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Oligosaccharides in feces of breast- and formula-fed babies

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ABSTRACT

So far, little is known on the fate of oligosaccharides in the colon of breast- and formula-fed babies. Using capillary electrophoresis with laser induced fluorescence detector coupled to a mass spectrometer (CE–LIF–MS^n), we studied the fecal oligosaccharide profiles of 27 two-month-old breast-, formula- and mixed-fed preterm babies. The interpretation of the complex oligosaccharide profiles was facilitated by beforehand clustering the CE–LIF data points by agglomerative hierarchical clustering (AHC). In the feces of breast-fed babies, characteristic human milk oligosaccharide (HMO) profiles, showing genetic fingerprints known for human milk of secretors and non-secretors, were recognized. Alternatively, advanced degradation and bioconversion of HMOs, resulting in an accumulation of acidic HMOs or HMO bioconversion products was observed. Independent of the prebiotic supplementation of the formula with galactooligosaccharides (GOS) at the level used, similar oligosaccharide profiles of low peak abundance were obtained for formula-fed babies. Feeding influences the presence of diet-related oligosaccharides in baby feces and gastrointestinal adaptation plays an important role herein. Four fecal oligosaccharides, characterized as HexNAc-Hex-Hex, Hex-[Fuc]-HexNAc-Hex, HexNAc-[Fuc]-Hex-Hex and HexNAc-[Fuc]-Hex-HexNAc-Hex-Hex, highlighted an active gastrointestinal metabolization of the feeding-related oligosaccharides. Their presence was linked to the gastrointestinal mucus layer and the blood-group determinant oligosaccharides therein, which are characteristic for the host's genotype.

1. Introduction

Dietary prebiotic carbohydrates, such as human milk oligosaccharides (HMOs) and galactooligosaccharides (GOS), have a considerable impact on the neonatal gastrointestinal development. They influence the intestinal microbial colonization, which is responsible for the absorption and metabolisation of food ingredients, the establishment of the immune system, act as receptor analogs for pathogenic bacteria and prevent constipation.[1–6] HMOs are a mixture of structurally complex, lactose-based oligosaccharides, composed of galactose (Gal), glucose (Glc), *N*-acetyl-glucosamine (GlcNAc) and frequently decorated by fucose (Fuc) and sialic acid (Neu5Ac).[7] HMOs are present in human milk to an extent of 3–19 g/L.[7,8] The qualitative and quantitative composition of HMOs in breast milk is individual-dependent. Human milk can be classified according to the presence and absence of α-(1→3/4)- and α-(1→2)-fucosylated oligosaccharides, which indicate the activity of the Lewis (Le)- and Secretor (Se)-gene of the mother, with the latter being responsible for the expression of α-(1→2)-fucosyltransferase FUT2.[5,8,9] Due to the complexity and individual-dependent profile of HMOs it has not been possible to reproduce HMOs for the use as prebiotic supplements of breast-milk replacers so far. Prebiotic GOS are structurally similar to HMOs, however, less complex. They are high in galactose content and carry a lactose unit at the reducing end. GOS are frequently added to infant formulas for term and preterm babies.[10] Especially preterm infants (born after a gestational age of 24–36 weeks) are vulnerable for infections due to their impaired immunity.[11] This results in frequent antibiotic use and this is one of the factors, which can distort the intestinal flora. Although human milk is the preferred feeding to these infants, human milk is not always available and substitution with preterm formula is important,[12] for example, in order to establish a balanced microflora.

Abbreviations: AHC, agglomerative hierarchical clustering; APTS, 9-aminopyrene-1,4,6-trisulfonate; CE–LIF–MS^n, capillary electrophoresis–laser induced fluorescence detection–mass spectrometry; DF-L, difucosyllactose; DF-LNH, difucosyllacto-N-hexaose; DS-LNT, disialyllacto-N-tetraose; FL, fucosyllactose; F-LNH, fucosyllacto-N-hexaose; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamin; Glc, glucose; GlcNAc, *N*-acetylglucosamin; Hex, hexose; HMO, human milk oligosaccharides; HPAEC, high performance anion exchange chromatography; LNDFH, lacto-N-difucosylhexaose; LNFP, lacto-N-fucopentaose; LNH, lacto-N-hexaose; LNT, lacto-N-tetraose; LnNT, lacto-N-neo-tetraose; Neu5Ac, sialic acid; SL, sialyllactose; S-LNT, sialyllacto-N-tetraose; TFA, trifluoroacetic acid; TF-LNH, trifucosyllacto-N-hexaose.

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Several studies on the intestinal flora composition of term and preterm born babies have been performed in order to evaluate the value of HMOs in breast milk and prebiotic oligosaccharides in infant formula.[13,14] However, little is known on the gastrointestinal fate of the individual oligosaccharide structures. Diet-related oligosaccharides can be traced back in baby feces, but they have rarely been studied in detail.[14–16] A more profound knowledge of the gastrointestinal fate of oligosaccharides would be helpful for the understanding of the structure–function relation of feeding-related oligosaccharides.

We recently introduced the hyphenation of capillary electrophoresis with laser-induced fluorescence detection to a mass spectrometer capable to perform multiple MS analysis (CE–LIF–MS^n) for the separation and characterization of HMOs in breast milk and feces of breast-fed babies.[17]

In the present study we applied CE–LIF–MS^n for a broad-range screening of oligosaccharides present in feces of breast-, formula- and mixed-fed preterm babies. The formula was either fortified with prebiotic galactooligosaccharides (GOS; 0.4 g/dL) or did not contain any prebiotic oligosaccharides. Agglomerative hierarchical clustering (AHC) was performed on the CE–LIF data points in order to observe general trends. Subsequently, CE–LIF–MS^n oligosaccharide profiles were described in order to study the individual-dependent and genetically determined gastrointestinal degradation of feeding-related carbohydrates. MS^n analysis was applied for the identification of unknown oligosaccharides.

2. Results

Twenty-seven fecal samples from preterm babies were analyzed. Thirteen babies received a feeding exclusively or predominantly composed of breast milk, with breast milk contributing to >60% of the total feeding, which the baby received during the study period. The other 14 babies received a feeding exclusively or predominantly composed of preterm infant formula (Frisolac prematuur), supplemented with 0.4 g/dL GOS or the same formula without GOS supplementation. The infant formula contributed to >45% to the total feeding (Table 1).

