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Oligosaccharides in Urine, Blood, and Feces of Piglets Fed Milk Replacer Containing Galacto-oligosaccharides

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ABSTRACT: Human milk oligosaccharides (HMOs) are absorbed into the blood (about 1% of the HMO intake) and subsequently excreted in urine, where they may protect the infant from pathogen infection. As dietary galacto-oligosaccharides (GOS) have partial structural similarities with HMOs, this study investigated the presence of GOS and oligosaccharides originating from milk replacer in blood serum, urine, and cecal and fecal samples of piglets, as a model for human infants. Using liquid chromatography–mass spectrometry and capillary electrophoresis with fluorescence detection, oligosaccharides originating from piglet diet including 3′-sialyllactose and specific GOS ranging from degree of polymerization 3 to 6 were detected in blood serum and in urine of piglets. In blood serum, GOS levels ranged from 16 to 23 μg/mL, representing about 0.1% of the GOS daily intake. In urine, approximately 0.85 g of GOS/g of creatinine was found. Cecum digesta and feces contained low amounts of oligosaccharides, suggesting an extensive GOS intestinal fermentation in piglets.

KEYWORDS: *GOS, pig, absorption, creatinine, prebiotics, intestine, liquid chromatography, capillary electrophoresis, mass spectrometry, fermentation*

INTRODUCTION

Human milk oligosaccharides (HMOs) are the third most abundant component in milk, after lactose and lipids.[1] About 200 different HMOs have been annotated, of which around 100 oligosaccharide structures have been elucidated.[1,2] After oral ingestion of human milk, about 1% of HMOs, both neutral and acidic, are reported to be absorbed in the small intestine of the infant, thereafter entering the blood circulation. So far, about 15 HMOs, including lacto-*N*-tetraose, lacto-difucosyl-pentaose, 3′- and 6′-sialyllactose, and 3′- and 6′-sialyl-*N*-acetyllactosamine, have been observed in the blood of infants.[3] Literature suggest a protective function of HMOs in the blood circulation by influencing leucocyte adhesion to endothelial cells and platelet–neutrophil interaction.[4−7] When systemic HMOs are cleared, they are excreted into the urinary system, thus being detectable in urine.[6−10] In addition to lactose, 13 HMOs, both neutral and acidic, have been found in the urine of infants.[7] One of the proposed functions of HMOs is an in situ protection against urinary tract infections in the infant, by blocking the adhesion of pathogens to the epithelial cell wall.[5,6,11] Around 99% of the HMOs reach the colon of infants, where they can be fermented by the colonic microbiota.[12,13] In the intestine, HMOs can exert prebiotic, immunomodulatory, and anti-infective functions.[14] To date, HMOs are not produced in the food-grade volumes required for infant nutrition. Therefore, other dietary ingredients that promote health and well-being and reduce the risk of diseases are of broad public interest.[15] Non-digestible oligosaccharides, such as galacto-oligosaccharides (GOS), belong to these health-promoting ingredients. Multiple preclinical studies have shown that GOS are a prebiotic fiber that selectively stimulates the growth of beneficial gut bacteria.[16−21] Different studies have shown that daily ingestion of GOS increases beneficial bacteria, such as Bifidobacteria, in the colon of adults and infants.[16,19,22] It is hypothesized that GOS lowers the intestinal pH and reduces the survival of pathogens.[16,23−25] Moreover, GOS fermentation by intestinal microbiota leads to the production of short-chain fatty acids, including butyrate.[20] Butyrate is known for decreasing inflammation, carcinogenesis, and oxidative stress in the intestine.[26,27] GOS also act as soluble ligands for pathogens, inhibiting, for example, the binding of *Escherichia coli* and *Salmonella typhimurium* to the intestinal mucosa layer.[28] In addition, GOS are associated with a lower risk of infections and diarrhea due to direct effects on the intestinal immune system.[20] Moreover, the microbiota of infants fed formula enriched with GOS or galacto-/fructo-oligosaccharides (FOS) resembled more the microbiota of breast-fed infants

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than the microbiota of infants fed GOS/FOS-free formula.[29] The observed GOS health-promoting effects are dose dependent and are a result of their structural characteristics, which have similarities with those of HMOs.[1,30,31]

GOS are industrially produced from lactose by β-galactosidase enzyme of microbial or fungal origin.[15] The composition of GOS mixtures varies depending on the enzyme source and the conditions used during the production process, such as temperature, pH, and substrate concentration.[16] GOS mixtures consist of galactose oligomers with a terminal glucose, varying in degree of polymerization (DP) and type of glycosidic linkage(s).[16] The DP ranges mostly between 2 and 8.[24] The complex mixture that is produced predominantly consists of structures with a reducing end, but also in minor amounts (7% of GOS-DP2) with a non-reducing end.[16,20]

Although beneficial health effects of GOS have been shown, GOS in vivo fate is not completely known.[20,28] In the current study, a pig model was used to investigate the absorption, excretion, and fermentation of orally ingested GOS, making use of biological sample being available from a piglet study addressing health benefits of GOS during a 26 day feeding trial.[32]

MATERIALS AND METHODS

Materials. Monosaccharides and oligosaccharides used as standards were D-(+)-xylose, D-(+)-glucose, D-(+)-galactose (Sigma-Aldrich, St. Louis, MO, USA); 3′- and 6′-sialyllactose (Dextra Laboratories, Reading, UK); (1−4)-β-D-galactobiose (Megazyme, Bray, Ireland); D-(+)-lactose and D-(+)-maltose (Merck, Darmstadt, Germany); and maltodextrine (dextrose equivalent 20) (AVEBE, Veendam, The Netherlands). Vivinal GOS syrup (DM 75%) was provided by FrieslandCampina DOMO (Borculo, The Netherlands). Specifications by the supplier were as follows: dry matter content of 75%, of which 59% was galacto-oligosaccharides, 21% lactose, 19% glucose, and 1% (w/w) galactose. Fractions of Vivinal GOS (degree of polymerization from 1 to 6) were obtained after size exclusion chromatography (SEC) as described elsewhere.[33] Sodium cyanoborohydride, trifluoroacetic acid (TFA), and ammonium hydroxide were purchased from Sigma (Sigma-Aldrich). UHPLC grade water and UHPLC-grade acetonitrile (ACN) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Millipore water was obtained from an Elix Integral water purification system (Millipore, Darmstadt, Germany), and it is referred to in the text as "water".

