**Supplementary methods**

*Analysis of human milk oligosaccharides*

The composition of each individual HM sample with regard to its HMOS was qualitatively and quantitatively determined using highly sensitive multiplexed capillary-gel-electrophoresis with laser-induced-florescence detection (xCGE-LIF) [1] as described in the Supplementary methods.

In brief and in accordance with the glyXera GmbH kit protocol (KIT-glyX-Quant-DPV, glyXera GmbH, Magdeburg, Germany), the pure HM samples were diluted 1:100 and then spiked with an internal standard (IS) (oligosaccharide quantification standard solution, OS-A5-N-1mL-01, glyXera GmbH, Magdeburg, Germany). For this step, 2 µL of an IS solution and 3 µL of ultrapure water [2] were added to 20 µL of each diluted HM sample. Subsequently, the IS spiked samples were treated with denaturation solution (C-DeNatSol-100uL-01, glyXera GmbH, Magdeburg, Germany). Next, the free oligosaccharides within the denaturized HM/IS samples, comprising the free HMOS and IS, were labeled with APTS dye (KIT-glyX-OSP-A-96-01, glyXera GmbH, Magdeburg, Germany). Afterwards, the excess of APTS (not reacted) and salt were removed using hydrophilic interaction chromatography solid phase extraction (HILIC-SPE, in accordance with the glyXera GmbH kit protocol KIT-glyX-OS.H-APTS-96-01, glyXera GmbH, Magdeburg, Germany). In the next step, the purified APTS-labeled oligosaccharides (HILIC-SPE eluates) were analyzed via xCGE-LIF in accordance with the glyXera GmbH measurement kit protocol (KIT-glyX-OS.M-96-01, glyXera GmbH, Magdeburg, Germany). Finally, data processing, analysis, and qualitative evaluation of the HMOS *Fingerprints* (normalized electropherograms) were performed via glyXtool™ software (version 5.3.1, glyXera GmbH, Magdeburg, Germany), and data analysis and quantitative evaluation of the HMOS *Fingerprints* was performed via glyXtoolGUI™ software (Beta v0.4 [r166+], glyXera GmbH, Magdeburg, Germany).

The limit of quantification (LOQ) was determined using the signal-to-noise ratio (S/N) of each HMOS *Fingerprint* that was calculated according to Ullsten et al. [3]. The LOQ was defined at an S/N of 10. The respective noise within each sample was determined after the migration time alignment of the unsmoothed data in the late migration time range (approximation range = degree of polymerization (DP) 18<DP<20). Consequently, the LOQ is specific with regard to each sample and measurement. All HMOS-peaks ≥LOQ were taken into account and their IS-normalized peak areas were calculated.

For the low-abundant GLs (i.e. β6'-, β4'-, β3'-, and α3'-GL), which were of particular interest in this study, the relative and absolute levels were determined down to the limit of detection (LOD), defined at an S/N of 3. Absolute concentrations (*c*) were determined according to the following formula: *c* = IS-normalized peak height of the analyte × concentration of IS × dilution factor × response factor (KIT-glyX-Quant-DPV, glyXera GmbH, Magdeburg, Germany).

**Supplemental Table S1.** Overview of the number of human milk (HM) samples collected at different time-points over the first 4 months (middle column) and for the subset of 24 Dutch women over the first 12 months (right column) of lactation.

| Lactation month | First 4 months Number of HM samples | First 12 months (Dutch subset) Number of HM samples |
|---|---|---|
| 1 month postpartum | 240 | 21 |
| 2 months postpartum | 92 | 24 |
| 3 months postpartum | 54 | 23 |
| 4 months postpartum | 249 | 23 |
| 5 months postpartum | - | 23 |
| 6 months postpartum | - | 21 |
| 7 months postpartum | - | 12 |
| 8 months postpartum | - | 9 |
| 9 months postpartum | - | 7 |
| 10 months postpartum | - | 4 |
| 11 months postpartum | - | 2 |
| 12 months postpartum | - | 2 |
| **Total** | 635 (371 donors) | 171 (24 donors) |