2.1. Sugar composition of the fecal extracts

For a first screening, the HMO-constituting sugars [fucose (Fuc), galactose (Gal), glucose (Glc), *N*-acetylglucosamine (GlcNAc), sialic acid (Neu5Ac)] and *N*-acetylgalactosamine (GalNAc) and mannose (Man), which are known to be supplementary present in the glycoconjugates of the human gastrointestinal mucosa,[7,18] were determined by high performance anion exchange chromatography (HPAEC) after acid hydrolysis of the fecal samples. The molar sugar proportions, are presented relative to Gal = 1 in Table 2, grouped according to the main feeding the babies received. Similar molar proportions were obtained for samples analyzed in duplicate but highly diverse proportions were found for the different samples. Nevertheless, mean values were calculated. The high standard deviations indicated variations in the fecal sugar compositions despite the similar feeding received. Overall, the sugar proportions found for the predominantly breast-fed babies (group A) can be described as Fuc, GlcNAc > Glc ≫ GalNAc, Man, Neu5Ac, whereas for predominantly formula-fed babies (group B) it was GlcNAc > Fuc > Glc, GalNAc > Man, Neu5Ac. The mean proportions of GlcNAc, GalNAc, Man and Neu5Ac for formula-fed babies were higher than for breast-fed babies. Some sub-groups could be observed within the feeding groups. The predominantly breast-fed babies A4–A10 showed similar fecal relative proportions of Fuc, Glc and GlcNAc, but sub-divisions of the sample sub-group were indicated by the different GalNAc-, Man- and Neu5Ac-proportions, which were low or even zero for samples A4, A9 and A10. Samples A1–A3 and A11–A13 showed highly diverse sugar proportions. The proportions of GalNAc, found for these specific breast-fed babies, were more comparable to what was found for formula-fed babies. The predominantly formula-fed babies B1–B2, who got GOS-supplemented formula, and B13–B14 could be distinguished within the formula-fed group due to the lowered proportions of all or several of the sugars Fuc, Glc, GlcNAc, GalNAc and Neu5Ac and may indicate an increased relative galactose content.

Table 1
Feeding groups, blood groups of babies and sample codes for fecal samples used in this study and examined by AHC and CE–LIF–MS

Composition of feeding (% of feeding to total feeding)	*n* (babies)	AHC codes (Fig. 1) (blood group indicated in brackets)
Breast-milk (100%)	5	A1(0), A4(A), A9(A), A11(A), A13(B)
Breast milk (>80%) and formula	6	A2(nd), A3(0), A5(0), A8(0), A10(A), A12(0)
Breast milk (>60%) and formula	2	A6*(A), A7(A)
Formula (>45%) and breast milk	8	B5(A), B6(A), B7(A), B8(A), B10(A), B12*(B), B13(A), B14(AB)
Formula (100%)	6	B1*(A), B2*(0), B3(B), B4(0), B9(A), B11(0)

nd: not defined.
* Formula supplemented with 0.4 g/dL GOS.

2.2. Agglomerative hierarchical clustering (AHC) of CE–LIF oligosaccharide profiles

Sugar composition analysis indicated variations within feeding groups and the possible presence of sub-groups with high similarities. Lower standard deviations were obtained by averaging the sugar proportions obtained for A5–A7, A9–A10 and B3–B12 (Table 2), compared to what was obtained for the total feeding group. These sample-groups thus represent sub-groups. The sugar building units, as determined in 2.1, can represent numerous, structurally different oligosaccharides. The fecal extracts were, therefore, analyzed for their oligosaccharide profiles by CE–LIF. AHC was then performed on the data points for all CE–LIF electropherograms in order to investigate the similarity between the samples. Two main clusters and one sample (A1), which did not match with any of the two clusters, are obtained (Fig. 1). The first cluster was composed of samples from exclusively and predominantly breast-fed babies (A2–A10). Samples A2 and A3 showed low similarity to samples A4–A10, which could be further divided into the sub-clusters A4/A5–A7 and A8/A9–A10. The second cluster was mainly composed of samples from exclusively and predominantly formula-fed babies. Three breast-fed babies (samples A11–A13) were also located in the second cluster. Several sub-clusters were observed for feces from the formula-fed group, with the most homogenous cluster being composed of samples B4–B11. In line with the exceptional sugar proportions observed for babies A1–A3, A11–A13, B1–B2 and B13–B14, low similarities to the other samples of the same feeding group were found with AHC for these samples as based on the CE–LIF profiles.

2.3. Characterization of the CE–LIF oligosaccharide profiles

2.3.1. Breast-fed babies showing fecal oligosaccharide profiles similar to breast milk

Fucosyl-lactose (FL), difucosyl-lactose (DF-L), lacto-N-neotetraose(LnNT)-isomers, lacto-N-tetraose (LNT), lacto-N-fucopentaose (LNFP)-isomers, lacto-N-difuco-hexaose(LNDFH)-isomers, fucosyl-lacto-N-hexaose(F-LNH)-isomers, di-fucosyl-lacto-N-hexaose(DF-LNH)-isomers and tri-fucosyl-lacto-N-hexaose(TF-LNH)-isomers

Table 2
Sugar composition of fecal samples, presented relative to gal = 1 and ordered according to the feeding groups of babies

	Fuc	Gal	Glc	GlcNAc	GalNAc	Man	Neu5Ac
Exclusively or predominantly breast-fed babies (A)							
A1	0.09	1.00	0.34	0.78	0.19	0.09	0.21
A2	1.07	1.00	0.32	0.84	0.23	0.00	0.07
A3	0.56	1.00	0.51	0.50	0.04	0.08	0.08
A4	0.97	1.00	0.69	0.69	0.00	0.00	0.01
A5	0.82	1.00	0.51	0.58	0.05	0.04	0.01
A6	0.81	1.00	0.43	0.63	0.13	0.05	0.03
A7	0.85	1.00	0.42	0.57	0.09	0.05	0.03
A8	0.72	1.00	0.38	0.70	0.10	0.05	0.10
A9	0.90	1.00	0.55	0.53	0.03	0.00	0.01
A10	0.86	1.00	0.51	0.51	0.05	0.00	0.05
A11	0.55	1.00	0.65	0.85	0.58	0.19	0.00
A12	0.63	1.00	0.32	0.83	0.36	0.23	0.18
A13	0.44	1.00	0.42	1.21	0.20	0.08	0.07
mean ± STDEV	0.71 ± 0.25	1.00	0.46 ± 0.11	0.71 ± 0.19	0.16 ± 0.15	0.07 ± 0.07	0.06 ± 0.06
Exclusively or predominantly formula-fed babies (B)							
B1	0.41	1.00	0.34	0.60	0.19	0.48	0.08
B2	0.43	1.00	0.71	0.80	0.15	0.13	0.14
B3	0.50	1.00	0.57	0.73	0.28	0.17	0.10
B4	0.43	1.00	0.71	1.44	0.35	0.37	0.26
B5	0.66	1.00	0.11	1.05	0.20	0.13	0.07
B6	0.57	1.00	0.12	0.97	0.35	0.19	0.09
B7	0.75	1.00	0.23	0.96	0.50	0.06	0.16
B8	0.56	1.00	0.26	0.97	0.23	0.05	0.12
B9	0.75	1.00	0.27	1.11	0.31	0.08	0.10
B10	0.58	1.00	0.20	1.09	0.22	0.07	0.08
B11	0.48	1.00	0.84	1.16	0.28	0.18	0.25
B12	0.67	1.00	0.30	1.02	0.30	0.11	0.14
B13	0.29	1.00	0.30	0.46	0.21	0.14	0.16
B14	0.33	1.00	0.24	0.85	0.32	0.25	0.00
mean ± STDEV	0.53 ± 0.14	1.00	0.37 ± 0.23	0.94 ± 0.24	0.28 ± 0.09	0.17 ± 0.12	0.12 ± 0.06
A5–A7							
mean ± STDEV	0.83 ± 0.01	1.00	0.45 ± 0.04	0.59 ± 0.02	0.09 ± 0.03	0.05 ± 0.01	0.02 ± 0.01
A9–A10							
mean ± STDEV	0.88 ± 0.02	1.00	0.53 ± 0.02	0.52 ± 0.01	0.04 ± 0.01	0.00 ± 0.00	0.03 ± 0.02
B3–B12							
mean ± STDEV	0.58 ± 0.11	1.00	0.37 ± 0.23	1.07 ± 0.17	0.29 ± 0.08	0.14 ± 0.09	0.13 ± 0.06