Experimental Animal Model and Diets Used. The piglet was used as a model to study the effects of GOS on the intestinal functions as described in detail elsewhere.[32] In short, 40 Landrace × Yorkshire piglets, separated from the mother sows 36−48 h post-partum, were selected for this study. The short pre-weaning period allowed the intake of colostrum and consequent protection by maternal antibodies. After being separated from the sow, the piglets were housed under conventional conditions at the animal housing facility of Utrecht University (Department of Farm Animal Health) and allocated randomly to two experimental groups receiving either the piglet milk replacer (control diet) alone or supplemented with GOS. The commercial piglet milk replacer (Milkiwean Babymilk Yoghurt, Nutreco, Amersfoort, The Netherlands) contained 965 g/kg dry matter, 200 g/kg crude protein, 200 g/kg fat, 0.10 g/kg crude fiber, 3.50 g/kg calcium, and 4.80 g/kg phosphorus. Part of the piglet milk replacer was supplemented for the experimental group with 0.8% GOS (Vivinal GOS syrup, FrieslandCampina Domo). From each group (control- and GOS-fed piglets) one subgroup of 10 animals was sacrificed 3 days after the start of the experiment (age of approximately 4−5 days and 3 days on the diet), whereas the other two subgroups (control- and GOS-fed piglets) of 10 animals were sacrificed 26 days after the start of the experiment (age of approximately 27−28 days and 26 days on the diet). All in vivo experimental protocols were approved by the Ethics Committee for Animal Experiments (reference no. DEC 2011.III.11.117) and were performed in compliance with governmental and international guidelines on animal experimentation.

Collection of Fecal, Blood, Digesta, and Urine Samples. Piglets, at days 3 and 26 of the experimental feeding period, were sacrificed within 2 h after the last feeding. Fecal samples per piglet were collected and directly stored at −80 °C. The gastrointestinal tract was removed, and the digesta from the cecum were collected and stored at −80 °C. Blood from the external jugular vein and urine from the bladder were collected and stored at −20 °C.

Prior to the analysis of GOS in serum samples, extraction and analytical methods, already established for milk and food liquid matrices, were tested on serum samples from two pigs (Faculty of Veterinary Science, Utrecht University) not belonging to the feeding trial.[15,34] The two pigs were fed a diet different from Milkiwean Babymilk with no GOS supplementation. Part of the two serum samples was spiked with Vivinal GOS (0.08% w/v), and after purification and carbohydrate extraction, they were compared with the original serum samples.

Oligosaccharide Extraction and Purification. *Extraction.* Three randomly chosen serum (500 μL) and urine samples (200 μL) and Vivinal GOS solution (200 μL, 1 mg/mL) were centrifuged (20000*g*, 5 min, 20 °C), and the supernatants were analyzed. Oligosaccharide extraction from feces and cecal digesta was performed as described elsewhere, with minor modifications.[35] Briefly, watery slurries of cecal digesta and fecal samples (50 mg/mL) were kept overnight at 4 °C, under head-over-tail rotation, to optimize the carbohydrate extraction. Next, the samples were centrifuged (20000*g*, 15 min, 4 °C), and the supernatants were filtered on a 0.22 μm cellulose acetate filter membrane (GE Healthcare, Pittsburgh, PA, USA). The filtrate was heated (5 min, 100 °C) and used as sample. The filtrates (digesta and fecal samples) and the supernatants (serum and urine samples) were diluted in 2 mL of water. Piglet formula Milkiwean Babymilk Yoghurt (2 mg) was suspended in water (1 mg/mL) and subsequently centrifuged as described above. This supernatant was used as control sample for further analysis.

Purification. Monosaccharides and salts present in all samples were removed by solid phase extraction (SPE) on a non-porous graphitized carbon cartridge (bed weight,150 mg; tube size, 4 mL; Alltech, Deerfield, IL, USA). The cartridges were conditioned with 1.5 mL of 80:20 (v/v) acetonitrile (ACN)/water containing 0.1% (v/v) TFA, followed by a washing step with 1.5 mL of water, as reported elsewhere.[36] Samples and GOS solution, obtained as described above, were loaded onto the cartridges. Salts and monosaccharides were removed by elution with 6 mL of water. Oligosaccharides, including disaccharides, were collected after elution with 3 mL of 40:60 (v/v) ACN/water containing 0.05% (v/v) TFA. The eluted oligosaccharide fraction was dried overnight under a stream of nitrogen at 20 °C and resolubilized in 500 μL of water (Vivinal GOS, milk replacer, urine, feces, and cecal digesta) or in 200 μL of water (serum samples). After SPE, the Vivinal GOS solution, presenting a reduced amount of monomers (glucose and galactose), is coded "GOS ref" and is used as a standard for comparative analysis within the current study.

Analysis of Oligosaccharides by Capillary Electrophoresis with Laser-Induced Fluorescence detection (CE-LIF). Fractions of GOS and oligosaccharides from serum, urine, feces, and cecal digesta samples of three piglets of each experimental group (from 3 and 26 days on control or GOS diet) were analyzed. Purified oligosaccharides were labeled as reported elsewhere using a Proteomelab Carbohydrate Labeling and Analysis Kit (Beckman Coulter, Fullerton, CA, USA).[33] Five nanomoles of xylose were added as internal standard to 100 μL of sample. According to the manufacturer's instructions, the labeled samples were diluted 40 times prior to CE-LIF analysis, and the electropherograms were normalized on the internal standard. Data analysis was performed with Chromeleon software 6.8 (Dionex, Sunnyvale, CA, USA). The degree of polymerization of individual GOS components was recognized by comparing the obtained CE-LIF profiles with known SEC GOS-DP fraction profiles.[37]

Analysis of Oligosaccharides by Hydrophilic Interaction Liquid Chromatography with Mass Detection (HILIC-MS^n). The

