Structure and fermentation of natural and manufactured lactose-based oligosaccharides

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Elisabetta Difilippo

Thesis

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To my grandfather Vincenzo/ A mio nonno Vincenzo

Abstract

At early stages of life, infant immature intestine is not fully developed, exposing the new-born to potential diseases. Compounds that can exert beneficial actions on the infant intestine are bioactive lactose-based oligosaccharides (LBOs). The natural source of LBOs is mother milk. When human milk is lacking, dietary supplementation with infant formula fortified with manufactured LBOs, such as galacto-oligosaccharides (GOS), is pursued. GOS have been shown to have several properties in common with HMOs. LBO composition and intestinal fate is extensively described for humans, whereas both aspects are hardly investigated for domestic animal. In this PhD thesis, composition of LBOs in equine and porcine colostrum were described and new structures were elucidated. The analysis were performed mainly using liquid chromatography with mass spectrometry and capillary electrophoresis with fluorescence detection. With these techniques novel milk oligosaccharides were found: seven equine milk oligosaccharides, of which four neutral and three acidic, and thirteen porcine milk oligosaccharides (PMOs), of which eight neutral and five acidic. High inter- and intra-individual variations were found for oligosaccharides present in equine and porcine milks. *In vivo* fermentation of PMOs was described analysing PMOs as found in fecal samples of piglets. The results were correlated to existing literature on HMOs. The presence of GOS and PMOs in blood, urine and fecal samples from an *in vivo* feeding trial with piglets was described. Intact GOS and milk oligosaccharides from the piglet diet, in addition to dietary oligosaccharides, were found in piglets' blood and urine samples, as suggested from studies on HMO presence in infants' blood and urine. All oligosaccharides were fermented/absorbed *in vivo*, not being detectable in the piglets' fecal samples. GOS *in vitro* fermentation by piglet inoculum delineate a unique fermentation profile regarding GOS size consumption compared with GOS *in vitro* fermentation by human fecal inoculum. Similar degradation profile regarding GOS linkage types was observed for GOS fermentation by piglet and human inocula.

List of abbreviations

DSL	Disialyllactose	LN(n)T	Lacto-N-(neo)tetraose
DSLNnT	Disialyllacto-N-neotetraose	LST	Sialyllacto-N-tetraose
FL	Fucosyllactose	SL	Sialyllactose
Fuc	Fucose	S-LNH	Sialyllacto-N-hexaose
Gal	Galactose		
Gal-LNnH	Galactose-sialyllacto-N-neohexaose		
GalNAc	N-Acetylgalactosamine		
GL	Galactosyllactose		
Glc	Glucose		
Hex	Hexaose		
HexNAc	N-Acetyl-hexosamine		
L	Lactose		
LNDFH	Lacto-N-difucohexaose		
LNFP	Lacto-N-fucopentaose		
LNH	Lacto-N-hexaose		
LN(n)H	Lacto-N-(neo)hexaose		

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Chapter 1

General introduction

The project

The immature gastro-intestinal barrier of the neonate is a health risk factor. The immaturity can lead to increased antigen transfer, local inflammatory reactions and impaired immunological development of the intestinal microflora. These effects are considered not only in terms of immediate disease, but also as factors increasing the chance of chronic diseases during the adulthood. Chronic diseases can include auto-immune diseases, allergies, asthma and atopic skin disorders as well as inflammatory bowel diseases.

Compounds that can influence the body response to immunological changes are designated as immuno-modulators. Many immuno-modulators have been identified as carbohydrates, and the most well studied class of carbohydrates are lactose-based oligosaccharides (LBOs). LBOs are present in human breast milk (HMO – human milk oligosaccharides) as a highly complex mixture, or they can be enzymatically manufactured, resulting in a galacto-oligosaccharide mixture (GOS).

A multidisciplinary project named 'Immuno-modulation of oligosaccharides from various sources' was conducted within the Carbohydrate Competence Center program. The project focused on the investigation of the chemical structure and function of LBOs, in relation to their immunological aspects. This thesis, as part of the project, aimed to study aspects of isolation, characterization and *in vitro* fermentation of LBOs from different sources, such as milk from various mammals and manufactured GOS.

Lactose-based oligosaccharides

Oligosaccharides are defined as carbohydrates with a degree of polymerization (DP) from 2 to about 10, according to the IUB-IUPAC nomenclature.[1] The main oligosaccharide with DP2 in mammalian milk and in manufactured GOS is represented by lactose (53-70 g/L in human milk).[2, 3] Lactose is an oligosaccharide hydrolysed by intestinal lactase and has been described aside from oligosaccharides.[4] Therefore, in this PhD thesis, LBO descriptions exclude lactose, while they include oligosaccharides with DP2 other than lactose.

Mammalian milk oligosaccharides

Mammalian milk oligosaccharides (MMOs) are the third largest component in human milk, after lactose and lipids.[4] The concentration of MMOs depends on the milk origin and the lactation stage of the mammal: 21-24 g/L and 12-13g/L for human milk oligosaccharides (HMOs) in human colostrum and in mature milk, respectively; about 1g/L and 0.05g/L in MMOs in cow colostrum and in mature milk, respectively; and 0.25-0.3 g/L MMOs in goat mature milk, and about 0.03 g/L in ovine mature milk.[5-7] Domestic animal MMOs are present overall in 100-1000 fold lower concentrations than HMOs.[8]

In general, MMOs have a lactose unit (Gal(β1-4)Glc) at their reducing end. In bovine, equine and caprine milks, lactose or N-acetyllactosamine (Gal(β1-4)GlcNAc) can be found as reducing unit.[4, 5, 9] Lacto-N-biose (Gal(β1-3)GlcNAc) and N-acetyllactosamine can elongate the above described units, forming type I or type II MMOs, respectively (Figure 1).[10] MMOs are elongated by glycosyltransferases present in the mammary gland, forming oligosaccharides up to around DP10.[4] Monomers that can elongate the MMO structure are the neutral sugars galactose, fucose, N-acetylglucosamine and, in domestic animal milk, N-acetylgalactosamine. Sialic acids, like N-acetyl-neuraminic acid for human milk, and both N-acetyl- and N-glycolyl-neuraminic acids for animal milk, can also elongate the MMO structure. In case of domestic animal milk, glucose and N-acetylglucosamine substituted with one phosphoric group was also reported as part of the MMO structure.[4, 11, 12] The core oligosaccharide structure of MMOs can be divided into 12 groups as represented in Figure 1. Classification is based on core structure of MMOs, containing β1-3, β1-4 and β1-6 linked neutral monomers. These core structures can be elongated by fucose, attached via α1-2, α1-3, α1-4 and/or by N-acetylneuraminic acid, attached via α2-3 and α2-6.[4, 13]

The compositions of the MMOs found in human milk and common domestic animal milks are summarized in Table 1. Not all MMOs found in human milk are found in domestic animal milks and vice-versa. The major HMOs reported are the neutral 2'-fucosyllactose (2'-FL), lacto-N-tetraose, fucosyllacto-N-pentaose-I, and lacto-N-difucosylhexaose-II (numbers 9, 13, 19, and 26, highlighted in bold in Table 1) next to the acidic 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), sialyllacto-N-tetraose-a, sialyllacto-N-tetraose-b, sialyllacto-N-tetraose-c, and disialyllacto-N-tetraose (numbers 33, 34, 44, 45, 46 and 48, highlighted in bold in Table 1).[4] Interestingly, only 3 of the major HMOs are present in domestic animal milk: 2'-FL is present in caprine and porcine milks (number 9, Table 1), while 3'-SL and 6'-SL are present in bovine, equine, caprine, ovine and porcine milk. The composition of MMOs depends on the milk origin, including animal breed and mammal lactation stage.[2, 14-17] Some HMOs seem to be present specifically in human milk and not in domestic animal milks. Sialylated tetramers (numbers 44-46, Table 1), mono-fucosylated pentamers (numbers 19 and 20, Table 1) and mono- or di-fucosylated hexamers (numbers 25, 26 and 29-31, Table 1) are present in high abundance in human milk, while they were not reported for domestic animal milk.

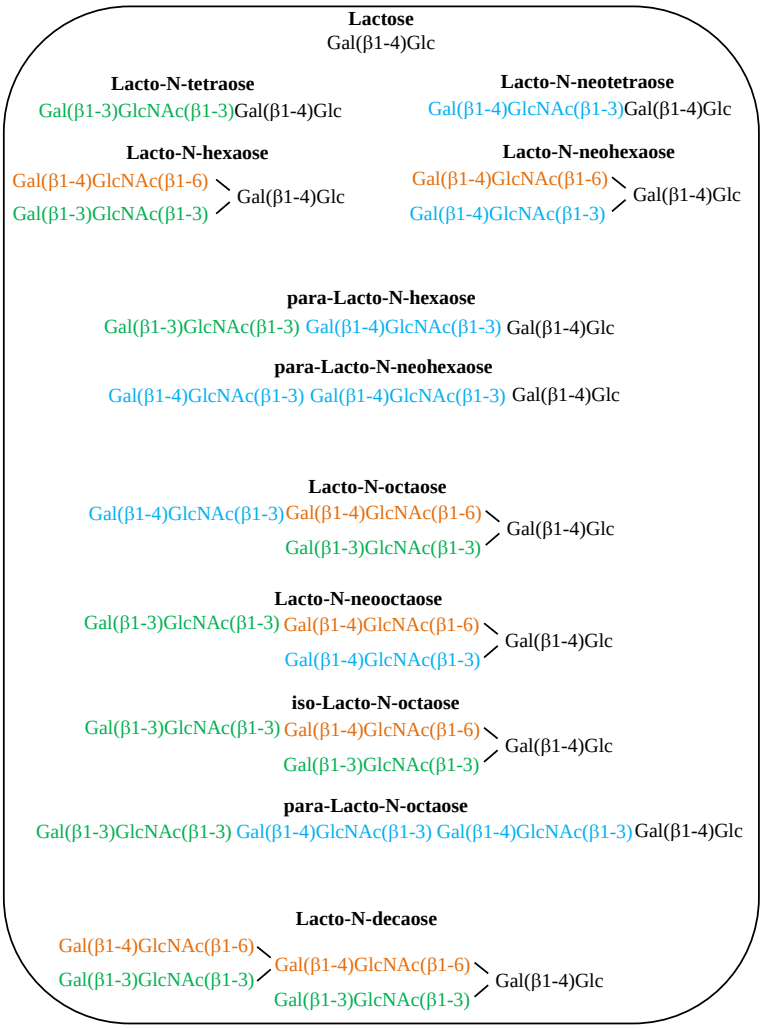

Figure 1. Core oligosaccharide structures as found in human milk. Colours indicate dimers presenting the same linkage type. Adapted from Haeuw-Fievre et al.[13] With Gal = galactose, Glc = glucose and GlcNAc = N-acetylglucosamine.

Table 1. Presence and concentration of oligosaccharides in human milk and domestic animal milk (bovine, equine, caprine, ovine and porcine).

Mammalian milk oligosaccharides									
				Concentration (mg/L)					
No	Neutral	Name	Abbreviation	Human[a]	Bovine[b]	Equine[c]	Caprine[d]	Ovine[e]	Porcine[f]
1	Gal(β1-4)GlcNAc	N-Acetyllactosamine		–	n.q.	–	–	–	–
2	GalNAc(β1-4)Glc	N-Acetylgalactosaminylglucose		–	n.q.	–	–	–	–
3	Gal(β1-3)Gal(β1-4)Glc	β3'-Galactosyllactose	β3'-GL	traces	n.q.	7.8	30-50	30.2	n.q.
4	Gal(β1-4)Gal(β1-4)Glc	β4'-Galactosyllactose	β4'-GL	traces	n.q.	–	–	–	n.q.
5	Gal(β1-6)Gal(β1-4)Glc	β6'-Galactosyllactose	β6'-GL	2-3	n.q.	4.8	48.5	51	–
6	Gal(α1-3)Gal(β1-4)Glc	Isoglobotriose		–	n.q.	–	1.6-50	21.4	–
7	GalNAc(β1-6)Gal(β 1-4)Glc	β6'-N-Acetylgalactosaminyllactose	6'-GLN	–	–	–	20-40	–	–
8	GalNAc(β1-3)Gal(β 1-4)Glc	β3'-N-Acetylgalactosaminyllactose	3'-GLN	–	1-65	–	–	–	–
9	**Fuc(α1-2)Gal(β1-4)Glc**	**2'-Fucosyllactose**	**2'-FL**	**1580-4130**	–	–	**0.95**	–	n.d*
10	Fuc(α1-3)Gal(β1-4)Glc	3'-Fucosyllactose	3'-FL	460	–	–		–	
11	Gal(β1-4)[Fuc(α1-3)]GlcNAc	3'-Fucosyllactosamine	3'-FLN	–	n.q.	–	–	–	–
12	GalNAc(α1-3)Gal(β1-4)Glc	α3'-N-acetygalactosaminyllactose		–	n.q.	–	–	–	–
13	**Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc**	**Lacto-N-tetraose**	**LNT**	**860**	–	–	–	–	–
14	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-N-neotetraose	LNnT	110	–	n.q.	–	–	n.q.
15	Gal(β1-4)GlcNAc(β1-6)Gal(β1-4)Glc	Iso-Lacto-N-tetraose	iso-LNT	–	–	n.q.	n.q.	–	–
16	Fuc(α1-2)Gal(β1-4)[Fuc(α1-3)]Glc	Difucosyllactose	DF-L	170	–	–	–	–	–
17	Fuc(?)Gal(β1-4)[Fuc(?)]Glc	Difucosyllactose isomer		–	–	–	–	–	n.q.
18	Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)] Gal(β1-4)Glc	Novo-lacto-N-pentaose-I	novo-LNP-I	–	n.q.	1.1	–	–	n.q.
19	**Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc**	**Fucosyllacto-N-pentaose-I**	**LNFP-I**	**670**	–	–	–	–	–
20	Gal(β1-3)[Fuc (α1-4)]GlcNAc(β1-3) Gal(β1-4)Glc	Fucosyllacto-N-pentaose-II	LNFP-II	200	–	–	–	–	–
21	Gal(β1-4)[Fuc (α1-3)]GlcNAc(β1-3) Gal(β1-4)Glc	Fucosyllacto-N-pentaose-III	LNFP-III	280	–	–	n.q.	–	–
22	Fuc (?)Gal(β1-4)GlcNAc(β1-6) [Gal(β1-3)]Gal(β1-4)Glc	Fucosyl-novolacto-N-pentaose-I	F-novoLNP-I	n.q.	–	–	–	–	n.q.
23	Gal(β1-3)GlcNAc(β1-6)Gal(β1-4) [Fuc (α1-3)]Glc			–	–	–	n.q.	–	–

(Table 1. continued)

Mammalian milk oligosaccharides									
				Concentration (mg/L)					
No	Neutral	Name	Abbreviation	Human[a]	Bovine[b]	Equine[c]	Caprine[d]	Ovine[e]	Porcine[f]
24	Gal(β1-3)[Gal(β1-4)]GlcNAc(β1-3) Gal(β1-4)Glc			–	–	–	n.q.	–	–
25	Fuc(α1-2)Gal(β1-3)[Fuc(α1-4)] GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-N-difucosylhexaose-I	LNDFH-I	580	–	–	–	–	–
26	**Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3) Gal(β1-4)[Fuc(α1-3)]Glc**	**Lacto-N-difucosylhexaose-II**	**LNDFH-II**	**250**	–	–	–	–	–
27	Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4) GlcNAc(β1-3)]Gal(β1-4)Glc	Lacto-N-neohexaose	LNnH	n.q.	–	n.q.	–	–	n.q.
28	Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3) GlcNAc(β1-3)]Gal(β1-4)Glc	Lacto-N-hexaose	LNH	130	–	–	n.q.	–	–
29	Gal(β1-4)GlcNAc(β1-6)[Fuc(α1-2) Gal(β1-3)GlcNAc(β1-3]Gal(β1-4)Glc		2'-F-LNH	140	–	–	–	–	–
30	Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6) [Gal(β1-3)GlcNAc(β1-3)]Gal(β1-4)Glc		3'-F-LNH	320	–	–	–	–	–
31	Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6) [Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)] Gal(β1-4)Glc		2',3'-DF-LNH	180	–	–	–	–	–
	Acidic								
32	Neu5Ac(α2-3)Gal	3'-Sialylgalactose		–	n.q.	–	–	–	–
33	**Neu5Ac(α2-3)Gal(β1-4)Glc**	**3'-Sialyllactose**	**3'-SL**	**144-310**	**95-1245**	**n.q.**	**30-50**	**n.q.**	**n.q.**
34	**Neu5Ac(α2-6)Gal(β1-4)Glc**	**6'-Sialyllactose**	**6'-SL**	**420-1310**	**18-243**	–	**50-70**	**n.q.**	**n.q.**
35	Neu5Gc(α2-3)Gal(β1-4)Glc	3'-N-glycolylneuraminyllactose		–	1	–	–	n.q.	–
36	Neu5Gc(α2-6)Gal(β1-4)Glc	6'-N-glycolylneuraminyllactose		–	n.q.	–	40-60	–	–
37	Neu5Ac(α2-3)Gal(β1-4)GlcNAc	3'-Sialyllactosamine	3'-SLN	–	–	–	–	–	n.q.
38	Neu5Ac(α2-6)Gal(β1-4)GlcNAc	6'-Sialyllactosamine	6'-SLN	–	47-239	–	–	–	n.q.
39	Neu5Gc(α2-6)Gal(β1-4)GlcNAc	6'-N-glycolylneuraminyllactosamine		–	n.q.	–	n.q.	–	–
40	Gal(β1-6)[Neu5Ac(α2-3)]Gal(β1-4)Glc	β6'-Sialylgalactosyllactose		–	–	–	n.q.	–	n.q.
41	Gal(β1-3)[Neu5Ac(α2-6)]Gal(β1-4)Glc	β3'-Sialylgalactosyllactose		–	–	–	n.q.	–	n.q.
42	Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc	3'-Sialyl-β3'-galactosyllactose		–	2	–	–	–	–
43	Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc	Disialyllactose	DS-L	–	28-283	–	1-5	–	n.q.
44	**Neu5Ac(α2-3)Gal(β1-3)GlcNAc (β1-3)Gal(β1-4)Glc**	**Sialyllacto-N-tetraose-a**	**LST-a**	**13**	–	–	–	–	–

(Table 1. continued)

Mammalian milk oligosaccharides									
				Concentration (mg/L)					
No	Acidic	Name	Abbreviation	Human[a]	Bovine[b]	Equine[c]	Caprine[d]	Ovine[e]	Porcine[f]
45	**Gal(β1-3)[Neu5Ac(α2-6)]GlcNAc (β1-3)Gal(β1-4)Glc**	**Sialyllacto-N-tetraose-b**	**LST-b**	**40**	–	–	–	–	–
46	**Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) Gal(β1-4)Glc**	**Sialyllacto-N-tetraose-c**	**LST-c**	**68**	–	–	–	–	–
47	Neu5Ac(?)Gal(β1-4)GlcNAc(β1-3) Gal(β1-4)Glc	Sialyllacto-N-neotetraose	SLNnT	–	–	–	–	–	n.q.
48	**Neu5Ac(α2-3)Gal(β1-3)[Neu5Ac(α1-4)] GlcNAc(β1-3)Gal(β1-4)Glc**	**Disialyllacto-N-tetraose**	**DS-LNT**	**100**	–	–	–	–	–
49	Neu5Ac(?)Gal(β1-4)GlcNAc(β1-6) [Gal(β1-3)]Gal(β1-4)Glc	Sialyl-novolacto-N-pentaose-I	novo-SLNP-I	–	–	–	–	–	n.q.
50	Neu5Ac(?)Gal(β1-4)GlcNAc(β1-6) [Gal(β1-4)GlcNAc(β1-3)]Gal(β1-4)Glc	Sialyllacto-N-neohexaose	SLnH	–	–	–	–	–	n.q.
51	[Fuc(?)]Neu5Ac(?)Gal(β1-4)GlcNAc(β1-6) [Gal(β1-4)GlcNAc(β1-3)]Gal(β1-4)Glc	Fucosylsialyl-novolacto-N-pentaose-I	novo-FS-LNP-I	–	–	–	–	–	n.q.
52	[Fuc(?)][Neu5Ac(?)]Gal(β1-4)GlcNAc(β1-6) [Gal(β1-4)GlcNAc(β1-3)]Gal(β1-4)Glc	Fucosylsialyllacto-N-neohexaose	FS-LNnH	–	–	–	–	–	n.q.
53	[Neu5Ac(?)]Neu5Ac(?)Gal(β1-4)GlcNAc (β1-6)[Gal(β1-4)GlcNAc(β1-3)]Gal(β1-4)Glc	Disialyllacto-N-neohexaose	DS-LNnH	–	–	–	–	–	n.q.
	Phosphorylated								
54	Gal(β1-4)Glc-3-PO_4	Lactose-3-phosphate		–	n.q.	–	–	–	–
55	Gal(β1-4)GlcNAc-1-PO_4	Lactosamine-1-phosphate		–	–	n.q.	–	–	–
56	Neu5Ac(α2-6)Gal(β1-4)GlcNAc-1-PO_4	6'-Sialyllactosamine-1-phosphate		–	2	–	–	–	–
57	Neu5Ac(α2-6)Gal(β1-4)GlcNAc-6-PO_4	6'-Sialyllactosamine-6-phosphate		–	0.7	–	–	–	–

Structures in bold: most abundant human milk oligosaccharides also present in domestic animal milk; n.q = present but not quantified; (?) = linkage not further specified; * = isomer not further specified; – = not present

[a] = Ref.[2, 3, 8]; [b] = Ref.[2, 7, 8, 18]; [c] = Ref.[7, 8, 21]; [d] = Ref.[6, 7, 22]; [e] = Ref.[7, 23, 24]; [f] = Ref.[15]

HMOs are reported to be highly fucosylated (covering 50-80% of the HMO structures) and poorly sialylated (accounting for 10-30% of the HMOs), whereas domestic animal milks showed the opposite trend.[4, 8] In general, domestic animal milks show a high level of sialylation (70, 13, 41, 50, and 62%, for bovine, equine, caprine, ovine and porcine milk, respectively, Table 2) and low levels of fucosylation (1-5, 0, 18, 0, and 24% for bovine, equine, caprine, ovine and porcine milk, respectively, Table 2) when comparing the number of structures reported so far. [4, 8, 16]

Table 2. Overall characteristics of mammalian milk oligosaccharides (MMOs) identified in milk of 6 mammals.

Mammalian milk oligosaccharides (MMOs)						
	Human[a,b]	Bovine[a,c]	Equine[c]	Caprine[c]	Ovine[c]	Porcine[c]
Number of MMOs						
structures identified	>100	22	8	17	6	21
neutral structures identified	>47	10	6	10	3	8
sialylated structures identified	>44	9	1	7	3	13
phosphorylated structures	0	3	1	0	0	0
Percentage based on MMO number						
sialylated structures	10-30	70	13	41	50	62
fucosylated structures	50-80	1-5	0	18	0	24
structural overlap with HMOs	–	23	63	41	67	38

HMOs = human milk oligosaccharides. With a = Ref.[8]; b = Ref. [4]; c = Ref.[16].

Another essential difference between human and domestic animal milk, is the type of sialic acid present. HMOs exclusively contain N-acetyl-neuraminic acid (Neu5Ac), while domestic animal milk oligosaccharides might contain either N-acetyl- or N-glycolyl-neuraminic acid (Neu5Gc) (e.g. numbers 36 and 39, Table 1).[9] The presence of Neu5Gc in domestic animal milk is considered an undesired characteristic when this milk would be applied in human nutrition, since Neu5Gc-antibodies were found upon several human diseases, such as chronic hepatitis and cancer.[18, 19]

Galacto-oligosaccharides

Galacto-oligosaccharides (GOS) are industrially produced from lactose by a β-galactosidase of microbial or yeast origine.[10] The composition of GOS strongly varies with enzyme origin and conditions used during GOS production, such as temperature, pH and substrate concentration.[20] GOS mainly consist of galactose oligomers with a terminal reducing glucose, mostly in the range of 2-8 (Table 3).[20] Glycosidic linkages that could exist between GOS monomeric units are β1-2, β1-3, β1-4 and β1-6, as exemplified in Table 3. Next to glucose at the reducing end of GOS structures, also non-reducing terminal

glucose or galactose presenting a β1-1 linkage can be found, accounting for about 7% (w/w) of the GOS-DP2. [20, 21]

Table 3. GOS-DP2-4 isomers identified in commercial GOS preparations. [3, 20, 22]

GOS-DP2-4 structures		
Glycosyl Substituent		
DP4	DP3	DP2
		Gal(1-3)Gal
		Gal(1-4)Gal
	Gal(1-4)–	Gal(1-2)Glc
	Gal(1-4)–	Gal(1-3)Glc
	Gal(1-4)–	Gal(1-4)Fru (lactulose)
	Gal(1-2)–	Glc(1-1)Gal
Gal(1-4)–	Gal(1-4)–	Glc(1-1)Gal
	Gal(1-6)–	Gal(1-4)Glc (lactose)
Gal(1-4)–	Gal(1-4)–	Gal(1-4)Glc (lactose)
	[Gal(1-2)]–	Gal(1-6)Glc (*allo*-lactose)
Gal(1-6)–	Gal(1-4)–	Gal(1-6)Glc (*allo*-lactose)
Gal(1-4)–	Glc(1-1)–	Gal(4-1)Gal

DP = degree of polymerization; Gal = galactose; Glc = glucose; Fru = fructose.