Dashed lines indicate sample groups for which similarities in sugar composition were observed. Mean values and their standard deviations (mean ± STDEV) are presented for the feeding groups. mean ± STDEV for A5–A7, A9–A10 and B3–B12 indicate similar sample-sub-groups.

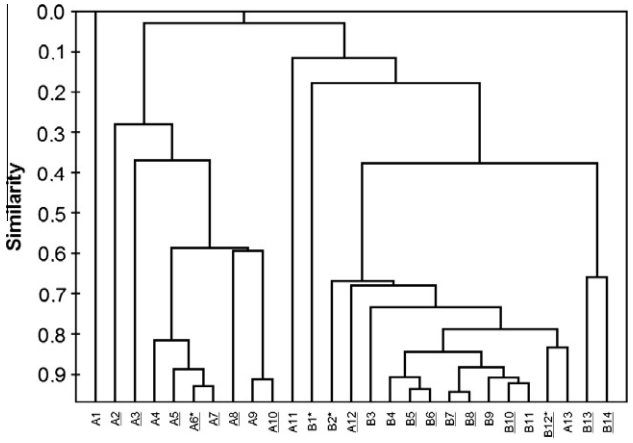

Figure 1. AHC dendrogram of CE–LIF datasets from the 27 fecal samples analyzed. A: Exclusively breast-fed babies. A: Predominantly breast-fed babies. B: Exclusively formula-fed babies. B: Predominantly formula-fed babies. *: Supplementation of formula with 0.4 g/dL GOS. See Table 1 for the assignment of the sample codes to the respective feeding group.

are HMOs and their degradation products, which were found to be present in individual-dependent proportions in the feces of two exclusively and five predominantly breast-fed babies (A4–A10), corresponding to what was previously reported.[17] Representative electropherograms for these sub-groups are shown in Figure 2. Peaks, which have been annotated by CE–LIF–MS^n and/or using standards are listed in Table 3. The oligosaccharide profiles could be discriminated according to their respective secretor-(A4–A8) or non-secretor-(A9, A10) fingerprint. The α-(1→2)-fucosylated HMOs, such as DF-L (α-Fuc-(1→2)-β-Gal-(1→4)-[α-Fuc-(1→3)]-Glc; peak 7), LNFP-I (α-Fuc-(1→2)-β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc; peak 18) or LNDFH-I (α-Fuc-(1→2)-β-Gal-(1→3)-[α-Fuc-(1→4)]-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc; peak 20) are characteristic for Le(a−b+)[9]-secretor-milk and were present in the fecal oligosaccharide profiles A4–A8. Two representative examples (A4, A7) are shown in Figure 2. Besides the well-known HMO-peaks and their degradation products[17] (Fig. 2-A4) also an unknown structure of *m/z* 328 (Fig. 2-A7, peak6) and two unknown structures of *m/z* 377 (Fig. 2-A7, peaks 12/13) could be present in the secretor-profiles of breast-fed babies, as exemplified by sample A7 in Figure 2. Strikingly, *m/z* 328 and *m/z* 377-I were never present in the HMO-profiles from exclusively breast-fed babies, but in some cases present in the profiles of mixed-fed babies.

Secretor-milk-specific HMOs containing α-(1→2)-fucose residues were absent in samples A9 and A10. This phenomenon is typical for non-secretor milk Le(a+b−).[5,9] CE–MS analysis indicated isomeric structures for F-LNH and DF-LNH in

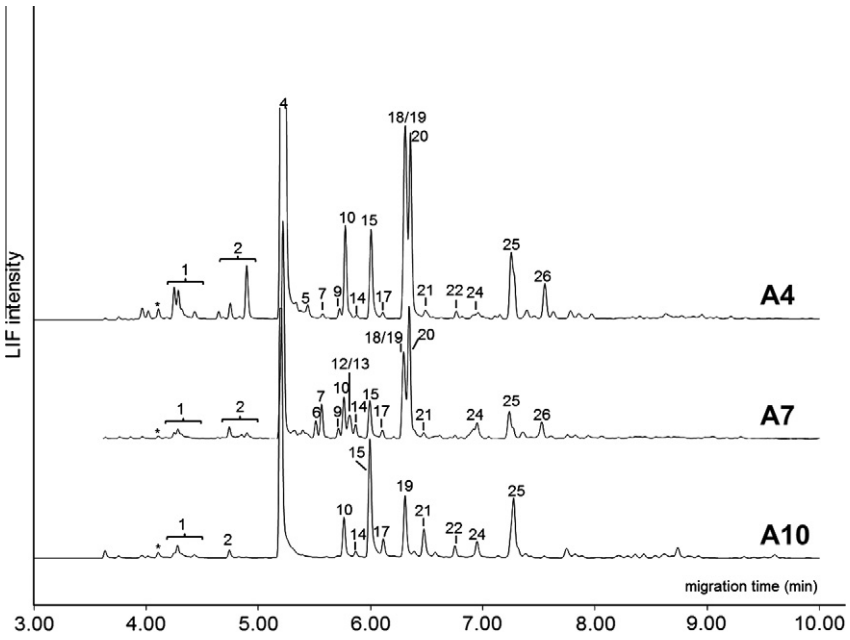

Figure 2. CE–LIF electropherograms of APTS-derivatized oligosaccharides extracted from baby feces. (A4) HMO secretor profile of exclusively breast-fed baby A4. (A7) HMO secretor profile including unknown oligosaccharides of mixed-fed baby A7. (A10) HMO non-secretor profile of mixed-fed baby A10. See Table 3 for peak annotation. * Internal standard xylose.

secretor-profiles, visible as shoulders (Fig. 2, peaks 24/25) which thus may contain α-(1→2)-fucose linkages. Only single-shaped peaks were observed for F-LNH and DF-LNH in these non-secretor profiles, as exemplified by sample A10 in Figure 2. On the other hand, LNFP-II and two not further specified LNDFH isomers contributed with a higher proportion to the total oligosaccharide content in non-secretor profiles (Fig. 2-A10, peaks 15/21/22).