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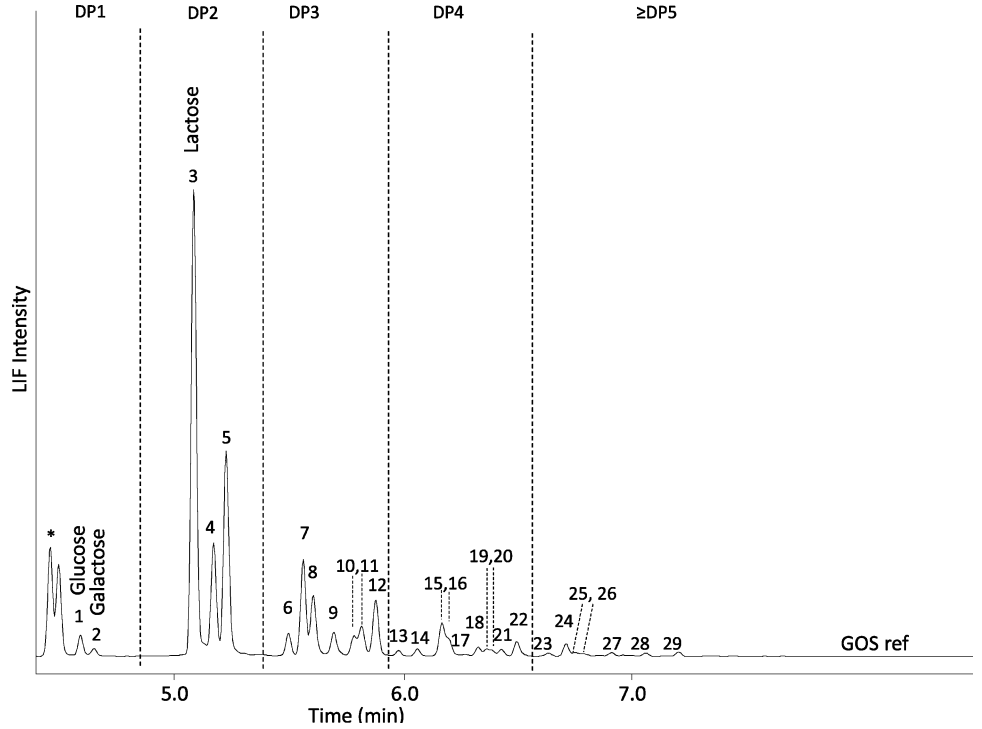

Figure 1. CE-LIF electropherogram of the GOS ref. The detected GOS peaks are numbered from 1 to 29, and the internal standard, xylose, is indicated with an asterisk (∗). DP, degree of polymerization.

oligosaccharide samples were solubilized 1:1 (v/v) in ACN and analyzed using an Accela Ultra High Pressure Liquid Chromatography (UHPLC) system (Thermo Scientific, Waltham, MA, USA). Chromatographic separation was performed on an Acquity HILIC BEH Amide column (1.7 μm, 2.1 mm × 150 mm) combined with a Van Guard precolumn (1.7 μm, 2.1 mm × 5 mm; Waters Corp., Milford, MA, USA). The flow rate was 300 μL/min, and the injection volume was 5 μL. The eluents were (A) water with 1% (v/v) ACN, (B) 100% (v/v) ACN, and (C) 200 mM ammonium formate (pH 4.5). Separation was achieved under the following conditions: 0−31 min, from 10 to 35% (v/v) A; 31−36 min, from 35 to 55% (v/v) A; 36−45 min, from 55 to 10% A. Eluent C was kept constant at 5% during the separation. Temperatures of the autosampler and column oven were set at 20 and 35 °C, respectively. In-line mass spectrometric analysis was performed using a Velos Pro mass spectrometer (Thermo Scientific) coupled to the UHPLC system described above. Mass data were acquired in negative ionization mode over a m/z scan range of 300−2000 Da. MS^2 and MS^3 fragmentation was performed on the most abundant ion in the MS and MS^2 spectra, respectively.

Oligosaccharide Estimation in Blood and Urine of Piglets. To roughly estimate the oligosaccharides absorption in blood and excretion in urine, several parameters were considered. Piglet blood was estimate to be 13% of body weight, and serum was estimated to be 60% of blood volume.[38] After 3 and 26 days of the GOS diet, the average serum volume was estimated to be 151 and 595 mL, respectively, with a piglet average body weight of 1.9 (3 days) and 7.2 kg (26 days). Piglets feedings were approximately 600 mL/piglet/day at the start of the experiment, increasing to approximately 1600 mL/piglet/day during the experimental period. Systemic GOS was expressed as percentage of GOS absorbed during the daily mean GOS intake. Urine samples are an easily available source for compound estimation in urine; however, variation in compound concentration in urine is present. Oligosaccharide urinary concentration was adjusted by relating it to the creatinine concentration in the same urine sample.[39] Creatinine is a metabolic product of muscle tissue and is almost constantly excreted in urine. Therefore, the urinary oligosaccharide concentration was expressed as grams of GOS per gram of excreted creatinine, calculated for each analyzed urinary sample.[39]

The amount of oligosaccharides in the biological samples was determined using CE-LIF. Quantification of APTS-labeled oligosaccharides was performed by converting the peak areas via amount of nanomoles to amount of micrograms.[33] The amount (μg) per DP and per individual compound was calculated relative to the total amount (μg) of GOS-DP3−DP6 present in the samples analyzed.

RESULTS AND DISCUSSION

Characterization of Vivinal GOS Syrup. Vivinal GOS syrup, used as source of GOS in the experimental feeding trial, was characterized prior to piglet sample analysis. GOS ref, GOS-SEC fractions, and relevant oligosaccharide standards were APTS-labeled and analyzed by CE-LIF. By comparison of GOS-SEC fraction electropherograms with previous literature, GOS peaks were recognized for their degree of polymerization (DP) and were numbered (1−29, Figure 1).[37] Peak numbers were used in further comparative data analysis in this paper. Peaks 1, 2, and 3 were assigned to glucose, galactose, and lactose, respectively. GOS-DP3 (peaks 6−12), GOS-DP4 (peaks 13−22), GOS-DP5 (peaks 23−28), and GOS-DP6 (peak 29) peaks were assigned on the basis of previous data.[37] The presence of seven GOS-DP3 with a free reducing end is in agreement with previous data.[16] Quantification of GOS having specific DP was performed on APTS-labeled Vivinal GOS syrup. The abundances of GOS-DP2−DP6 were 40, 24, 11, 4, and <1% (w/w), respectively, in accordance with the literature.[16,37] Hence, it can be concluded that GOS were reliably separated and quantified by CE-LIF, allowing further analysis of biological samples.