**Supplemental Table S2**. Total peak area of the selected human milk oligosaccharides (HMOS) (nTPA) and relative peak area (rPA, %) of the HMOS (median (Q1-Q3)) according to maternal human milk (HM) group [4–6] in HM samples (n=171) collected longitudinally over the first year postpartum from 24 Dutch mothers (Dutch subset).

| | HM Group I (*n* = 126 Samples, 18 Donors) *median (Q1-Q3)* | HM Group II (*n* = 21 Samples, 3 Donors) *median (Q1-Q3)* | HM Group III (*n* = 15 Samples, 2 Donors) *median (Q1-Q3)* | HM Group IV (*n* = 9 Samples, 1 Donor) *median (Q1-Q3)* |
|---|---|---|---|---|
| nTPA[1] | 11.4 (9.33-13.4)[a] | 9.30 (7.53-10.8)[b] | 9.95 (8.51-10.7)[a,b] | 6.22 (5.27-9.83)[b] |
| **rPA (%)** | | | | |
| 2'-FL | 22.2 (15.0-29.5)[a] | 0.00 (0.00-0.00)[2 b] | 42.0 (35.5-53.0)[c] | 0.00 (0.00-0.00)[2 b] |
| 3-FL | 11.7 (5.53-21.4)[a] | 35.9 (26.4-45.3)[b] | 0.00 (0.00-3.38)[c] | 6.78 (3.04-7.31)[d] |
| DFL | 1.86 (1.29-2.50)[a] | 0.00 (0.00-0.00)[2 b] | 1.01 (0.62-1.48)[a] | 0.00 (0.00-0.00)[2 b] |
| LNT | 10.6 (7.87-13.1)[a] | 15.3 (11.3-20.6)[b] | 9.77 (6.50-13.8)[a] | 51.8 (47.8-59.3)[c] |
| LNnT | 0.98 (0.67-1.48)[a] | 0.66 (0.00-0.91)[b] | 1.21 (0.83-1.78)[a] | 1.35 (0.94-1.89)[a,b] |
| LNFP I+V | 7.56 (3.29-12.5)[a] | 1.51 (1.36-1.63)[2 b] | 18.8 (15.6-21.5)[c] | 1.29 (1.16-1.55)[2 b] |
| LNFP II | 5.79 (3.31-9.01)[a] | 19.7 (17.1-21.2)[b] | 0.00 (0.00-0.00)[2 c] | 0.00 (0.00-0.00)[2 c] |
| LNFP III | 7.52 (5.66-9.12)[a] | 9.28 (8.06-10.6)[b] | 8.94 (6.99-12.3)[a,b] | 15.6 (10.1-18.4)[b] |
| LNDFH I | 16.3 (13.5-19.1)[a] | 0.00 (0.00-0.00)[2 b] | 0.00 (0.00-0.00)[2 b] | 0.00 (0.00-0.00)[2 b] |
| LNDFH II | 0.00 (0.00-0.00)[2 a] | 3.50 (2.87-4.16)[b] | 0.00 (0.00-0.00)[2 a] | 0.00 (0.00-0.00)[2 a] |
| 3'-SL | 1.08 (0.78-1.43)[a] | 1.37 (1.04-1.91)[b] | 1.18 (1.04-2.15)[a,b] | 1.76 (1.05-2.21)[c] |
| 6'-SL | 0.83 (0.47-1.64)[a] | 0.70 (0.50-1.64)[a] | 0.91 (0.37-1.62)[a] | 1.74 (0.66-2.71)[a] |
| DSLNT | 1.97 (1.40-2.47)[a] | 3.20 (2.86-3.71)[b] | 2.95 (1.94-4.10)[a,b] | 5.53 (5.00-8.83)[c] |
| LSTa | 0.00 (0.00-0.20)[a] | 0.00 (0.00-0.21)[a] | 0.00 (0.00-0.21)[a] | 0.78 (0.75-0.93)[b] |
| LSTb | 0.92 (0.68-1.08)[a] | 1.43 (1.29-1.89)[b] | 0.64 (0.51-0.98)[a] | 2.36 (2.18-2.72)[c] |
| LSTc | 0.34 (0.00-0.84)[a] | 0.28 (0.00-0.77)[a] | 0.26 (0.00-0.57)[a] | 0.99 (0.27-1.60)[a] |
| 3'-F-LNH | 2.38 (1.42-3.85)[a] | 3.65 (0.76-6.23)[ab] | 2.51 (1.44-3.94)[a] | 7.46 (4.94-8.41)[b] |
| 2'-F-LNH | 0.00 (0.00-0.00)[2 a] | 0.00 (0.00-0.00)[2 a] | 0.00 (0.00-0.00)[2 a] | 0.00 (0.00-0.00)[2 a] |
| β3'-GL[3] | 0.074 (0.049-0.12)[a] | 0.10 (0.080-0.13)[a] | 0.11 (0.00-0.13)[a] | 0.42 (0.38-0.51)[b] |
| β6'-GL[3] | 0.36 (0.25-0.53)[a] | 0.52 (0.44-0.61)[b] | 0.42 (0.38-1.01)[ab] | 1.07 (0.87-1.36)[c] |
| α3'-GL[3] | 0.00 (0.00-0.00)[2 a] | 0.00 (0.00-0.00)[2 a] | 0.00 (0.00-0.00)[2 a] | 0.00 (0.00-0.00)[2 a] |