2.3.2. Breast-fed babies showing fecal oligosaccharide profiles not similar to breast milk

For three exclusively and three partially breast-fed babies (samples A1–A3, A11–A13), which stood out in the clustering profile due to their low similarity, fecal oligosaccharide profiles were found, that did not depict a characteristic HMO pattern with CE–LIF (Fig. 3). Exclusively acidic HMOs predominated in sample A1 (Fig. 3-A1). In human milk, acidic HMOs are present in a 10-fold lower concentration compared to neutral oligosaccharides.[7] SL, three sialyl-lacto-N-tetraose(S-LNT)-isomers (Fig. 3-A1, peak 8/11/17) and one disialyl-lacto-N-tetraose(DS-LNT)-isomer (Fig. 3-A1, peak 16) were found in this oligosaccharide profile by CE–LIF–MS analysis. Their origin from breast milk was confirmed by their presence in the acidic fraction obtained by anion exchange chromatography of HMOs (results not shown). Remarkably, in sample A12 solely SL was found (results not shown).

Only a few, but dominant HMOs were found for A2 and A3, which were LNFP II and LNDFH I as shown for A2 (Fig. 3-A2, peak 15/20). An unknown structure of *m/z* 377 was present as well (Fig. 3-A2, peak 12), which corresponded to peak 12 in Figure 2-A7, assigned as 377-I. No oligosaccharides were found to be present in the profile of sample A13 (results not shown).

Table 3
Structural composition of oligosaccharides detected in CE–LIF electropherograms (Fig. 2/Fig. 3/Fig. 4)

Peaks (Fig. 2/Fig. 3/Fig. 4)	*m/z* (APTS 3-)	Hexose	GlcNAc	Fucose	Neu5Ac	Name (abbreviation)	Peaks (Fig. 2/Fig. 3/Fig. 4)	*m/z* (APTS 3-)	Hexose	GlcNAc	Fucose	Neu5Ac	Name (abbreviation)
*						Xylose ISTD	15	431	3	1	1	0	LNFP II
1						Monomers	16	576	3	1	0	2	DS-LNT
2						Dimers	17	479	3	1	0	1	S-LNT*
3	357	2	0	0	1	SL*	18	431	3	1	1	0	LNFP I
4	309	2	0	1	0	FL*	19	431	3	1	1	0	LNFP III
5	382	3	1	0	0	LnNT Y	20	479	3	1	2	0	LNDFH I
6	328	2	1′	0	0	*m/z* 328	21	479	3	1	2	0	LNDFH*
7	358	2	0	2	0	DF-L	22	479	3	1	2	0	LNDFH*
8	479	3	1	0	1	S-LNT*	23	498	3	2	1′	0	*m/z* 498
9	382	3	1	0	0	LnNT	24	552	4	2	1	0	F-LNH*
10	431	3	1	1	0	LNFP Y	25	601	4	2	2	0	DF-LNH*
11	479	3	1	0	1	S-LNT*	26	650	4	2	3	0	TF-LNH*
12	377	2	1‘	1	0	*m/z* 377-I	III[a–d]	314	3	0	0	0	Hexo-trioses*
13	377	2	1‘	1	0	*m/z* 377-II	IV[a–c]	368	4	0	0	0	Hexo-tetraoses*
14	382	3	1	0	0	LNT							

The oligosaccharides are attached to an APTS-molecule and are present in their threefold negative charge in the ESI-MS profiles.
*: Isomers not further specified; ′: GlcNAc = HexNAc.

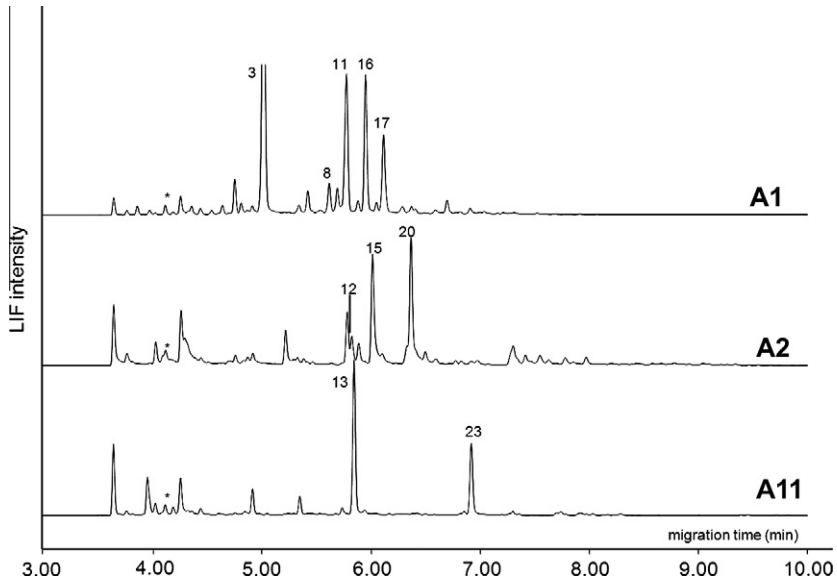

Figure 3. CE–LIF electropherograms of APTS-derivatized oligosaccharides extracted from baby feces. (A1) Exclusively acidic HMOs of exclusively breast-fed baby A1. (A2) Accumulation of LNFP-II/LNDFH-I of mixed-fed baby A2. (A11) Accumulation of structures with *m/z* 377-II/498 of exclusively breast-fed baby A11. See Table 3 for peak annotation. * Internal standard xylose.

Interestingly, an oligosaccharide, previously assigned as 377-II in the fecal profiles of breast-fed babies showing secretor-HMO-profiles (Fig. 2-A7), was found to prevail in the profile of the breast-fed baby A11 (Fig. 3-A11, peak 13), together with a structure of *m/z* 498 (Fig. 3, A11, peak 23). Besides the presence of *m/z* 377-II and *m/z* 498, the oligosaccharide level found for sample A11 was quite low.

