

## Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation

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Using reverse-phase HPLC after pyridylamination, we quantified the concentrations of major neutral oligosaccharides in the milk of sixteen Japanese women collected at 4, 10, 30 and 100 d postpartum. In colostrum and mature milk (30 d lactation), lacto-N-fucopentaose (LNFP) I was the most abundant oligosaccharide, followed by 2'-fucosyllactose (2'-FL) + lacto-N-difucotetraose (LNDFH I), LNFP II + lacto-N-difucohexaose II (LNDFH II), and 3-fucosyllactose (3-FL). Together these accounted for 73 % of the total weight of neutral oligosaccharides in colostrum and mature milk. Changes in concentration occurred during the course of lactation. LNFP I and 2'-FL + LNDFH I increased from 4 to 10 d postpartum, and then declined by 100 d. LNFP II + LNDFH II steadily increased during the first 30 d and then declined. In contrast, 3-FL increased steadily throughout the entire 100 d of study. Large differences were observed between our data and previously published data in Italian women, in terms of both the concentration and temporal changes of each oligosaccharide. These differences may be caused by different assay methodology, although racial differences cannot be ruled out.

### Human milk oligosaccharides: Lactation period: Pyridylamination: High-performance liquid chromatography

The oligosaccharide fraction of human milk is the third largest solid component following lactose and lipid (Newburg & Neubauer 1995). Mature human milk contains 12–14 g oligosaccharides/l while human colostrum contains 20–24 g/l. Using matrix-assisted laser desorption/ionization MS (Stahl *et al.* 1994) and other methods, approximately 130 oligosaccharides have been found in human milk, with about eighty of them characterized. Based on their core structure, they are classified in twelve families. The secretor and Lewis phenotypes of lactating woman determine the structure of the terminal group of these carbohydrate chains, and have been used to characterize the

Lewis a and Lewis b blood group determinants (Watkins, 1966; Thurl *et al.* 1997).

Breast-fed infants appear to have fewer or less severe gastrointestinal and respiratory infections during the first year of life than formula-fed infants. Milk oligosaccharides may play a protective role. In the first instance, they act as a growth factor for intestinal bacteria such as *Bifidobacterium bifidum* (György *et al.* 1954; Yoshioka *et al.* 1983; McVeagh & Miller, 1997; Newburg, 2000). Human milk oligosaccharides containing N-acetylglucosamine are essential for the growth of *B. bifidum* (György & Rose, 1955). Bifidobacteria produce lactic acid by lactose fermentation,

**Abbreviations:** 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; LNDFH, lacto-N-difucohexaose; LNDFH I, lacto-N-difucotetraose; LNFP, lacto-N-fucopentaose; LNHI, lacto-N-hexaose; LNnH, lacto-N-neohexaose; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose.

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increasing the acidity of the intestinal environment, and thereby inhibiting the growth of pathogenic organisms.

Second, milk oligosaccharides act as direct inhibitors to pathogenic organisms (Andersson *et al.* 1986; Coppa *et al.* 1990, 2000; Dai *et al.* 1990; Cravioto *et al.* 1991; Kunz & Rudloff, 1993; Cervantes *et al.* 1995; Idota *et al.* 1995; Newburg, 1996, 1997, 1999; Zopf & Roth, 1996; McVeagh & Miller, 1997; Portelli *et al.* 1998; Miller & McVeagh, 1999; Newburg *et al.* 1999). It is known that some human milk oligosaccharides have structures analogous to glycoproteins and glycolipids on cell surface receptors for pathogens and that they bind competitively to pathogens in the gastrointestinal, respiratory, and urinary tract.

The amounts of oligosaccharides increase or decrease in human milk during the course of lactation to protect infants from viral or bacterial attack, but quantitative data for individual oligosaccharides are scarce (Coppa *et al.* 1999; Nakhla *et al.* 1999; Erney *et al.* 2000; Chaturvedi *et al.* 2001). Coppa *et al.* (1999) reported changes in several oligosaccharides in the milk of Italian women at different stages of lactation, but it is not known if this is representative of all women.