[1] Displayed as the internal standard (IS)-normalized total peak area of selected HMOS (nTPA); [2] '0.00' does not necessarily mean that the respective HMOS is not present at any level, but it means that any amount of HMOS present is below the limit of quantification (LOQ); [3] For β6'-GL, β3'-GL and α3'-GL, the limit of

detection was defined at a signal-to-noise ratio (S/N) of 3. For all other HMOS, the limit of quantification was defined at a S/N of 10; [a,b,c and d] Different superscript letters indicate statistical significance (p<0.05). Significance values have been adjusted by the Bonferroni correction for multiple tests.

Abbreviations used: 2'-FL: 2'-fucosyllactose; 3-FL: 3-fucosyllactose; DFL: difucosyllactose; LNT: lacto-N-tetraose; LNnT: lacto-N-neotetraose; LNFP I: lacto-N-fucopentaose I; LNFP II: lacto-N-fucopentaose II; LNFP III: lacto-N-fucopentaose III; LNFP V: lacto-N-fucopentaose V; LNDFH I: lacto-N-difucohexaose I; LNDFH II: lacto-N-difucohexaose II; 3'-SL: 3-sialyllactose; 6'-SL: 6-sialyllactose; DSLNT: disialyllacto-N-tetraose; LSTa: sialyl-lacto-N-tetraose a; LSTb: sialyl-lacto-N-tetraose b; LSTc: sialyl-lacto-N-tetraose c; 2'-F-LNH: 2'-fucosyllacto-N-hexaose; 3'-F-LNH: 3'-fucosyllacto-N-hexaose; β3'-GL: β3'-galactosyllactose; β6'-GL: β6'-galactosyllactose; α3'-GL: α3'-galactosyllactose.

**Supplemental Table S3.** Percentage of human milk (HM) samples (n=635) in which the specific human milk oligosaccharide (HMOS) was detected (Presence, % of total) and relative peak area (rPA, %) of the selected HMOS (median (Q1-Q3)) according to maternal HM group [4-6]. HM samples were collected in the first 4 months postpartum from 371 donors. Three samples could not be assigned to a HM group and are for this reason not depicted in this table.