2.3.3. Fecal oligosaccharide profiles of formula-fed babies

Similar fecal oligosaccharide profiles were obtained for 10 formula-fed or predominantly formula-fed babies (B2, B4–B12), irrespective of having received formula-feeding supplemented with 0.4 g/dL GOS. Representative electropherograms are shown in Figure 4 for GOS-fed baby B12 and formula-fed baby B5. By means of CE–MS, the two main oligosaccharide peak-clusters were identified as hexose trimers and tetramers (Fig. 4, peak III^{a-d} and IV^{a-c}, respectively). The oligosaccharide profiles of samples B1, B3, B13 and B14 could be distinguished from the other peak profiles of formula-fed babies due to the abundant presence of an unknown structure of *m/z* 328, as represented by baby B3, (Fig. 4-B3, peak 6), which was as well present as a minor component in the oligosaccharide profiles of breast-fed babies showing a secretor-profile (Fig. 2-A7, peak 6).

2.3.4. Annotation of the unknown peaks *m/z* 377-I/-II, 328 and 498

As described above, four unknown compounds of the masses *m/z* 328, *m/z* 377 (isomers I and II) and *m/z* 498 were observed in

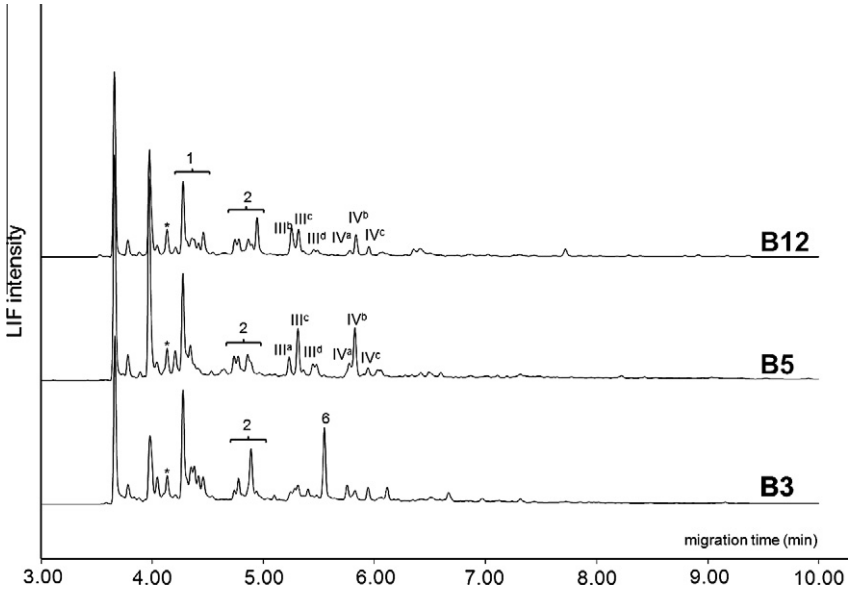

Figure 4. CE–LIF electropherograms of APTS-derivatized oligosaccharides extracted from baby feces. (B12) Mixed-fed baby B12 (GOS-supplemented formula). (B5) Mixed-fed baby B5 (control formula). (B3) Exclusively formula-fed baby B3 (control) showing abundant oligosaccharide of *m/z* 328. See Table 3 for peak annotation. * Internal standard xylose.

several fecal extracts. Although having the same *m/z* values and migrating closely together in CE–LIF, the MS fragmentation patterns of *m/z* 377-I (found for several partially breast-fed babies (Fig. 2/Fig. 3, peak 12)) and *m/z* 377-II (found for several exclusively and partially breast-fed babies (Fig. 2/Fig. 3, peak 13)) showed marked differences (Fig. 5A).

The compound *m/z* 377-I was annotated as Hex-[Fuc]-HexNAc-Hex-APTS (Fig. 5A), as supported by its mass fragments Hex-APTS, HexNAc-Hex-APTS, Fuc-HexNAc-Hex-APTS and Hex-HexNAc-Hex-APTS (Table 4).

For compound 377-II, the presence of the fragments Hex-APTS, Hex-Hex-APTS, Fuc-Hex-Hex-APTS and HexNAc-Hex-Hex-APTS, as summarized in Table 4, led to an annotation of HexNAc-[Fuc]-Hex-Hex-APTS (Fig. 5A).

The fragmentation pattern of *m/z* 328 (found as minor component for several partially breast-fed babies and as dominant peak for several formula-fed babies (Fig. 2/Fig. 4, peak 6)) lacked the fragment Hex-APTS, although showing an abundant Hex-Hex-APTS fragment (Fig. 5B, Table 4). This characteristic fragmentation was also found for a mixture of the single-substituted, Hex-Hex-based structures 3′SL (α-Neu5Ac-(2→3)-β-Gal-(1→4)-Glc) and 6′SL (α-Neu5Ac-(2→6)-β-Gal(1→4)-Glc) (data not shown). HexNAc-Hex-Hex-APTS was thus annotated for *m/z* 328.

The MS fragments, which resulted for *m/z* 498 (Fig. 5B) (breast-fed baby A11 (Fig. 3-A11, peak23)) are summarized in Table 4. The presence of fragments Fuc-Hex-HexNAc-Hex-Hex-APTS and HexNAc-Hex-HexNAc-Hex-Hex-APTS provide complementary information on the substitution of the terminal hexose unit with Fuc as well as HexNAc. This led to the annotation of HexNAc-[Fuc]-Hex-HexNAc-Hex-Hex-APTS for *m/z* 498. Unfortunately, the definitive identification of the new structures by NMR was not possible due to the low sample amounts available.

3. Discussion

3.1. Breast-fed babies

Three out of the five exclusively breast-fed babies showed the absence of a characteristic HMO profile after approximately two months of life. On the other hand, for the majority of predominantly, but not exclusively, breast-fed babies (five out of eight), fecal HMO-profiles were obtained. The individual-dependent, genetically determined HMO-profiles of secretor- [Le(a−b+)]- and non-secretor- [Le(a+b−)] breast milk[5] were mirrored in these fecal HMO-profiles and resulted in different degrees of similarities among the samples.