Physiological fate and properties of lactose-based oligosaccharides

Fermentation

It has been shown *in vitro* that GOS resist to acidic stomach conditions and hydrolysis by mammalian enzymes.[23] Also HMOs have been found to resist *in vitro* pancreatic and brush border enzyme digestion.[8] *In vivo*, more than 90% of dietary GOS and HMOs are reported to resist metabolization and to reach the colon.[10, 24, 25] In some cases, GOS and HMOs are still detectable in fecal samples.[10, 24, 25] LBOs are defined prebiotics, since they reach the colon and they can influence intestinal microbiota composition resulting in beneficial effects for the host.[8, 21, 23, 26] Both GOS and HMOs have been shown to be substrates for bifidobacteria fermentation, promoting bifidobacteria growth. It was shown that babies fed with human milk had higher amounts of bifidobacteria in their intestine, when compared with formula (not containing GOS) fed infants.[27] In general, a shift towards high numbers of bifidobacteria in the large intestine has been reported to be favourable for the host.[28] Being competitive species for both spatial adhesion to the intestinal mucosa and nutrient supply, bifidobacteria may give protection against harmful bacteria,.[8, 28] Moreover, bifidobacteria as diet supplementation decrease symptoms of chronic enterocolitis and lymphoid hyperplasia, and lowers the level of blood cholesterol.[28] As a consequence of

MMOs and GOS fermentation by colonic microbiota, short chain fatty acids are produced. Among short chain fatty acids produced, butyrate has been shown to play and important role in the intestinal health. It decreases intestinal inflammation, carcinogenesis and oxidative stress of the intestine.[21, 29, 30] Due to their structural similarities with HMOs, GOS can replicate their bifidogenic effect, explaining their role as fortifier in infant formula.[21]

Pathogen inhibitor

LBOs can exert protective functions in the intestine, being soluble ligands for pathogens in the receptor-mediated pathogen infection. GOS resemble glyco-conjugate cell surface receptors and can prevent colonization of pathogen on the intestinal surface.[21] It was shown that acidic HMOs can prevent the attachment of *Escherichia coli* and *Helicobacter pylori* to the intestinal mucosa, while both acidic and fucosylated HMOs can prevent the attachment of *Campylobacter jeuni*, being the main cause of bacterial diarrhoea and infant mortality.[31, 32]

Immunomodulation

Recent studies suggest that HMOs can directly interact with intestinal cells, leading to host protection against bacterial infection.[8] 3'-Sialyllactose has been reported to decrease *in vitro* gene expression of sialyltransferases, thus reducing sialic acid glycan decoration on the cell surface.[8, 33] Therefore, adhesion of the pathogenic *Escherichia coli*, binding a sialic acid moiety on the cell surface as first step for infection, was inhibited.[33] HMOs are suggested to directly act as ligand for specific sugar-receptors present on immuno-cells.[34] Interaction of HMOs with the immuno-system has been shown to have protective functions (*in vivo*), leading to leucocyte (white blood cells) recruitment to inflammation site, balanced T helper (Th) 1 to Th2 ratio's and an decrease of Th2 immuno-response to allergens.[42] Also GOS have been reported to possibly protect infants from allergic disorders during the first five years of their lives.[35] Therefore, dietary intervention in early stages of life with LBOs can lead to prevention of immunological and allergic diseases in later stages of life.[34]

Systemic absorption and excretion

Although it has been demonstrated that major parts of LBOs reach the colon, also a partial adsorption of HMOs into the blood circulation (systemic adsorption) has been shown.[8] In infants, about 1% of both neutral and acidic dietary HMOs have been found present systemically.[36, 37] It has been shown *in vitro* that neutral HMOs pass the intestinal cells through both para-cellular and receptor-mediated trans-cytosis pathway, while acidic HMOs pass the intestinal cells only through para-cellular pathway.[38] It was suggested that HMOs containing sialic acid and being present systemically, could help brain development,

as dietary-sialic acid has been reported to increase the learning speed and memory abilities in piglets.[39]

After intestinal absorption, HMOs can be excreted by the urinary system, where they were also suggested to have protective functions.[18, 40-42] Literature reports on sialylated HMOs as protective agents against urinary *Escherichia coli* infection and cystitis.[18, 43] As described for HMOs, also GOS have been reported to reach the intestinal tract for more than 90%, being possibly in minor part absorbed.[21, 26] *In vitro* study described GOS passage through cell line monolayer, depending on their size and structure, indicating their possible absorption *in vivo*.[44]

Pig as a model for human intestinal study

Intestinal fermentation and intestinal microbiota modulation of LBOs have a key role in human and animal health.[45] Therefore, *in vivo* and *in vitro* models have been developed in order to study the intestinal fate of LBOs.

In vivo pig model

In vivo models can be based on animal and human intervention studies.[46] For obvious ethical reasons, it is not possible to collect contents from the different gastro-intestinal regions of infants, while *in vivo* animal models provide a valuable alternative for sample collection. Mice are small and easy to handle experimental animals. Nevertheless, they show differences in bifidobacteria population and eating behaviour compared with humans.[46] Pig intestinal functions, anatomy and nutritional requirements are comparable with those of humans.[47] Pigs are considered to be a good model, not only to mimic human intestine, but also to mimic immunological intestinal changes.[48] Pig models can be used to investigate infant pathologies associated with intestinal microbiota, such as diarrhea supported by *Escherichia coli* and rotavirus, necrotising enterocolitis and obesity.[47] Pigs as well as humans are omnivorous species, with comparable digesta transit times and comparable digestive and absorptive processes.[47, 49, 50] Both species are colon fermenters and their colonic microbiota have analogous compositions.[50] Both species present as major bacterial groups Firmicutes, Bacteriodes.[51] Nevertheless, as for mice, pigs present differences in level and type of bifidobacteria when compared with human intestinal bifidobacteria population. [47, 52, 53] Moreover, pig has been shown already in the cecum an extensive fermentation, while cecal fermentation has been shown to be limited in humans.[46] Overall, it has been stated that pig provide a valuable alternative to human in order to study fermentation and metabolization phenomena in different regions of the gastro-intestinal tract.[46, 47]

In vitro fermentation model

Invasive sampling techniques, high costs and ethical reasons are the drawbacks of *in vivo* human and animal trials, which are, therefore, not always feasible. In order to overcome these restrictions, *in vitro* models using human or animal fecal inocula can be used. *In vitro* models are easy to set and may have a high throughput.[3, 54, 55] *In vitro* models can include fermentation in 1 batch or in a 3-container system, where the pH can be set in each container individually in order to mimic the pH-changes along the gastrointestinal tract.[56, 57] A more complex intestinal model has been described as the TIM-2 (TNO gastro-intestinal model) that represents the intestine from stomach untill the colon.[58-60] The TIM-2 model represent the *in vivo* physiology better than batch *in vitro* model, since absorption of small molecules such as nutrients, drugs and water is simulated. However, the low throughput makes its use still quite expensive, making batch fermentation still a valuable alternative.

Since the pig digestive system is described to be a suitable *in vivo* model for the human digestion system, studies have been conducted in order to compare fermentation of non-digestible oligosaccharides by human and by pig inocula *in vitro*.[54, 61] In these studies differences in gas, SCFAs production and remaining soluble carbohydrates content were highlighted.[54] Overall, human microbiota was able to degrade a larger variety of fibers than pig microbiota.[54, 61] Saccharolytic fermentation stopped earlier during fermentation by pig inocula than by human inocula.[54, 61] SCFAs production was higher during fermentation by human inocula than by pig inocula.[54, 61] In these studies, it was suggested that microbiota of humans and pigs were adapted to the different types of fibers: humans consumed a large variety of fibers, including retrograded starch and pectin, while pigs mainly consumed maize-, barley- and wheat- fibers.[54, 61] Therefore, differences in *in vitro* fermentation characteristics are expected when using human or pig inocula.

Analysis of lactose-based oligosaccharides

LBOs are usually present as complex mixtures. The complexity is caused by the wide diversity in composition, size and presence of isomers both in HMOs and in GOS.[4, 21] The higher the degree of polymerization, the higher the number of isomeric structures that are possibly present in the mixture. For these reasons, LBO separation and characterization represent a challenge that can be approached with several techniques.

Liquid chromatography

Liquid chromatography (LC), such as high-performance anion-exchange chromatography (HPAEC) and (ultra) high performance liquid chromatography ((U)HPLC) are often used in order to separate LBOs.[7, 20, 62-64] For example, hydrophilic interaction liquid chromatography, porous graphitized carbon column and C18 columns have been introduced for the separation of LBOs.[65-67] In order to avoid the occurrence of anomeric peaks in LC

analysis of oligosaccharides, labelling techniques were implemented, allowing a good separation and at the same time sensitive quantification of oligosaccharides using fluorescent detection.[68, 69] The labels mostly used are 2-aminopyridine, 2-aminobenzamide and 2-aminobenzoic acid, which all reacted through condensation with the reducing end of the oligosaccharides.[4, 68, 70, 71] The labelling procedures requires conditions (e.g. incubation at 65°C for 2h) that are sufficiently mild for most oligosaccharides, however they may enhance the release of fucose and sialic acid residues from specific MMO structures.[4, 66]

Mass spectrometry

Mass spectrometry allows LBO characterization and may even allow the complete elucidation of (isomeric) MMOs. After separation by chromatographic and/or non-chromatographic techniques, quantification in mass spectrometry of LBOs is possible with the use of selected ion monitoring, multiple reaction monitoring, or standard calibration curves.[17] Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), both in positive and negative ionization mode, has been often use in order to characterize LBOs, mainly HMOs.[24, 67, 72] GOS are also detectable by MALDI-TOF-MS resulting in one peak per DP. With MALDI-TOF-TOF-MS, a screening of the structures present in MMOs was performed by mass fragmentation analysis and isomeric structures of acidic HMOs were elucidated.[72]

Non-chromatographic techniques

Capillary electrophoresis (CE) with laser induced fluorescent detection has been successfully used to both characterize and quantify LBOs after oligosaccharide labelling at their reducing end.[10] Despite high sensitivity and good peak separation and high resolution, this technique requires the use of standards for characterization, and it doesn't allow the analysis of non-reducing oligosaccharides, present in minor amounts in GOS.[20] CE can be coupled to a mass spectrometer (MS) enabling structure identification, independently from standards. CE-MS, nevertheless, results in a decreased sensitivity and peak separation and resolution.[73] Nuclear magnetic resonance, both on proton and carbon, has been successfully used to elucidate specific LBOs structures, obtained after chromatographic separation, although is limited to relatively pure and concentrated samples.[13, 74-76]

Thesis outline

Human milk oligosaccharides (HMOs) have been reported to be highly beneficial for the infants, therefore composition of HMOs has been extensively investigated. Contrarily, milk oligosaccharides present in domestic animal milks have been investigated to a lesser extent. As demonstrated for HMOs, milk oligosaccharides composition depends on the milk origin and on the mammal stage of lactation. Therefore, in this thesis we describe LBO structures

and concentrations present in different mammalian milks with emphasis on inter-individual variation of milk oligosaccharides. Moreover, nowadays when breastfeeding is not possible, dietary supplementation with infant formula fortified with manufactured galacto-oligosaccharides (GOS) is pursued. GOS intestinal fermentation represent a crucial event for their beneficial effects. Therefore, GOS and dietary oligosaccharides fermentation *in vitro* was investigated. In addition, as described from HMO presence in infant blood and urine, also GOS and dietary oligosaccharides were expected to be present systemically, hypothesizing that they could have the same metabolic fate as HMOs.

In **Chapter 2** equine milk was investigated for its oligosaccharides content. Equine milk oligosaccharides (EMOs) were described highlighting their differences in composition between breeds as well as per individual. EMOs identified were compared for their structural similarities including the bifidogenic-factor with oligosaccharides present in milk from human and other domestic animals.

In **Chapter 3** porcine milk was evaluated, describing porcine milk oligosaccharides (PMOs) as found in different colostrum and mature milk samples. PMO concentration in colostrum, mature milk and in fecal samples from suckling piglets was evaluated.

The pig intestinal system is considered to be a good model for human intestinal system. In **Chapter 4**, an experiment is described, in which piglets fed for 3 or 26 days on a diet enriched with GOS were sacrificed and biological samples (blood and urine) were collected. The biological samples were analysed with respect to dietary oligosaccharides and GOS presence in blood and urine of piglets, as well as in feces.

Oligosaccharides were fully consumed/absorbed during the *in vivo* piglet feeding trial, hindering conclusion on individual oligosaccharide utilization by intestinal microbiota. In **Chapter 5**, *in vitro* fermentation of PMOs and GOS was described using piglet fecal inocula. PMO and GOS fermentation was evaluated for individual oligosaccharide consumption and organic acid production.

Finally, the results obtained in the research are discussed in **Chapter 6** and their impact on future research on LBOs is reflected.

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Chapter 2

Comparison of milk oligosaccharides pattern in colostrum of different horse breeds

Abstract

Colostrum oligosaccharides are known to exhibit prebiotic and immunomodulatory properties. Oligosaccharide composition is species-specific, and equine colostrum has been reported to contain unique oligosaccharides. Therefore, equine oligosaccharides (EMOs) from colostrum from different horse breeds were analyzed by CE-LIF, CE-MS^n, HILIC-MS^n, and exoglycosidase degradation. Sixteen EMOs were characterized and quantified, of which half were neutral and half were acidic. EMOs showed about 63% structural overlap with human milk oligosaccharides, known for their bioactivity. Seven EMOs were not reported before in equine oligosaccharides literature: neutral Gal(β1–4)HexNAc, Gal(β1-4)Hex-Hex, β4'-galactosyllactose, and lactose-N-hexaose, as well as acidic 6'-Sialyl-Hex-Ac-HexNAc, sialyllacto-N-tetraose-a, and disialylacto-N-tetraose (isomer not further specified). In all colostrum samples, average oligosaccharide concentration ranged from 2.12 to 4.63 g/L; with β 6'and 3'- galactosyllactose, 3'-sialyllactose, and disialyllactose as the most abundant of all oligosaccharides (27-59, 16-37, 1-8, and 1-6%, respectively). Differences in presence and in abundance of specific EMOs were evident; not only between the four breeds, but also within the breed.

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Introduction

Mammalian milk and colostrum contain many structures, which are essential for the nutrition and health of the suckling neonate. Throughout the past decades, specific oligosaccharides, which have been identified in milk and colostrum, have been shown to beneficially affect health.[4] For example, human milk oligosaccharides (HMOs) carrying the so-called bifidogenic factor (Gal(β1-3)GlcNAc) have been illustrated to enhance the growth of bifidobacteria in the intestine of neonates.[5] When bifidobacteria selectively ferment HMOs, they produce organic acids, leading to a pH decrease in the intestine. The lowered pH in the intestine creates a less favorable environment for the growth of the pathogens and stimulates the production of mucin which in turn, enhance the host's resistance to infections.[6] The production of SCFA also leads to a stimulation of the immune system through a direct interaction with the immune cells of the intestine.[6, 7] Moreover, increasing evidence has come forward to demonstrate direct immunomodulatory properties of oligosaccharides derived from milk and colostrum. Some properties could derive from interaction with specific receptors on intestinal epithelial cells and immune cells, such as carbohydrate receptors and Toll-like receptors.[7-9] In addition, milk oligosaccharides serve as soluble ligands for pathogens, preventing viral, and bacterial attachment to the intestinal mucosa.[8] The core structure of mammalian milk oligosaccharides (MMOs) is, in most cases, a lactose unit (Gal(β1-4)Glc) at the reducing end. However, in cow's, horse's and goat's milk, N-acetyllactosamine (Gal(β1-4)GlcNAc) is also found at the reducing end instead of the lactose unit.[1, 10] Starting from these disaccharides, glycosyltransferases can elongate the core structure with neutral and charged sugars. The neutral sugars that can be attached to the core structure are D-galactose (Gal), L-fucose (Fuc), N-acetylglucosamine (GlcNAc) and, with the exception of HMOs, also N-acetylgalactosamine (GalNAc).[10] The charged sugars that can decorate the core structure are sialic acids, both N-acetyl- and N-glycolyl-neuraminic acids, and glucose or N-acetylglucosamine substituted with one phosphoric group (e.g., in milk oligosaccharides of horses and cattle).[1, 10, 11] Depending on the linkage present in their chains, the MMOs are divided into two groups: Type I group contains Gal(β1-3)GlcNAc, and Type II group contains Gal(β1-4)GlcNAc.[12] The reported mature-milk oligosaccharide concentration is 12-13 g/L for human milk and approximately 0.05 g/L for cow's milk.[1, 13] The oligosaccharide concentration in colostrum is higher than the concentration in mature milk: 24 g/L in human colostrum and more than 1 g/L in cattle's colostrum.[10, 14] So far, 43 equine milk oligosaccharides (EMOs) have been reported of which 31 are neutral and 12 are acidic.[1, 10, 15] Compared to the HMOs, the EMOs have been reported to have a higher degree of sialylation (20-30% and 60%, respectively)[13] and a lower degree of fucosylation (70% and 5%, respectively).[13] The previously documented EMOs showed a 17% overlap with oligosaccharides present in human milk. Although not reported so far, the presence and relative abundance of individual EMOs may vary between individual animal breeds,

comparable to the variations in HMOs in different human populations.[16-18] This study focuses on the analysis of EMOs in colostrum samples of four different horse breeds: Dutch Warmblood horse, Shetland pony, Crossbred Arabian/New Forest pony and Friesian horse. After isolation from the colostrum samples, the EMOs were identified and quantified, highlighting the inter-breed (EMOs for the different horse breeds) and the intra-breed (EMOs for the same breed) variations.

Materials and methods

Materials

Colostrum samples of horses belonging to four different breeds were obtained from private Dutch owners with consent. Five colostrum samples were available for the Crossbred Arabian/New Forest pony (Nederlands Rijpaarden and Pony Stamboek), four colostrum samples were available for the Dutch Warmblood horse (Royal Warmblood Studbook of the Netherlands), two colostrum samples were available for the Friesian horse, and one colostrum sample was available for the Shetland pony. Each colostrum was collected within 12 hours postpartum and frozen (-20^{o} C) until use. The standards 3'-, and 6'-sialyllactose, 3'-, and 6'-sialyl-N-acetyllactosamine, lacto-N-tetraose, sialyllacto-N-tetraose-a, and lacto-N-hexaose were purchased from Dextra Laboratories (Reading, UK). The standards β 3'-, 4'- and 6'- galactosyllactose, lacto-N-tetraose, and lacto-N-neotetraose were bought from Carbosynth (Compton, UK). The labelling of the oligosaccharides was accomplished using the Carbohydrate Labelling & Analysis Kit (Beckman Coulter, Fullerton, CA, USA). All other chemicals used were of analytical grade. Millipore water (Millipore, Darmstadt, Germany) was referred to as water in the text.

Extraction of equine colostral sugars

The Dutch Warmblood colostrum samples were available in larger quantities than those of other breeds. Therefore, a mixture of the Dutch Warmblood colostrum samples was used to optimize the extraction method and to obtain enough material to be loaded in the preparative Size Exclusion Chromatography. Specifically, 1.25 mL of each colostrum sample was pooled together and mixed with a vortex for 2 minutes. After method optimization, EMOs were extracted from 1mL of the 12 individual colostrum samples. The optimized sugar extraction from the colostrum samples was performed by modification of the method of Nakamura *et al*.[15] Briefly, colostrum (5 mL) was treated with 4 volumes of chloroform. After two hours head over tail mixing, the sample was centrifuged (5000 *x g*, 30 min, 5 ^{o}C) and the supernatant was treated with 2 volumes of methanol. After the same mixing and centrifugation procedure, the methanol-water solution was concentrated by vacuum evaporation. The remaining water solution was freeze-dried.

Purification and fractionation of EMOs

Size Exclusion Chromatography (SEC) was used to remove lactose from the extracted carbohydrates of the pooled Dutch Warmblood colostrum, in order to obtain pools containing EMOs with difference degrees of polymerization (DP). In total four pools were obtained and they were analyzed for the characterization of the EMOs. The fractionation was carried out at 35 ºC on three Superdex 30 Hiload 26/60 preparative grade columns (GE Healthcare, Pittsburgh, PA, USA) connected in series on an AKTA Purifier (GE Healthcare). After manual injection, the EMOs (200 mg) were eluted with water containing 0.5% (v/v) EtOH and collected in 9 mL fractions. The fractions were pooled as follows: pool 1: 290- 492 mL, pool 2: 493- 688 mL, pool 3: 689 – 730 mL, and pool 4: 731- 758 mL. Using water solution as eluent, charged oligosaccharides were excluded from the column material and were eluted first. Neutral oligosaccharides were instead fractionated based on their size. Lactose and monomers were the last to be eluted and were excluded from further analysis. The neutral oligosaccharides were pooled according to the retention times shown by a mixture of neutral galacto-oligosaccharides (DP from 1 to 6) (Vivinal® GOS syrup) (FrieslandCampina Domo, Borculo, The Netherlands), which was considered a reference for its retention time. After freeze drying, the resulting powders were rehydrated with 1mL of water prior to analysis. Solid Phase Extraction (SPE) was used to reduce the lactose content from the 12 individual colostrum samples. The extracted carbohydrates (1 mg) were solubilized in 1mL of water and loaded onto activated graphitized carbon cartridge (150 mg bed weight, 4 mL tube size; Grace, Deerfield, IL, USA). Elution with water (1.5 mL) was used to remove salts and 2% (v/v) acetonitrile (ACN) (1.5 mL) was used in order to elute monomers and lactose. The EMOs, still bound to the graphitized carbon cartridge, were recovered with 1.5 mL of 40/60% (v/v) ACN/water solution containing 0.05% (v/v) trifluoroacetic acid. The obtained EMOs were dried overnight under a stream of nitrogen and subsequently solubilized in 0.5mL of water.

Capillary electrophoresis with laser induced fluorescent and mass spectrometry detection

The EMOs fractionated either by SEC or SPE were labelled with a fluorescent label (9-aminopyrene-1,4,6-trisulfonate, APTS) for their analysis by capillary electrophoresis (CE) with laser induced fluorescence (LIF) detection as reported previously with minor modifications.[19] Briefly, 100 µL of sample containing 5 nmol of xylose, as internal standard, was dried and labelled with APTS. Ten nmol of each oligosaccharide standard, containing 5 nmol of xylose, was labelled with the APTS and run together with the EMOs samples. The labelled samples were diluted 40 times before the analysis. The experiment was performed using a ProteomeLab PA 800 system (Beckman Coulter), equipped with a LIF detector (Beckman Coulter). During the run, the sample was loaded hydrodynamically (7 s at 0.5 psi)

into a polyvinyl alcohol (NCHO) coated capillary (50 μm x 50.2 cm (Beckman Coulter). Resultant peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA, USA). In the CE technique, the oligosaccharides are linked in a molar ratio of 1:1 with the fluorescent, negatively-charged label (APTS). The resulting migration times of the linked oligosaccharides depend on their size and on their net charge. The SEC-fractionated, labelled EMOs were also analyzed for their masses by the PA 800 plus system (Beckman Coulter) coupled to a Velos Pro mass spectrometer (LTQ Velos Pro ion trap MS, Thermo Scientific, Waltham, MA, USA), after 20 times dilution. The CE-MS^n experiment conditions were performed as reported elsewhere. [20]

HILIC-ESI-MS^n

The EMOs pools, derived from SEC, were also analyzed through hydrophilic interaction liquid chromatography (HILIC) with mass spectrometry detection (MS^n) as described elsewhere [21] with some minor modifications. Briefly, an Accela UHPLC system (Thermo Scientific) coupled to a mass spectometer (LTQ Velos Pro ion trap MS, Thermo Scientific) was used. The 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 Corporation, Milford, MA, USA). The acquisition time was 72 min and the eluents had a flow rate of 600 μL/min. The injection volume was 5μL. The composition of the three mobile phases were (A) water with 1% (v/v) ACN, (B) 100% (v/v) ACN and (C) 200 mM ammonium formate (pH 3.0). The elution program was performed as follows: 2 min isocratic 80% B; 58 min linear gradient from 80% to 30% B; followed by 12 min of column washing with a linear gradient from 30% to 20% B and column re-equilibration form 20 to 80% B. The eluent C was kept at 5% during the elution.

Exoglycosidase degradation

To determine the linkage type of the sialic acid decorating the EMOs, two types of sialidases were used: Sialidase S and Sialidase T (ProZyme, Hayward, CA, USA). Sialidase S is specific for the α2-3 linked neuraminic acid, while Sialidase T splits α 2-3 and α 2-6 linked neuraminic acid. For the structural analysis of the neutral EMOs Glyco(β1-3)galactosidase, Glyco(β1-4)galactosidase, Glyco(β1-4,6)galactosidase and Glycoβ-N-acetylhexosaminidase (ProZyme) were used. All reactions were performed as suggested by the manufacturer. After the enzyme degradation at 37 ºC overnight, the solutions were centrifuged (5000 *x g*, 5 min, 25 ºC) and the supernatants were analyzed by HILIC-MS^n.

Quantification of the EMOs

In CE-LIF the quantification of the oligosaccharides was enabled by the use of an internal standard and by a 1:1 stoichiometry between the oligosaccharide and the APTS molecule. A

linear correlation between concentration and CE-LIF peak area has been shown.[19] CE-LIF peak areas were converted to the corresponding EMOs nanomoles and their concentrations in the colostrum samples (g/L). The samples were run in duplicate and the quantification was validated with the help of labelled neutral and acidic oligosaccharides standards. The quantification of 3'-sialyl-N-acetyllactosamine (3'-SLN) and Gal(β1-4)Hex-Hex, annotated by HILIC-MS^n and not assigned in the CE-LIF electropherograms, was performed with HILIC-MS^n. A calibration curve was made based on the peak areas in mass spectrometry of the 3'-SLN, and β 4'-galactosyllactose (β 4'-GL) standards, respectively. The curves fitting the 3'-SLN and β 4'-GL standards had a linear correlation with R^2 of 0.997 and 0.998, respectively.

Results and discussion

Structural characterization of the equine milk oligosaccharides

In order to elucidate the structure of the EMOs, a colostrum mixture was prepared using Dutch Warmblood colostrum. After sugar extraction, part of the oligosaccharides was fractionated using Size Exclusion Chromatography (SEC). A lactose-free EMO mixture was obtained pooling part of the SEC fractions. Lactose-free EMO mixture and four SEC pools obtained were labelled with the fluorescent dye APTS and subsequently analyzed by CE-LIF and CE-MS^n. In Figure 1, CE electropherograms of labelled lactose-free EMO mixture and SEC pools are shown. The migration times of labelled EMOs were compared with available standards and with HMO elution patterns, that have been described in literature.[22] In total, twenty-four peaks were counted of which eleven peaks were annotated (numbers 2-14, Figure 1). Together with remaining lactose after SEC (symbol #, Figure 1), the annotation of five oligosaccharides was confirmed by CE-MS^n analysis. Acidic oligosaccharides were the first eluted from the SEC columns and they were mainly pooled in pool 1. Pool 2 contained sialyllactose together with neutral pentamer and hexamer oligosaccharides. Pools 3 and 4 contained neutral tetramer and trimer, respectively. In the CE-MS^2, the APTS-labelled oligosaccharides showed a specific fragmentation behavior due to the fluorescent molecule attached at their reducing end and a multiple charge state in their fragments spectrum. One example of CE-MS^n identification is given for lacto-N-novo-pentaose I, previously identified in equine milk, and shown in Figure 2.[1, 3] APTS-lacto-N-novo-pentaose I loses one terminal galactose yielding a APTS-tetramer fragment, with m/z 573 and m/z 382, having charge states of -2 and -3, respectively.