| | HM Group I (n = 420 Samples, 241 Donors) | | HM Group II (n = 137 Samples, 83 Donors) | | HM Group III (n = 53 Samples, 32 Donors) | | HM Group IV (n = 22 Samples, 14 Donors) | |
|---|---|---|---|---|---|---|---|---|
| | Presence % of total[1] | rPA (%) median (Q1-Q3) | Presence % of total[1] | rPA (%) median (Q1-Q3) | Presence % of total[1] | rPA (%) median (Q1-Q3) | Presence % of total[1] | rPA (%) median (Q1-Q3) |
| 2′-FL | 100 | 23.2 (18.4-29.0)[a] | 0 | - | 100 | 40.9 (34.2-48.9)[b] | 0 | - |
| 3-FL | 95 | 7.76 (4.17-14.5)[a] | 100 | 28.4 (17.2-35.8)[b] | 15 | 2.86 (2.47-4.18)[c] | 100 | 6.25 (4.03-11.3)[a] |
| DFL | 100 | 1.61 (1.13-2.20)[a] | 4.4 | 0.44 (0.25-0.65)[b] | 89 | 0.68 (0.54-1.15)[c] | 4.5 | 1.29 (1.29-1.29)[b] |
| LNT | 100 | 11.9 (9.20-14.6)[a] | 99 | 19.3 (14.6-26.5)[b] | 100 | 11.8 (9.10-13.8)[a] | 100 | 51.8 (46.4-56.7)[c] |
| LNnT | 99 | 1.45 (1.03-2.06)[a] | 85 | 0.78 (0.54-1.26)[b] | 98 | 1.10 (0.83-1.54)[c] | 95 | 1.04 (0.45-1.50)[a,b,c] |
| LNFP II | 100 | 5.14 (3.58-7.34)[a] | 100 | 20.6 (17.6-25.4)[b] | 3.8 | 0.18 (0.13- .)[2 c] | 0 | - |
| LNFP III | 100 | 6.81 (4.62-8.83)[a] | 100 | 9.25 (7.30-11.3)[b] | 100 | 6.44 (3.85-8.49)[a] | 100 | 14.3 (9.58-18.0)[c] |
| LNFP I+V | 100 | 9.69 (6.00-14.0)[a] | 100 | 1.65 (1.39-1.97)[b] | 100 | 24.9 (19.3-31.5)[c] | 100 | 1.60 (1.32-2.36)[b] |
| LNDFH I | 100 | 17.4 (14.9-20.2)[a] | 1.5 | 1.44 (0.043- .)[2 b] | 3.8 | 0.59 (0.46- .)[2 b] | 0 | - |
| LNDFH II | 2.1 | 1.25 (0.99-1.59)[a] | 100 | 3.77 (2.84-4.88)[b] | 0 | - | 0 | - |
| 3′-SL | 100 | 0.89 (0.70-1.07)[a] | 100 | 1.00 (0.82-1.25)[b] | 98 | 0.95 (0.77-1.22)[a,b] | 100 | 1.45 (1.09-1.63)[c] |
| 6′-SL | 100 | 1.22 (0.76-2.23)[a] | 100 | 1.58 (0.92-2.67)[a,b] | 98 | 1.35 (0.89-2.52)[a] | 100 | 2.42 (1.80-3.45)[b] |
| DSLNT | 100 | 1.71 (1.33-2.27)[a] | 99 | 2.37 (1.72-3.14)[b] | 98 | 1.94 (1.33-2.48)[a,b] | 100 | 4.09 (3.22-5.05)[c] |
| LSTa | 60 | 0.24 (0.18-0.30)[a] | 58 | 0.29 (0.23-0.44)[a] | 45 | 0.33 (0.27-0.45)[a] | 95 | 0.48 (0.34-0.78)[b] |
| LSTb | 99 | 0.80 (0.61-1.04)[a] | 99 | 1.21 (1.04-1.50)[b] | 92 | 0.76 (0.62-0.93)[a] | 100 | 2.21 (1.89-2.75)[c] |
| LSTc | 96 | 0.79 (0.46-1.42)[a] | 93 | 0.84 (0.42-1.33)[a] | 92 | 0.71 (0.47-1.71)[a] | 95 | 1.02 (0.76-1.82)[a] |
| 3′-F-LNH | 98 | 3.46 (2.40-4.61)[a] | 99 | 4.63 (2.77-7.18)[b] | 98 | 2.71 (2.05-4.76)[a] | 100 | 10.5 (8.48-11.3)[c] |
| 2′-F-LNH | 12 | 1.72 (1.17-2.45)[a] | 0 | - | 36 | 1.69 (1.04-3.19)[a] | 0 | - |