Extraction and Detection of GOS in Serum Samples. Previous studies have indicated that neutral oligosaccharides could be purified by SPE when present in liquid food matrices, urine, digesta, and feces, although to our knowledge it was never investigated for serum samples.[36,37,40] Therefore, extraction and purification of GOS from the serum of two

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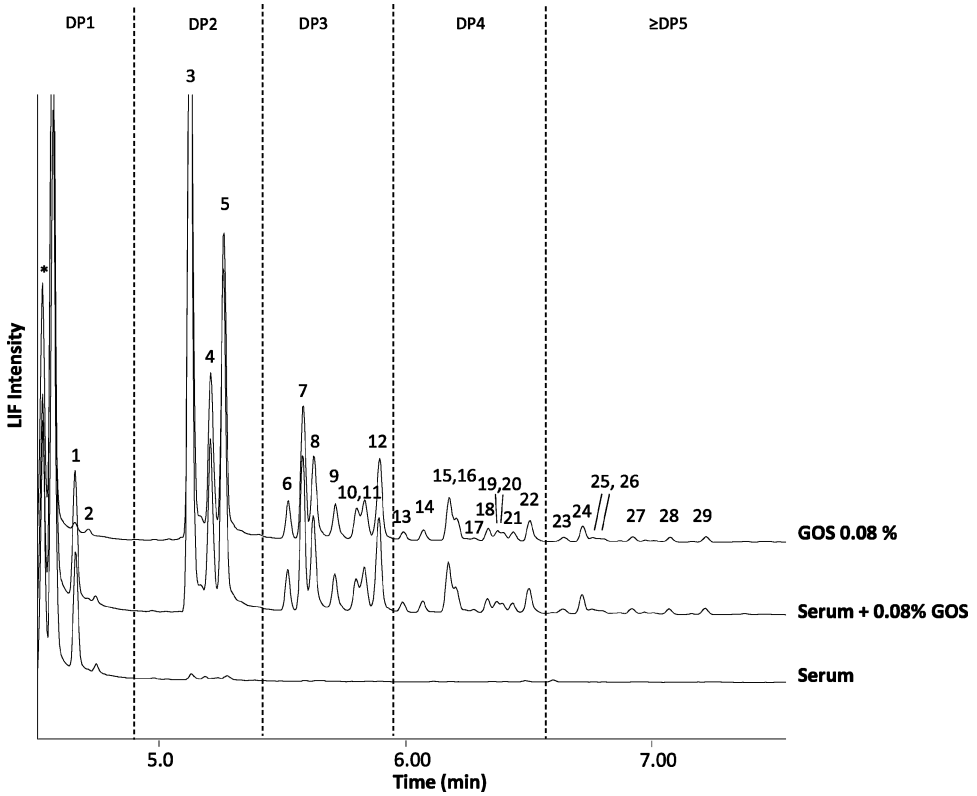

Figure 2. CE-LIF electropherograms of oligosaccharides from piglet serum, from piglet serum spiked with 0.08% of GOS, and from GOS 0.08%. The electropherograms are normalized on the internal standard, xylose (∗), and GOS peaks are numbered from 1 to 29 as in Figure 1. DP1−DP5, degree of polymerization.

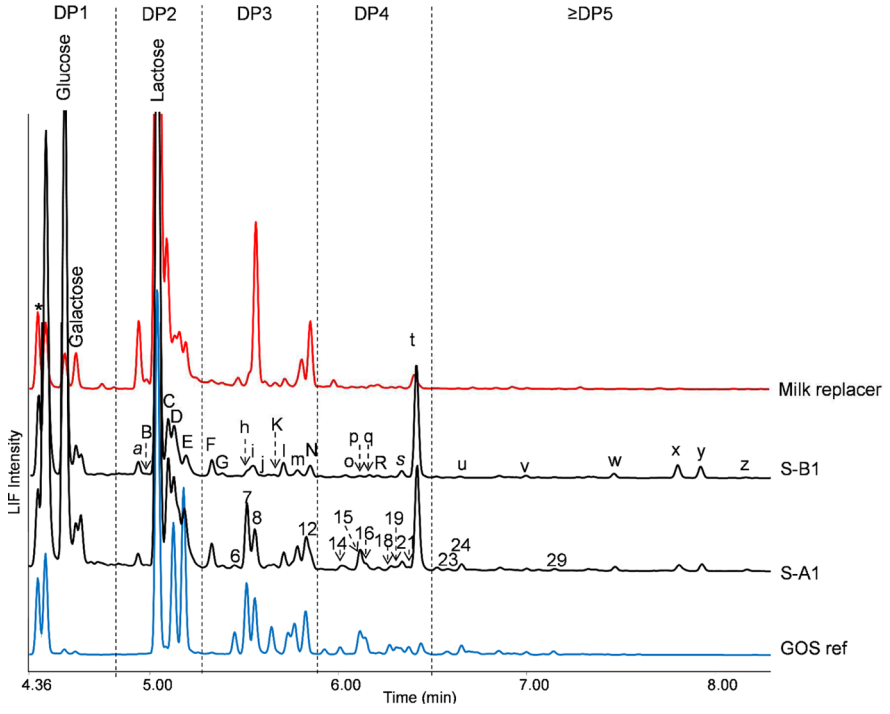

Figure 3. CE-LIF electropherograms of GOS ref, milk replacer, oligosaccharides present in serum at day 3 of GOS diet (S-A1), and control diet (S-B1). DP1−DP5, degree of polymerization based on GOS. The electropherograms are normalized on the internal standard, xylose (∗); 6−29 are peaks corresponding to GOS; a−z are peaks as found in S-B1, and capital letters represent oligosaccharides possibly derived from the milk replacer.

piglets not belonging to the piglet feeding trial were performed prior to serum sample analysis from the feeding trial. Recovery of GOS was examined by spiking piglet sera with Vivinal GOS (0.08% w/v). As shown in Figure 2, the CE electropherograms of the spiked serum samples and GOS ref were comparable. The total GOS peak areas were compared, and a recovery of

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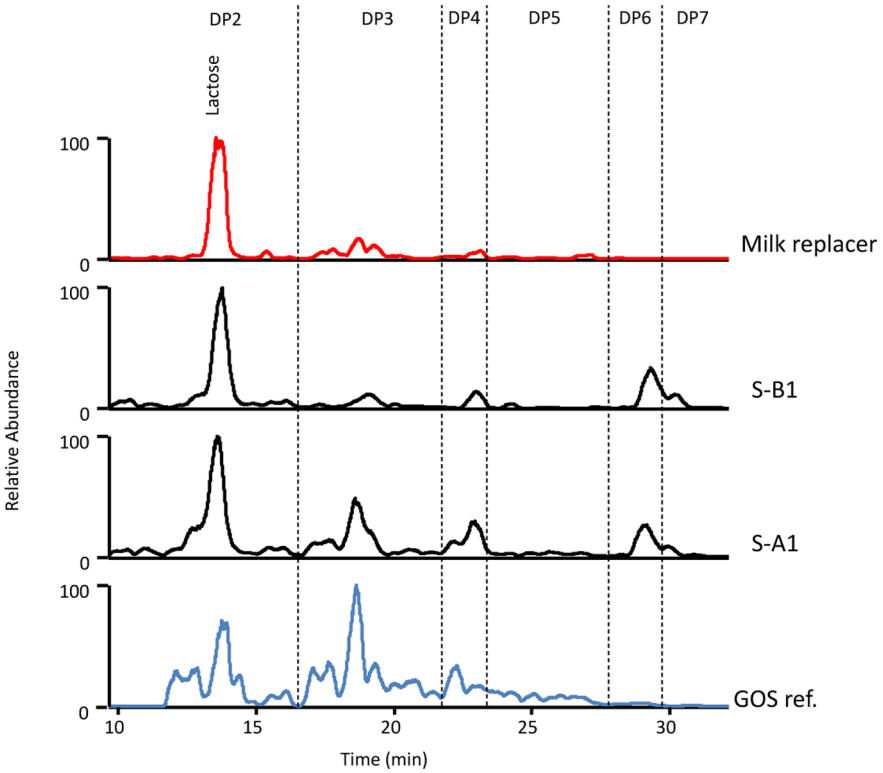