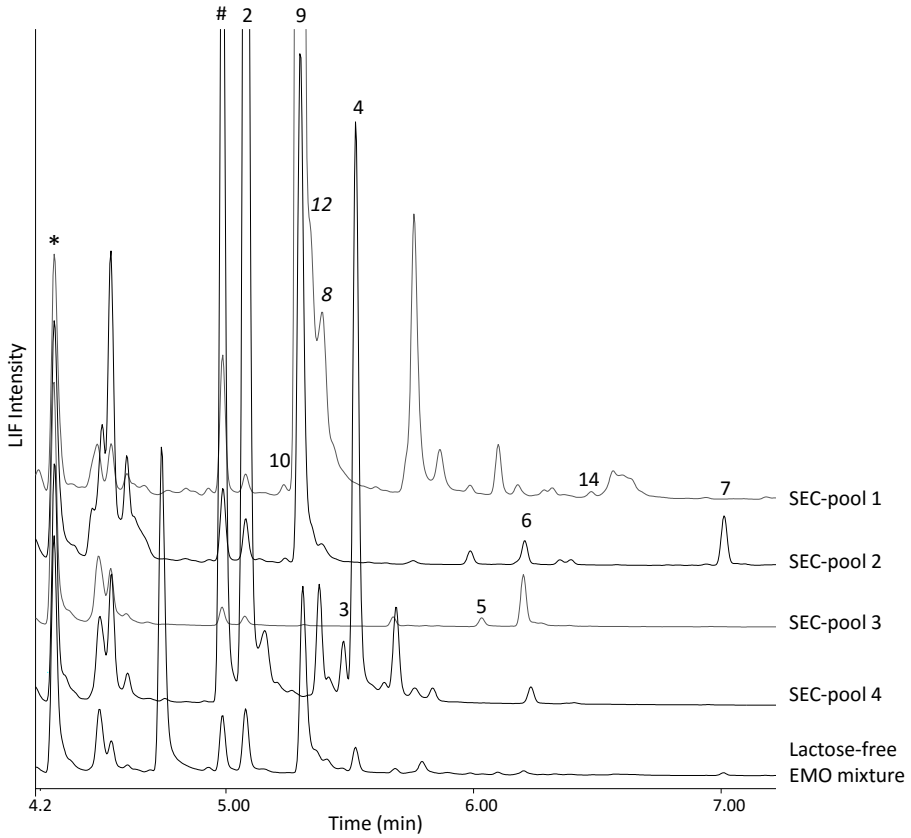

Figure 1. CE-LIF electropherograms of lactose-free EMO mixture and the EMO SEC pools1-4. *: Internal standard xylose, #: Lactose, 1-14: identified peaks as named in Table 1.

The APTS-tetramer fragment loses a terminal galactose producing the fragment GlcNAc-Gal-Glc-APTS with m/z 492 and 328, having charge states -2 and -3, respectively. The GlcNAc-Gal-Glc-APTS subsequently forms APTS-lactose, with m/z 391, having charge state -2, via the loss of the terminal N-acetyl-glucosamine. From the fragmentation of the APTS-lactose, a molecule of APTS-glucose was formed, with m/z 310, having charge state -2. In order to enhance the characterization of the EMOs, the mass analysis was also performed with HILIC-MS^n with non-labelled oligosaccharides obtained after SEC fractionations. With this technique five more EMOs were annotated. All molecules found had a charge state of -1. One example for HILIC-MS^n identification of 3'-sialyl-N-acetyllactosamine (3'-SLN) is shown in Figure 3. Two peaks are highlighted for the sialyl-N-acetyllactosamine (Figure 3a).

The highest peak refers to 3'-SLN, while the lowest peak refers to 6'-SLN. Each of the molecules were found as a double peak. It is hypothesized that the double peaks correspond to the α/β conformation of the reducing end.[23] The MS^2 fragmentation of the 3'-SLN was compared with existing literature and with MS^2 fragmentation of available standard.[24]

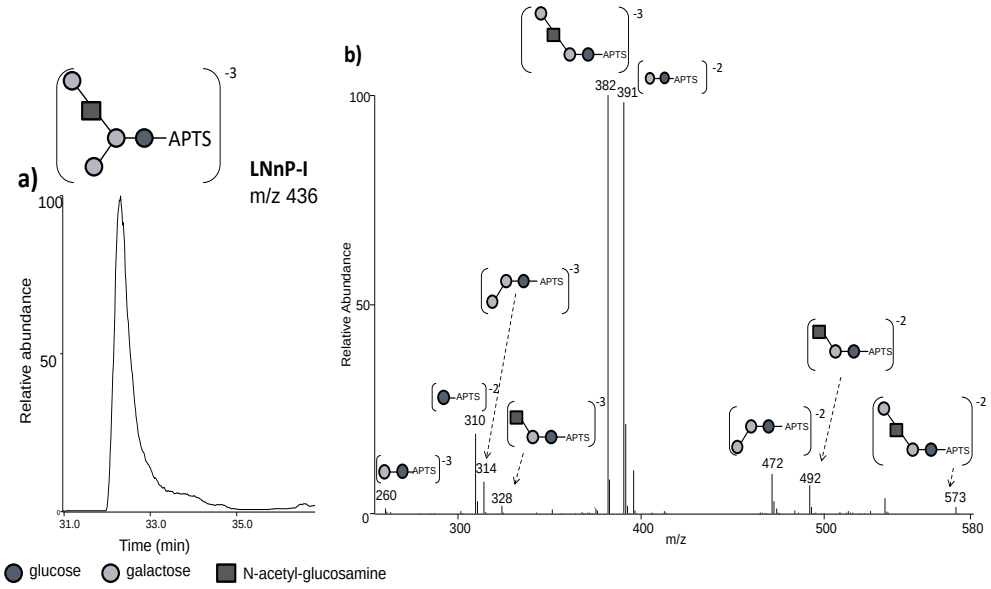

Figure 2. Selected base peak for the lacto-N-novo-pentaose-I (LNnP-I) in CE-MS^n as present in lactose-free EMO mixture (a). Corresponding MS2 fragmentation patterns and structural composition of LNnP-I (b). m/z 436 precursor ion in CE-MSn. [-2], [-3]: charge state of the molecules formed after fragmentation, APTS: 9-aminopyrene-1,4,6-trisulfonate.

The 3'-SLN loses water (Figure 3b), forming thereby the fragment with m/z 655. A characteristic intra-ring fragmentation of the molecule leads to the formation of the fragment with m/z 572. The fragment with the highest intensity refers to the N-acetylneuraminic acid, with m/z 290. For four milk oligosaccharides enzymatic degradation was necessary to determine their linkages. The neutral dimer Gal(β1–4) HexNAc and the trimer Gal(β1-4)Hex-Hex could be characterized only by using Glyco-galactosidases specific for β1-3, 1-4 and 1-4, 6 linkages, in addition using the Glycoβ-N-acetylhexosaminidase. The acidic 6'-sialyl-Hex[Ac]-HexNAc and 3'-sialyllacto-N-tetraose a (LSTa), could be characterized only by using Sialidase S (specific for the α2-3 linked N-acetylneuraminic acid), Sialidase T (specific for α2-3 and 6 linked N-acetylneuraminic acid) and Glycoβ-N-acetylhexosaminidase. After their enzyme degradation, the samples were analyzed by HILIC-MS^n. As example, Figure 4 illustrates the enzymatic degradation of Gal(β1–4)HexNAc and LSTa prior to analysis by HILIC-MS^n. The Gal(β1–4)HexNAc was

found as double peak (Figure 4a), as consequence of α/β conformation of the reducing end. Using the enzymes tested, Gal(β1−4)HexNAc was digested by Glyco(β1-4)galactosidase (Figure 4b), while neither Glyco(β1-3)galactosidase nor Glyco-N-acetylhexosaminidase showed any activity, indicating the presence of a β1-4 linked, terminal galactose.

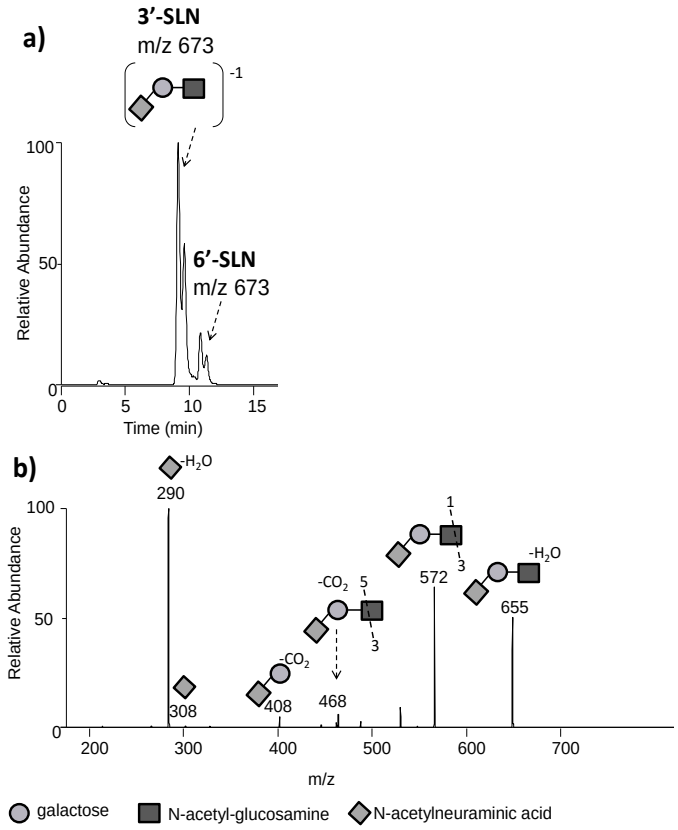

Figure 3. Selected base peak for the 3'-sialyl-N-acetyllactosamine (3'-SLN) in HILIC-MS^n as present in lactose-free EMO mixture (a). Corresponding MS^2 fragmentation patterns and structural composition of 3'-SLN as found in the lactose-free EMO mixture (b). m/z 673 precursor ion in HILIC-MS^n. [-1]: charge state of molecules.

Table 1. Presence and concentration of equine milk oligosaccharides for the four breeds' colostrum samples.

Degree of polymerization	EMO structures	Abbreviation	MW	Peak CE-LIF (Figure 1)	CONCENTRATION OF EQUINE MILK OLIGOSACCHARIDES											
					Dutch Warmblood					Crossbred Arabian/ New Forest ponies				Shetland pony	Friesian	
					A1	A2	A3	A4	B1	B2	B3	B4	B5	C1	D1	D2
	NEUTRAL															
DIMER	**Gal(β 1-4)NAcHex**		383	1	0,11	0,14	0,05	0,08	0,14	0,11	0,38	0,09	0,51	0,22	0,29	0,13
	Gal(β 1-4)Hex-Hex			na	++	++	++	+	+	+	+	++	+	+	–	–
TRIMER	β6'-Galactosyllactose	β6'-GL	504	2	0,98	2,08	0,65	0,84	0,83	0,55	1,64	0,83	3,15	1,91	1,2	0,88
	β4'-Galactosyllactose	β4'-GL		3	tr	tr	tr	tr	tr	tr	–	–	–	–	tr	tr
	β3'-Galactosyllactose	β3'-GL		4	0,16	0,16	0,10	0,09	0,23	0,08	0,04	0,14	0,23	0,11	0,05	0,05
TETRAMER	Lacto-N-neotetraose	LNnT	708	5	0,02	tr	0,13	0,02	0,39	0,21	0,13	0,26	0,09	0,10	0,07	0,03
PENTAMER	Lacto-N-neopentose I	LNnP I	870	6	0,03	0,08	0,09	tr	0,05	0,04	0,04	0,04	0,11	0,06	tr	tr
HEXAMER	**Lacto-N-hexaose**	LNH	1073	7	0,03	0,05	0,02	tr	0,04	0,03	0,02	0,04	0,07	0,04	tr	0,01
	ACIDIC															
TRIMER	6'-Sialyl-N-acetyllactosamine	6'-SLN	674	8	0,07	–	–	0,01	0,12	0,02	0,06	0,01	0,02	0,02	0,07	0,19
	3'-Sialyl-N-acetyllactosamine	3'-SLN		na	0,05	0,03	0,02	0,02	0,01	0,003	0,01	0,02	0,03	0,02	–	–
	3'-Sialylactose	3'-SLN	633	9	1,00	1,16	0,71	0,29	0,48	0,38	0,69	0,44	1,75	1,73	0,53	0,33
	6'-Sialyllactose	6'-SLN		10	0,1	0,15	0,07	0,03	0,12	0,06	0,12	0,03	0,23	0,09	0,09	0,02
	6'-sialyl-Hex[Ac]-HexNAc		716	11	tr	tr	tr	tr	0,1	0,05	0,01	0,06	0,02	0,03	tr	0,02

(Table 1. continued)

Degree of polymerization	EMO structures	Abbreviation	MW	Peak CE-LIF (Figure 1)	CONCENTRATION OF EQUINE MILK OLIGOSACCHARIDES											
					Dutch Warmblood					Crossbred Arabian/ New Forest ponies				Shetland pony	Friesian	
					A1	A2	A3	A4	B1	B2	B3	B4	B5	C1	D1	D2
ACIDIC																
TETRAMER	Disialyllactose	DSL	924	12	0,16	0,13	0,04	0,02	0,09	0,06	0,21	0,02	0,4	0,23	0,13	0,07
PENTAMER	**Sialyllacto-N-tetraose-a**	LSTa	999	13	0,02	0,03	0,06	0,02	0,27	0,16	0,83	0,16	0,06	0,07	0,05	tr
HEXAMER	**Disialyllacto-N-tetraose***	DS-LNT	1290	14	0,02	tr	tr	tr	0,15	0,06	0,03	0,05	0,04	tr	tr	0,03
TOTAL OLIGOSACCHARIDE CONCENTRATION					2,75	4,01	1,94	1,42	3,02	1,81	4,21	2,19	6,71	4,63	2,48	1,76
AVERAGE OLIGOSACCHARIDE CONCENTRATION PER BREED						2,52					3,58			4,63	2,12	

na= peak not assigned in CE-LIF and quantified by HILIC-MS^n, ++= 0.001-0.002; += 0.001-0.0002 g/L; tr=detectable but not quantifiable; — = not detected, * = isomer not further specified. Structures in **bold:** oligosaccharides novel for the equine milk literature.[1-3] .

The LST molecule, as found in the mare colostrum samples, was digested by Sialidase S (Figure 4f), proving the presence of a terminal N-acetylneuraminic acid α 2-3 linked. Moreover, the identification of LST-a was confirmed by its migration time in CE, similar to commercially available LST-a standard (Table S1, supporting information).

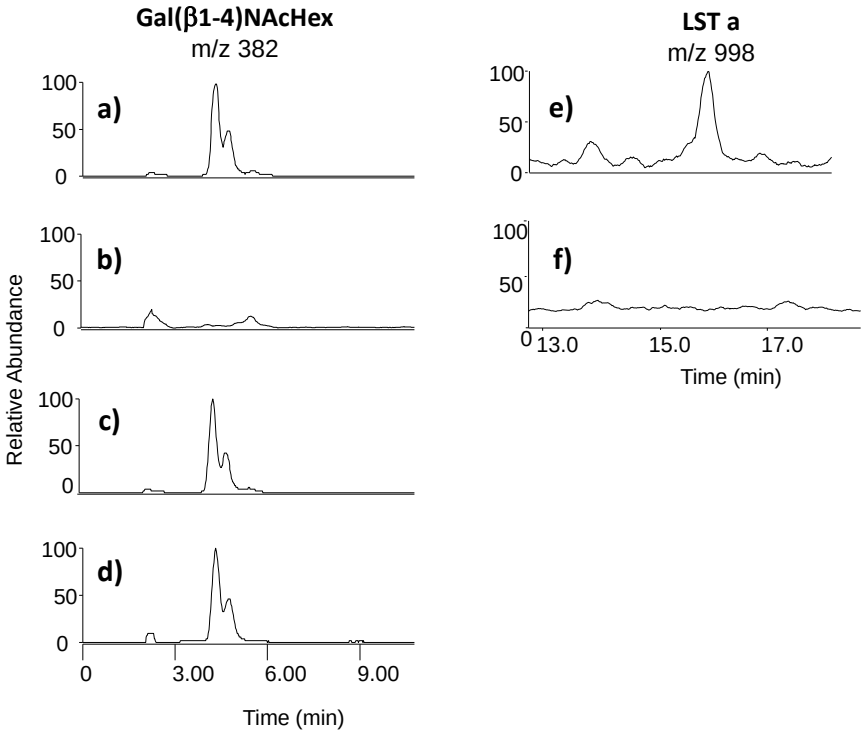

Figure 4. Selected base peak for the Gal(β1-4)HexNAc in HILIC- MS^n (a) as present in lactose-free EMO mixture. Corresponding base peak after degradation by Glyco-galactosidases specific for β1-4 linkage (b), by Glyco-galactosidases specific for β1-3 linkage (c), by Glycoβ–N-acetylhexosaminidase (d). Selected base peak for the 3'-sialyllacto-N-tetraose-a (LSTa) in HILIC- MS^n as present in lactose-free EMO mixture (e). Corresponding base peak after degradation by Sialidase S specific for the α2-3 linkage (f). m/z 382 precursor ion for Gal(β1-4)HexNAc in HILIC-MS^n; m/z 998 precursor ion for LSTa in HILIC-MS^n.

Through the combination of CE and HILIC mass analysis and enzyme degradation of the SEC pools, it was possible to identify 16 EMOs. All identified EMOs are listed in Table 1, and an overview of methods used in order to characterize them (CE migration time, mass over charge values, and retention times in CE-MS^n, and HILIC-MS^n, and enzymes) are described in supporting information (Table S1). Comparing the outcomes of this study with previous data, seven EMOs were not reported previously, and were presented in Table 1 in

bold: the neutral oligosaccharides Gal(β1−4)HexNAc, Gal(β1-4)Hex-Hex, β4'-galactosyllactose, and lactose-N-hexaose, as well as the acidic oligosaccharides 6'-sialyl-Hex-Ac-HexNAc, LST-a, and disialylacto-N-tetraose (isomer not further specified).[1, 10] Full identification was possible for six of the novel EMOs, while partial identification was possible for three of the novel EMOs. For 6'-sialyl-Hex-Ac-HexNAc, the presence of an additional acetyl group (Ac) was confirmed by mass fragmentation. An additional acetyl group linked to acidic milk oligosaccharides has previously been reported in equine colostrum.[1] The additional acetyl group was reported to be linked to the sialic acid group, while in our study it was found to be linked to the hexose moiety.

Comparison of the equine milk oligosaccharides per breed

After annotation of the EMOs, peaks in the CE-LIF electropherogram of the lactose-free EMO mixture were assigned. The peaks assigned were used as a reference for the identification of EMOs in the CE profiles of the 12 colostrum samples of the four horse breeds. In Figure 5, the EMO profiles of the four breeds are shown, including the peak assignments. The CE-LIF electropherograms of the labelled EMOs showed high reproducibility and high peak resolution.

Inter-breed EMOs comparison

The comparison of the EMO profiles for the four breeds (A, B, C and D in Figure 5) reveals that the presence and relative abundance of the different oligosaccharides varied depending on the breed. Quantification was carried out after manual integration of CE-LIF peaks area. For two compounds, Gal(β1-4)Hex-Hex and 3'-sialyl-N-acetyllactosamine, the assignment in the CE-LIF EMO profiles was not possible. For these two molecules the quantification was obtained with HILIC-MS^n. The total concentrations of EMOs varied between the different colostrum samples: 4.63 g/L for the Shetland pony, 1.81-6.71 g/L for the Crossbred Arabian/New Forest ponies, 1.42-2.75 for the Dutch Warmblood horses, and 1.76-2.48 g/L for the Friesian horses (Table 1). Oligosaccharide concentrations found in this study are higher than concentrations found in cow colostrum (about 1 g/L).[1] For other animals, the oligosaccharide concentration has only been reported for mature milks with levels (0.02 to 0.25 g/L), as expected, lower than the values obtained in this study for equine colostrum.[25] Although a large variation in the total concentration of EMOs was observed, it was noticed that the ponies' colostrum samples contained the highest concentration of EMOs, followed by the Dutch Warmblood horses' and the Friesian horses' colostrum samples. Twelve out of the 16 EMOs were present in all colostrum samples. Gal(β1-4)Hex-Hex and 3'-sialyl-N-acetyllactosamine were absent in the Friesian horses' colostrum samples, β4'-galactosyllactose was absent in the Crossbred ponies' colostrum samples, and 6'-sialyl-N-acetyllactosamine was absent in the Dutch Warmblood horses' colostrum samples (Table 1).

In all breeds, the most abundant oligosaccharides were β3'-galactosyllactose, β6'-galactosyllactose, 3'-sialyllactose, and disialyllactose (numbers 2, 4, 9 and 12 in Table 1).

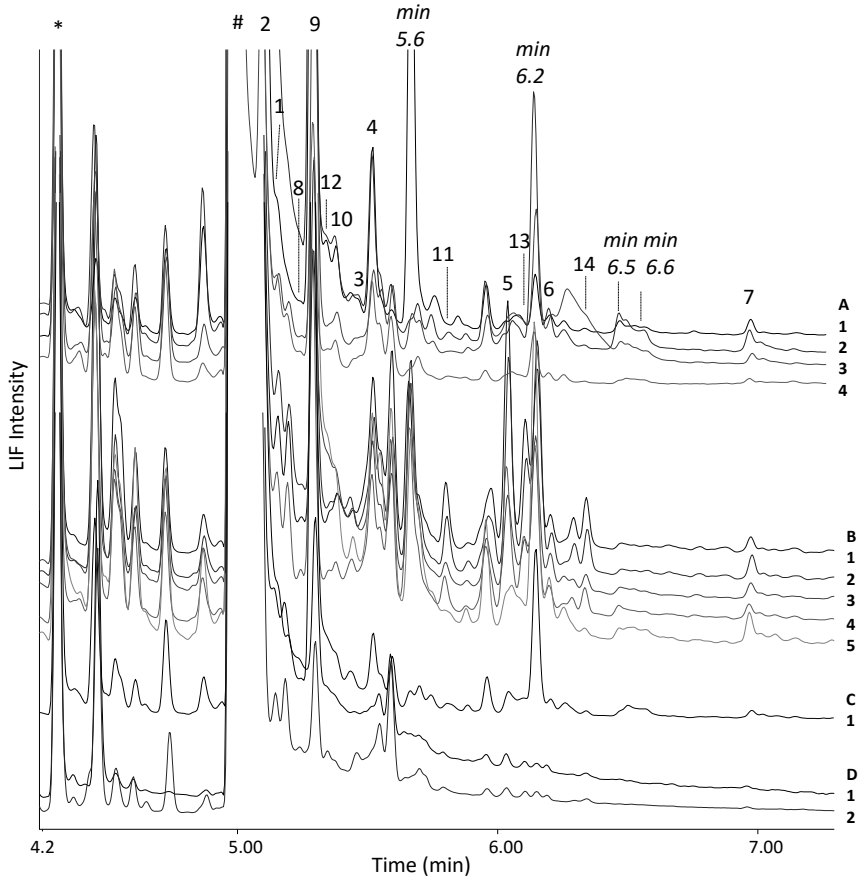

Figure 5. CE-LIF electropherograms of APTS-derivatised oligosaccharides for the four breeds. A: Dutch Warmblood horse; B: Crossbred Arabian/New Forest pony; C: Shetland Pony; D: Friesian horse. *: Internal standard xylose, #: Lactose, 1-14: identified peaks as named in Table 1.

The highest concentrations of β6'-galactosyllactose, β3'-sialyllactose, and disialyllactose were found in the colostrum sample of the Shetland pony (concentrations of 1.91 g/L, 1.73 g/L, and 0.23 g/L, respectively). These values for EMOs present in Shetland pony colostrum are only an indication, since only one colostrum sample was available for the analysis. The highest concentration of the β3'-galactosyllactose was found in the colostrum samples of the Crossedbred ponies, with an average concentration of 0.14 g/L. In profiles A1, and B1-5 (Figure 5) high concentrations of non-characterized EMOs, migrating at 5.6 and 6.2 min., were observed. From the CE migration time it could be suggested that these are neutral

trimers and pentamers, respectively. The unidentified peaks with a retention time of 6.5, and 6.6 min (Figure 5), suggesting acidic or neutral hexamers, were present in all colostrum samples. The oligosaccharide with the lowest observed concentration in all colostrum samples was 3'-sialyl-N-acetyllactosamine with an average concentration of 0.02 g/L.