[1] Percentage of HM samples in which the specific HMOS was detected (≥limit of quantification (LOQ)); [2] In only two samples, the respective HMOS was present and hence Q3 could not be calculated; [a,b,c and d] Different superscript letters indicate statistical significance (p<0.05). Significance values have been adjusted by the Bonferroni correction for multiple tests.

Abbreviations used: 2′-FL: 2′-fucosyllactose; 3-FL: 3-fucosyllactose; DFL: difucosyllactose; LNT: lacto-N-tetraose; LNnT: lacto-N-neotetraose; LNFP I: lacto-N-fucopentaose I; LNFP II: lacto-N-fucopentaose II; LNFP III: lacto-N-fucopentaose III; LNFP V: lacto-N-fucopentaose V; LNDFH I: lacto-N-
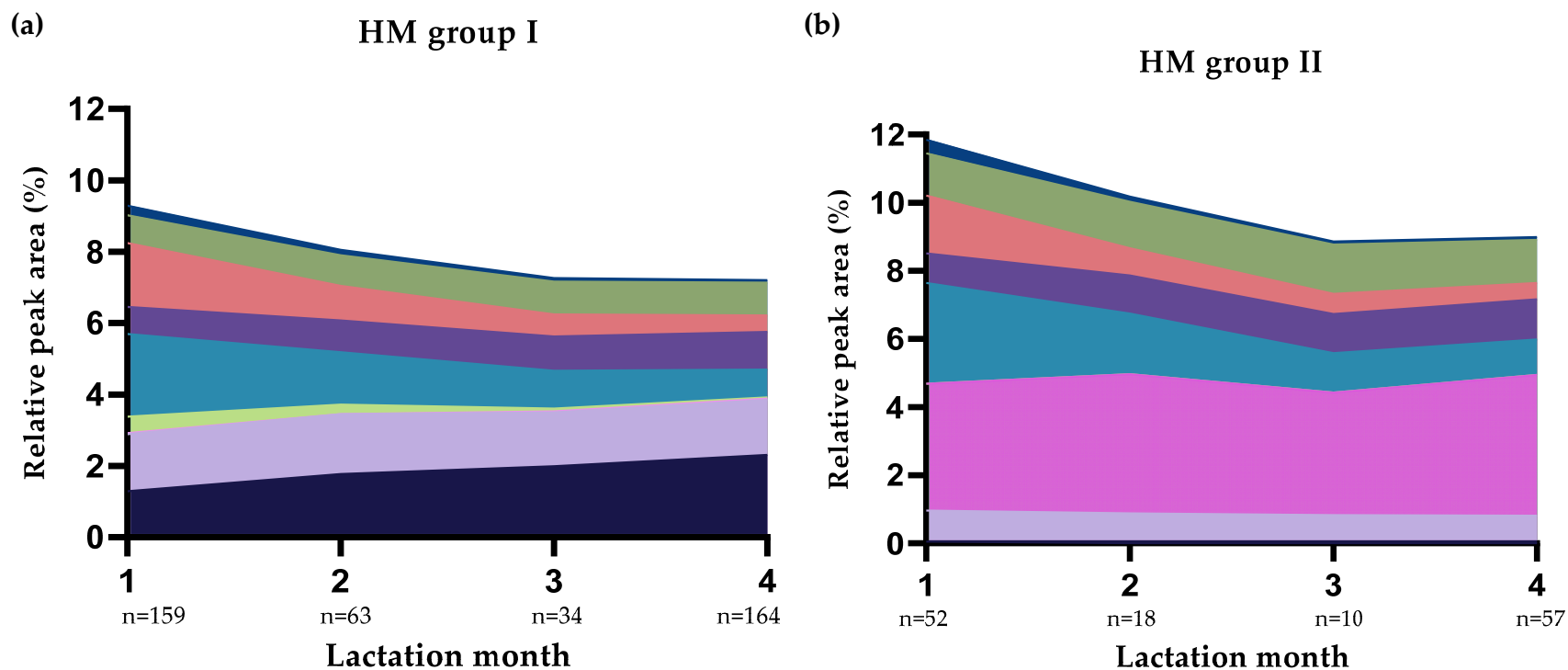