Figure 4. Base peak in HILIC-MS^n for oligosaccharides as found in GOS ref, in serum at day 3 of GOS diet (S-A1) and control diet (S−B1), and in milk replacer.

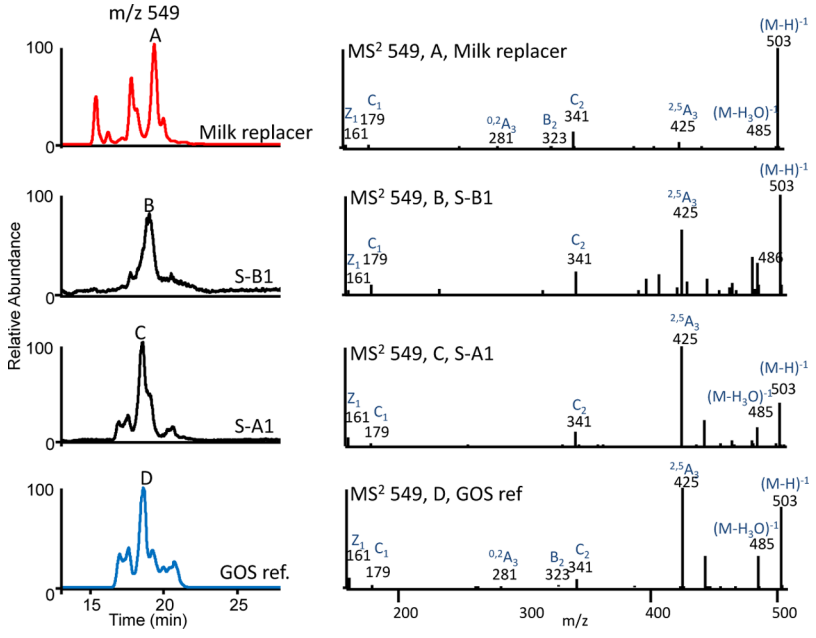

Figure 5. Selected base peak in HILIC-MS^n for trimers as found in GOS ref, in serum at day 3 of GOS diet (S-A1) and control diet (S−B1), and in milk replacer. Corresponding MS^2 fragmentation patterns and fragment annotation, as reported by Domon and Costello, of the trimers named A, B, C, and D; m/z 549 precursor ion in HILIC-MS^n.[41]

~97% of added GOS to serum samples was achieved. In the non-spiked serum samples, next to glucose and galactose, only trace amounts of oligosaccharides (eluting from 5 to 6 min, Figure 2) were observed. Consequently, it can be concluded that SPE can be successfully used to extract and purify GOS from serum samples prior to CE-LIF analysis.

GOS and Dietary Oligosaccharides in the Biological Samples. *Detection of Oligosaccharides in Serum at Day 3 by CE-LIF.* In Figure 3, CE-LIF profiles of APTS-labeled oligosaccharides from serum samples of piglets fed 3 days on GOS or control diet (S-A1 and S-B1, respectively) are compared with those of GOS ref and milk replacer control. In both S-A1 and S-B1 samples, peaks representing reducing

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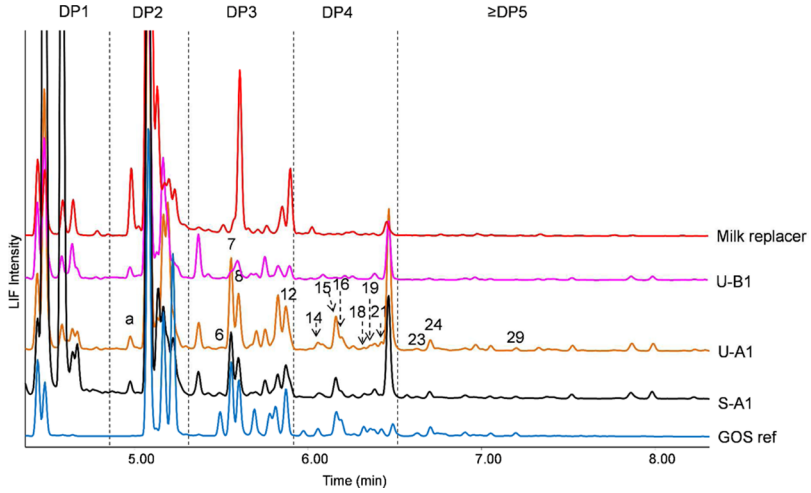

Figure 6. CE-LIF electropherograms of GOS ref, milk replacer, oligosaccharides from serum and urine at day 3 of GOS diet (S-A1 and U-A1, respectively), and oligosaccharides from urine at day 3 of control diet (U-B1). DP1−DP5, degree of polymerization based on GOS. The electropherograms were normalized on the internal standard xylose (∗); 6−29 are peaks corresponding to GOS; peak a is 3′-silayllactose.

oligosaccharides were observed: in S-A1, besides dimers including lactose, peaks were assigned specifically to GOS structures (numbers 6−29, Figure 3). Peaks 6−12, 14−21, and 23−29 in Figure 3 were assigned to GOS-DP3, -DP4, and -DP≥5, respectively. The abundance of the detected GOS will be discussed below. In addition, dietary acidic oligosaccharide 3′-sialyllactose (3′-SL) present in the milk replacer was recognized in serum samples of piglets fed control and GOS diet (peak a, Figure 3). 3′-SL, reported to be one of the most abundant oligosaccharides in cow's milk (95 mg/L), represented approximately 0.8 and 1.3% of the total oligosaccharides, excluding lactose, present in serum samples of piglets fed 3 days on GOS or control diet, respectively.[15] None of the oligosaccharide peaks assigned to GOS were present in the serum samples of piglets fed control diet (S-B1). Surprisingly, S-B1 showed the presence of an additional 25 reducing oligosaccharides (peaks b−z, Figure 3). Most of them were present in very low abundance, although peak t was present in high abundance. Comparing migration times, it was hypothesized that, beside 3′-SL, 11 of the 25 peaks were specifically originating from the milk replacer (capital letters in Figure 3). Peak 7 in S-A1, being present in a comparable relative amount to peak as observed in the GOS ref, was assigned to a GOS structure. However, peak 7 overlapped with the unresolved peak h present in low abundance in serum S-B1 of the control group.