Intra-breed comparison of EMOs

Evaluating the EMOs profiles within the same breed, differences were also noticed. Specifically, for the Dutch Warmblood profiles, β6'-galactosyllactose and 3'-sialylactose (numbers 2 and 9 in Table 1) were the most abundant oligosaccharides and their concentrations varied by a factor 3.2 and 4, respectively. β6'-galactosyllactose was present with a concentration of 0.65-2.08 g/L, while 3'-sialylactose with was present with a concentration of 0.29-1.16 g/L. Looking at the overall electropherograms, the Crossbred ponies' colostrum samples contained the same EMOs structures, although they highly varied in concentration. β4'-Galactosyllactose, however, was not detected in three colostrum samples (B3-5, Table 1). In the Crossbred ponies' colostrum samples, similarly to the Dutch Warmblood horses' colostrum samples, the most abundant oligosaccharides were the β6'-galactosyllactose and the 3'-sialylactose. The β6'-galactosyllactose was present in a concentration of 0.55-3.15 g/L, while the 3'-sialylactose with was present in a concentration of 0.38-1.75 g/L, exhibiting a 5.7 and a 4.6 factor, respectively. Intra-breed analysis showed relatively high variation in EMOs concentration (Table 1). Most probably the variation in EMOs concentration could be dependent on the intrinsic variation in composition and in quality of colostrum among individuals, as shown before for cow milk and colostrum.[26, 27]

Profile of equine milk oligosaccharide structures in comparison with those in milk of other mammals

Ten out of the 16 EMOs found in this study were also found in milk samples from other domestic animals (Table 2). So far, β6'- and 3'- galactosyllactose, 3'-sialylactose, and 3'-sialyl-N-acetyllactosamine have been found in bovine, caprine, and ovine milk, while 6'-sialyllactose and disialyllactose have been found both in bovine and caprine milk.[10, 28] Lacto-N-hexaose was characteristic for caprine milk, while β4'-galactosyllactose, lacto-N-novo-pentaose I, and 6'-sialyl-N-acetyllactosamine were present in bovine milk.[10, 11, 28] Ten out of 16 milk oligosaccharide structures present in equine colostrum were previously reported to be present in human milk: the neutral trimers, tetramer, and hexamer, and the acidic 3'-, 6'-sialyllactose, tetramer, pentamer, and hexamer.[12, 22] The presence of milk oligosaccharides carrying the bifidogenic factor (Gal(β1-3)GlcNAc) (number 6, 7, and 13 in Table 1) and the presence of eight EMOs decorated with sialic acids suggest bioactivity of the EMOs, such as being bifidogenic and soluble ligands for intestinal pathogens.[5, 29-32] In this study, EMOs

decorated with N-glycolylneuraminic acid (Neu5Gc) were not detected, while they were reported to count for less than 1% in previous study.[1]

Table 2. EMOs found in the Dutch Warmblood horses' colostrum and their presence in other mammalian milk samples.

DEGREE OF POLYMERIZATION	EMO structures (Dutch Warmblood milk)[a]	MILK			
		COW[b]	SHEEP[c]	GOAT[d]	HUMAN[e]
NEUTRAL EQUINE MILK OLIGOSACCHARIDES					
DIMER	**Gal(β1-4)HexNAc**	–	–	–	–
	Gal(β1-4)Hex-Hex	–	–	–	–
TRIMER	β6'-Galactosyllactose	X	X	X	X
	β4'-Galactosyllactose	X	–	–	X
	β3'-Galactosyllactose	X	X	X	X
TETRAMER	Lacto-N-neotetraose	–	–	–	X
PENTAMER	Lacto-N-novo-pentaose I	X	–	–	–
HEXAMER	**Lacto-N-hexaose**	–	–	X	X
ACIDIC EQUINE MILK OLIGOSACCHARIDES					
TRIMER	6'-Sialyl-N-acetyllactosamine	X	–	–	–
	3'-Sialyl-N-acetyllactosamine	X	X	X	–
	3'-Sialylactose	X	X	X	X
	6'-Sialyllactose	X	–	X	X
	6'-Sialyl-Hex-Ac-HexNAc	–	–	–	–
TETRAMER	Disialyllactose	X	–	X	X
PENTAMER	**Sialyllacto-N-tetraose-a**	–	–	–	X
HEXAMER	**Disialyllacto-N-tetraose***	–	–	–	X

X = present; – = not present, * = isomer not further specified. **Structures in bold:** oligosaccharides novel for the equine milk literature.[1-3] a = Ref. [1, 3, 12]; b = Ref. [11, 12]; c = Ref [12, 28]; d = Ref. [12, 28]; e = Ref. [12, 22].

The presence of Neu5Gc linked to milk oligosaccharides is not rare in oligosaccharides derived from domestic animals. For example, such oligosaccharides account for about 6% of the oligosaccharides in cows' colostrum.[1] On the contrary, milk oligosaccharides decorated with Neu5Gc are absent in human colostrum, which is considered as beneficial since blood

antibodies against Neu5Gc are present in several diseases such as acute and chronic hepatitis, hyperacute transplant rejection, and cancer. [12, 32-34]

In summary, oligosaccharide profile of colostrum from horses showed significant differences between breeds as well as per individual. EMOs identified showed quite some overlap with oligosaccharides present in milk from human and other mammals, although also horse specific structures were identified. The presence of EMOs carrying the bifidogenic factor and/or the N-acetylneuraminic acid, together with low concentrations of EMOs with N-glycolylneuraminic acid, could make equine colostrum a good source for further research into in vitro bioactive effects of milk oligosaccharides.

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Supporting information

Table S1. analytical techniques used for the identification of EMOs in the Dutch Warmblood horses' colostrum, and liquid chromatography and mass characteristics of EMOs.

DEGREE OF POLYMERIZATION	EMOS structures (Dutch Warmblood milk)	ANALYTICAL TECHNIQUES						ENZYMES[#]
		CE-LIF Migration Time (minutes)		CE-MS^n		HILIC-MS^n		
		HMOS	Oligosaccharides standard	m/z (with z = -3)	t_R (minutes)	m/z (with z = -1)	t_R (minutes)	
NEUTRAL EQUINE MILK OLIGOSACCHARIDES								
DIMER	**Gal(β1-4)HexNAc**					382	4.74	Glyco(β1-4)galactosidase
TRIMER	**Gal(β1-4)Hex-Hex**					503	11.05	Glyco(β1-4)galactosidase
	β6'-Galactosyllactose		5.08			503	10.18	
	β4'-Galactosyllactose		5.45	314	30.55-32.06	503	9.85	
	β3'-Galactosyllactose		5.52			503	10.54	
TETRAMER	Lacto-N-neotetraose	6.05	6.05	382	30.73			
PENTAMER	Lacto-N-novopentaose I	6.20		435	32.25	869	16.59	
HEXAMER	**Lacto-N-hexaose**	6.95	6.95	503	35.38	1072	18.22	
ACIDIC EQUINE MILK OLIGOSACCHARIDES								
TRIMER	6'-Sialyl-N-acetyllactosamine		5.25			673	11.2	Sialidase T
	3'-Sialyl-N-acetyllactosamine					673	9.46	Sialidase S
	3'-Sialyllactose	5.30	5.3	357	31.55	632	11.36	Sialidase S
	6'-Sialyllactose		5.4			632	12.58	Sialidase T
	6'-Sialyl-Hex-Ac-HexNAc					715	5.93	Sialidase T
TETRAMER	Disialyllactose					923	15.14	Sialidase T
PENTAMER	**Sialyllacto-N-tetraose-a**		6.1			998	16.84	Sialidase S
HEXAMER	**Disialyllacto-N-tetraose***	6.35						

t_R = retention time, * : isomer not further specified; [#] : enzymes used for confirmation of oligosaccharides linkage type. Enzyme descriptions are given in material and methods section. Structures in **bold**: oligosaccharides novel for the equine milk literature.

Chapter 3

Milk oligosaccharide variation in porcine milk and their fermentation in piglet intestine

Abstract

Porcine milk oligosaccharides (PMOs) were analyzed in six colostrum and two milk samples from Dutch Landrace sows. In total, 35 PMOs were recognized of which 13 were novel for the PMO literature: neutral HexNAc-Hex, β4'-galactosyllactose, putative GalNAc(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)GlcNAc(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. Inter-individual variation regarding PMO presence and concentration was observed between porcine milks. Moreover, during 1 week lactation period, a 43% decrease of the major PMOs was found. In particular, Gal(α1-3)Gal(β1-4)Glc, 3'-sialyllactose and sialyllacto-N-hexaose (isomer not further specified) decreased. Some other PMOs such as β3'-galactosyllactose, lacto-N-neotetraose and sialyl-lacto-N-tetraose-a increased in concentration. PMOs were also monitored in fecal samples of suckling piglets. In feces of 1-2 days old piglets few intact PMOs were found, indicating considerable PMO fermentation at early stage of life.

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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.[1, 5, 7, 8] 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 pig brain shows similarities with that of human infants, suggesting dietary sialic acid contribution to 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 decrease from about 24 to 12 g/L, while in bovine milk, oligosaccharides concentration decreased 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's milks.[1, 7, 17]

Bio-activity of MMOs is suggested to be closely related to their structure.[5] Oligosaccharide composition in milk varies due to diet, health, lactation stage, 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 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 were investigated, with attention to PMO inter-individual variation and changes in PMO abundance during 1 week of sow lactation. The fate of PMOs in the intestine of piglets after 1-2 days and 1 week of nursing was also investigated. PMOs were characterized and quantified using a combination of capillary electrophoresis with fluorescence or using mass spectrometry detection and liquid chromatography with mass spectrometry 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, USA). Lacto-N-tetraose, β3'-, β4'- and β6'- galactosyllactose were bought from Carbosynth (Compton, UK). Lactose-N-neotetraose, 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, USA). All other chemicals were of analytical grade. Millipore water (Millipore, Darmstadt, Germany) was referred to in the text as water.

Colostrums and fecal samples

Eight milk samples from Dutch-Landrace sows were collected within 0.5-2 days and at 1 week *postpartum*, and frozen (-20^{o}C) until use. Milk samples collected within 0.5-2 days postpartum are referred to as colostrum, while milk samples collected at 1 week *postpartum* are referred to as mature milk. Two colostrum samples were donated by Animal Nutrition (ANU) group (Wageningen University, Wageningen, The Netherlands), while six samples, of which 4 colostrum and 2 mature milk samples, matching the corresponding colostrum samples, were donated by proefaccommodatie de Tolakker (Utrecht University, Utrecht, The Netherlands). Individual fecal samples were collected from three piglets, 1-2 days and 1 week old (proefaccommodatie de Tolakker), and stored at -80 ^{o}C until use.

Extraction, purification and fractionation of PMOs

Milk samples

In order to obtain a representative sample containing PMOs, colostrum samples (5ml) were pooled and the carbohydrates were extracted as reported previously.[1] After carbohydrate extraction, solutions were freeze-dried. Two methods were used in order to remove lactose from the extracted carbohydrates: size exclusion chromatography (SEC) for qualitative analysis and solid phase extraction (SPE) for quantitative analysis.

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, USA) 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 PMOs mixture with reduced lactose content (lactose-free PMOs). Another part of SEC pools, as well as lactose-free PMOs were labeled with 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 (1mL of 1 mg/mL solution) were loaded onto an activated graphitized carbon cartridge (150 mg bed weight, 4 mL tube size; Grace, Deerfield, IL, USA). Activation of the SPE cartridges, lactose removal and oligosaccharides elution was performed as reported previously.[1] The fractions obtained containing PMOs were dried overnight under a stream of nitrogen and afterwards 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 (4ºC) and afterwards they were centrifuged (15 min, 15000*g*, 4ºC). The supernatants were collected and filtered through a 0,22 μm cellulose acetate membrane (GE Healthcare). Fecal enzyme inactivation was performed by boiling the solutions (5 min at 100ºC), and samples were purified using SPE as reported elsewhere.[18] The fractions obtained from fecal samples were dried overnight under a stream of nitrogen and afterwards 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 9-aminopyrene-1,4,6-trisulfonate (APTS) and analyzed by CE-LIF as reported elsewhere.[19] CE-LIF peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA, USA). APTS-labeled PMOs were quantified using xylose as internal standard. A linear correlation between CE-LIF peak areas and oligosaccharide molar concentrations has been demonstrated previously.[1, 19] After manual peak integration, the peak areas were converted into the corresponding PMO concentrations (g/L).

Capillary electrophoresis with mass spectrometer 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, USA). The CE-MS^n analysis was performed as previously reported.[19]

Hydrophilic interaction liquid chromatography with mass spectrometry detection (HILIC-MS^n)

In order to analyze PMOs present in minor abundances in the milk samples, non-labeled SEC PMO pools were analyzed by HILIC-MS^n as previously described, with some modification.[1] The mobile phases used were: A = water + 1% (v/v) acetonitrile (ACN), B = ACN, 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 85-60% B; 31-35 min from 60-40% B; 35-36 min isocratic 40% B; 36-36.1 min from 40-85% B; 36.1-45 min isocratic 85% B. The mobile phase C was kept constant at 5% during the entire gradient. The auto-sampler 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 *m/z* range of 300-2000. The mass spectrometer was tuned using maltotriose (0.3 mg/mL) in 70:30 (v/v) ACN/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 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 1, where the novel 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 novel: neutral HexNAc-Hex, β4'-galactosyllactose, putative GalNAc(α/β1−3)Gal(β1−4)Glc, lacto-N-fucopentaose-II, lact-N-tetraose, galactose linked to lacto-N-neohexaose, lacto-N-hexaose and difucosyl-lacto-N-hexaose, and acidic Neu5Ac(α2-6)GlcNAc(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 1) 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 is 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 24% of all PMO structures and about 70% of HMOs.[20] Fucosylated PMOs are numbers 2, 3, 8, 13, 14, 19, 32 in Table 1.[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] 19 of the 35 PMOs showed structural overlap with structures present in human milk, as indicated in Table 1. Eight PMOs carried the so-called bifidogenic factor (Gal(β1-3)GlcNAc) and, therefore, were considered potential prebiotic (PMOs numbers 10, 13, 14, 16, 19, 27, 28, 32 in Table 1).[20] An overview of PMO characteristics is presented in Table 2. One molecule of lactose carrying a phosphorylated group was observed (PMO number 35 in Table 1). 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, 21] This is the first time that it has been identified in porcine milk.[7, 16, 17]

Table 1. Overview of porcine milk oligosaccharides found in this study.

Milk oligosaccharides in porcine milk				
No.	proposed structure	molecular weight	Ref. PMOs	Ref. HMOs
	neutral			
1	**HexNAc-Hex**	383		
2	2'-FL	488	*7*	*20*
3	3'-FL	488	*7*	*20*
4	β3'-GL	504	*7, 16*	*20*
5	**β4'-GL**	504	*16*	*20*
6	β6'-GL	504	*7*	*20*
7	*Gal(α1-3)Gal(β1-4)Glc*	504	*7*	
8	3'-FLN	529	*7*	
9	**GalNAc(α/β1-3)Gal(β1-4)Glc**	545		
10	**LNT**	707		*20, 22*
11	LNnT	707	*7, 16*	*20, 22*
12	*Novo-LNP-I*	869	*7, 16*	
13	**LNFP- II**	852		*20, 22*
14	LNFP- III/V	852	*7*	*20, 22*
15	**Gal-LNnH**	1234		
16	**LNH**	1072		*20, 22*
17	LNnH	1072	*7, 16*	*20*
18	Gal(α1-3)+Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)]Gal(β1-4)Glc	1031	*7*	
19	**LNDFH**	1364		*20*
	sialylated			
20	3'-SL	633	*7, 16*	*20*
21	6'-SL	633	*7, 16*	*20*
22	3'-SLN	674	*7, 16*	
23	6'-SLN	674	*7, 16*	
24	Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc	795	*7*	
25	Neu5Ac(α2-6)GlcNAc(β1-6)Gal(β1-4)Glc	836	*7*	
26	**Neu5Ac(α2-6)GlcNAc(β1-3)Gal(β1-4)Glc**	836		
27	**LSTa**	998		*20*
28	**LSTb**	998		*20*
29	LSTc	998	*16*	*20*
30	**Neu5Ac-Neu5Ac-Hex-Hex-Hex**	1086		
31	*S-novo-LNP-I*	1160	*7, 16*	
32	**S-LNFP-II**	1307		
33	S-LNH*	1363	*7, 16*	*20*
34	FS-LNH*	1509	*16*	*20*
	phosphorylated			
35	**P+lactose**	421		

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

Table 2. Overview of characteristics of porcine milk oligosaccharides.

Pig milk oligosaccharides	
No. structures identified	35
No. new structures identified	13
neutral structures	54%
sialylated structures	43%
fucosylated structures	23%
phosphorylated structure	3%
structural overlap with HMOs	54%

HMOs = human milk oligosaccharides; percentages refer to total number of structures identified.

PMO pattern for different porcine colostrum samples

The CE-LIF has been recognized as a suitable method for oligosaccharide quantification due to its good peak resolution and separation. Therefore, CE-LIF was chosen for quantification of PMOs in porcine colostrum samples.[19] In the CE-LIF profile, 17 peaks were annotated (Figure 1) using migration times of available oligosaccharides standards, of characterized PMO-SEC pools (current study) and of previously characterized HMOs.[22] 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.[23] The most abundant acidic PMO was 3'-sialyllactose (3'-SL), similarly as in horse and cow colostrum.[1, 21, 24] 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, 25] Using the same annotation as in Figure 1, peaks were identified in Figure 2 for all 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 quantified 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, 26] Acidic PMOs represented the most abundant structures,

accounting for 77% of the total amount of PMOs quantified. This finding correspond with previous data, in which 82% peak abundance was reported for acidic PMOs.[16]

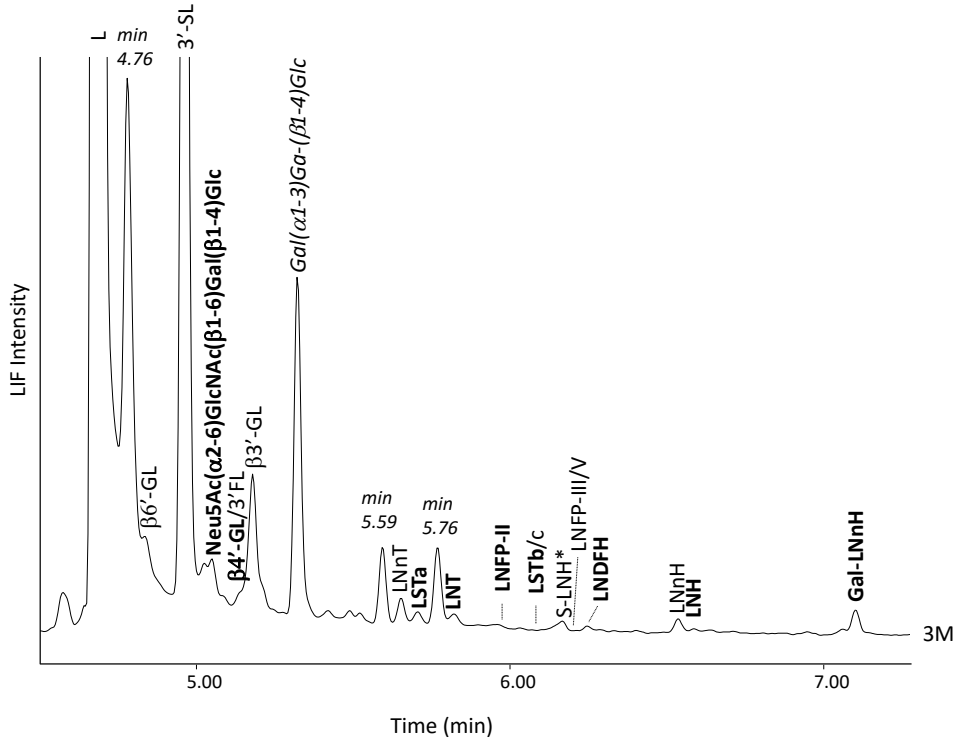

Figure 1**.** CE-LIF electropherogram of PMOs in porcine colostrum (3M). * = PMOs isomer not further specified, structures in *Italic* = putative isomeric PMOs structure. Structures in **bold**: PMOs novel for the porcine milk literature.[7, 16, 17]. L= lactose, β3'-, 4'-, 6'-GL= β3'-, 4'- ,6'-Galactosyllactose, 3'-FL = 3'-fucosyllactose, LN(n)T = lacto-N-(neo)tetraose, LNFP-II, III, V = lacto-N-fucopentaose-II, III, V, LSTa, b, c = sialyl-lacto-N-tetraose-a, -b, -c, LNDFH = lacto-N-difucohexaose, S-LNH = sialyllacto-N-hexaose, LN(n)H = lacto-N-(neo)hexaose, Gal-LNnH= galactose-sialyllacto-N-neohexaose.

The relative 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, 27] In all porcine 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 quantified 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, 25]

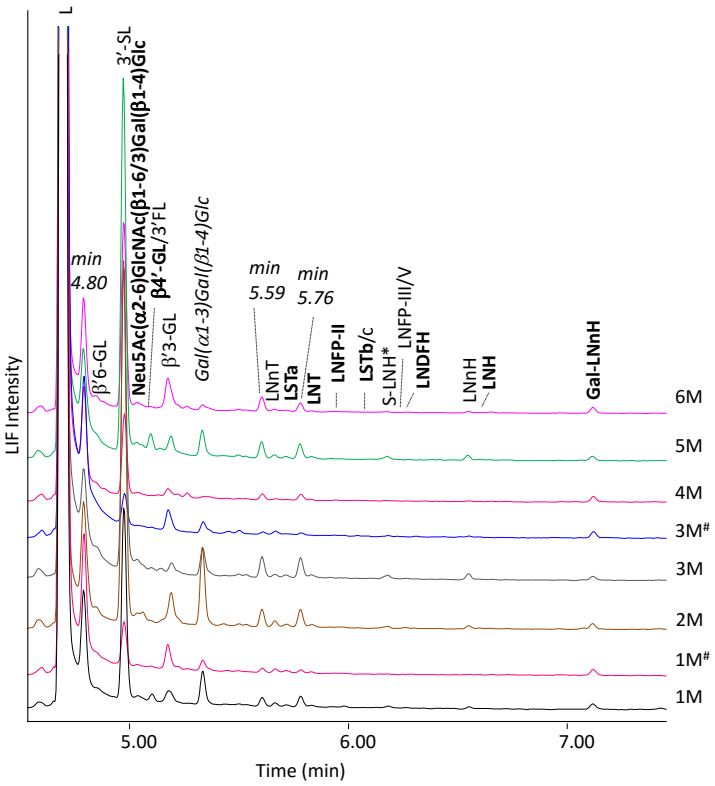

Figure 2. CE electropherograms of PMOs from six porcine milk samples. 1-6 M= porcine colostrum, 1 and 3 M$^{\#}$ = porcine mature milks. * = PMOs isomer not further specified, structures in *Italic* = putative isomeric PMOs structure. Structures in **bold**: PMOs novel for the porcine milk literature.[7, 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, V = lacto-N-fucopentaose-II, III, V, LSTa, b, c = sialyl-lacto-N-tetraose-a, -b, -c, LNDFH = lacto-N-difucohexaose, S-LNH = sialyllacto-N-hexaose, LN(n)H = lacto-N-(neo)hexaose, Gal-LNnH = galactose-sialyllacto-N-neohexaose.

Variation of PMOs during lactation

Having two porcine mature milks available matching the corresponding colostrum samples, a first inventory comparing PMO composition of colostrum and mature milk was conducted (1M-1M$^{\#}$ and 3 M-3M$^{\#}$, for colostrum and mature milk, respectively, Table 3). Overall, the PMO concentrations decreased from 11.86-12.19 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 days of lactation.[17, 26] In those studies no information on the decrease of individual oligosaccharides was reported.[15, 28]

Table 3**.** Presence and concentration (g/L) of PMOs in colostrum and mature milk (#) of six sows.

PMOs concentration in colostrum and mature milk								
	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(α1-3)Gal(β1-4)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(α2-6)GlcNAc(β1-6)Gal(β1-4)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	11.86	6.98	29.35	12.19	6.82	7.38	23.28	14.87

* = PMOs isomer not further specified, structure names in *Italic* = putative isomeric PMO structure. Structures in **bold**: PMOs novel for the porcine milk literature.[7, 16, 17] ; – = not present.

In the current study, levels of individual PMOs highly varied depending on the milk analyzed. Nevertheless, abundance of some PMOs showed comparable trend in concentration from colostrum to mature milk samples: putative (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, 29] 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 days *postpartum*.[15]

Fermentation of PMOs

In order to investigate PMO fermentation, fecal samples from piglets were collected after 1-2 days and after 1 week of nursing. Oligosaccharides present in fecal samples were analyzed by CE-LIF and HILIC-MS^n and their levels were 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}, f-6_{a-c}) fed for 1 day on the corresponding porcine colostrum (1M and 6M) are shown. CE profiles of oligosaccharides of fecal samples showed only five intact PMOs: 3'-SL, LSTa, LNT, LNnT and LNFP-II, with quite some individual variation in

content. Next to the identified PMOs, several oligosaccharides with DP3 and DP4, as suggested from their CE peak migration times (min 5-6, Figure 3), were observed.

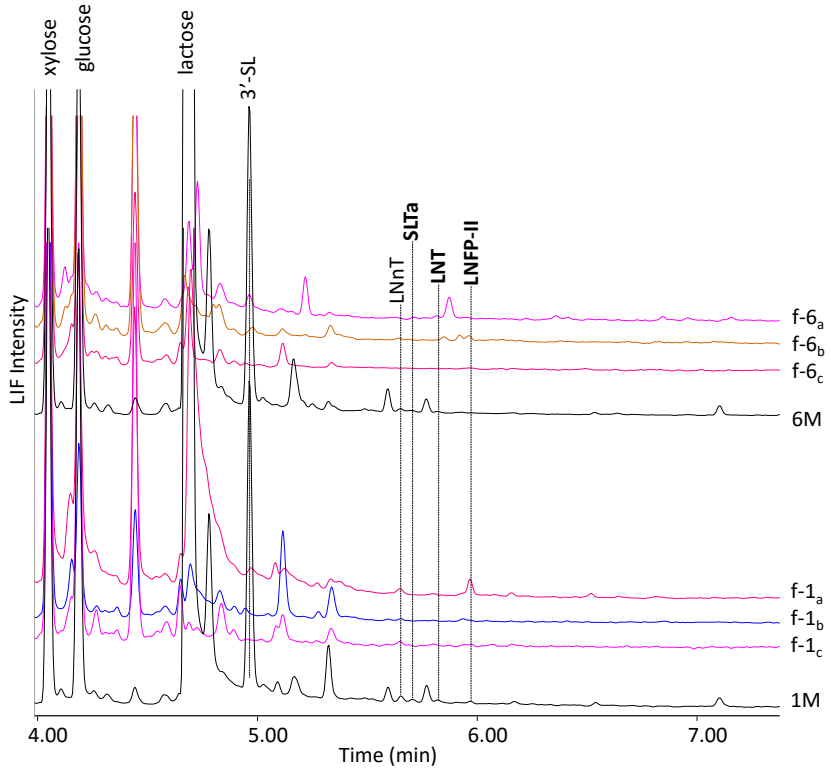

Figure 3**.** CE electropherograms of fecal PMOs (f-$1_{a\text{-}c}$, and f-$6_{a\text{-}c}$) from 3 piglets fed 1 day on corresponding porcine colostrum (1M, and 6M). Structures in **bold**: oligosaccharides novel for the porcine milk literature.[7, 16, 17] 3'-SL = 3'-sialyllactose, LN(n)T = lacto-N-(neo)tetraose, LSTa, = sialyl-lacto-N-tetraose-a, LNFP-II = lacto-N-fucopentaose -II.