In the present study we report quantitative data on several neutral oligosaccharides in the milk of Japanese women throughout the course of lactation. In some quantitative methods of sugars utilizing pre-column derivatization (Chaturvedi *et al.* 1997; Yasuno *et al.* 1999), we used reductive amination with 2-aminopyridine and subsequent HPLC to determine each oligosaccharide content in the milk, because the procedures are easy and the derivatives are very sensitive to being quantified in HPLC analysis. These are well-established methods for the analysis and determination of some oligosaccharides and carbohydrate moieties of glycoproteins (Hase, 1994; Saito *et al.* 1998; Yanagida *et al.* 1999). The pyridylation reaction proceeds in two steps; a coupling reaction (Schiff base formation) and reduction of the Schiff base. It has been reported that the methods have high recovery and reproducibility ( $\pm 2\%$ ) and allow the determination of picomole levels of neutral and amino sugars (Takemoto *et al.* 1985). We used only a small quantity of milk (1 ml) to determine each oligosaccharide content with this method.

## Materials and methods

### Materials

Human milk samples were collected from sixteen women at 4, 10, 30 and 100 d postpartum. Two samples, one collected at 10 d and one at 100 d postpartum, were excluded from the analysis. All samples (approximately 3–5 ml) at each lactation period were collected by hand into sterilized tubes, which were then stored at  $-40^{\circ}\text{C}$  until use. The ABO blood group of the donors was determined: A, three; B, five; O, seven; AB, one. The Rhesus blood group of all donors was positive.

3-Fucosyllactose (3-FL) and lacto-N-fucopentaose (LNFP) III were purchased from Funakoshi Co., Tokyo, Japan, and 2'-fucosyllactose (2'-FL), lacto-N-difucohexaose (LNDFH) II and lacto-N-hexaose (LNH) were from Seikagaku Co., Tokyo, Japan. Lacto-N-tetraose (LNT),

lacto-N-neotetraose (LNnT), lacto-N-difucohexaose (LNDFH), LNFP I, LNFP II, LNDFH I and lacto-N-neohexaose (LNnH) were obtained from Sigma, St Louis, MO.

### Preparation of each oligosaccharide fraction from human milk

The milk samples were thawed and a portion of each (1 ml) was extracted with 4 volumes of chloroform–methanol (2:1, v/v). The emulsion was centrifuged at 3500 rpm for 30 min at  $4^{\circ}\text{C}$  and the lower chloroform layer and denatured protein were discarded. The upper layer was collected and the fraction was evaporated and then freeze-dried. The resulting powders were called the 'carbohydrate fraction'.

The carbohydrate fraction was dissolved in water (1 ml) and the solution passed through a Bio Gel P-2 (Extra fine,  $<45\ \mu\text{m}$ ; Bio Rad Laboratories, USA) column ( $2.6 \times 100\ \text{cm}$ ) which had been calibrated with 2 mg of each of galactose (monosaccharide), lactose (disaccharide), raffinose (tri-saccharide). Elution was done with water at a flow rate of 15 ml/h and ninety-five fractions of 5 ml were collected. Aliquots (0.25 ml) of each fraction were analysed for hexose using the phenol– $\text{H}_2\text{SO}_4$  method (Dubois *et al.* 1956) and for sialic acid with the periodate–resorcinol method (Jourdain *et al.* 1971). Peak fractions were pooled and freeze-dried.

Of fractions separated by the gel chromatography, the fraction, which was positive by the periodate–resorcinol method, was dissolved in 1 ml of 50 mM-Tris-hydroxyaminomethane (Tris)–HCl buffer (pH 8.7) and subjected to anion exchange chromatography with the DEAE Sephadex A-50 (Amersham Pharmacia Biotech UK Ltd., UK) column ( $1.0 \times 40\ \text{cm}$ ) equilibrated with the same buffer. The unadsorbed components containing the neutral oligosaccharide fraction were eluted with the same buffer (100 ml) and the adsorbed components containing the acidic oligosaccharide fraction were eluted with 300 ml of linear gradient from 0 to 1.0 mM-NaCl in 50 mM-Tris-HCl buffer (pH 8.7). Elution was done at a flow rate of 15 ml/h and fractions of 5 ml were collected. Aliquots (0.25 ml) of each fraction were analysed for hexose. Peak fractions were pooled and freeze-dried. The neutral oligosaccharide in the unadsorbed fraction was dissolved in water (1 ml) and passed through a Bio Gel P-4 (Bio Rad, Extra fine,  $<45\ \mu\text{m}$ ) column ( $2.6 \times 100\ \text{cm}$ ) under the same condition as in the gel chromatography with Bio Gel P-2. Peak fractions were pooled and freeze-dried.