difucohexaose I; LNDFH II: lacto-N-difucohexaose II; 3′-SL: 3-sialyllactose; 6′-SL: 6-sialyllactose; DSLNT: disialyllacto-N-tetraose; LSTa: sialyl-lacto-N-tetraose a; LSTb: sialyl-lacto-N-tetraose b; LSTc: sialyl-lacto-N-tetraose c; 2′-F-LNH: 2′- fucosyllacto-N-hexaose; 3′-F-LNH: 3′-fucosyllacto-N-hexaose
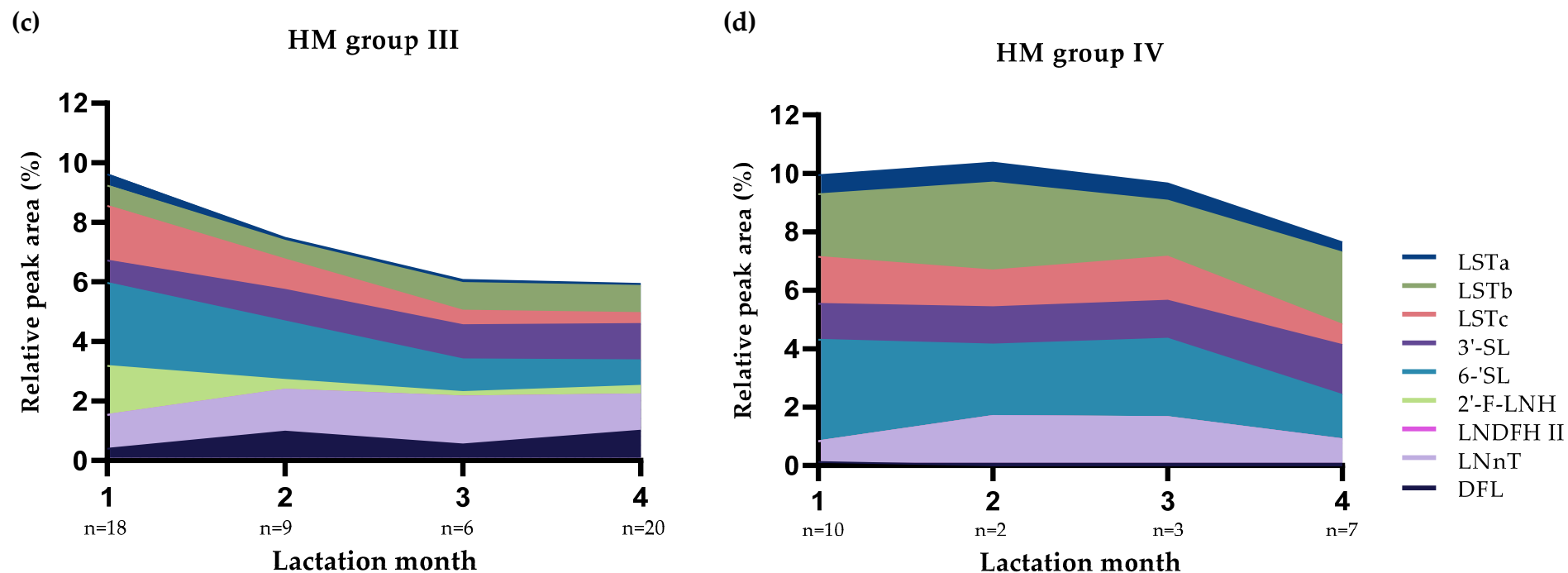
**Supplemental Table S4**. Percentage of human milk (HM) samples (n=171) in which the specific human milk oligosaccharide (HMOS) was detected (Presence, % of total) and relative peak area (rPA, %) of the selected HMOS (median (Q1-Q3)) according to maternal HM group [4–6]. HM samples were collected longitudinally over the first year postpartum from 24 Dutch mothers.