To the best of our knowledge, this is the first time that GOS and oligosaccharides present in a piglet milk replacer have been detected in piglet blood samples. The systemic presence of HMOs and neutral oligosaccharides from cow's milk after ingestion of infant formula has been described previously.[3,4,8] In this study, oligosaccharides added to the piglet formula were found to be absorbed and excreted, comparable to HMOs in infants.

Detection of Oligosaccharide in Serum at Day 3 by HILIC-MS^n. Because this is the first time that GOS have been detected in serum samples, HILIC-MS^n was used to confirm these results. In Figure 4, profiles of oligosaccharides in serum of piglets fed 3 days on GOS or control diet (S-A1 and-B1, respectively), GOS ref, and milk replacer control are shown. On the basis of profile comparison, especially GOS DP3 and DP4 were present in serum of GOS-fed piglets (S-A1). Due to their extremely low abundance, GOS-DP5 present in the serum samples could not be assigned to specific oligosaccharides. In serum sample profiles, peaks representing oligosaccharides eluting around 30 min were also detected. Nevertheless, their masses did not correspond to oligosaccharides with DP6 or DP7, as expected from their elution times. From MS^2 fragmentation analysis, they were suggested to present two neutral hexose trimers and one unsaturated hexose monomer. In Figure 5, an example of mass analysis of oligosaccharides observed in serum samples of piglets fed 3 days on GOS or control diet (S-A1 and B1, respectively), the GOS ref and milk replacer control are shown. MS^2 fragmentation patterns and structural composition of the trimers named A, B, C, and D are also presented, and fragments were annotated as suggested elsewhere.[41] All of the hexose trimers, having formate adduct, presented a mass-over-charge (m/z) of 549. The chromatograms of serum of piglets fed GOS diet (S-A1) showed a specific peak overlap with GOS ref peaks (15−22 min). On the contrary, in the profile of serum from piglets fed control diet (S−B1), only one trimer was observed (19 min). The highest peak for each sample was compared for its fragmentation pattern. The highest intensity peaks, C (S-A1) and D (GOS ref), were attributed to a fragment with m/z 425 referring to intra-ring hexaose fragmentation. Fragments with m/z 161, and 179, representing monomeric units, moreover, showed a comparable relative abundance (ratio 161:179 m/z of 6.5 and 5.3, respectively). In contrast, highest intensity peaks in A (milk replacer) and B (S−B1) were attributed to a m/z 503 referring to trimers deprived of the formate adduct. Fragments with m/z 161 and 179 showed relative ratios of 0.2 and 0.04, for peaks A and B, respectively, both not comparable with the ratio reported above for peaks C and D. Subsequent mass analysis of DP4 oligosaccharides proved the GOS contribution to the oligosaccharide profile in serum (data not shown) and confirmed that GOS-DP4 was present in the serum of GOS-fed piglets. HILIC-MS^n supported our CE-LIF findings, confirming the presence of GOS in the serum of piglets fed GOS diet (S-A1).

In the milk replacer used in this study, cow's milk oligosaccharides and neutral hexoses were found. Identified cow's milk oligosaccharides were 3′- and 6′-sialyllactose, disialyllactose, and *N*-acetylgalactosaminyllactose.[15] After mass

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Table 1. Presence, Concentration, and Relative Percentage of GOS Structures As Detected by CE-LIF in GOS Ref, in Serum, and in Urine at Day 3 of GOS Diet of Piglets

	piglet serum (3 days) concn (μg/mL)					piglet urine (3 days) concn (g/g creatinine)		
peak	GOS ref	S-A1	S-A2	S-A3	S-A4	U-A1	U-A2	U-A3
6	14.7	0.6	0.1	0.2		0.004	0.003	0.002
7	49.1	10.1	1.2	8.7	5.9	0.3	0.3	0.2
8	41.3	6.3	1.1	5.5	4.2	0.2	0.2	0.1
9	18.4							
10	13.9							
11	25.1							
12	31.5	5.7	0.8	4.8	4.2			
concn GOS-DP3	193.9	22.6	3.2	19.1	14.2	0.5	0.5	0.4
rel %	60.9	66.5	59.6	61.5	67.1	57.8	58.3	56.4
13	4.6							
14	6.3	1.2	0.1	0.9	0.8	0.03	0.03	0.02
15	23.6	4.6	0.8	4.7	3.1	0.16	0.16	0.11
16	14.4	1.2	0.4	1.8	0.8	0.05	0.06	0.05
17	1.4							
18	8.5	0.8	0.2	0.5		0.01	0.01	0.00
19	6.7	0.4	0.1	0.5		0.02	0.02	0.02
20	4.2							
21	7.0	0.4	0.2	0.5	0.8	0.03	0.02	0.02
22	11.6							
concn GOS-DP4	88.4	8.7	1.9	8.8	5.5	0.3	0.3	0.2
rel %	27.8	25.6	35.5	28.3	25.9	31.6	31.8	33.3
23	4.4	0.5	0.1	0.6		0.01	0.01	0.01
24	11.4	1.6	0.1	2.0	1.0	0.07	0.06	0.04
25	4.8							
26	2.6							
27	3.9							
28	3.5							
conn GOS-DP5	30.7	2.1	0.2	2.5	1.0	0.1	0.1	0.05
rel %	9.6	6.1	4.9	8.1	4.6	8.3	7.9	7.6
29								
concn GOS-DP6	5.2	0.6		0.7	0.5	0.02	0.02	0.02
rel %	1.6	1.8		2.2	2.5	2.3	2.0	2.7
total GOS concn	318.2	34.1	5.3	31.1	21.2	0.9	0.9	0.6
av total GOS concn	22.9					0.8		

analysis, 3′-sialyllactose was observed in serum of piglets fed GOS and control diet, as expected from CE-LIF analysis. In in vivo studies, the systemic presence of neutral and acidic oligosaccharides from human milk has been reported for suckling human neonates.[4,7,13] Moreover, in vitro studies showed the passage of neutral and acidic milk oligosaccharides through intestinal epithelial cells.[13,42] 6′-Sialyllactose, disialyllactose, and *N*-acetylgalactosaminyllactose detected in the milk replacer were not found in serum samples. They are reported to be present in low abundance in cow's milk and, therefore, they were probably below the detection limit.[15] Another possibility refers to discrimination in oligosaccharide intestinal absorption or in early oligosaccharide fermentation in the small intestine, as suggested by investigation on the prebiotic fructo-oligosaccharides.[43−45]