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 fermentation products derived from PMOs. 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.[22] As reported previously, already within 12 h after piglet birth, bacteria colonize the colon of piglets (10^9 -10^{10} bacteria/g colonic content) and within 2 days piglet microbiota could already be established.[30, 31] A decrease of about 41% in CE-LIF peak area was found for oligosaccharides present in feces of 1 week 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 week 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 quantified individually showing high inter-individual PMO variation, both regarding their presence and abundance in porcine milk samples. 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 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 few intact PMOs, which are not present anymore in fecal samples of 1 week old piglets. Fecal oligosaccharides different from PMOs were recognized, indicating PMO intestinal fermentation products.

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Chapter 4

Oligosaccharides in urine, blood and feces of piglets fed on milk replacer containing galacto-oligosaccharides

Abstract

Human milk oligosaccharides (HMOs) are absorbed into the blood (about 1% of the HMOs intake) and subsequently excreted in urine, where they may protect the infant from pathogen infection. As dietary galacto-oligosaccharides have partially structural similarities with HMOs, this study investigated the presence of galacto-oligosaccharides (GOS) and oligosaccharides originating from milk replacer in blood serum, urine, cecal and fecal samples of piglets, as model for 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 16-23 □g/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.

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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 were observed in blood of infants.[3] Literature suggest a protective function of HMOs in the blood circulation by influencing leucocyte adhesion to endothelial cells and platelets-neutrophils interaction.[4-6] When systemic HMOs are cleared, they are excreted into the urinary system, thus being detectable in urine.[5-8] Next to lactose, 13 HMOs, both neutral and acidic, have been found in urine of infants.[6] 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.[4, 5] Around 99% of the HMOs reach the colon of infants, where they can be fermented by the colonic microbiota.[9, 10] In the intestine, HMOs can exert prebiotic, immuno-modulatory and anti-infective functions.[11] To date, HMOs are not produced in the food-grade volumes required for infant nutrition. Therefore, other dietary ingredients that promote health and wellbeing, and reduce the risk of diseases are of broad public interest.[12] Non-digestible oligosaccharides, such as galacto-oligosaccharides (GOS), belong to these health promoting ingredients. Multiple preclinical studies have shown that GOS is a prebiotic fiber that selectively stimulates the growth of beneficial gut bacteria.[13-18] Different studies have shown that daily ingestion of GOS increases beneficial bacteria, such as bifidobacteria, in the colon of adults and infants.[13, 16, 19] It is hypothesized that GOS lowers the gastrointestinal pH and reduces the survival of pathogens.[13, 20-22] Moreover, GOS fermentation by intestinal microbiota leads to the production of short chain fatty acids, including butyrate.[17] Butyrate is known for decreasing inflammation, carcinogenesis and oxidative stress in the intestine.[23, 24] GOS are also acting as soluble ligands for pathogens, inhibiting for example the binding of *Escherichia coli* and *Salmonella typhimurium* to the intestinal mucosa layer.[25] In addition, GOS are associated with a lower risk of infections and diarrhea due to direct effects on the intestinal immune system.[17] Moreover, the microbiota of infants fed on formula enriched with GOS or galacto-/fructo-oligosaccharides (FOS) resembled more the microbiota of infants breast-fed, than microbiota of infants fed on GOS/FOS-free formula.[26] 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, 27, 28] GOS are industrially produced from lactose by β-galactosidase enzyme of microbial or fungal origin.[12] 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.[13] GOS mixtures consist of galactose oligomers with a terminal glucose, varying in degree of polymerization (DP) and type of glycosidic linkage(s).[13] The DP ranges mostly between 2 to 8.[21] 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.[13, 17] Although beneficial health effects of GOS have been shown, GOS *in vivo* fate is not completely known.[17, 25] In the current study, a pig model was used to investigate 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 days feeding trial.[29]

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, U.K.), (1-4)-β-D-galactobiose (Megazyme, Bray, Ireland), D-(+)-lactose, 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). Specification by the supplier were: dry matter content of 75%, of which 59% 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.[30] Sodium cyanoborohydride, trifluoracetic acid (TFA) and ammoniumhydroxide 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 in the text to 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.[29] In short, 40 Landrace × Yorkshire piglets, separated from the mother sows 36-48 h *postpartum*, 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), allocated *ad random* 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 number: 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 matrixes, were tested on serum samples from 2 pigs (Faculty of Veterinary Science, Utrecht University) not belonging to the feeding trial. The 2 pigs were fed on a diet different from Milkiwean Babymilk with no GOS supplementation. A part of the 2 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, 1mg/mL) were centrifuged (20000g, 5 min., 20 ºC) and the supernatants were analyzed. Oligosaccharide extraction from feces and cecal digesta was performed as described elsewhere, with minor modifications.[31] Briefly, watery slurries of cecal digesta and fecal samples (50 mg/mL) were kept overnight at 4 °C under head over tail rotation, in order to optimize the carbohydrate extraction. Next, the samples were placed into centrifugal filter unit (0.22 μm PVDF, Merck Millipore) and centrifuged (20000*g*, 15 min, 4 °C). 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 water. Piglet formula Milkiwean Babymilk Yoghurt (2 mg) was suspended in water (1 mg/mL)

and subsequently was 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 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) trifluoroacetic acid (TFA), followed by a washing step with 1.5 mL water, as reported elsewhere.[32] 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 re-solubilized in 500 µL water (Vivinal GOS, milk replacer, urine, feces and cecal digesta) or in 200 µL 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 3 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).[30] Five nanomoles of xylose were added as internal standard to 100 µL of sample. According to 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.[33]

Analysis of oligosaccharides by Hydrophilic Interaction Liquid Chromatography with mass detection (HILIC-MS^n)

The 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 Corporation, Milford, MA, USA). The flow rate was 300

μL/min and the injection volume was 5μL. 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 condition: 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 auto-sampler and column oven were set at 20 °C 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 were performed on the most abundant ion in the MS and MS^2 spectrum, respectively.

Oligosaccharides estimation in blood and urine of piglets

In order 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.[34] After 3 and 26 days GOS-diet, the average serum volume was estimated to be 151 and 595 ml, respectively, with a piglet body average 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.[35] 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/gram of excreted creatinine, calculated for each analyzed urinary sample.[35] Amount of oligosaccharides in the biological samples was determined using CE-LIF. Quantification of APTS-labeled oligosaccharides was performed converting the peak areas via amount of nanomoles to amount of micrograms.[30] The amount (μg) per DP, and per individual compound was calculated relative to the total amount (μg) of GOS-DP3-6 present in the samples analyzed.

Results and Discussions

Characterization of Vivinal GOS syrup

Vivinal GOS syrup, used as source of GOS in the experimental feeding trial, was characterized prior to piglet samples analysis. GOS ref., GOS-SEC fractions and relevant oligosaccharide standards were APTS-labeled and analyzed by CE-LIF. Comparing GOS-SEC fraction electropherograms with previous literature, GOS peaks were recognized for their degree of polymerization (DP) and were numbered (1-29, Figure 1).[33] 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 based on previous data.[33] The presence of seven GOS-DP3 with a free reducing end is in agreement with previous data.[13] Quantification of GOS having specific DP was performed on APTS-labeled Vivinal GOS syrup. The abundances of GOS-DP2-6 were 40, 24, 11, 4, and <1% (w/w), respectively, in accordance with literature.[13, 33] Hence, it can be concluded that GOS were reliably separated and quantified by CE-LIF, allowing further analysis of biological samples.

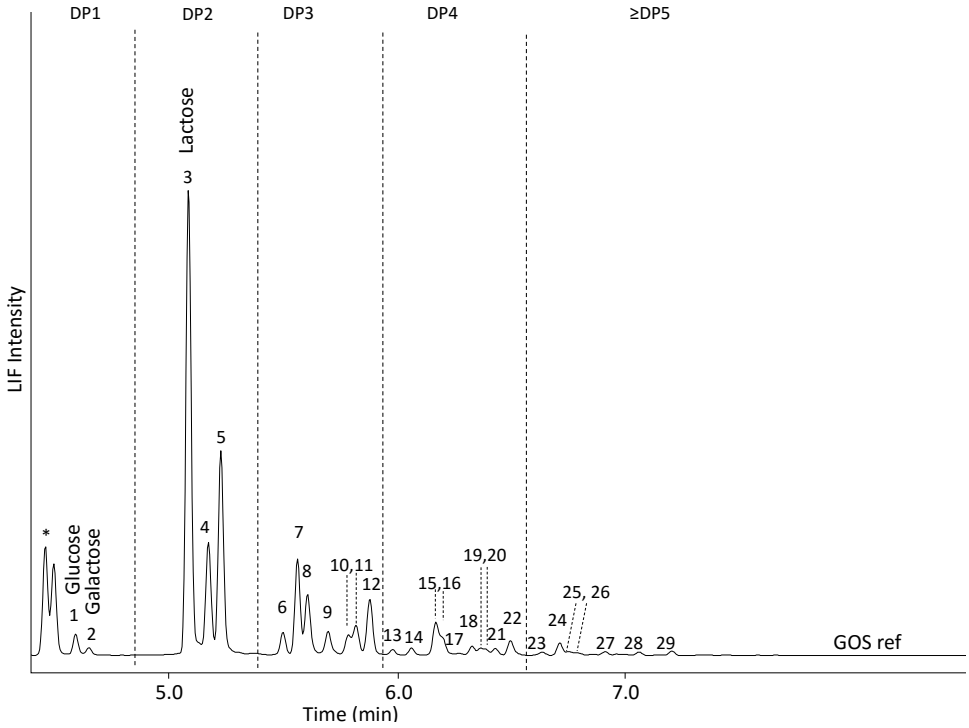

Figure 1. CE–LIF electropherogram of the GOS ref. The detected GOS peaks are numbered from 1-29, and the internal standard xylose was indicated with a star (*). DP = degree of polymerization.

Extraction and detection of GOS in serum samples

Previous studies have indicated that neutral oligosaccharides could be purified by solid phase extraction (SPE) when present in liquid food matrices, urine, digesta and feces, although to our knowledge it was never investigated for serum samples.[32, 33, 36] Therefore, extraction and purification of GOS from the serum of 2 piglets not belonging to the piglet feeding trial were performed prior to serum samples 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 area were compared and a recovery of ~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 is a successful method to extract and purify GOS from serum samples prior to CE-LIF analysis.

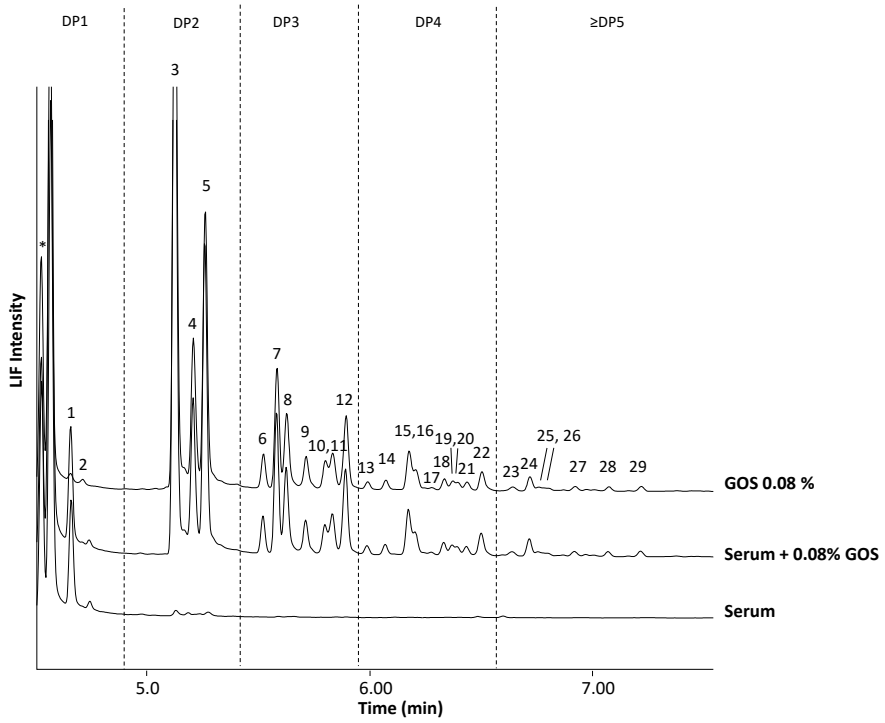

Figure 2. CE-LIF electropherograms of oligosaccharides from piglet serum, from piglet serum spiked with 0.08% of GOS, and from GOS 0.08%. The electropherograms were normalized on the internal standard xylose (*) and GOS peaks were numbered from 1 to 29 as in Figure 1. DP = degree of polymerization.

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 piglet 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. Both in S-A1 and S-B1 sample, peaks representing reducing oligosaccharides were observed: in S-A1, besides dimers including

lactose, peaks were assigned specifically to GOS structures (numbers 6-29, Figure 3). Peak numbers 6-12, 14-21, and 23-29 in Figure 3 were assigned to GOS-DP3, -DP4, and ≥ -DP5, respectively. Abundance of the detected GOS will be discussed below.

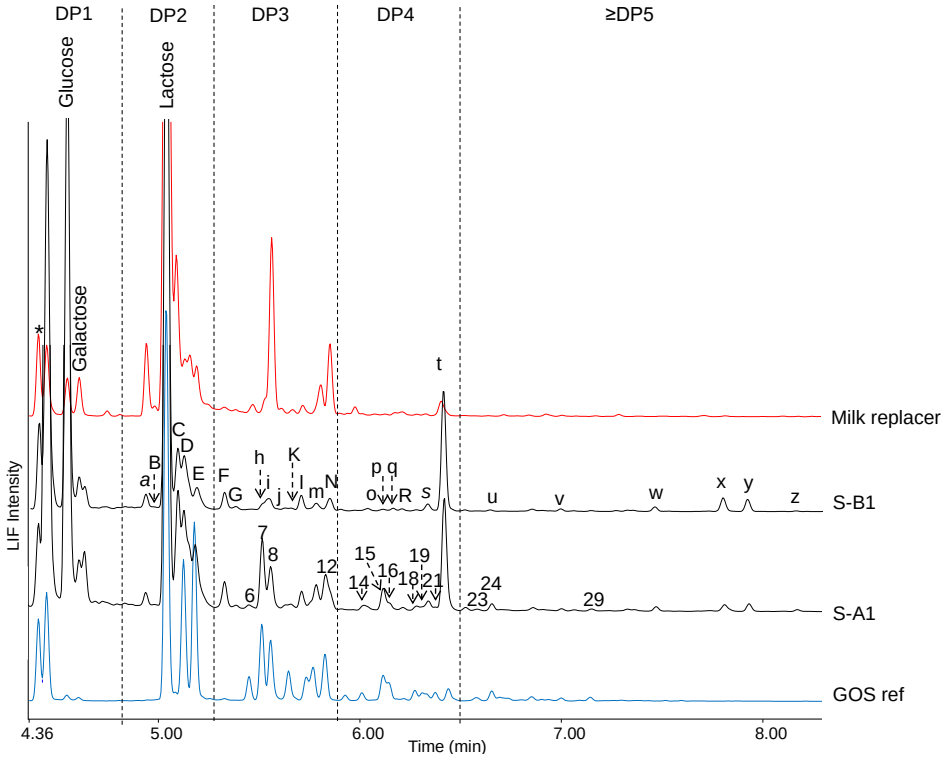

Figure 3. CE-LIF electropherograms of GOS ref., milk replacer; oligosaccharides present in serum at day 3 of GOS diet (S-A1), and control diet (S-B1). DP = degree of polymerization based on GOS. The electropherograms were normalized on the internal standard xylose (*); 6-29: peaks corresponding to GOS;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

In addition, dietary acidic oligosaccharide 3'-sialyllactose (3'-SL) present in the milk replacer was recognized in serum samples of piglets fed on control and on 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.[12] None of the oligosaccharide peaks assigned to GOS were present in the serum samples of piglet fed on control diet (S-B1). Surprisingly, S-B1 showed the presence of additional 25 reducing oligosaccharides (peaks named 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 was overlapping with the un-resolved peak-h present in low abundance in serum S-B1of the control group. To the best of our knowledge, this is the first time that GOS and oligosaccharides present in a piglet milk replacer are detected in piglet blood samples. Systemic presence of HMOs and neutral oligosaccharides from cow milk after ingestion of infant formula have been described previously.[3] 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

Since 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. Based on 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 minutes were also detected. Nevertheless, their masses did not correspond to oligosaccharides with DP6 or 7, as expected from their elution times. From MS^2 fragmentation analysis, they were suggested to present 2 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.[37] All the hexose trimers, having formate adduct, presented a mass-over-charge (m/z) of 549. The chromatograms of serum of piglets fed on 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 on 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 fragment with m/z 425 referring to intra-ring hexaose fragmentation. Fragments with a 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 a relative ratio 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.

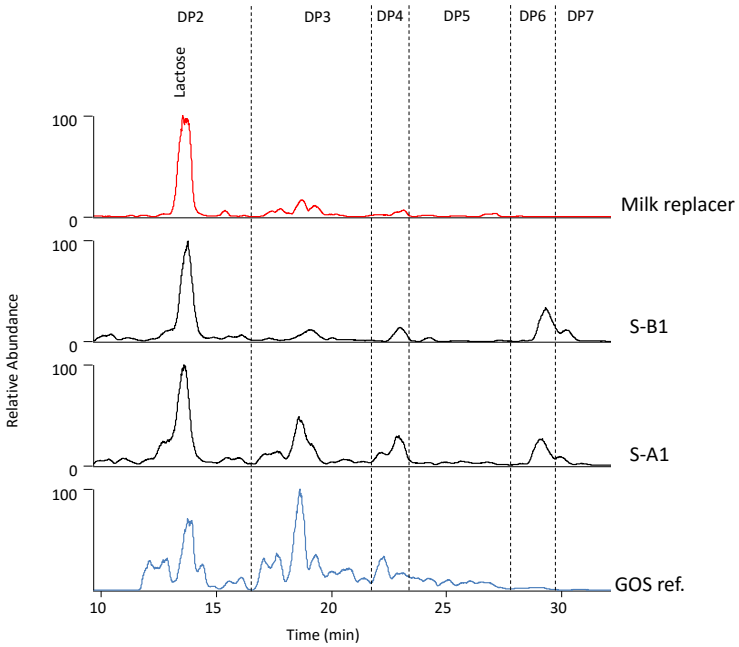

Figure 4. Base peak in HILIC-MS^n for oligosaccharides as found in GOS ref., in serum at day 3 of GOS diet (S-A1) and control diet (S-B1), and in milk replacer. DP = degree of polymerization.

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 serum of GOS-fed piglets. HILIC-MS^n supported our CE-LIF findings, confirming the presence of GOS in serum of piglets fed on 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.[12] After mass analysis, 3'-sialyllactose was observed in serum of piglets fed on GOS and control diet, as expected from CE-LIF analysis. In *in vivo* studies, systemic presence of neutral and acidic oligosaccharides from human milk has been reported for suckling human neonates.[6, 10] Moreover, *in vitro* studies showed the passage of neutral and acidic milk oligosaccharides through intestinal epithelial cells.[10, 38] 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. 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.[12]

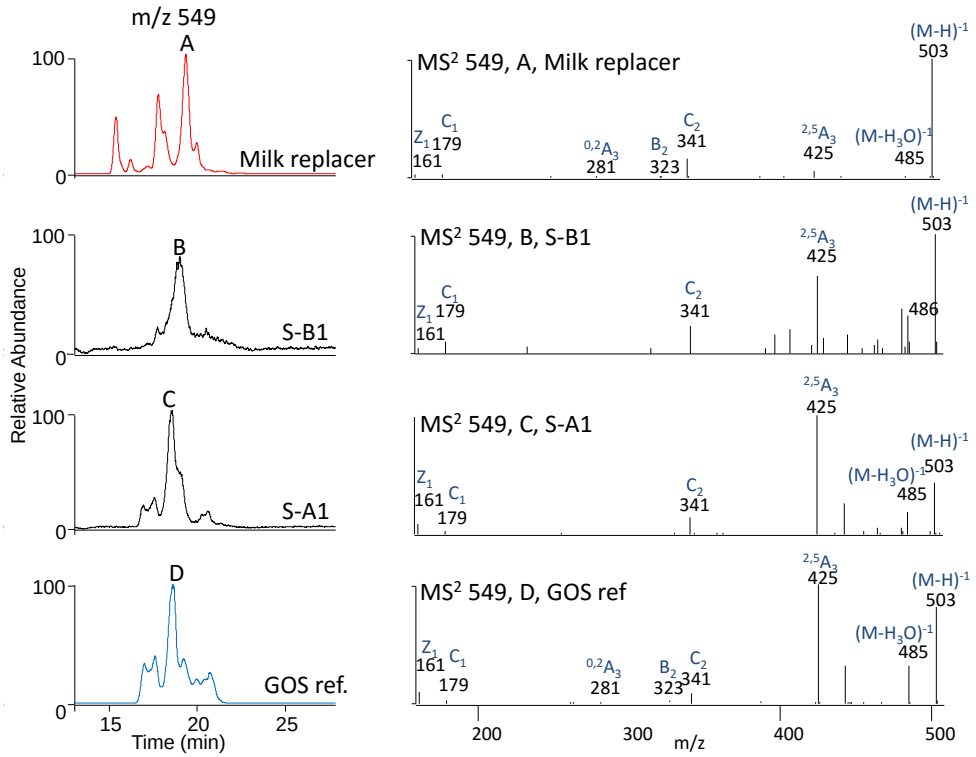

Figure 5. Selected base peak in HILIC-MS^n for trimers as found in GOS ref., in serum at day 3 of GOS diet (S-A1) and control diet (S-B1), and in milk replacer. Corresponding MS^2 fragmentation patterns and fragment annotation, as reported by Domon et al, of the trimers named A, B, C, and D; m/z 549 precursor ion in HILIC-MS^n.[37]

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 3 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 the 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-DP3-4 was confirmed by liquid chromatography mass spectrometry, in which mass fragmentation showed GOS origin of oligosaccharides in piglet serum and urine (data not

shown). 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 fructo-oligosaccharides was described for urine of infants and adults, respectively.[7, 8, 39, 40] Concerning HMOs, both neutral (fucosyllactose, lacto-N-tetraose, lacto-N-fucopentaose and lacto-difucosylpentaose) and acidic (3', and 6'-SL, 3'-, and 6'-sialyl-N-acetyllactosamine) dietary HMOs were found in urine samples of infants, indicating HMOs systemic absorption.[3, 6]

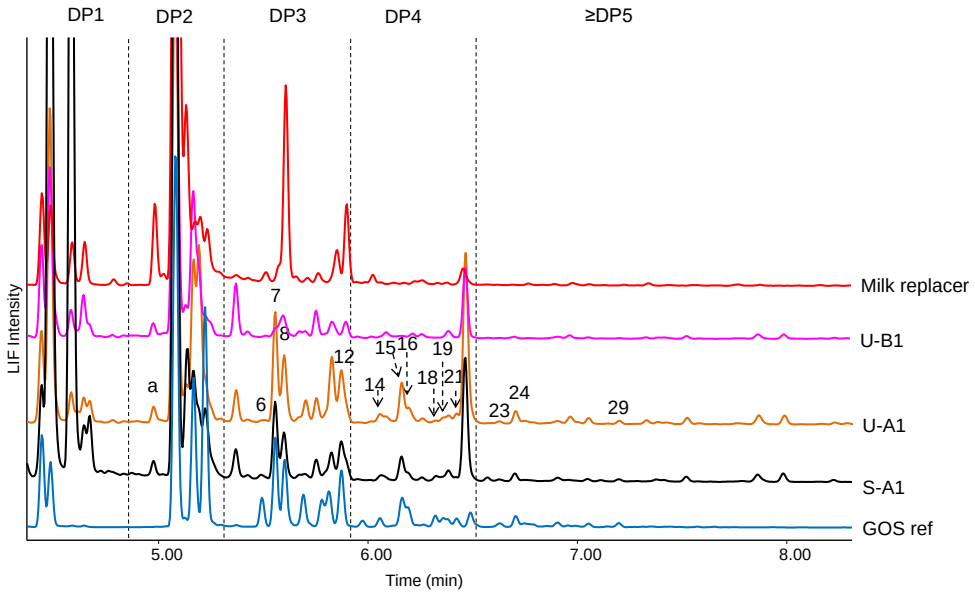

Figure 6. CE-LIF electropherograms of GOS ref, milk replacer, oligosaccharides from serum and urine at day 3 of GOS diet (S-A1 and U-A1, respectively) and urine at day 3 of control diet (U-B1). DP1-5 = degree of polymerization based on GOS. The electropherograms were normalized on the internal standard xylose (*); 6-29: peaks corresponding to GOS, peak-a = 3'-silayllactose.

Quantification of GOS and other oligosaccharides

Concentration of oligosaccharides in serum and urine at day 3

In order 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 on a GOS diet (S-A1-3 and U-A1-3, respectively), and serum (S-A4) lacking the corresponding urine sample are reported. The total GOS concentration in sera was: 34.1, 5.3, 31.1, and 21.2 μg/mL (S-A1-4, respectively). The GOS concentration in

urine was 0.9, 0.9 and 0.6 g/g creatinine (U-A1-3, 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. Literature describes *in vitro* passage of GOS through cell monolayer depending on GOS molecular size and structure, supporting our findings.[14] Nevertheless, non-digestible oligosaccharides are reported to be fermented already in the upper tract of piglets intestine, while in human, metabolization of prebiotic fructo-oligosaccharides in the last part of the small intestine was described.[40-42]

Detection and concentration of oligosaccharides in piglet serum and urine at 26 days

In order to investigate the absorption and excretion of oligosaccharides in a more mature intestinal system, 3 serum and urine samples from piglets fed 26 days on GOS or control diet were analyzed. In Table 2, presence, concentration (μg/ml) and relative proportion of GOS structures in serum and urine samples of piglets fed on GOS diet for 26 days (S-B1-3, U-B1-3, respectively) detected by CE-LIF are shown. Piglets serum samples showed an average concentration of GOS of 16.1 μg/ml, while 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, since on average 70.6% of GOS-DP3 on total GOS was detected. GOS-DP4-6 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-DP3-4 was confirmed by mass analysis (data not shown). Since 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, both at day 3 and 26 of the feeding trial. In this study, presence of dietary oligosaccharides in piglets body fluids was proven using two different techniques. Specific oligosaccharides from GOS supplementation, and milk replacer were detected in serum and urine of piglets at day 3 and 26 of experimental feeding. It was therefore

hypothesized that oligosaccharides of DP3-6 were absorbed by the piglet intestine, while early breakdown of specific dietary oligosaccharide structures in the small intestine could not be excluded.