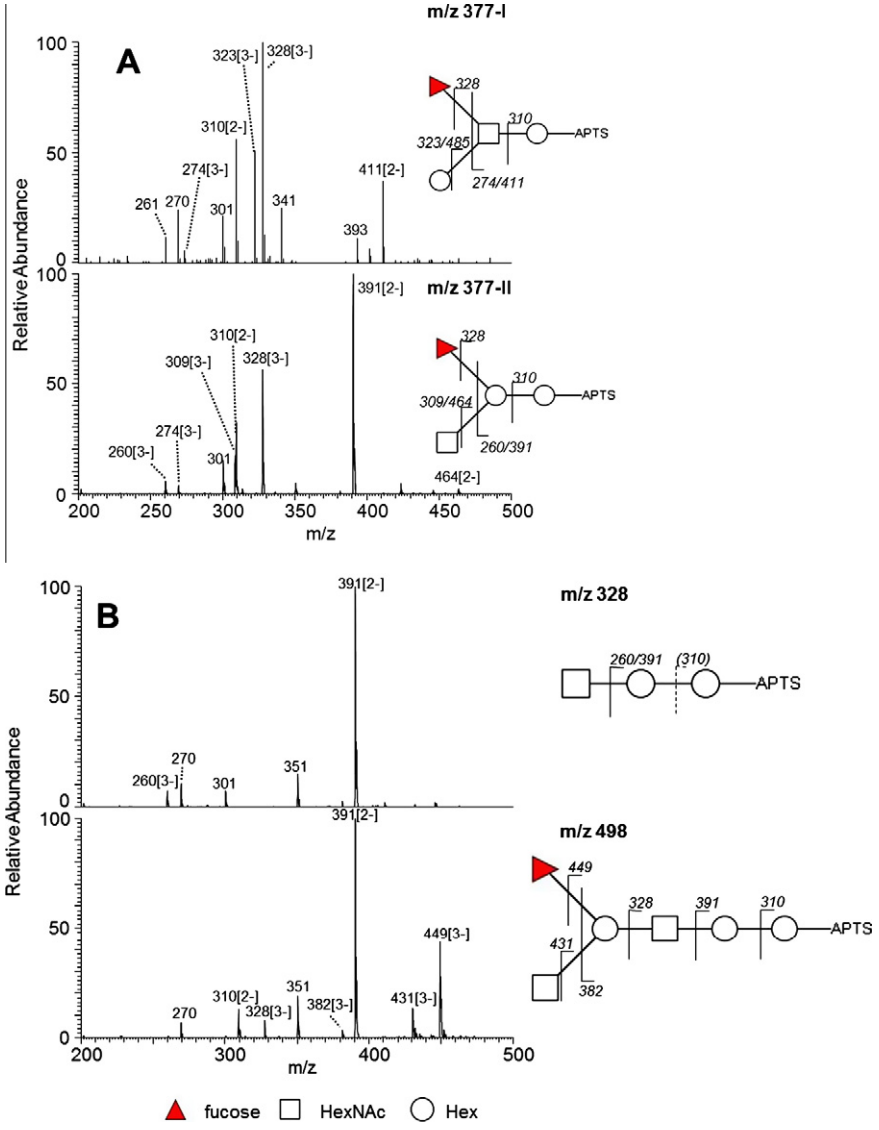

Figure 5. MS^2 fragmentation patterns and structural composition of (A) *m/z* 377-I/-II. (B) *m/z* 328 and *m/z* 498. [2−] [3−]: charge state of APTS-molecule.

Table 4
Presence and annotation of MS^2 fragments of *m/z* 328 (Table 3, peak 6), 377-I/II (Table 3, peaks 12/13) and 498 (Table 3, peak 23)

		m/z 328	*m/z* 377-I	*m/z* 377-II	*m/z* 498
m/z Fragment pair	Structural composition				
m/z 207[3−]/310[2−]	Hex-APTS	–	+	+	+
m/z 255[3−]/383[2−]	Fuc-Hex-APTS	–	–	–	–
m/z 260[3−]/391[2−]	Hex-Hex-APTS	+	–	+	+
m/z 274[3−]/411[2−]	HexNAc-Hex-APTS	–	+	–	–
m/z 309[3−]/464[2−]	Fuc-Hex-Hex-APTS	–	–	+	–
m/z 323[3−]/485[2−]	Fuc-HexNAc-Hex-APTS	–	+	–	–
m/z 328[3−]/492[2−]	HexNAc-Hex-Hex-APTS/Hex-HexNAc-Hex-APTS	–	+	+	+
m/z 382[3−]/574[2−]	Hex-HexNAc-Hex-Hex-APTS	–	–	–	+
m/z 431[3−]/647[2−]	Fuc-Hex-HexNAc-Hex-Hex-APTS	–	–	–	+
m/z 449[3−]/674[2−]	HexNAc-Hex-HexNAc-Hex-Hex-APTS	–	–	–	+

Mixed feeding can thus delay an advanced HMO-degradation and metabolization at two months after birth. A lower adaptation to the feeding and a microbiota not exclusively specified in the degradation and metabolization of HMOs for these babies was indicated.

The presence of exclusively acidic oligosaccharides, such as SL, S-LNT, DS-LNT-isomers, as observed in the feces of the breast-fed baby A1 (Fig. 3-A1), the accumulation of specific HMOs (Fig. 3-A2) or unknown structures (Fig. 3-A11) and the absence of specific oligosaccharides (sample A13) can thus be interpreted as biomarkers for an advanced gastrointestinal and gastromicrobial development and explain the diversity found for these samples with CE–LIF, expressed by their low similarities in AHC. The increased presence of GlcNAc and the non-HMO sugars GalNAc and Man, which are known to be present in the glycoconjugates of the human gastrointestinal mucosa,[18] may indicate an involvement of mucin oligosaccharides in the gastrointestinal re-design of diet-related HMOs. To understand the gastrointestinal bioconversion of HMOs, more study is needed.

3.2. Blood group A-specific metabolization products in the feces of breast-fed babies

Breast-fed babies, for whom HexNAc-[Fuc]-Hex-Hex-APTS (*m/z* 377-II) and HexNAc-[Fuc]-Hex-HexNAc-Hex-Hex-APTS (*m/z* 498) were annotated in their fecal oligosaccharide profiles, all had blood group A (Table 1), as was exemplified by babies A7 and A11 in Figure 2/Figure 3. α-GalNAc-(1→3)-[α-Fuc-(1→2)]-Gal is the antigenic determinant structure of blood group A,[19] which corresponds to the non-reducing HexNAc-[Fuc]-Hex-group found for *m/z* 377-II and *m/z* 498 and is further supported by the increased presence of the non-HMO-sugar GalNAc in the respective samples. The oligosaccharides α-GalNAc-(1→3)-[α-Fuc-(1→2)]-β-Gal(1→4)-Glc (corresponding to *m/z* 377-II) and α-GalNAc-(1→3)-[α-Fuc-(1→2)]-β-Gal(1→3)-β-GlcNAc(1→3)-β-Gal-(1→4)-Glc (corresponding to *m/z* 498) were previously identified in the feces of a single, eight weeks old, prematurely born breast-fed blood group A-secretor baby.[15,16] Blood group characteristic oligosaccharides are not expected to occur in human milk.[15,20] However, the occurrence of trace amounts has previously been suggested but the structural composition of these oligosaccharides has not been proven.[21] The gastrointestinal mucosa is known to carry a broad range of cell-surface glycoconjugates, which are built up from Gal, Glc, GalNAc, GlcNAc, Man, Fuc and Neu5Ac.[18] The antigenic determinant structures of these glycoconjugates are, among other factors, determined by the host's ABO histo-blood group type.[5] A sole microbial degradation of these structures from mucus epitopes[22,23] was not expected. Our results indicate a gastrointestinal bioconversion of the HMOs, with an involvement of these blood group specific mucus epitopes. This is similar to what has been hypothesized in previous studies in view of the high yield of these fecal oligosaccharides detected in the respective sample.[15,16] Our assumption of bioconversion is further supported by the fact that neither *m/z* 377-II nor *m/z* 498 was detected in the feces of any of the formula-fed babies.