| | HM Group I (n = 126 Samples, 18 Donors) | | HM Group II (n = 21 Samples, 3 Donors) | | HM Group III (n = 15 Samples, 2 Donors) | | HM Group IV (n = 9 Samples, 1 Donor) | |
|---|---|---|---|---|---|---|---|---|
| | Presence % of total[1] | rPA (%) Median (Q1-Q3) | Presence % of total[1] | rPA (%) Median (Q1-Q3) | Presence % of total[1] | rPA (%) Median (Q1-Q3) | Presence % of total[1] | rPA (%) Median (Q1-Q3) |
| 2'-FL | 100 | 22.3 (15.0-29.9)[a] | 0 | - | 100 | 42.0 (35.5-53.0)[b] | 0 | - |
| 3-FL | 96 | 12.2 (6.29-21.7)[a] | 100 | 35.9 (26.4-45.3)[b] | 33 | 3.61 (3.04-4.40)[c] | 100 | 6.78 (3.04-7.31)[d] |
| DFL | 100 | 1.86 (1.29-2.50)[a] | 0 | - | 100 | 1.01 (0.62-1.48)[a] | 0 | - |
| LNT | 100 | 10.6 (7.87-13.1)[a] | 100 | 15.3 (11.3-20.6)[b] | 100 | 9.77 (6.50-13.8)[a] | 100 | 51.8 (47.8-59.3)[c] |
| LNnT | 94 | 1.03 (0.73-1.51)[a] | 71 | 0.73 (0.55-1.13)[b] | 93 | 1.22 (0.96-1.80)[a] | 100 | 1.35 (0.94-1.89)[a,b] |
| LNFP I+V | 100 | 7.56 (3.29-12.5)[a] | 100 | 1.51 (1.36-1.63) | 100 | 18.8 (15.6-24.5)[b] | 100 | 1.29 (1.16-1.55) |
| LNFP II | 100 | 5.79 (3.31-9.01)[a] | 100 | 19.7 (17.1-21.2)[b] | 0 | - | 0 | - |
| LNFP III | 100 | 7.52 (5.66-9.12)[a] | 100 | 9.28 (8.06-10.6)[b] | 100 | 8.94 (6.99-12.3)[a,b] | 100 | 15.6 (10.1-18.4)[b] |
| LNDFH I | 100 | 16.3 (13.5-19.1)[a] | 0 | - | 0 | - | 0 | - |
| LNDFH II | 6.3 | 1.20 (1.04-1.85)[a] | 90 | 3.91 (3.09-4.19)[b] | 0 | - | 0 | - |
| 3'-SL | 100 | 1.08 (0.78-1.43)[a] | 100 | 1.37 (1.04-1.91)[a] | 100 | 1.18 (1.04-2.15)[a] | 100 | 1.76 (1.05-2.21)[a] |
| 6'-SL | 95 | 0.88 (0.52-1.66)[a] | 95 | 0.74 (0.51-1.71)[a] | 100 | 0.91 (0.37-1.62)[a] | 100 | 1.74 (0.66-2.71)[a] |
| DSLNT | 100 | 1.97 (1.40-2.47)[a] | 95 | 3.30 (2.91-3.75)[b] | 100 | 2.95 (1.94-4.10)[a,b] | 100 | 5.53 (5.00-8.83)[c] |
| LSTa | 46 | 0.20 (0.17-0.28)[a] | 33 | 0.26 (0.20-0.31)[a] | 27 | 0.23 (0.21-0.42)[a] | 100 | 0.78 (0.75-0.93)[b] |
| LSTb | 99 | 0.93 (0.69-1.08)[a] | 100 | 1.43 (1.29-1.89)[b] | 100 | 0.64 (0.51-0.98)[a] | 100 | 2.36 (2.18-2.72)[c] |
| LSTc | 73 | 0.57 (0.27-1.08)[a] | 57 | 0.70 (0.32-1.20)[a] | 60 | 0.53 (0.29-0.77)[a] | 89 | 1.17 (0.51-1.64)[a] |
| 3'-F-LNH | 93 | 2.49 (1.59-3.90)[a] | 86 | 4.06 (1.23-6.82)[a,b] | 87 | 2.76 (1.75-4.37)[a] | 100 | 7.46 (4.94-8.41)[b] |
| 2'-F-LNH | 16 | 1.00 (0.61-2.14)[a] | 0 | - | 20 | 0.90 (0.32- .)[3 a] | 0 | - |
| β3'-GL[2] | 83 | 0.087 (0.062-0.15)[a] | 95 | 0.11 (0.081-0.14)[a] | 73 | 0.12 (0.11-0.13)[a] | 100 | 0.42 (0.38-0.51)[b] |
| β6'-GL[2] | 100 | 0.36 (0.25-0.53)[a] | 100 | 0.52 (0.44-0.61)[b] | 100 | 0.42 (0.38-1.01)[a,b] | 100 | 1.07 (0.87-1.36)[c] |
| α3'-GL[2] | 1.6 | 0.18 (0.13- .)[3 a] | 0 | - | 0 | - | 0 | - |