Table 1. Presence, concentration (µg/ml in urine and g GOS/g creatinine in urine), 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)				piglet urine (3 days)		
	GOS ref.	S-A1	S-A2	S-A3	S-A4	U-A1	U-A2	U-A3
		concentration						
peak number		µg /mL				g/g creatinine		
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	–	–	–
concentration GOS-DP3	193.9	22.6	3.2	19.1	14.2	0.5	0.5	0.4
relative %	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	–	–	–	–	–	–	–
concentration GOS-DP4	88.4	8.7	1.9	8.8	5.5	0.3	0.3	0.2
relative %	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	–	–	–	–	–	–	–
concentration GOS-DP5	30.7	2.1	0.2	2.5	1.0	0.1	0.1	0.05
relative %	9.6	6.1	4.9	8.1	4.6	8.3	7.9	7.6
29								
concentration GOS-DP6	5.2	0.6	–	0.7	0.5	0.02	0.02	0.02
relative %	1.6	1.8	–	2.2	2.5	2.3	2.0	2.7
total GOS concentration	318.2	34.1	5.3	31.1	21.2	0.9	0.9	0.6
average total GOS concentration		22.9				0.8		

Table 2. Presence, concentration (μg/ml in urine and g GOS/g creatinine in urine), and relative percentage of GOS structures as detected by CE-LIF in serum and urine at day 26 of GOS diet of 3 piglets.

		piglet serum (26 days)			piglet urine (26 days)		
	GOS ref.	S-B1	S-B2	S-B3	U-B1	U-B2	U-B3
		concentration					
peak number		μg/mL			g/g creatinine		
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	–	–	–
concentration GOS-DP3	193.9	15.1	8.9	9.9	0.4	0.7	0.7
relative %	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	–	–	–	–	–	–
concentration GOS-DP4	88.4	5.6	3.1	3.0	0.2	0.3	0.3
relative %	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	–	–	–	–	–	–
concentration GOS-DP5	30.7	1.3	0.4	0.6	0.04	0.1	0.1
relative %	9.6	5.8	3.3	4.5	6.8	5.1	5.3
29							
concentration GOS-DP6	5.2	0.3	–	0.2	0.01	0.01	0.01
relative %	1.6	1.4	–	1.4	1.3	1.0	1.2
total GOS concentration	318.2	22.3	12.3	13.7	0.7	1.0	1.1
average total GOS concentration			16.1			0.9	

Intestinal fermentation of dietary oligosaccharides at day 3 and 26

It has been demonstrated that the major part of GOS reaches the colon, where they are fermented by colonic microbiota.[12, 20, 23] 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 large intestine at day 3 and 26 of the piglet experimental feeding trial. Therefore, piglets fecal samples (at day 3 and 26) and cecal digesta samples (at day 26) were collected and analyzed. In Figure 7, CE-LIF profiles of oligosaccharides present in feces from piglets fed 3 days on GOS or control diet (f-A1-3 and f-B1-3, respectively) are shown. Overall, no intact dietary oligosaccharides were detected in fecal samples using CE-LIF and HILIC-MS^n. Based on CE-LIF analysis (Figure 7) low abundance of oligosaccharides was found in the fecal samples both at day 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. [29]

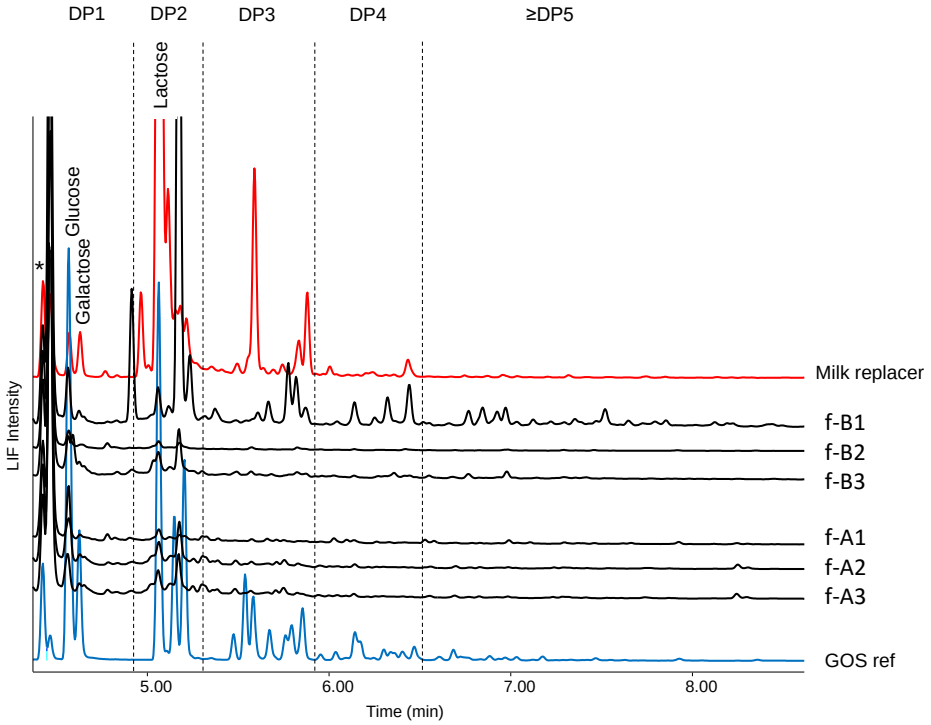

Figure 7. CE-LIF electropherograms of GOS ref., oligosaccharides from feces at day 3 of GOS or control diet (f-A1-3 and f-B1-3, respectively), and milk replacer, DP = degree of polymerization based on GOS. The electropherograms were normalized on the internal standard xylose (*).

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.[29] However, one profile (f-B1, Figure 7) showed high oligosaccharide abundance, 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 GOS. 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.[43] For all samples, efficiency regarding oligosaccharide fermentation and accumulation in the fecal and cecum digesta samples was evaluated. Amount of oligosaccharides per gram of fecal slurries or cecal content was estimated, assuming the presence of exclusively neutral oligosaccharides. The concentration of oligosaccharides was 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. Both in 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 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), both at day 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 piglets caecum digesta and feces, low levels of oligosaccharides were detected, suggesting extensive intestinal GOS fermentation. The discovery of GOS and dietary oligosaccharides in blood and urine of piglets now promote further evaluation of the systemic role of oligosaccharides in humans and animals.

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Chapter 5

In vitro fermentation of porcine milk- and galacto-oligosaccharides using piglet fecal inoculum

Abstract

In this study, the *in vitro* fermentation by piglet fecal inoculum of galacto-oligosaccharides (GOS) and porcine milk oligosaccharides (PMOs) was investigated in order to identify possible preferences for individual oligosaccharide structures by piglet microbiota. Firstly acidic PMOs and GOS with degree of polymerization 4-7 were depleted within 12 h of fermentation, whereas fucosylated and phosphorylated PMOs were partially resistant to fermentation. GOS structures containing β1-3 and β1-2 linkages were preferably fermented over GOS containing β1-4 and β1-6 linkages. Upon *in vitro* fermentation, acetate and butyrate were produced as the main organic acids. GOS fermentation by piglet inoculum showed unique fermentation pattern when compared with GOS *in vitro* fermentation by human fecal inoculum with respect to preference of GOS size and organic acids production. In both fermentations, the same GOS linkage preference was noticed.

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Introduction

Milk is the first food source for infants, providing not only energy but also offering protection against pathogens.[1] The third largest class of compounds present in human milk are oligosaccharides (HMOs).[1] Numerous health related effects of HMOs, involving direct interactions with pathogen and/or indirect effects upon their fermentation by the intestinal microbiota have been reported.[2] When human milk is lacking during the early stage of life of infants, cow milk based infant formula is used.[3, 4] However, cow milk has a low content of milk oligosaccharides, therefore in many cases infant formulas are fortified with prebiotics such as galacto-oligosaccharides (GOS).[5, 6] Prebiotics are defined as non-digestible food ingredients that can influence the intestinal microbiota composition resulting in beneficial effects for the host.[5, 7, 8] GOS are produced from lactose using β-galactosidases of microbial or yeast source.[9] The enzymatic reaction results in a GOS mixture containing oligosaccharides varying in degree of polymerization, mostly from 2 to 8, and in types of glycosidic linkages present.[5] Commonly, GOS have a glucose monomer at their reducing end to which multiple galactose monomers with different linkages (β1-2, β1-3, β1-4 and β1-6) are attached.[10] GOS exhibit less structural complexity than HMOs. The major part of GOS resist degradation in the upper intestinal tract, consequently reaching the colon, as is the case for HMOs.[8] In the colon, GOS enhance bifidobacteria growth, potentially protecting the infant against infections and pathogen development.[2, 11] Moreover, upon GOS fermentation, short chain fatty acids are produced, among which butyrate.[12] Butyrate has been shown to be anti-inflammatory and anti-carcinogenic, also enhancing (*in vitro*) the intestinal barrier function.[5, 13, 14] Recent study showed that *in vivo* GOS fermentation in young pigs (piglets) was extensive, not being conclusive on microbiota utilization towards individual GOS structures.[15] In the present study, *in vitro* fermentation by piglet fecal inoculum was used in order to control different stages of fermentation, thereby focusing on the fate of individual oligosaccharides and organic acids produced. Oligosaccharides investigated were from porcine milk as present as primary feed source at early stage of piglet life and GOS.

Materials and methods

Acetic acid, acetonitrile (ACN), ammonium formate, formic acid, chloroform and methanol were purchased from Biosolve BV (Valkenswaard, The Netherlands). Butyric acid, lactic acid, succinic acid, maltotriose, 2-(N-morpholino)ethanesulfonic acid, 2-ethylbutyric acid, sodium chloride, 2-picoline borane complex and sodium hydroxide were purchased from Sigma Aldrich (Steinheim, Germany). Propionic acid, oxalic acid and 2-aminobenzamide were purchased from VWR International (Amsterdam, The Netherlands). β3'-, β4'- and β6'-Galactosyllactose were purchased from Carbosynth (Compton, UK), while 3'- and

6'- sialyl-N-acetylactosamine, 3'-fucosyllactose, lacto-N-(neo)tetraose and lacto-N-(neo)hexaose were purchased from Dextra (Reading, UK). 3'- and 6'-Sialyllactose and lacto-N-fucosylpentaose were purchased from Sigma Aldrich. Water was filtered using a Milli-Q water purification system (Millipore, Darmstadt, Germany) and it was referred to as water in the text. Vivinal GOS Easy Drying Syrup (FrieslandCampina Domo, Borculo, The Netherlands), dry matter (DM) 75%, with GOS 72%, lactose 23%, glucose and galactose together 5% on DM, is referred to in the text as GOS.

Oligosaccharide extraction and separation

Porcine milk oligosaccharides (PMOs) from porcine colostrum (37 mL) (Proefaccommodatie de Tolakker, Utrecht University, Utrecht, The Netherlands) were extracted, freeze-dried and dissolved in water (70 mg/mL), and subsequently separated using Size Exclusion Chromatography (SEC) as reported elsewhere.[16] Collection of 103 fractions (7 mL each) started after 0.27 column volume (CV) and fractions were collected during 0.8 CV, as described before.[16] In total, 4 runs were performed with an injection volume of 2.86 mL each injection. Afterwards, the fractions were pooled and freeze dried: pool 1: fraction 290 - 500 mL, pool 2: fraction 501 - 697 mL, pool 3: fraction 698 - 719 mL, pool 4: fraction 720- 741 mL, pool 5: fraction 742 - 770 mL. The freeze-dried pools were dissolved in 36 mL water. For each pool, 1 mL was used for oligosaccharide characterization, while the remaining volumes were mixed and freeze dried again to obtain porcine milk oligosaccharides (PMOs) with a reduced amount of lactose.

Fermentation of GOS and PMOs by piglet fecal inoculum

Culture medium

The culture medium was based on a modified simulated ileal environment medium (SIEM), not containing carbohydrates prepared as reported elsewhere.[17] The pH was set at 6.0 using MES buffer. All the culture medium components were provided by Tritium Microbiology (Veldhoven, The Netherlands).

Fecal inoculum

Inoculum for the *in vitro* fermentation was obtained from fecal material from 3 Dutch Landrace piglets (age of 3 weeks). The piglets were fed on porcine milk over the feeding period, combined with pig formula (de Heus, Ede, The Netherlands) for the 2nd day to the 8th day of life. From the 9th day of life until the end of the 3 weeks the piglets received pellets (de Heus). Immediately after defecation, fecal material from each piglet was collected in eppendorf tubes, which was immediately put into an insulated box with crushed ice and afterwards stored at -80°C. The piglet fecal inoculum was prepared as described elsewhere, with some minor modifications.[18] Feces (± 100mg) of three piglets were pooled

after defrosting in an anaerobic cabinet (gas phase: 96% N_2 and 4% H_2). The pooled feces were diluted 6 times (w/v) with sterilized 0.9% (w/v) NaCl solution and subsequently homogenized using a vortex. The homogenization was facilitated by the addition of sterile glass beads. The fecal solution was added to the culture medium in a 20 mL flasks at a 5:82 (v/v) ratio, in order to obtain the final inoculum. Afterwards, the bottle was closed with rubber stoppers and aluminum caps to maintain anaerobic conditions. Next, the inoculum was kept overnight into an incubator shaker (Innova 40, New Brunswick Scientific, Nijmegen, The Netherlands) (39°C, 100 rpm) to activate the bacteria.

In vitro fermentation

GOS and PMOs, obtained as described above, were used as substrates for the fermentation by piglet fecal inoculum. The *in vitro* fermentation experiment was performed as described elsewhere with minor modifications.[18] GOS or PMOs were added to the culture medium with a final substrate concentration of 11.1 mg/mL. The fecal inoculum was added to the solution containing substrate and medium in a 1:10 ratio (v/v). Control samples consisting of culture medium without substrate were used to monitor the background fermentation. All procedures were performed in an anaerobic cabinet. Flasks were closed with rubber stoppers and aluminum caps, and they were put into an incubator shaker (Innova 40) (100 rpm, 39 °C). After 9, 10, 12, 24 and 48 hours of the fermentation, two times 70 µL were taken from the fermentation bottles with a syringe and transferred to eppendorf tubes. In order to inactivate fecal enzymes, eppendorf tubes were put into a boiling water bath for 5 min and stored afterwards at -20^{o}C.

Characterization and quantification of GOS and PMOs

Samples after fermentation were cleaned prior to analysis by hydrophilic interaction liquid chromatography with mass spectrometry (HILIC-ESI-MS^{n}). The samples after fermentation were diluted 10 times in water and 150 µL were placed into a polyvinylidene fluoride centrifugal filter unit (0.22 µm, Merck Millipore, Amsterdam, The Netherlands) and centrifuged (5 min, 20000*g*, 20^{o}C). Afterwards, ACN was added to the filtrate in a ratio of 1:1 (v/v) and the samples were centrifuged (5 min, 20000*g*, 20^{o}C). Supernatants were analyzed on a Thermo Accela Ultra High Liquid Chromatography (UHPLC) system (Thermo Scientific, Waltham, MA, USA.) as described elsewhere.[19] In order to recognize GOS linkage type, HPAEC-PAD was used and the HPAEC profiles were compared with profiles as reported elsewhere.[10, 12] Oligosaccharides standards were used for PMO characterization and quantification by HILIC-MS^{n}. Calibration curves of the corresponding milk oligosaccharide standards and GOS (0.002 to 0.5 mg/mL respectively) were used in order to quantify PMOs and GOS by mass spectrometry. Area and concentration of individual GOS-DP were obtained by selecting the mass range for each GOS-DP and taking into account the relative amount of a given GOS-DP in the total GOS sample. If no

corresponding standards for neutral PMOs were available, GOS with a comparable DP was used for the calibration curve. The curves fitting the PMO standards showed a linear correlation with R^2 of 0.90-0.99, while the curves fitting the GOS-DPs showed a linear correlations with R^2 of 0.98-0.99.

Organic acid analysis

In order to quantify acetate, propionate and butyrate produced during the fermentation, GC analysis was performed as described elsewhere with minor modifications.[12] Samples after fermentation were diluted 10 times with water. Aliquots (50 µL) and short chain fatty acids (SCFAs) standards (0.01-3 mg/mL) were mixed with 0.15 M oxalic acid (50 µL). The solutions were kept for 30 min before addition of 0.025 mg/mL 2-ethyl butyric acid as internal standard in water (143 µL). In order to analyze succinic and lactic acid, high performance liquid chromatography with refractive index detection was performed as described elsewhere.[12]

Results and discussion

Fermentation of porcine milk oligosaccharides (PMOs)

As a result of rapid colonic bacterial colonization, piglets can extensively ferment dietary fibers.[19-23] GOS and porcine milk oligosaccharides (PMOs) present in the diet of piglets are almost completely fermented in the colon, resulting in trace amounts of oligosaccharides present in the feces other than intact GOS or PMO structures.[15, 19] Similarly as found for dietary fibers, piglets intestinal fermentation of GOS is suggested to start already in the small intestine and to continue extensively in cecum and colon.[22, 23] In order to study any preference and differentiation in speed of fermentation among GOS and PMO structures, *in vitro* fermentation was performed using piglet fecal inoculum: PMOs was chosen since they are present in the piglets diets through sow nursing, and GOS was chosen as it has been used in previous piglets feeding trial.[15, 21] Prior to monitor their degradation profiles, the main PMOs were identified and quantified by HILIC-MS^n.[16] Through single ion monitoring, in total thirteen main PMOs representing about 77% of the total peak area, were identified and monitored during the *in vitro* fermentation. Using previously gained knowledge,[19] PMOs identified were the neutral β3'- and β6'- galactosyllactose (β3'- and β6'-GL), lacto-N-neotetraose (LNnT), lacto-N-pentaose-I (LNP-I), lacto-N-neohexaose (LNnH), 2'- and 3'- fucosyllactose (2'- and 3'-FL); and the acidic 3'- and 6'- sialyllactose (3'- and 6'-SL), 3'- and 6'- sialyl-N-acetyllactosamine (3'- and 6'-SLN), phosphorylated lactose (P+ lactose) and Neu5Ac(α2-6)GlcNAc(β1-3)Gal(β1-4)Glc. The degradation of individual PMOs during fermentation was indirectly expressed via the abundance of remaining oligosaccharides present (Figure 1). Overall, a decrease in concentration upon fermentation was observed for each PMO, with individual differences in degradation rate

(Figure 1). One exception was phosphorylated lactose, which increased in concentration during the first 12 h of fermentation, while being depleted completely afterwards (Figure 1 C). The lactose present was attributed partially to the small amount of lactose still present after purification with SEC and partially to lactose released by bacterial degradation of PMOs. Lactose was the first carbohydrate to be fully fermented by the porcine microbiota, decreasing approximately 98% during the first 9 h of fermentation. Trimers, such as β3'-GL, β6'-GL, 2'-FL and 3'-FL were all fermented with a different rate.

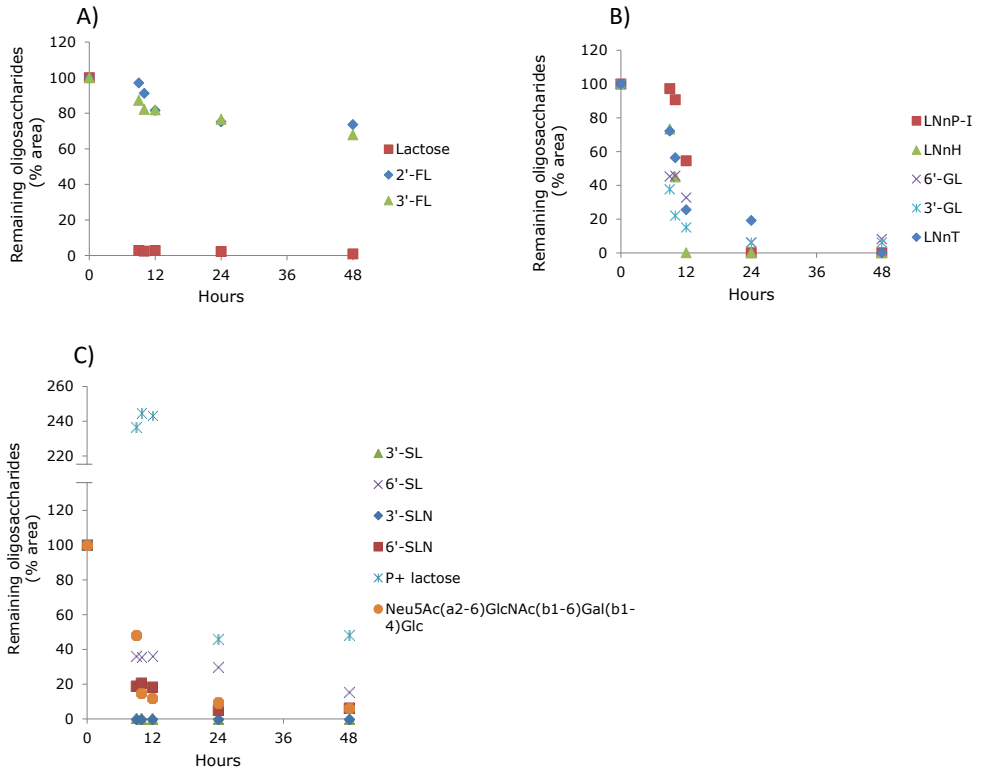


Figure 1. Proportion (%) of remaining PMOs during *in vitro* batch fermentation by piglet fecal inoculum. Concentration per individual PMOs at t = 0 was set to 100%. In A) lactose and 2'-, and 3'-fucosyllactose (2'-, and 3'-FL) are shown; in B) β 3'-, and 6'- galactosyllactose (β 3'-, and 6'-GL), lacto-N-neotetraose (LNnT), lacto-N-pentaose-I (LNP-I), and lacto-N-neohexaose (LNnH) are shown; while in C) 3'-, and 6'- sialyllactose (3'-, and 6'-SL), 3'-, and 6'- sialyl-N-acetyllactosamine (3'-, and 6'-SLN), phosphorylated lactose (P+ lactose), and Neu5Ac(α2-6)GlcNAc(β1-3)Gal(β1-4)Glc are shown.

The trimers β3'-GL and β6'-GL diminished about 50% of their initial concentrations in the first 9 h of fermentation, whereby β3'-GL is more quickly fermented than β6'-GL (Figure 1 B). LNnH is fermented faster than LNnT and LNnP-I, decreasing 80% of its original concentration during 12 h of fermentation. All the neutral PMOs are almost completely fermented within 24 h, whereas LNnT still was not consumed completely. Most probably, part of the LNnT present at 24 h is released by the degradation of larger PMOs. LNnT is totally consumed in the subsequent 24 h by the piglet colonic microbiota. Acidic PMOs are fermented for 50% during the first 12 h and are fermented faster than neutral PMOs (Figure 1, C). Surprisingly, the phosphorylated lactose (P+lactose) more than doubled in concentration during the first 12 h of fermentation, with a subsequent decrease to about 50% of its starting concentration. Hypothetically, the increase of phosphorylated lactose could be derived from fermentation of larger phosphorylated PMOs present, although they were not reported so far to be present in domestic animal milk samples.[19, 24] Overall, fermentation of acidic and neutral PMOs was almost complete in 48 h, with the exception of fucosylated PMOs and phosphorylated lactose. In conclusion, *in vitro* fermentation with piglet microbiota showed a rapid consumption of PMOs, with different fermentation rates detected for individual PMOs. Piglet microbiota firstly ferment small PMOs, such as neutral and acidic trimers, and afterwards neutral larger PMOs, each with its own specific rate.