### Quantification of each neutral oligosaccharide

The pyridylaminated derivative of each neutral oligosaccharide fraction was prepared according to the method of Kondo *et al.* (Kondo *et al.* 1990; Suzuki *et al.* 1991) as described in the analysis of galactosyloligosaccharides in Yakult products (Kimura *et al.* 1995) and commercial cows' milk (Kimura *et al.* 1997). Each sample was dried in a screw-cap tube ( $16 \times 100\ \text{mm}$ ) under reduced pressure at room temperature. Coupling reagent (100  $\mu\text{l}$ ) (1.1 g 2-aminopyridine (Wako Pure Chemical Industries Ltd., Osaka, Japan) in 650  $\mu\text{l}$  acetic acid) was added to the

residue in the tube. The tube was sealed with a screw cap and heated at 90°C for 1 h, and then 100 µl of freshly prepared reducing reagent (195 mg borane–dimethylamine complex (Aldrich Chemical Company Inc., USA) in 1 ml acetic acid) was added. The tube was sealed again and heated at 80°C for 50 min. The resulting pyridylaminated derivatives were separated from the reaction mixture by electro dialysis using a Microacilyzer model G-1 (Asahi Kasei Co., Kanagawa, Japan) with a 120-10 cartridge, followed by freeze-drying. The detail of the electro dialysis procedure has been described elsewhere by Kimura *et al.* (1995).