Detection of Oligosaccharides in the Urine at Day 3 by CE-LIF and Mass Analysis. After confirmation of GOS presence in serum samples, it was investigated whether GOS were excreted in the urinary system of the piglets. Similarly to serum, urine samples of three piglets per feeding group were analyzed by CE-LIF. In Figure 6, CE-LIF electropherograms of oligosaccharides present in serum and urine from piglets fed 3 days on GOS diet (S-A1 and U-A1, respectively) are compared with urine from piglets fed 3 days on control diet (U-B1), GOS, and control diet. From the CE profiles, besides dimers including lactose and dietary 3′-sialyllactose (peak a, Figure 3), GOS structures were found in urine, which were comparable to the GOS structures in serum samples. In the CE-LIF urine profile, peaks assigned specifically to GOS were annotated with numbers (peaks 6−29, Figure 6). The CE-LIF outcome for GOS-DP3−DP4 was confirmed by liquid chromatography−mass spectrometry, in which mass fragmentation showed the GOS origin of oligosaccharides in piglet serum and urine (data not shown). The abundance of the detected GOS structures will be discussed in the next paragraph. To our knowledge, excretion of GOS structures in urine of GOS-fed animals has not been shown before. Nevertheless, the presence of dietary HMOs and prebiotic

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fructo-oligosaccharides was described for urine of infants and adults, respectively.[9,10,45,46] With regard to HMOs, both neutral (fucosyllactose, lacto-*N*-tetraose, lacto-*N*-fucopentaose, and lacto-difucosylpentaose) and acidic (3′- and 6′-SL and 3′- and 6′-sialyl-*N*-acetyllactosamine) dietary HMOs were found in urine samples of infants, indicating HMOs systemic absorption.[3,7,8]

Quantification of GOS and Other Oligosaccharides. *Concentration of Oligosaccharides in Serum and Urine at Day 3.* To investigate the absorption of oligosaccharides through the porcine intestinal system, quantification of GOS structures observed in serum and urine samples was conducted using CE-LIF. In Table 1, GOS ref, serum, and corresponding urine samples of three piglets fed a GOS diet (S-A1−A3 and U-A1−A3, respectively) and serum (S-A4) lacking the corresponding urine sample are reported. The total GOS concentrations in sera were 34.1, 5.3, 31.1, and 21.2 μg/mL (S-A1−A4, respectively). The GOS concentrations in urine were 0.9, 0.9, and 0.6 g/g creatinine (U-A1−A3, respectively), as shown in Table 1. The most abundant GOS structures referred to trimers (59.6−67.1 and 56.4−58.3% w/v in serum and in urine, respectively, Table 1), followed by tetramers (25.6−35.5 and 31.6−33.3%), pentamers (4.6−8.1 and 7.6−8.3%), and hexamer (1.8−2.5 and 2.0−2.7%). The concentration of GOS in serum of piglet A2 was lower when compared to other serum samples. This could be possibly explained by a different timing of sample collection or different eating behavior of this piglet. GOS concentration in the corresponding urine sample showed GOS concentration with the same order of magnitude as the other analyzed urine samples. Although the relative amounts of GOS DP structures observed in the biological samples could be related to GOS composition as present in the piglet diet, not all GOS structures as present in GOS ref were detected in piglet serum and urine. These observations can suggest absorption and/or early breakdown of specific GOS structures in the piglet small intestine. The literature describes in vitro passage of GOS through cell monolayer depending on GOS molecular size and structure, supporting our findings.[17] Nevertheless, non-digestible oligosaccharides are reported to be fermented already in the upper tract of piglet intestine, whereas in humans, metabolization of prebiotic fructo-oligosaccharides in the last part of the small intestine was described.[43−45]

Detection and Concentration of Oligosaccharides in Piglet Serum and Urine at 26 Days. To investigate the absorption and excretion of oligosaccharides in a more mature intestinal system, three serum and urine samples from piglets fed 26 days on GOS or control diet were analyzed. In Table 2, the presence, concentration (μg/mL), and relative proportion of GOS structures in serum and urine samples of piglets fed GOS diet for 26 days (S-B1−B3, U-B1−B3, respectively) detected by CE-LIF are shown. Piglet serum samples showed an average concentration of GOS of 16.1 μg/mL, whereas in urine 0.9 g GOS/g creatinine was detected, comparable with values at day 3. GOS-DP3 were present in larger proportion in serum and urine samples from day 26 compared to day 3, because on average 70.6% of GOS-DP3 on total GOS was detected. GOS-DP4−DP6 at day 26 were present in the same order of magnitude in serum and urine samples, confirming the trend of samples at day 3. Also at day 26, the presence of GOS-DP3−DP4 was confirmed by mass analysis (data not shown). Because no marker was included in the feeding trial, accurate quantification of GOS absorbed in the small intestine could not be finalized. However, taking the estimated feed intake per day into account, a very rough estimation of the GOS absorption could be obtained. Overall, the absorbed GOS in the circulation was estimated to be approximately 0.1% of the daily GOS intake, at both days 3 and 26 of the feeding trial.

Table 2. Presence, Concentration, and Relative Percentage of GOS Structures As Detected by CE-LIF in Serum and Urine at Day 26 of GOS Diet of Three Piglets