Fermentation of galacto-oligosaccharides (GOS)

GOS showed to be a not easy fermentable substrate for the piglet microbiota, since 20-60% of the GOS-DP3-7 were still present after 12 h of fermentation. Dimers were fermented first, as expected from a previous investigation on GOS fermented by human fecal inoculum.[12] Lactose was fermented faster than non-lactose GOS-DP2 structures (Figure 2). Lactose was introduced in the pig diet not only by nursing, but also by the cow milk based pig formula, presumably leading to adaptation of the piglet microbiota to lactose. On the contrary, GOS-DP2 were not present at any phase of the piglet diet. Hence, a slower adaptation of the microbiota than for lactose was expected. For GOS-DP3-7 (Figure 2), longer fermentation times compared with fermentation times found for dimers were observed (9-12 h), with different utilization rates depending on the DP. In the first 12 h of *in vitro* fermentation by piglet fecal inoculum, GOS-DP5-7 were mostly degraded (about 30% remaining oligosaccharides), while GOS-DP3 was still present in relatively high abundance (60% remaining oligosaccharides) (Figure 2). This was not observed during 12 h of *in vitro* fermentation of GOS by human fecal inoculum, where about 70% of remaining GOS-DP4-6 and about 50% of remaining GOS-DP3 were observed.[12] After 24h of fermentation, all GOS-DPs showed to be fully fermented without DP discrimination, implying piglet microbiota adaptation to the GOS substrate. As expected from GOS fermentation by human fecal inoculum, also piglet fecal microbiota showed preference

regarding specific GOS structures.[12] Using HPAEC-PAD analysis, not only the decrease during fermentation time of GOS-DP, but also the decrease of individual GOS isomers was monitored. In Figure 3, GOS peaks are assigned with numbers as reported previously.[25] Isomeric GOS structures were degraded differently depending on their structures as can be seen for lactose and for GOS-DP2 such as Gal(1-3)Gal, Gal(1-3)Glc and Gal(1-2)Glc (Figure 3, numbers 2.3, 2.5 and 2.6, respectively). This observation was similar to that observed in a previous study, reporting that β1-2 and β1-3 linkages were more easily degradable than β1-4 linkages during GOS-DP2 fermentation by human fecal inoculum.[12] As expected from the HILIC-MS^n profile, GOS-DP3 were harder to degrade than GOS-DP2. The trimers indicated by numbers 3.8 and 3.9 were easier to ferment by piglet inoculum than trimers numbers 3.4, 3.5, 3.6 and 3.7 (Figure 3), as expected from GOS fermentation by human fecal inoculum.[25]

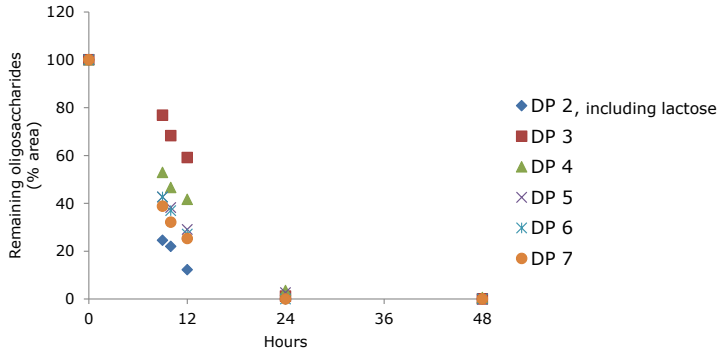
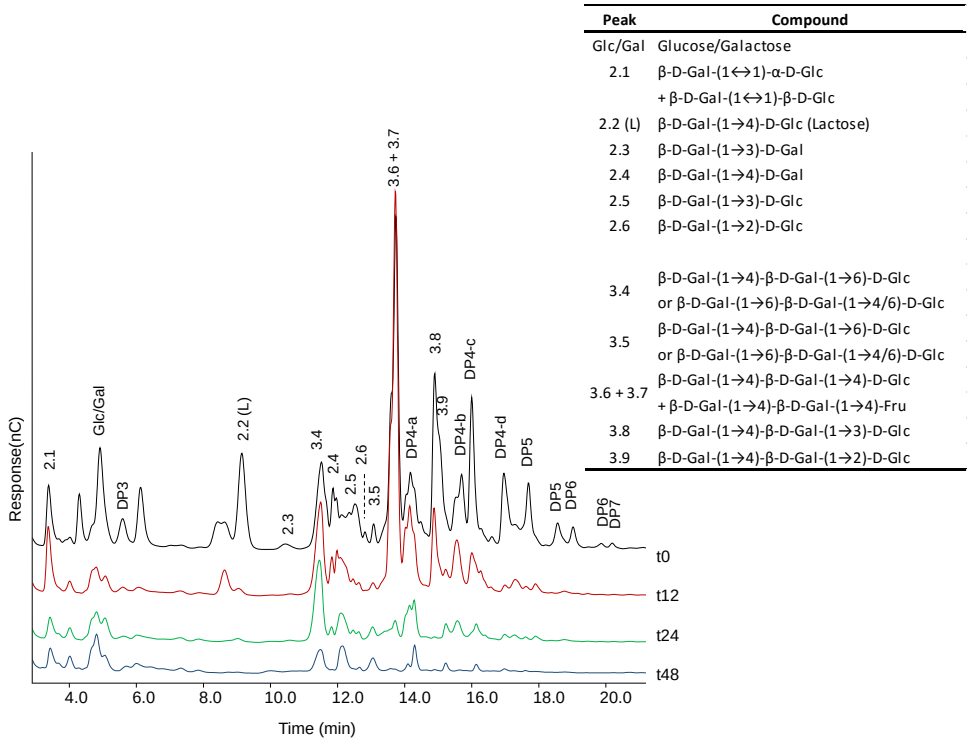


Figure 2. Proportion (area %) of remaining GOS during in vitro fermentation by piglet fecal inoculum as measured by HILIC-MS^n. Concentrations per PMOs at t=0 were set to 100%. DP = degree of polymerization.

A relevant difference between piglet and human inocula, was noticed for Gal(β1-4)Gal(β1-4)Glc and Gal(β1-4)Gal(β1-4)Fru (numbers 3.6 + 3.7, Figure 3), which accumulated with an increase of 17% during the first 12 h of fermentation by piglet fecal inoculum, while they were easily fermentable substrates for the human microbiota.[12] A similar preference towards the degradation of specific linkages was noticed when comparing piglet and human *in vitro* fermentation: β1-3/β1-2 linkages were preferred over β1-4/β1-6 linkages for GOS-DP2-3. Literature describes human and piglet microbiota composition to be similar, mainly consisting of Firmicutes and Bacteriodes.[26] On the other hand, differences were reported to occur in bifidobacteria species, relevant for GOS degradation.[26] Studies reported on extremely low amounts of bifidobacteria species present in the gastro-intestinal tracts of piglets (< 1% of the total bacteria present). [26-28] When detected, certain bifidobacteria species present in piglet intestine could differ from the species reported in human intestinal

tract.[26, 29] Our results indicate a difference in microbiota composition based on the fermentation characteristics of oligosaccharides.



Peak	Compound
Glc/Gal	Glucose/Galactose
2.1	β-D-Gal-(1↔1)-α-D-Glc
	+ β-D-Gal-(1↔1)-β-D-Glc
2.2 (L)	β-D-Gal-(1→4)-D-Glc (Lactose)
2.3	β-D-Gal-(1→3)-D-Gal
2.4	β-D-Gal-(1→4)-D-Gal
2.5	β-D-Gal-(1→3)-D-Glc
2.6	β-D-Gal-(1→2)-D-Glc
3.4	β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc or β-D-Gal-(1→6)-β-D-Gal-(1→4/6)-D-Glc
3.5	β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc or β-D-Gal-(1→6)-β-D-Gal-(1→4/6)-D-Glc
3.6 + 3.7	β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc + β-D-Gal-(1→4)-β-D-Gal-(1→4)-Fru
3.8	β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc
3.9	β-D-Gal-(1→4)-β-D-Gal-(1→2)-D-Glc

Figure 3. HPAEC-PAD chromatograms of GOS fermentation with piglet fecal inoculum, with structures of oligosaccharides as present in the GOS mixture (2.1-3.9) (according to Ladirat et al.).[25] DP= degree of polymerization. Peak numbers correspond to structures in the insert.

Organic acid production upon PMOs and GOS fermentation by piglet fecal inoculum

The degradation of PMOs and GOS by piglet fecal inoculum resulted in the production of organic acids: acetate (A), succinate (S), butyrate (B), lactate (L) and propionate (P) as shown in Figure 4. The molar ratios A:S:B:L:P observed after 24 h of fermentation were of 49:1:20:2:27 and 45:1:21:4:29 for PMOs and GOS, respectively. Acetate was the most abundant organic acid detected after 24 h of PMOs and GOS fermentation by piglet fecal inoculum. Similarity acetate was the most abundant organic acid produced for GOS *in vitro* fermentation by human fecal inoculum.[30] Next to acetate, also propionate and butyrate (Figure 4, A and B) were produced for both GOS and PMOs fermentation by piglet fecal inoculum, whereas mainly succinate and propionate were co-produced for GOS

fermentation by human fecal inoculum.[12, 13] For 24 h of *in vitro* fermentation, GOS fermentation with piglet and human inoculum showed differences in A:S:B:L:P molar ratios: 45:1:21:4:29 and 75:10:6:2:8, for piglet and human inoculum, respectively.[12, 13] Overall, after fermentation for 24 h, butyrate was produced in higher abundance upon GOS fermentation by piglet microbiota than by human microbiota.

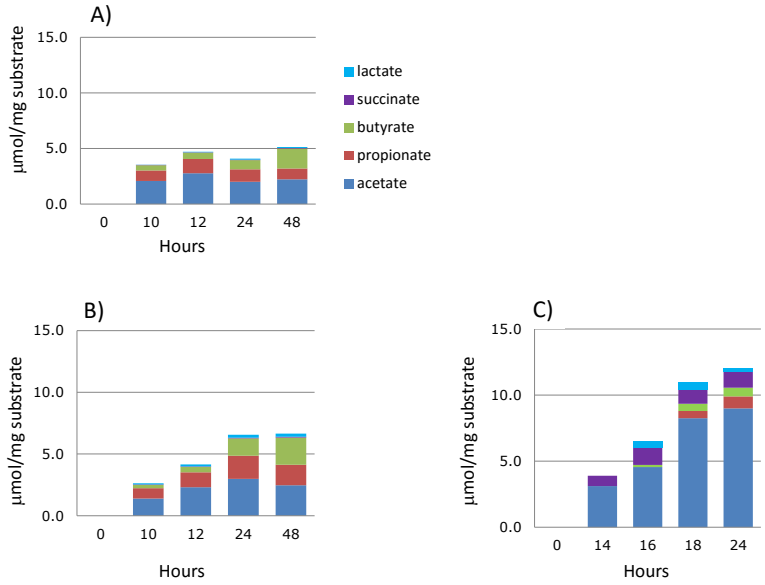


Figure 4. Organic acid amount and relative concentration during *in vitro* fermentation of GOS (A) and PMOs (B) by piglet fecal inoculum and of GOS by human fecal inoculum (C); adapted from Ladirat et al.[12]

The absolute amount of butyrate was of 1.4 and 0.7 µmol/mg substrate for GOS fermented by piglet and human inoculum, respectively.[12, 13] Comparing butyrate production upon GOS fermentation, it can be hypothesized that microbiota from piglet inoculum contain more butyric-producing bacteria than human microbiota that can ferment GOS. During fermentation for 24 h by piglet inoculum, about 7 and 4 µmol organic acids per mg of substrate were produced for GOS and PMOs, respectively. Fermentation of GOS by human fecal inoculum resulted in about 12 µmol/mg organic acids production in 24 h of fermentation.[12] Overall, in the intestinal tract, $10^{10\text{-}11}$ and 10^{14} bacteria per gram of intestinal content for piglets and human are present, respectively.[26, 31]

In summary, differently from *in vivo* piglet fermentation, *in vitro* fermentation provide the opportunity to observe the preference of piglet microbiota on fermentation of individual oligosaccharides. During *in vitro* fermentation, acidic PMOs and neutral PMO trimers were the first to be degraded followed by larger neutral PMOs. Fucosylated and phosphorylated

PMO were the most resistant to fermentation. Interestingly, while PMO trimers were quickly degraded, GOS trimers showed a slow apparent degradation rate for the first 12 h of fermentation. GOS trimers degradation rate was hypothesized to be influenced not only by utilization by the microbiota, but also by the release of trimeric degradation products upon fermentation of larger GOS. In addition, GOS containing β1-3 and β1-2 linkages were faster fermented than GOS containing β1-4 and β1-6 linkages. Major products upon PMOs and GOS fermentation by piglet microbiota were acetate, propionate and butyrate.

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Chapter 6

General discussion

Research aim and approach

Lactose-based oligosaccharides (LBOs) are reported to have beneficial biological and immunological effects for new-borns. LBOs are present in milk from mammals, as mammalian milk oligosaccharides (MMOs), or are manufactured using enzymes, as galacto-oligosaccharides (GOS). A lot of research has been devoted to reveal the composition and intestinal fate of LBOs from mother milk, the so-called human milk oligosaccharides (HMOs). The composition of LBOs from other milk sources originating for example from domestic animals and LBO fermentative fate in animal digestive tract has been hardly investigated. The aims of this PhD thesis are to describe the composition of MMOs from equine and porcine milk and to determine the fate of GOS and of MMOs from porcine milk during *in vitro* fermentation. Furthermore, LBOs in blood, urine and fecal samples from *in vivo* piglet experiments were analysed. The research described in this PhD thesis was part of a multidisciplinary project named 'Immuno-modulation of oligosaccharides from various sources'. The approach used for this PhD thesis was the characterization of GOS and MMOs form equine and porcine milk using hydrophilic interaction liquid chromatography with mass spectrometry and capillary electrophoresis with laser induced fluorescence or mass spectrometric detection. The individual MMOs and GOS utilization and formation of their degradation products was also monitored during an *in vitro* batch fermentation using piglet fecal inoculum. The knowledge gained provides a basis for further research on the structure-function relationships of LBOs including beneficial immunological effects in mammal small and large intestine and their utilization by the colonic microbiota.

Mammalian milk oligosaccharides (MMOs)

In this PhD study, oligosaccharides from equine and porcine milk were investigated, comparing them with structures present in human milk. Milk samples were derived from sows from one breed and from mares of different breeds. One of the main conclusions of this thesis is that MMO structures strongly vary on the milk origin, not only with respect to different mammalian species, but also regarding inter-individual variation within one mammal species (Chapters 2 and 3). Nevertheless, some MMO structures are preserved through all mammalian milks. Another outcome of this PhD study is that neutral and acidic trimers are the most abundant milk oligosaccharides in colostrums analysed: 3'-sialyllactose in both equine and porcine milk (about 1 and 12 g/L, respectively), β6'-galactosyllactose (1.3 g/L) in equine colostrum and the putative Gal(α1-3)Gal(β1-4)Glc (1.1 g/L) in porcine colostrum (Chapters 2 and 3).

MMO structural variation per species

In total, 16 equine milk (EMOs) and 35 porcine milk (PMOs) oligosaccharides were characterized and when possible quantified. In addition, structure and quantity were compared with oligosaccharides from human milk (Table 1). Human milk has been reported to have more than 200 different HMO structures, of which more than 100 oligomers were fully structurally elucidated.[1] In Figure 1, CE-LIF profiles indicating the main oligosaccharides as present in human, porcine and equine colostrum are shown. HMOs appear to be a more complex mixture, both in number and concentration of oligosaccharides compared to EMOs and PMOs. When comparing the number of structures elucidated in EMOs and PMOs (Chapters 2 and 3), neutral and acidic structures represent each about 50% of the total number of characterized oligosaccharides (Table 1). During the MMO characterization, 7 and 13 novel structures for EMOs and PMOs ranging from DP3 to DP6 were identified, respectively. In colostrum samples, MMO concentrations ranged from about 2 to 5 g/L; 7 -29 g/L, and 20-25 g/L, for EMOs, PMOs and HMOs, respectively (Chapters 2 and 3).[1] MMOs were found to be present in equine and porcine colostrums: β6′- and β3′- galactosyllactose, 3′-sialyllactose, lacto-N-neotetraose (LNnT), and lacto-N-neohexaose (LNnH). These oligomers from equine and porcine milk have not been reported so far to be predominantly present in human milk. Different MMO structures and levels present in equine an porcine milk also suggest different health-related needs from the new-borns, depending on the species.[2]

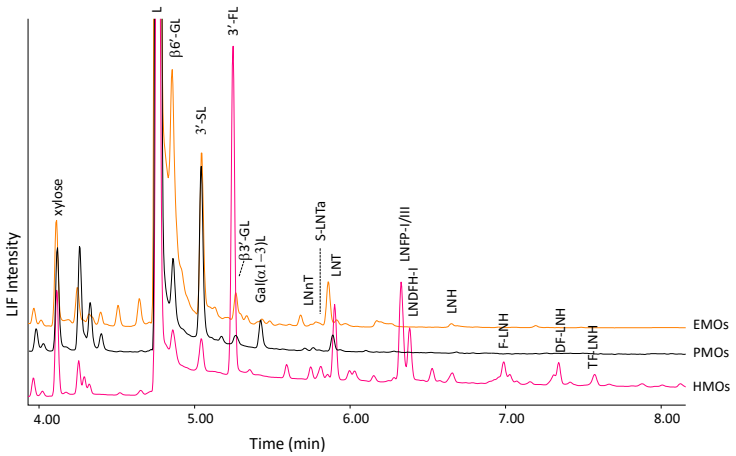

Figure 1. CE-LIF profiles of equine, porcine and human milk oligosaccharides (EMOs, PMOs and HMOs, respectively). With xylose as internal standard, L = lactose, β3'-, β6'-GL = β3'-, β6'-galactosyllactose, 3'-SL = 3'-sialyllactose, 3'-FL = 3'- fucosyllactose, Gal(α1-3)L = α-galactosyllactose, LN(n)T = lacto-N-(neo)tetraose, S-LNTa = sialyllacto-N-tetraose-a, LNFP-I/III = lacto-N-fucopentaose-I/III, LNDFH-I = lacto-N-difucohexaose-I, LNH = lacto-N-hexaose, (T) or (D)F-LNH = (tri) or (di)fucosyllacto-N-hexaose.

In EMOs and PMOs, a higher abundance of Type II chain (Gal(β1-4)GlcNAc) oligosaccharides than type-I chain (e.g. Gal(β1-3)GlcNAc; lacto-N-biose) was observed, both in number and concentration. This is the opposite to what has been found for HMO samples.[2] Differentiation is also observed regarding the degree of sialylation and fucosylation of the MMOs. On the total number of structures, EMOs and PMOs were found to be sialylated for 50% and 43%, respectively, while HMOs were reported to be sialylated for 10-30%. Fucosylation of MMOs highly vary, depending on the specie: 0%, 23% and 50-80% for EMOs, PMOs and HMOs, respectively (Table 1). Significant variations in abundance of sialylation and fucosylation for HMOs have been reported.[2-4]

Table 1. Overview of equine, porcine and human milk oligosaccharide characteristics.

Mammalian milk oligosaccharides (MMOs)			
	EMOs	**PMOs**	**HMOs**
Number of MMOs			
structures identified	16	35	>100[a]
structures overlapping with HMOs	10	19	—
Percentage based on MMO number			
sialylated structures	50%	43%	10-30%[a]
fucosylated structures	0%	23%	50-80%[a]
phosphorylated structures	0%	3%	0%[a]
bifidogenic factor (Gal(β1−3)GlcNAc)	19%	23%	72%[b]
Concentration (g/L)			
total oligosaccharides	2-5	7-29	20-25[a]
bifidogenic factor MMOs	0.25	0.21	—
decrease from colostrum to mature milk	—	≈50%	≈50%

EMOs = equine milk oligosaccharides, PMOs = porcine milk oligosaccharides and HMOs = human milk oligosaccharides, a = Ref.[1], b = Ref.[2], — = not applicable.

Porcine, equine and also bovine, caprine and ovine colostrums contain oligosaccharide structures comparable to HMOs, but also rather unique structural unit, like phosphorylated monosaccharides and glycolylneuraminic acid could be present.[5, 6] PMOs were found to contain phosphorylated lactose (Chapter 3), which is absent in HMOs. Overall, this PhD study shows that porcine colostrum have higher concentration and larger variation in oligosaccharides compared with equine colostrum. Moreover, contrarily to EMOs, some PMOs present fucosylation in their structures.

MMO level variation per species

When looking at the milk oligosaccharide literature, quantification of oligosaccharides is not simple and unambiguous in most cases. Frequently, just a signal response as obtained by a specific technique is given in order to describe MMO quantity.[6-8] Therefore, it is challenging to compare MMOs described from different studies and to be conclusive on MMO levels. In this study an overview was made on the most abundant oligosaccharides as present in equine, porcine (Chapter 2 and 3) and human colostrum (literature).[2] Overall, both neutral and acidic trimers were found to be the most abundant oligosaccharides in equine and porcine colostrum. Trimers have been reported to be the most abundant HMOs as well (as shown in Table 1, Chapter 1). The abundance of trimeric structures may vary depending on the species analysed. Neutral β6'-galactosyllactose (1.3 g/L), putative Gal(α1-3)Gal(β1-4)Glc (1.1 g/L) and 2'-fucosyllactose (average 2.8 g/L) were the most abundant oligosaccharides in EMOs, PMOs and HMOs, respectively. 3'- Sialyllactose was the most abundant acidic trimer in EMOs and PMOs (1 and 12 g/L, respectively), while both 3'-, and 6'-sialylactose (average of 0.2 and 0.9 g/L, respectively) were most abundant in HMOs (Chapters 2 and 3). Sialylated oligosaccharides have been found to be important towards pathogen protection, and may contribute to allergy prevention.[9, 10] The bifidogenic factor Gal(β1-3)GlcNAc chain (type-I chain or Lacto-N-biose) was present in EMOs and PMOs, covering 19% and 23% of the total number of oligosaccharides found respectively (Table 1). It has been shown that bifidobacteria growth preferentially on HMOs type-I chain structures when compared with other bacteria such as clostridia, enterococci and lactobacilli.[11-13] Among HMO structures reported by Urashima et al., 72% of all HMOs contain the so-called bifidogenic factor.[2]

MMO variation in time

When investigating MMOs, the stage of lactation of the mother should be taken into account. In general, the highest MMOs concentration is found during the first few days of lactation, while in time the concentration tend to decrease.[2] In this PhD study, a decrease of about 43% in the concentration of PMOs in porcine milk was found within 1 week lactation of the sow (Chapter 3). This value is comparable with decreases described elsewhere: 50% for both human and cow milk.[3, 7] High concentrations of MMOs in the early stage of life are expected, since they play a key role in the health of new-borns, exerting intestinal protecting functions and intestinal immune system maturation.[1] Lower MMO concentrations in mature milk, nevertheless, could be compensated by an increased milk intake by the suckling neonate, and to a more developed and stable intestinal system. It was shown for two porcine milk samples, that PMO concentration decreases within 1 week nursing time (Chapter 3). Interestingly, while the total PMO concentrations decrease in time, not all PMOs decrease accordingly. Relative abundance of acidic PMOs decreased on

average 28%, while the relative abundance of neutral PMOs increased 34% within 1 week nursing time. This trend was comparable for bovine milk oligosaccharides, in which the predominantly present acidic milk oligosaccharides decreased 30% while the neutral oligosaccharides increased 50% in abundance.[7] Nevertheless, more studies should be conducted towards the variation of MMO concentration and composition in time in order to have a better understanding of the role of the MMOs during different stages of nursing.

Fate of lactose-based oligosaccharides (LBOs) in piglet

Systemic fate of LBOs is hardly investigated. A small part of the LBOs are found in blood serum and subsequent in urine of the new-born as was already shown for HMOs in blood and urine of infants.[1, 14, 15] An important conclusion from the piglet *in vivo* trial is that GOS as well as neutral and acidic oligosaccharides derived from the piglet diet were found for the first time both in blood and urine of GOS-fed piglets (Chapter 4). So far, literature reports on LBOs entering into the colon, where they are fermented by microbiota leading to an increase of the bifidobacteria population, lowering the pH and contributing to the protection of the host towards pathogen infection.[16-19] In the *in vitro* fermentation experiment in which GOS was fermented by piglet inoculum, the preference of the utilisation of specific GOS structures was observed. GOS containing β1-3 and β1-2 linkages were more rapidly fermented than GOS containing β1-4 and β1-6 linkages. Among PMOs, 3'-linked sialylated PMOs were preferred over the 6'-linked sialylated PMOs by piglet microbiota (Chapter 5).

GOS in blood and urine

Literature report on the presence of diverse HMOs in bloodstream and subsequently in urine of infants.[20-22] In this thesis, the presence of GOS in blood and urine (body fluids) of piglets was proven (Chapter 4). Upon GOS diet (for 3 and 26 days) and subsequent animal sacrifice, piglet body fluids were analysed. Specific GOS were found in piglet body fluids of GOS-fed piglets, suggesting selective adsorption of GOS (Chapter 4). During their passage though the intestinal tract, GOS seem to be absorbed for a small part (0.1% of the GOS daily intake) and subsequently excreted by the urinary system. Absorption of components in the small intestine is possible by para-cellular and/or trans-cellular pathways. Para-cellular pathway is aspecific and based on a concentration gradient.[23] Trans-cellular pathways are active, receptor-mediated transportations and they allowed the passage of specific structures through the cell membrane.[24] This hypothesis is in line with literature reporting the *in vitro* passage of GOS through cell monolayer depending on GOS molecular size and structure.[25] Seen that comparable structures and relative abundances of GOS were found back in the blood for both 3 and 26 days GOS-fed piglets (Table 2), both active and passive transport from the small intestine to the blood circulation was

hypothesized, as shown for neutral HMOs.[24, 26] A stereospecific and saturable uptake of neutral HMOs into the cells by trans-cellular pathway (trans-cytosis), together with para-cellular transport (through tight junctions) was shown *in vitro* using Caco-2 monolayer.[24, 26] Having a pore size of around 4 Å, the tight junctions would allow structurally diverse oligosaccharides to pass the intestinal barrier.[27, 28] So far, from the more than 200 annotated structures in human milk, only 7 neutral HMOs (fucosyllactose, lacto-N-tetraose, fucosyllacto-N-tetraose, difucosyllacto-N-tetraose, lacto-N-fucosylpentaose-I and-II, and difucosyllacto-N-hexaose) and 4 acidic (3', and 6'-sialyllactose, 3'-, and 6'-N-acetyllactosamine) HMOs were found in blood and urine samples from babies.[27, 28] In this PhD thesis, it is suggested that GOS can pass the intestinal barrier through both the para- and trans-cellular pathway (Chapter 4), as suggested for HMOs. Since piglets have been shown to have oligosaccharides fermentation already in the upper intestinal tract [29, 30], it can be hypothesized that specific structures have been already utilized in the ileum (distal small intestine), resulting in a slight modified oligosaccharide composition when compared with oligosaccharide composition in the proximal small intestine. Therefore, depletion before absorption of specific GOS could not be excluded.

Table 2. Overview of average GOS-DP relative abundance referred to total GOS-DP3-6 found in serum and in urine, of 3 and 26 days GOS fed piglets.

		GOS feeding			
		3 days		26 days	
	GOS ref.	serum	urine	serum	urine
	average relative % GOS				
DP3	60.9	63.7	57.5	70.6	64.6
DP4	27.8	28.8	32.2	24.0	28.5
DP5	9.6	5.9	7.9	4.5	5.7
DP6	1.6	2.2	2.3	1.4	1.1

DP = degree of polymerization.

Excretion of GOS in urine was also monitored. Comparing the relative abundance of urinary GOS-DPs with GOS-DPs present in the feed as well in the blood, the same order of magnitude was found as exemplified in Table 2. HMOs presence in urine has been suggested to have protective function against urinary tract infections by blocking pathogen adhesion to the epithelial cell wall.[4, 31] Although the relative abundance of GOS-DPs was comparable, some GOS individual structures present in the blood were not present in urine samples (Chapter 4). It could be hypothesized that either the concentration of the specific structure was under the limit of detection for the techniques used, or that specific structures were utilized systemically. The mechanisms of GOS absorption and excretion remain still open for future investigation, although it can be hypothesized that GOS can follow similar fate as neutral HMOs.

