S. J.; Bovee-Oudenhoven, I. M.; Feitsma, A. L.; van Hoffen, E.; Schoterman, M. H. Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. *Nutr. Rev.* **2014**, *72*, 377−89.
(29) Chaturvedi, P.; Warren, C. D.; Altaye, M.; Morrow, A. L.; Ruiz-Palacios, G.; Pickering, L. K.; Newburg, D. S. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology* **2001**, *11*, 365−72.
(30) Nakamura, T.; Kawase, H.; Kimura, K.; Watanabe, Y.; Ohtani, M.; Arai, I.; Urashima, T. Concentrations of sialyloligosaccharides in bovine colostrum and milk during the prepartum and early lactation. *J. Dairy Sci.* **2003**, *86*, 1315−20.
(31) Dotz, V.; Rudloff, S.; Meyer, C.; Lochnit, G.; Kunz, C. Metabolic fate of neutral human milk oligosaccharides in exclusively breast-fed infants. *Mol. Nutr. Food Res.* **2015**, *59*, 355−64.
(32) Alizadeh, A.; Akbari, P.; Difilippo, E.; Schols, H. A.; Ulfman, L. H.; Schoterman, M. H. C.; Garssen, J.; Fink-Gremmels, J.; Braber, S. The piglet as a model for studying dietary components in infant diets: effects of galacto-oligosaccharides on intestinal functions. *Br. J. Nutr.* **2016**, *115*, 605−18.
(33) Swords, W. E.; Wu, C. C.; Champlin, F. R.; Buddington, R. K. Postnatal changes in selected bacterial groups of the pig colonic microflora. *Neonatology* **1993**, *63*, 191−200.

DOI: 10.1021/acs.jafc.6b00497
J. Agric. Food Chem. 2016, 64, 2087−2093