	piglet serum (26 days) concn (μg/mL)				piglet urine (26 days) concn (g/g creatinine)		
peak	GOS ref	S-B1	S-B2	S-B3	U-B1	U-B2	U-B3
6	14.7	0.9	0.2	0.9	0.03	0.02	0.05
7	49.1	6.0	3.1	3.5	0.22	0.31	0.35
8	41.3	3.8	3.7	3.4	0.15	0.34	0.32
9	18.4						
10	13.9						
11	25.1						
12	31.5	4.4	1.8	2.1			
concn GOS-DP3	193.9	15.1	8.9	9.9	0.4	0.7	0.7
rel %	60.9	67.7	71.7	72.3	60.1	67.9	65.9
13	4.6						
14	6.3	0.4	0.3	0.2	0.03	0.04	0.03
15	23.6	2.5	1.3	1.4	0.09	0.11	0.14
16	14.4	1.2	0.6	0.7	0.06	0.06	0.08
17	1.4						
18	8.5	0.6	0.3	0.2	0.02	0.01	0.01
19	6.7	0.4	0.2	0.2	0.01	0.02	0.02
20	4.2						
21	7.0	0.4	0.3	0.1	0.01	0.02	0.02
22	11.6						
concn GOS-DP4	88.4	5.6	3.1	3.0	0.2	0.3	0.3
rel %	27.8	25.2	25.0	21.8	31.9	26.0	27.6
23	4.4	0.5	0.2	0.2	0.02	0.02	0.02
24	11.4	0.8	0.2	0.5	0.03	0.03	0.04
25	4.8						
26	2.6						
27	3.9						
28	3.5						
concn GOS-DP5	30.7	1.3	0.4	0.6	0.04	0.1	0.1
rel %	9.6	5.8	3.3	4.5	6.8	5.1	5.3
29							
concn GOS-DP6	5.2	0.3		0.2	0.01	0.01	0.01
rel %	1.6	1.4		1.4	1.3	1.0	1.2
total GOS concn	318.2	22.3	12.3	13.7	0.7	1.0	1.1
av total GOS concn	16.1				0.9		

In this study, the presence of dietary oligosaccharides in piglet body fluids was proven using two different techniques. Specific oligosaccharides from GOS supplementation and milk replacer were detected in the serum and urine of piglets at days 3 and 26 of experimental feeding. It was therefore hypothesized that oligosaccharides of DP3−DP6 were absorbed by the piglet

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intestine, whereas early breakdown of specific dietary oligosaccharide structures in the small intestine could not be excluded.

Intestinal Fermentation of Dietary Oligosaccharides at Days 3 and 26. It has been demonstrated that the major part of GOS reaches the colon, where they are fermented by colonic microbiota.[15,23,26] The results in this study confirm this, as it was shown that only a small amount of GOS was absorbed systemically. Hence, it was investigated whether GOS could be still determined in the large intestine at days 3 and 26 of the piglet experimental feeding trial. Therefore, piglet fecal samples (at days 3 and 26) and cecal digesta samples (at day 26) were collected and analyzed. In Figure 7, CE-LIF profiles of

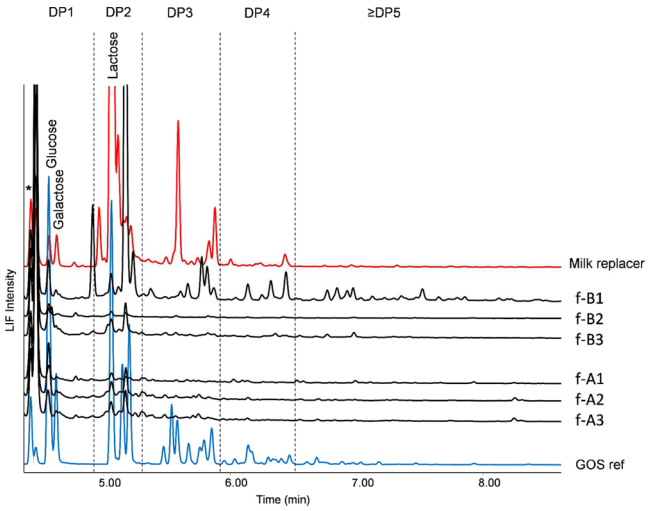

Figure 7. CE-LIF electropherograms of GOS ref, oligosaccharides from feces at day 3 of GOS or control diet (f-A1−A3 and f-B1−B3, respectively), and milk replacer. DP1−DP5, degree of polymerization based on GOS. The electropherograms are normalized on the internal standard xylose (∗).

oligosaccharides present in feces from piglets fed 3 days on GOS or control diet (f-A1−A3 and f-B1−B3, respectively) are shown. Overall, no intact dietary oligosaccharides were detected in fecal samples using CE-LIF and HILIC-MS^n. On the basis of CE-LIF analysis (Figure 7) low abundance of oligosaccharides was found in the fecal samples at both days 3 and 26. The few peaks detected were not overlapping with peaks as found in GOS ref (data not shown), possibly indicating an established and developed microbiota that could efficiently ferment GOS and other dietary oligosaccharides as shown in a previous study.[32] Alizadeh et al. reported a high bacterial load in fecal samples of piglets used in feeding trial described in this paper, already at the first day of piglet life.[32] However, one profile (f-B1, Figure 7) showed high oligosaccharide abundance and, thus, low fermentable capability. Analysis with HILIC-MS^n for this sample confirmed the presence of neutral hexose oligosaccharides ($3 \leq DP \geq 5$) hypothesized to be fermentation products of oligosaccharides present in the milk replacer. As reported for in vitro GOS fermentation by human fecal inocula, the high abundance of oligosaccharides could be explained by a difference in microbiota composition, accumulating oligosaccharides structures.[47] For all samples, efficiency regarding oligosaccharide fermentation and accumulation in the fecal and cecum digesta samples was evaluated. The amount of oligosaccharides per gram of fecal slurries or cecal content was estimated, assuming the presence of exclusively neutral oligosaccharides. The concentrations of oligosaccharides were 15 and 11 mg/g fecal slurry for piglets fed 3 days on GOS or control diet, respectively. For samples obtained at day 26, oligosaccharide levels of 16 and 9 mg/g cecal content and 12 and 7 mg/g fecal slurry were found for GOS and control diet, respectively. In both fecal and cecum digesta samples, trisaccharides were the most abundant structures. Due to the low abundance of the oligosaccharides, it was not possible to determine whether they were originated from milk replacer or GOS, using HILIC-MS^n.

In conclusion, oligosaccharides, such as GOS and 3′-sialyllactose, were found in the serum and urine of piglets fed 3 and 26 days on milk formula enriched with GOS. Not all GOS as present in the diet were detected in blood and urine samples, suggesting absorption and/or consumption of specific GOS in piglet small intestine. As expected from human milk oligosaccharide behavior, GOS were estimated to be absorbed in small quantities (about 0.1% of GOS daily intake), at both days 3 and 26 of the feeding trial. Subsequently, GOS was found to be excreted via the urinary system, with GOS-DPs relative abundance in urine samples comparable with serum samples. Moreover, in piglet cecum digesta and feces, low levels of oligosaccharides were detected, suggesting extensive intestinal GOS fermentation. The discovery of GOS and dietary oligosaccharides in the blood and urine of piglets now promotes further evaluation of the systemic role of oligosaccharides in humans and animals.

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