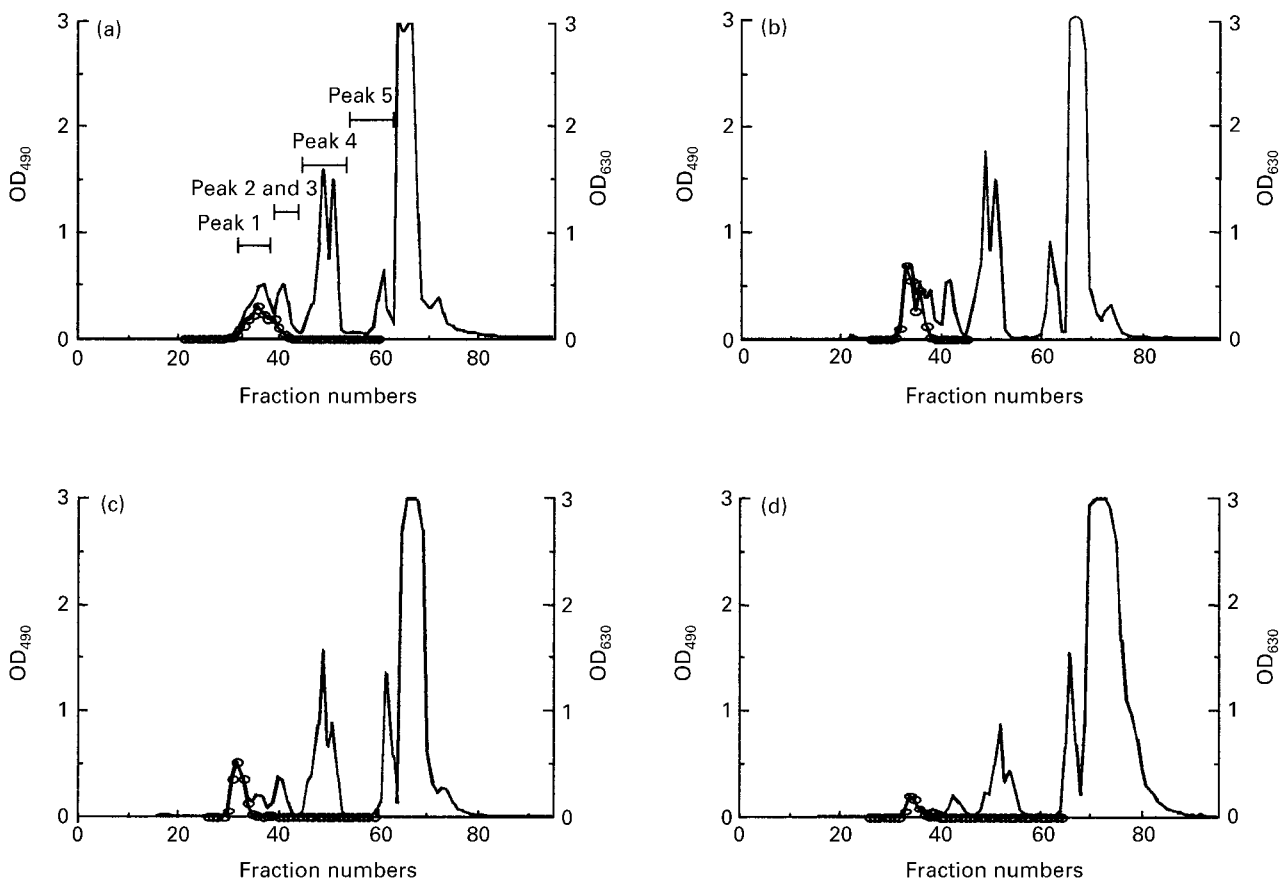
Pyridylaminated oligosaccharide fractions were subjected to reverse-phase HPLC. The analysis was performed with an Inertsil ODS-3V column (4.6 × 250 mm, packed with 5 µl particles having 10 nm pore size; GL Sciences Inc., Tokyo, Japan) at 25°C in a column oven (CO-8020; Tosoh Co., Tokyo, Japan). The eluate was controlled by a multi-pump (CCMP-II; Tosoh) equipped with a CCP controller (PX-8020; Tosoh). Ammonium acetate (a/M pH 5.5) was used as solution A and 5% (v/v) methanol in 0.1 M-ammonium acetate solution (pH 5.5) as solution B. Elution was done at a flow rate of 1 ml/min and with a linear gradient from 0 to 100% of solution B in 60 min. Detection was done with a MCPD-3600 Photodiode Array Detector (Otsuka Electronics Co. Ltd,

Osaka, Japan) by monitoring the optical density of the eluates at 310 nm. Pyridylaminated oligosaccharides were dissolved in water (1 ml) followed by filtration through a DISMIC-13CP membrane (0.45 µm, cellulose acetate membrane; Advantec Toyo, Tokyo, Japan), and 10 µl was injected. Identification was performed by corresponding the relative retention time of each peak to isomaltotetraose with that of the standard. The quantitative value of each pyridylaminated oligosaccharide was obtained by calculation of the ratio of the peak area compared with that of the standard.

The recovery of several oligosaccharides in this determining method has been described by K Kimura, C Ishihara and A Miyagi (unpublished results) in a Japanese patent report (potent number 3231103), and will be published in another paper.

## Results

The representative Bio Gel P-2 chromatograms of the carbohydrate fractions extracted from the milk at each lactation stage are shown in Fig. 1. The fractions were separated into peaks 1 to 5. The fractions containing both neutral and acidic oligosaccharides were further subjected to anion exchange chromatography with DEAE Sephadex A-50. The neutral oligosaccharides fraction was separated



**Fig. 1.** Gel chromatograms of the carbohydrate fractions separated on a Bio Gel P-2 (2.6 × 100 cm) column. Each fraction was analysed for hexose using the phenol–H<sub>2</sub>SO<sub>4</sub> method at 490 nm (—) and for sialic acid with the periodate–resorcinol method at 630 nm (---). OD<sub>490</sub>, optical density at 490 nm; OD<sub>630</sub>, optical density at 630 nm. The carbohydrate fractions were separated from human milk at (a) 4, (b) 10, (c) 30 and (d) 100 d post partum.