Blood-group specific oligosaccharides can be absent in the feces of breast-fed blood group A secretor babies (Fig. 2-A4), they can be present additionally to HMOs (Fig. 2-A7, peak 13) or they can be present as exclusive components (Fig. 3-A11, peaks 13/23). Blood-group A non-secretor babies (A9 and A10; Fig. 2-A10) lacked the presence of theses oligosaccharides, due to the presence of a terminal α-(1→2)-Fuc-linkage for these structures.[15,24] The extent to which blood-group specific oligosaccharides are present in feces depends on the secretor status of the baby and may depend on the presence of secretor-breast milk and on the state of development and adaption of the gastrointestinal microbiota to the HMOs provided.

3.3. Presence of unknown oligosaccharide *m/z* 377-I in the feces of breast-fed babies

Hex-[Fuc]-HexNAc-Hex-APTS (*m/z* 377-I), which was found for several predominantly but not exclusively, breast-fed babies (Fig. 2-A7 and Fig. 3-A2) seems to be dependent on the gastrointestinal presence of HMOs, but does not depend on the respective blood group (Table 1). The compound *m/z* 377-I may result from the gastrointestinal degradation of the *para*-isomers of higher-molecular weight HMOs, which carry a terminal β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-unit (e.g., F-*para*-LNH I, β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc).

Another possibility is the origin from gastrointestinal mucin-type-O-glycans, for which repeats of Gal-GlcNAc-units were described.[25] The presence of intestinal bacteria-/enzyme-sets able to degrade these structures for these HMO-fed babies has to be assumed in that case.

3.4. Formula-fed babies

Ten out of fourteen fecal extracts from exclusively and predominantly formula-fed babies showed similar fecal CE–LIF patterns, independent of feeding a preterm formula without GOS or a preterm formula supplemented with 0.4 g/dL GOS, as was represented in Figure 4 by samples B12 and B5. The hexo-trioses and hexo-tetraoses, which are present in a low abundance in these fecal profiles, as well as the numerous un-identified peaks were, therefore, assumed to originate from the gastrointestinal mucus layer and not from the supplemented GOS, as also indicated by the high GlcNAc-, GalNAc- and Man-proportions found for these samples (Table 2). A higher contribution of monomeric Gal was expected for the exclusively GOS-fed babies (B1, B2), due to the low proportions of the other sugars determined in these two samples relative to Gal (Table 2). However, no conclusions could be drawn from the CE–LIF profiles due to the fact that monomeric sugars were partly

removed during sample-preparation for CE–LIF–MS. For fecal samples originating from 24 day old preterm-born babies who received formula supplemented with 0.5 g/dL, we found hexose-oligosaccharides related to the supplemented GOS (unpublished results). In addition, GOS and FOS were recovered in the feces of 1 month old babies, who received a formula supplemented with a mixture of 0.8 g/dL GOS:FOS (9:1) by Moro et al.[14] In the present study, the fecal samples were taken at the age of two months and the fecal flora may be more developed and adapted to GOS than in the study of Moro et al.[14] and able to completely degrade GOS. The fecal recovery of supplemented oligosaccharides to infant formula may thus depend on their concentration and on the stage of gastrointestinal development of the baby.

3.5. Presence of unknown oligosaccharide *m/z* 328 in the feces of breast- and formula-fed babies

HexNAc-Hex-Hex-APTS (*m/z* 328) was found in minor proportions for several mixed-fed babies showing secretor-HMO profiles and as major component for the four formula-fed babies B1, B3, B13, B14 (exemplified by Fig. 2-A7, Fig. 4-B3). The presence of HexNAc-Hex-Hex is not dependent on the gastrointestinal presence of HMOs or the type of infant formula. Furthermore, this unknown structure was not present in any of the fecal profiles of exclusively breast-fed babies and babies showing non-secretor HMO-profiles. A mucosal origin is assumed for HexNAc-Hex-Hex and the pronounced expression of HexNAc-Hex-Hex for formula-fed babies may indicate an enhanced mucus-degradation. This may point to lack of intestinal feeding and thus to the need of the microbiota to degrade sugar units from the gastrointestinal mucus layer. The observed dependency of HexNAc-Hex-Hex on the presence of secretor-breast milk in case of breast-feeding may implicate the involvement of α-(1→2)-fucosylation prior to the gastrointestinal release of this compound.

4. Concluding remarks

Overall, the fecal recovery of oligosaccharides from human milk and formula seems to be dependent on the adaptation of the gastrointestinal microbiota to the respective feeding and the concentration of oligosaccharides herein. The host's genotype plays also an important role in the gastrointestinal oligosaccharide metabolisation and was identified as a determinant factor responsible for the presence of the blood group characteristic oligosaccharides GalNAc-[Fuc]-Gal-Glc, GalNAc-[Fuc]-Gal-GlcNAc-Ga-Glc, and may be involved in the formation of the structures annotated as Hex-[Fuc]-HexNAc-Hex and HexNAc-Hex-Hex in the feces.

Fecal oligosaccharide profiles contain valuable information. To understand the gastrointestinal bioconversion of HMOs, more study is needed.