GOS fermentation in vivo

It was already shown by *in vitro* experiments that GOS can directly affect intestinal epithelial cells by improving the intestinal barrier function via accelerating tight junction assembly.[32] *In vitro* experiments, however, represent an extreme simplification of the mammalian intestinal epithelial barrier. Therefore, it was evaluated whether the neonatal piglet could be an appropriate model to study GOS intestinal fate and function.[33] After birth and 24-48 h sow nursing, piglets were fed on a formula diet with or without GOS for 3 or 26 days.[33] After sacrificing the piglets, samples from saliva and the intestinal tract were analysed. From this study, we can conclude that GOS represent a bioactive mixture with multiple functions (yet unpublished results[33]). GOS were shown to be a specific substrate for bifidobacteria, to improve the intestinal architecture and to affect barrier integrity, whereas GOS can also influence the mucosal immune system by increasing salivary IgA (immunoglobulin-A) production. After 26 days fed on a GOS diet, bifidobacteria species in piglet faeces were not decreasing in number, as was observed for the piglets fed on the control diet. Interestingly not all bifidobacteria grew on GOS while among the species stimulated to grow, also intestinal human type-bifidobacteria were found. This finding is in line with data from experiments with HMOs illustrating that *B. infantis* grew on HMOs while *B. longum* did not.[1]

Extensive GOS *in vivo* fermentation was correlated with extremely low amounts of oligosaccharides in fecal and cecal samples (Chapter 4). GOS-fed piglets had a lower cecal pH after 26 days than at day 0 of GOS-diet, potentially protecting the piglets from pathogenic bacteria and increasing mucin production.[19, 33] The lower cecal pH after 26 days of GOS diet indicates organic acid production including butyrate. The content of the latter was found to be higher when compared with the control group (about 1280 and 950 μmol/L for GOS-fed and control-fed piglets, respectively).[33] Next to effects of GOS on the intestinal microbiota composition, also an effect of GOS on the intestinal wall was shown: after 26 days on the GOS-diet, piglets intestinal-villi were found to be thicker than villi of piglet fed on control diet, possibly improving digestion/adsorption of nutrients.[33] GOS improved the intestinal barrier function, directly up-regulating gene expression of tight junction proteins, helping the intestinal integrity of different parts of the intestine.[33] Moreover, in saliva of piglets fed 26 days on GOS-diet, an increased IgA concentration was found, showing an influence of GOS on the immune-system.[33] In this PhD thesis, it was shown that piglet was a successful model to investigate the effect of GOS in the intestinal system. However, one limitation of the pig as a model was that the intestinal fermentation of GOS was so extensive that it was not possible to investigate any preference of the microbiota for specific GOS structures. For this reason, the pig *in vivo* model was extended with an *in vitro* model to monitor intestinal microbiota fermentation behaviour.

GOS fermentation in vitro

Piglet intestine colonization was shown to be influenced by mode of delivery, environment and feeding.[26, 34, 35] Therefore, the *in vitro* batch fermentation was carried out with pooled piglet fecal inoculum since a pooled fecal inoculum can better represent larger and random microbiota communities.[18] Pooling microbiota will lower the expected high inter-individual variation and can create also the possibility to investigate the bacteria competition for the same substrate and cross-feeding interactions.[18] Cross-feeding is the base on which butyrate production lies: bifidobacteria themselves have no genetic potencial concerning butyrate production pathways. However, bifidobacteria carbohydrate-fermentation create metabolites such as acetic acid, that can enhance the growth of butyric-producing bacteria.[36] In this way, GOS exert both an bifidogenic and butyrogenic effect. During 24 h GOS *in vitro* fermentation with piglet inoculum, acetate was the most abundant product among the organic acids (3 μmol/mg substrate) (Chapter 5). Acetic acid has been also reported to be the major fermentation product for GOS *in vitro* fermentation by human fecal inoculum (9 μmol/mg substrate).[37] At the same fermentation time point, butyrate was abundantly present (about 1.4 μmol/mg substrate) for fermentation by piglet inoculum. The high abundance of butyrate together with a consumption of acetate during the last 24 h of fermentation of GOS with piglet inoculum could be an indication of the presence of bacteria capable to use external acetate for butyrate production.[36] The fecal microbiota composition during fermentation should be investigated in order to have a better understanding of the microbiota changes in time upon GOS fermentation in piglets. Although during the *in vivo* experiment all oligosaccharides were utilized very efficiently, our *in vitro* fermentation study showed the preferred utilization of individual GOS and PMOs by the piglet microbiota. Moreover, during *in vitro* fermentation studies using piglet microbiota, the preference of GOS containing β1-3 and β1-2 linkages above β1-4 and β1-6 linkages and of 3'-sialylated PMOs above 6'-sialylated PMOs was shown (Chapter 5).

Characterization of lactose-based oligosaccharides

In this thesis capillary electrophoresis and liquid chromatography were used in order to characterize lactose-based oligosaccharides (LBOs). Technique related characteristics are shown in Table 3. One of the main conclusions of this PhD study is that hydrophilic interaction liquid chromatography (HILIC) with on line UV and mass spectrometry detection is the preferable technique when compared with capillary electrophoresis with fluorescence and mass spectrometry detection when analysing complex oligosaccharides mixtures. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF) was investigated by Albrecht et al, and has been shown to have good peak resolution, sensitivity and reproducibility, being a valuable method to quantify reducing oligosaccharides.[3] Nevertheless, CE-LIF peak identification strongly depends on available standards that are

currently still complex to synthetize.[38] When coupled to mass spectrometry (MS), the capillary is increased in length, consequently decreasing peak separation and increasing peak broadening. Moreover, the label used (APTS) present 3 possible charge states of the ionised oligosaccharides, hindering oligosaccharide characterization during mass (fragmentation) analysis. The CE technique, therefore, required a compromise between mass data acquisition and (isomer) separation. HILIC using a BEH-amide column has been shown to be a suitable tool for acidic oligosaccharides isomer separation and characterization in previous studies.[39, 40]

Table 3. Overview of the techniques used in this study for characterization of oligosaccharides.

	CE-LIF		CE-MS		HILIC-MS*		HILIC-UV-MS*	
		note		note		note		note
Oligosaccharides								
labelled	yes	APTS	yes	APTS	no		yes	2-AB
reducing	yes		yes		yes		yes	
non-reducing	no		no		yes		no	
Technique								
isomer separation	+++		–		++		++	
peak resolution	+++		–		++		+++	
structural characterization	+	with standards	++	only reducing OS	++	reducing end recognition still challenging	+++	simpler spectra compared with HILIC-MS
multiple peak per compound	no		yes	multiple charged OS, APTS dependent	no		no	
MS ionization	n.a.		++	multiple charged OS, APTS dependent	++	OS structure dependent	+++	
quantification	absolute, molar		relative		relative and absolute	OS structure and standard availability dependent	absolute, molar	UV based

n.a. = not applicable, − = poor, + = sufficient, ++ = good, +++ = very good, and * = conditions as described in Chapter 4, OS = oligosaccharide.

Therefore, within this PhD project, the separation of both neutral and acidic LBOs on a BEH-amide column has been investigated (Chapters 3, 4 and 5). The GOS separation was shown to depend on the pH and gradient used and GOS isomers could be partly separated, while on-line MS (fragmentation) analysis enabled the identification of GOS (isomers) without any pre-fractionation. In order to obtain an optimal GOS-isomer separation, different conditions were tested, as shown in Figure 2. From different conditions tested, pH 4.5 showed to be most optimal for isomer separation. Porcine milk oligosaccharides

(PMOs) could be separated successfully using the same gradient and separation conditions as used to separate GOS. Characterization of GOS isomers still remain challenging in HILIC-MS^n, due to quite some peak overlap at DP≥4. Mammalian milk oligosaccharides (MMOs), on the other hand, being built up by different types of monomers, results in a structure-specific mass fragmentation. This facilitates isomeric recognition of MMOs, even when peak overlap occurs. MS quantification of LBOs still represents a challenge, since even isomeric structures appear to have a different ionization behavior.[41, 42] Moreover, when acidic and neutral molecules are both present in a mixture, as in case of MMOs, selective-suppression of the signal in MS is possible.[43]

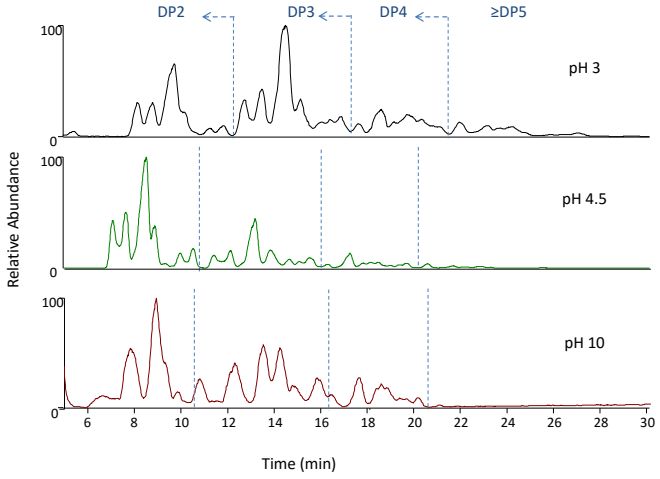

Figure 2. GOS separation by HILIC-MS using a BEH-amide column at pH 3, 4.5 and 10. DP = degree of polymerization.

During mass spectrometry measurements in positive mode, for example, acidic structures would give lower signals than neutral structures.[43] In this way the use of specific standards is needed for quantification, while such standards are not always commercially available. In order to overcome ionization- and standard-dependent quantification in liquid chromatography, labelling procedures involving fluorescent labels have been developed. As mentioned in Chapter 1, different labels have been reported for oligosaccharides analysis and all labelling mechanisms rely on reductive amination. GOS were labelled with 2-aminobenzamide (2-AB), using labelling conditions as reported elsewhere.[44] 2-AB is suitable for fluorescent detection, nevertheless it was also shown to be suitable for UV detection (254 nm).[45] As shown in Figure 3, peak shape and resolution of labelled GOS isomers make their separation on a BEH-amide column at pH 4.5 successful. The GOS shown in Figure 3 had a reduced amount of glucose, galactose and lactose compared with GOS presented in Figure 2, and was therefore called ‘purified GOS'.

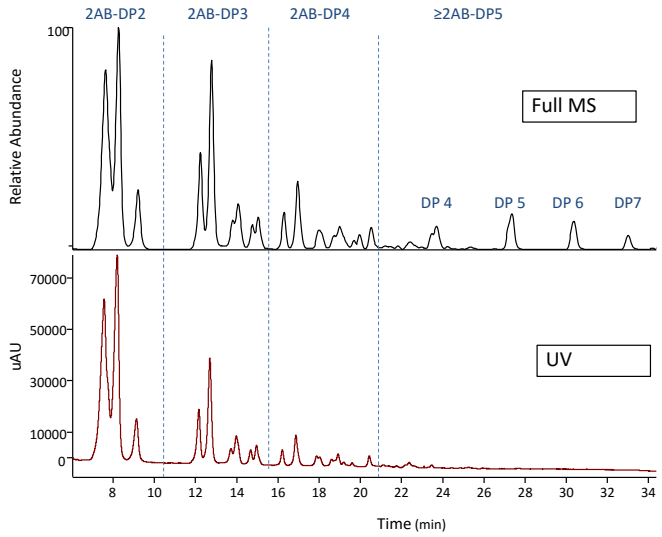

Figure 3. Separation of 2-AB labelled purified GOS on HILIC-MS using a BEH-amide column pH 4.5. 2-AB-DP refers to labelled GOS of a given degree of polymerization (DP), while DP refers to unlabelled GOS of the given DP.

From both their elution time and from MS analysis, unlabelled GOS-DP4-7 were identified in the labelled GOS sample (Figure 3), probably indicating the presence of 1-1 linked oligosaccharides that indeed could not be labelled. In order to evaluate 2-AB GOS labelling efficiency, different oligosaccharide concentrations (5-1000 nmol), temperatures and labelling times were tested with respect to labelling efficiency. Labelling efficiency didn't change dramatically when using different conditions. Therefore, in future oligosaccharide investigations using 2AB-labelling, a minor amount of substrate (100 nmol) and a lower temperature (40°C) when compared with literature (200 nmol and 65°C) should be used, accepting the obvious fact that non-reducing oligosaccharides will not be labelled.[6, 46] As summarized and exemplified in Table 3, currently used techniques have both advantages and drawbacks. At the moment, HILIC-(UV)-MS seems to be the most flexible and robust analytical tool for LBO analysis.

Future perspectives

In this thesis, MMOs different from human milk oligosaccharides (HMOs) were reported. High inter-individual variation regarding the presence and concentration of milk oligosaccharides in equine and porcine milk have been described. This information can be taken into account in future experiments involving domestic animal milk oligosaccharide analysis, and may even be used to evaluate current literature with another view. The approaches and methods developed during this thesis research are now available to

fingerprint milk samples from various origin in a semi-high throughput manner in order to make an inventory on type and level of oligosaccharides present. While such information on breed and animal variation level has been made available for equine colostrum in this study, for colostrum and milk from other animals this information is still lacking. This also holds for the milk oligosaccharide composition during the weaning period of new-born animals.

As was demonstrated in our research, the direct analysis of complex MMO mixtures is challenging due to the low abundance of some oligosaccharides. Although present in low abundance, specific oligosaccharide structures might be relevant both for triggering biological functions and for being (preferably) fermented by colonic microbiota. Pre-fractionation on size prior to identification showed to be quite successful, but separation on charge should also be considered. In addition, an ongoing search for improving the oligosaccharide separation methods developed should be pursued to face the complexity and natural variation of mammal milks. An issue to be addressed is the separation and mass spectrometry investigation of phosphorylated oligosaccharides, since this class of oligosaccharides has been hardly investigated.

Methods presented in this thesis research also revealed the importance of being able to monitor fermentation products in animal digesta and *in vitro* fermentation liquids. However, due to the presence of various types of dietary oligosaccharides in milk and feed, in addition to unknown/novel glycosidic degradation products from milk and feed, method development and fine-tuning could be necessary here as well. By investigating colonic fate of dietary oligosaccharides, also the composition and variation in time and per individuals of the microbiota could be addressed and explained.

During our research, the presence of dietary oligosaccharides in blood and urine of piglets was shown for the first time. However, the complete identification and accurate quantification of all different oligosaccharides in blood and urine is needed to fully understand both absorption and secretion mechanisms, and also to make progress in understanding their systemic functions. Progress being made in mass spectrometry (both quantitative MS and enhanced understanding of mass fragmentation mechanisms) will contribute in the understanding of the presence of oligosaccharides and their metabolites in blood and urine.

Awareness of the important role of milk oligosaccharides with respect to their biological and immunological functions in new-born intestinal and systemic health increased by the enormous scientific research done on milk so far. Therefore, efforts have been dedicated in manufacturing oligosaccharide mixtures resembling the composition of the natural milk oligosaccharides better. As example, a mixture containing N-acetylgalactosaminyl-lactose (GlcNAc(β1-3)Gal(β1-4)Glc), representing a MMO precursor, has been synthetized from lactose using β-N-acetylhexosaminidases, whereas a mixture containing GOS decorated

with sialic acid has been manufactured using a trans-sialidase enzyme.[47, 48] Analytical tools for identification and quantification of several types of oligosaccharides within complex oligosaccharide mixtures developed and investigated in our research can be used to investigate the novel manufactured oligosaccharide mixtures.

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Summary

Summary

Lactose-based oligosaccharides (LBOs), including mammalian milk oligosaccharides (MMOs) and galacto-oligosaccharides (GOS), have been shown to have beneficial effects on the intestine of infants. In **Chapter 1**, the structural characteristics, concentration and variation of MMOs of different mammalian species are described, in addition to some health benefits. Human milk has been shown to contain the highest number of structures as well as the highest concentrations of oligosaccharides compared with some domestic mammalian milks. Milk from domestic animals have been shown to have a unique MMO pattern. The composition of MMOs depend on the mammal lactation stage and concentrations of MMOs becomes lower in time as is the case for human milk. However, the variation in MMO composition for milk from domestic animals in time, between breed and between individuals has been hardly investigated.

In **Chapter 2**, MMOs present in equine milk is described. Equine milk oligosaccharides (EMOs) were analysed by capillary electrophoresis with fluorescence detection (CE-LIF), by hydrophilic interaction liquid chromatography with mass spectrometry (HILIC-MS^n) in combination with enzymatic digestion using exo-glycosidases. In total, 16 EMOs were recognized, seven of which were novel for EMO literature. EMOs found showed a 63% overlap with structures present in human milk. EMOs present in colostrum samples from 4 different horse breeds ranged from 2.12 to 4.63 g/L. Trimeric oligosaccharides were shown to be most abundant structures in all colostrum samples from all breeds. Interestingly, differences were found for the composition in milks from different breeds. A large inter-individual EMO variation was also found between animals within the same breed.

Also porcine milk oligosaccharides (PMOs), as present in pig colostrum and mature milk, were investigated as described in **Chapter 3**. In porcine colostrum, in total 35 PMOs were recognized of which 13 have not been reported for pig colostrum before. As expected from the horse milk study, variation regarding PMO type and concentration was observed among the colostrum samples. During the first week of lactation, a 43% decrease in PMO concentration was found. Interestingly this decrease was not found for all oligosaccharides in the same way: where neutral PMOs increased in concentration in time, the opposite was observed for acidic oligosaccharides. After PMO ingestion by piglets, it was investigated whether it was still possible to detect intact PMOs in piglet fecal samples. Overall, already after one day of birth, only very few intact PMOs were found in fecal samples of piglet suggesting extensive PMO fermentation already at early stage of piglet life.

In **Chapter 4** the presence and relative abundance of dietary oligosaccharides in blood, urine and fecal samples obtained from an *in vivo* piglet feeding trial are described. Oligosaccharides were investigated by liquid chromatography mass spectrometry and capillary electrophoresis with fluorescence detection. Overall, 3'-sialyllactose naturally occurring in the piglet diet and specific GOS structures with a degree of polymerization

(DP) from 3 to 6 were detected in piglet blood and urine. Despite the fact that during the *in vivo* trial no digestion marker was used, estimation of GOS present in the samples was attempted. At day 3 and 26 of GOS diet, about 0.1% of the GOS daily intake was found in piglet blood samples. Therefore, only a minor part of GOS intake was present systemically. GOS structures found in blood were also traced back in urine with the same relative abundance. Most of dietary GOS will end up in the large intestine where they can be fermented by the microbiota. In piglet fecal samples, all GOS were found to be fully fermented, illustrating an extensive GOS intestinal fermentation.

The quick PMO and GOS fermentation *in vivo* in piglets did not provide any information on the preference of utilisation of individual GOS by the colonic microbiota. In **Chapter 5,** the *in vitro* batch fermentation of PMOs and GOS using piglet fecal inocula is described. Overall, acidic PMOs and neutral trimeric PMOs were the first structures to be utilised by the microbiota. In addition, fucosylated and phosphorylated PMOs were shown to be more resistant to fermentation. In the first 12 h of fermentation, GOS with DP5-7 were depleted about 70% their initial concentrations, whereas GOS-DP3 showed to be depleted for about 40% their initial concentration. The relatively high abundance of GOS-DP3 was attributed to the fermentation of larger GOS structures. During GOS fermentation, piglet microbiota firstly fermented GOS having β1-2 and β1-3 linkages and afterwards GOS having β1-4 and β1-6 linkages. Similar GOS linkage preference has been shown for *in vitro* batch fermentation using human fecal inoculum. PMOs and GOS fermentation resulted in organic acids production, with acetate and butyrate as the major products.

In **Chapter 6**, novel information acquired during the PhD thesis are discussed and future perspectives on MMOs and GOS investigation are described. Oligosaccharides from equine and porcine milk are compared with oligosaccharides as found in human milk and other domestic animal milks. Possible EMO and PMO bio-functions are addressed, relating them to bio-functions reported for human milk. The relevance of oligosaccharide analyses in blood and urine and the observed presence of oligosaccharides in these biological samples are discussed. Since HMOs were also reported to be present in blood and urine of infants, knowledge gained on GOS in piglet blood and urine is correlated with HMO literature. GOS and PMOs are described regarding their *in vitro* fermentation patterns and organic acid production and compared with existing knowledge on non-digestible oligosaccharides fermentation using pig and human inocula. It is concluded that the *in vitro* fermentation studies, piglet inoculum showed a unique oligosaccharide fermentation pattern, increasing the knowledge on oligosaccharide intestinal fate in piglets. At last, methods developed in this thesis research as well as recent developments for the analysis of oligosaccharides in milk and mammal biological samples are discussed and advantages and disadvantages highlighted. Liquid chromatography with on line UV and mass spectrometry detection is considered to be the most promising analytical method for LBOs analysis.

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About the author

Curriculum vitae

Elisabetta Difilippo was born on September 24th, 1985 in Terlizzi, Italy. After graduating from high school (Liceo Classico Matteo Spinelli, Giovinazzo, Italy) in 2004 she was admitted at the University of Bari, Italy, and studied Pharmaceutical Chemistry and Technology. She completed her MSc thesis entitled 'Azaplatinacyclobutanes: steric distinction in their formation reaction' at the Laboratory of Bioinorganic Chemistry, directed by Prof. Giovanni Natile. In 2010, she completed an internship as pharmacist at Farmacia Comunale s.p.a, Giovinazzo, Italy. The same year, she obtained her MSc degree in Pharmaceutical Chemistry and Technology and her European Pharmacist Licence. As part of her curriculum she left to the Netherlands in 2011, as she obtained the opportunity to work as PhD student at Laboratory of Food Chemistry, Wageningen University, under the supervision of Prof. dr. Henk Schols and Prof. dr. ir. Harry Gruppen. The results of her PhD research are presented in this thesis.

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List of publications

Difilippo E., Willems H.A.M., 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, 4805-4814).

Difilippo, E., Bettonvil M., Willems H.A.M., Braber S., Fink-Gremmel J., Schoterman M.H.C. , Gruppen H., Schols H.A., Oligosaccharides in urine, blood, and feces of piglets fed a milk replacer containing galacto-oligosaccharides (accepted for publication in J Agric Food Chem, 2015, DOI: 10.1021/acs.jafc.5b04449).

Difilippo E., Pan F., Logtenberg M., Willems H.A.M., Braber S., Fink-Gremmels J., Schols H.A., Gruppen H., Milk oligosaccharides variation in porcine milk, and fermentation in piglets' intestine (submitted in J Agric Food Chem, 2015).

Difilippo E., Pan F., Logtenberg M., Willems H.A.M., Braber S., Fink-Gremmels J., Schoterman M.H.C., Schols H.A., Gruppen H., *In vitro* fermentation of pig milk- and of galacto-oligosaccharides using pig fecal inoculum (submitted in J Agric Food Chem, 2015).

Kiskini A., **Difilippo E.**, Oligosaccharides in goat milk: structure, health effects and isolation (Cell Mol. Biol. (Noisy-le-grand) 2013, 59, 25−30.).

Alizadeh A., Akbari P., **Difilippo E.**, Schols H.A., Ulfman L. H., Schoterman M.H.C., Garssen J., Fink-Gremmels J., Braber S., Piglet as a model for studying dietary components in infant diets: effects of galacto-oligosaccharides on intestinal functions (accepted for publication in Br J Nutr, 2015).

Akbari P., Fink-Gremmels J., Willems H.A.M., **Difilippo E.**, Schols H.A.,. Schoterman M.H.C, Garssen J., Braber S., Characterizing microbiota-independent effects of oligosaccharides in intestinal epithelial cells: insight into the role of structure and degree of polymerization (submitted for publication in Br J Nutr, 2015).

Nyffenegger C., Thorbjørn Nordvang R., Zeuner B., Łężyk M., **Difilippo E.**, Logtenberg J. M., Schols H. A., Meyer S. A., Dalgaard Mikkelsen J., Backbone structures in human milk oligosaccharides: trans-glycosylation by metagenomic β-N-acetylhexosaminidases (Appl Microbiol Biotechnol 2015, 99(19), 7997-8009).

Overview of completed training activities

Discipline specific activities

Courses

- Summerschool glycosciences, VLAG, Wageningen, The Netherlands, 2011
- Advanced Food Analysis, VLAG, Wageningen, The Netherlands, 2011
- Food and biorefinery enzymology, VLAG, Wageningen, The Netherlands, 2011
- British Society for Immunology Summer School, BSI Team, Edinburgh, Scotland, 2014

Conferences and meetings

- CCC scientific days on carbohydrates and (gut) health[§¥], Groningen, The Netherlands, 2011
- CCC scientific days on Dutch perspective on the bio-based economy and CarboHealth, Groningen[§¥], The Netherlands, 2013
- Fibers in food and feed symposium, VLAG, Wageningen, The Netherlands, 2013
- Developments in Carbohydrate Analysis, EUROFINS, Amsterdam, The Netherlands, 2013
- Conference in Food Digestion, INFOGEST, Wageningen, The Netherlands, 2014
- Symposium on functionality of Human Milk Oligosaccharides[¥], Wageningen, The Netherlands, 2014

General courses

- PhD Competence assessment, WGS, 2012
- Interpersonal Communication for PhD Students, WGS, 2013
- Project and Time management, WGS, 2013
- Philosophy and Ethics of Food, WGS, 2013
- Science and Technology Scientific Integrity, WGS, 2013
- Techniques for Writing and Presenting a Scientific Paper, WGS, 2014
- Career perspectives, WGS, 2015

Optional

- Preparation PhD research proposal
- FCH PhD trip to Singapore and Malaysia[§¥], WU (FCH), 2012
- FCH PhD trip to Germany, Denmark, Sweden and Finland[§¥], WU (FCH), 2014
- BSc/MSc thesis student presentations and colloquia, WU (FCH), 2011-2015
- PhD presentations, WU (FCH), 2011-2015

- Project meetings – consortium, 2011-2015

[§]poster presentation, [¥]oral presentation

Abbreviations used

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Science
BSI: British Society Immunology
CCC: Carbohydrate Competence Center
WU: Wageningen University
FCH: Laboratory of Food Chemistry

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