[1] Percentage of HM samples in which the specific HMOS was detected (≥limit of quantification (LOQ)); [2] For β6'-GL, β3'-GL and α3'-GL, the limit of detection was defined at a signal-to-noise ratio (S/N) of 3. For all other HMOS, the limit of quantification was defined at an S/N of 10; [3] In only two samples, the respective HMOS was present and hence Q3 could not be calculated; [a, b, c and d] Different superscript letters indicate statistical significance ($p < 0.05$). Significance values have been adjusted by the Bonferroni correction for multiple tests.

(a) HM group I

(b) HM group II

**Supplemental Figure S1.** Median relative peak areas (rPA) of low-abundant human milk oligosaccharides (HMOS) in the human milk (HM) samples (n=635) collected in the first 4 months of lactation according to HM group [4–6] and lactation stage. (**a**) HM group I; (**b**): HM group II; (**c**): HM group III and (**d**): HM group IV. Three samples could not be assigned to a HM group and are for this reason not depicted in these figures.

## References

1. Kottler R, Mank M, Hennig R, Müller-Werner B, Stahl B, Reichl U, et al. Development of a high-throughput glycoanalysis method for the characterization of oligosaccharides in human milk utilizing multiplexed capillary gel electrophoresis with laser-induced fluorescence detection. Electrophoresis. 2013;34(16):2323-36.
2. Melnik LA, Krysenko DA. Ultrapure Water: Properties, Production, and Use. J Water Chem Tech. 2019;41:143-50.
3. Ullsten S, Danielsson R, Backstrom D, Sjoberg P, Bergquist J. Urine profiling using capillary electrophoresis-mass spectrometry and multivariate data analysis. Journal of chromatography A. 2006;1117(1):87-93.
4. Stahl B, Thurl S, Henker J, Siegel M, Finke B, Sawatzki G. Detection of four human milk groups with respect to Lewis-blood-group-dependent oligosaccharides by serologic and chromatographic analysis. Advances in experimental medicine and biology. 2001;501:299-306.
5. Thurl S, Henker J, Siegel M, Tovar K, Sawatzki G. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. Glycoconj J. 1997;14(7):795-9.
6. Thurl S, Munzert M, Henker J, Boehm G, Müller-Werner B, Jelinek J, et al. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. Br J Nutr. 2010;104(9):1261-71.