5. Experimentals

5.1. Set-up study and sample collection

Twenty-seven fecal samples of approximately two month old preterm infants (born after 27–35 weeks of gestation and a birth weight between 770 g and 2285 g) were selected from a clinical study on the investigation of the effect of the supplementation of preterm infant formula with prebiotic GOS or lactoferrin on the performance of preterm babies, which was set up and performed prior to our research intention. The study was performed at the level III neonatal intensive care unit of the Isala clinics, Zwolle, The Netherlands. Enteral feeding of the babies was started after birth as soon as possible and full enteral feeding was established 5–19 days after birth. Fecal samples were taken 6 weeks after full enteral feeding was established and samples were frozen at −20 °C until analysis. The babies received either exclusively expressed human milk from their own mother ($n = 5$) or preterm infant formula ($n = 6$) [supplemented with 0.4 g/dL GOS (Frisolac prematuur, FrieslandCampina DOMO, Zwolle, The Netherlands), or without GOS (Frisolac prematuur, GOS replaced by maltodextrin), or supplemented with 0.1 g/dL lactoferrin]. Sixteen babies got mixed feeding (breast milk and formula). Breast milk meals contributed to >80%, >60% or <55% of the total number of feedings, which these babies got during the study period (Table 1). The babies were randomly assigned to one of the three formula groups. The study was performed double-blind and was approved by the medical ethical review board of the hospital. Written informed consent was obtained from all the parents. Except for baby A2, the blood group of the babies was known (Table 1). Among the 27 babies studied were two twin pairs and one triplet pair, but they were not further specified in this study. Lactoferrin-supplementation was not of interest for the present study on the analysis of fecal oligosaccharides and no differences were observed in the fecal oligosaccharide profiles of formula-fed babies with and without lactoferrin supplementation. Babies who got preterm infant formula supplemented with lactoferrin (A7, B4, B7, B8, B14) were thus assigned to the control group and not further specified.

5.2. HMO reference material and chemicals

2'Fucosyllactose, lacto-N-tetraose, lacto-N-fucopentaose I–III, lacto-N-difucohexaose, 3'monofucosyl-lacto-N-hexaose and a mixture composed of 3'sialyllactose, 6'sialyllactose and 6'sialyl-*N*-acetyllactosamine were purchased from Dextra Laboratories (Reading, UK). All other chemicals used were of analytical grade.

5.3. Oligosaccharide extraction from feces

Oligosaccharides from feces were extracted according to Moro et al.[14] Watery fecal slurries (50 mg/mL) were kept overnight at 4 °C, centrifuged (15 min, 3500×*g*) and filtered through a 0.22 μm membrane. Fecal enzymes were inactivated by heat (5 min, 100 °C).

The carbohydrates extracted from fecal extracts were purified by solid phase extraction on graphitized carbon column cartridges (150 mg bed weight, 4 mL tube size; Alltech, Deerfield, Il, USA) as described previously.[17,26] In short, the cartridges were washed with 80/20 (v/v) acetonitrile (ACN)/water containing 0.1% (v/v) trifluoroacetic acid (TFA) followed by millipore water. After loading the sample extract onto the cartridge, monomers and lactose were largely removed by eluting with an aqueous 2% (v/v) ACN solution. The remaining carbohydrates on the cartridge were eluted with 40/60 (v/v) ACN/water containing 0.05% (v/v) TFA. The solution was dried under a stream of air and the dried sample was then rehydrated with millipore water.

5.4. Monosaccharide composition analysis

The crude fecal extracts (not purified on graphitized carbon columns) were hydrolyzed with 2 M TFA for 1 h at $T = 121$ °C followed by evaporation and repeated washing with MeOH. The hydrolysates were re-dissolved in Millipore water and analyzed with HPAEC–PAD for their monosaccharide composition.[27] For the determination of sialic acid, mild sample hydrolysis with 0.1 M TFA for 40 min at $T = 80$ °C was applied[18] before HPAEC analysis.[27]

5.5. CE–LIF

For CE–LIF analysis, the carbohydrates present in the fecal extracts were derivatized with the fluorescent 9-aminopyrene-1,4,

6-trisulfonate (APTS) overnight at room temperature as reported elsewhere.[28,29] One nmol xylose was added as internal standard and mobility marker. CE–LIF was performed on a ProteomeLab PA 800 characterization system (Beckman Coulter, Fullerton, CA, USA), equipped with a laser induced fluorescence detector (LIF) (excitation: 488 nm, emission 520 nm) (Beckman Coulter) and a polyvinyl alcohol coated capillary (50 μm ID × 50.2 cm (Beckman Coulter), detector after 40 cm), kept at 25 °C. Samples were loaded hydrodynamically (4 s at 0.5 psi, representing approx. 14 nL sample solution) on the capillary. Separation was performed in the reversed polarity mode (30 kV, 20 min) in a 25 mM acetate buffer containing 0.4% polyethylene oxide provided in the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter). Due to the low pK_a of the sialic acid residues (pK_a 2.6) and in order to provide the same buffer-pH as for CE–MS analysis, the separation buffer was adjusted to pH 2.4 by adding 1.2% (v/v) formic acid.[17] Peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA, USA).

5.6. CE–LIF–ESI-MS^n

CE–LIF–ESI-MS^n experiments were performed on a P/ACE™ System MDQ (Beckman Coulter) according to Albrecht et al.[17] For the fluorescent detection (excitation: 488 nm, emission 520 nm), a Picometrics ZetaLIF discovery system was used (Picometrics, Toulouse, France). Separation in 0.3% (v/v) formic acid (pH 2.4) was performed on a fused silica capillary (50 μm ID × 85 cm (Beckman Coulter), capillary window fitted with an ellipsoid for LIF detection after 60 cm) in reversed polarity mode (20 kV, T = 15 °C, 40 min). Samples were injected at 10 psi for 2 s. LIF signals were sent to Beckmann 32Karat software via a SSXL4002 converter (Agilent Technologies, Santa Clara, CA, USA). The ESI-MS (LTQ ion trap, Thermo Fisher Scientific Inc., Waltham, MA, USA) was operated in the negative mode using a spray voltage of 1.9 kV and an MS-capillary temperature of 190 °C. The end of the CE capillary was installed in front of the ESI source by leading it through a T-part designed in our laboratory[30] and provided the coaxial addition of a sheath liquid (50/50 isopropanol/water) at 2 μL/min. Mass spectra were acquired from m/z 300 to 2000. MS^n was performed in the data dependent mode using a window of 1 m/z and collision energy of 35%. For increasing the S/N ratio, ions of m/z 311, 314 and 329 were excluded from detection in MS^n experiments (mass exclusion list). MS^n data were interpreted using Xcalibur software 2.0.7 (Thermo).

5.7. Statistical analysis

The areas of the peaks in the CE–LIF electropherograms were normalized on the internal standard xylose. Agglomerative hierarchical clustering (AHC) was performed on the CE–LIF data points of all the fecal extracts using XLSTAT 2010.2.03 (Addinsoft, New York, NY, USA). Data points starting from the migration time of the internal standard xylose (t = 4.1 min) were included in the statistical analysis. 3334 data points were subjected to AHC per sample electropherogram. The similarities for AHC were determined by the Pearson correlation coefficient and agglomerated using the unweighted pair group method. The results are presented as dendrograms, showing clustering patterns and the similarities among the clusters.

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