**Table 1.** The neutral oligosaccharides quantified in human milk at several stages of lactation\*

Oligosaccharide	Structure
3-FL	Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc
2'-FL	Fuc $\alpha$ 1-2Gal $\beta$ 1-4Glc
LNDFT	Fuc $\alpha$ 1-2Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc
LNT	Gal $\beta$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc
LNnT	Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc
LNFP I	Fuc $\alpha$ 1-2Gal $\beta$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc
LNFP II	Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc
LNFP III	Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc
LNDFH I	Fuc $\alpha$ 1-2Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ 1-3Gal $\beta$ 1-4Glc
LNDFH II	Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ 1-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc
LNH	Gal $\beta$ 1-3GlcNAc $\beta$ 1-3(Gal $\beta$ 1-4GlcNAc $\beta$ 1-6)Gal $\beta$ 1-4Glc
LNnH	Gal $\beta$ 1-4GlcNAc $\beta$ 1-3(Gal $\beta$ 1-4GlcNAc $\beta$ 1-6)Gal $\beta$ 1-4Glc

3-FL, 3-fucosyllactose; 2'-FL, 2'-fucosyllactose; LNDFT, lacto-N-difucotetraose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP, lacto-N-fucopentaose; LNDFH, lacto-N-difucohexaose; LNH, lacto-N-hexaose; LNnH, lacto-N-neohexaose; Gal, galactose; Fuc, fucose; Glc, glucose; Ac, acetyl.

\* For details of procedures, see p. 62.

by anion exchange chromatography and Bio Gel P-4 column chromatography, and designated as peak 3.

The area of peak 3, which contained the higher oligosaccharides, decreased relative to the others from 4 to 10 d postpartum. Subsequently, there was little change up to 100 d postpartum. The area of peak 4, which contained tetra- to hexa-saccharides, increased relative to the other peaks from 4 to 10 d postpartum, and then decreased up to 100 d postpartum. On the other hand, the area of peak 5, which contained tri- to tetra-saccharides, increased up to 100 d postpartum.

The oligosaccharides quantified in the present study are listed in Table 1. In recovery studies, greater than 90% of each pyridylaminated oligosaccharide was recovered. It

was shown that each peak contained the following oligosaccharides by HPLC of peak 3 to 5: LNH and LNnH in peak 3; LNT, LNnT, LNFP I, LNFP II, LNFP III, LNDFH I and LNDFH II in peak 4; 3-FL, 2'-FL and LNDFT in peak 5 (Fig. 2) LNFP II and LNDFH II in peak 4, and 2'-FL and LNDFT in peak 5 were not separated from each other by the HPLC. Because peak 3 had too many peaks derived from higher oligosaccharides, we could not quantify LNH and LNnH.

The quantitative value of each and the mean concentration of each oligosaccharide in each lactation period are shown in Table 2. Characteristic changes in each oligosaccharide can be seen. The total oligosaccharide concentration calculated by summing all the compounds in the

**Table 2.** Concentration of each oligosaccharide (mg/l) in human milk at 4, 10, 30 and 100 d postpartum\* (Mean values with their standard errors, and ranges)

	Day of lactation							
	4 (n 16)		10 (n 15)		30 (n 16)		100 (n 15)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
3-FL	232.5	68.1	280.9	55.3	427.5	105.1	452.0	127.5
Range	13.8–1009.3		3.7–766.0		23.0–1423.6		0.0–1491.8	
2'-FL+LNDFT	403.5	199.8	685.5	265.0	580.6	209.4	89.4	39.3
Range	0.0–2482.5		0.0–3448.2		0.0–2448.7		0.0–599.1	
LNT	347.2	79.1	360.9	78.5	259.6	45.6	96.1	40.4
Range	8.7–988.7		11.6–830.2		6.7–565.4		2.9–648.2	
LNnT	211.4	42.0	208.8	51.3	155.5	58.3	44.4	13.0
Range	10.6–516.7		0.0–718.2		0.0–768.7		0.0–146.0	
LNFP I	968.7	205.6	1137.5	319.9	742.7	204.7	268.1	109.1
Range	62.1–2772.5		0.0–4168.4		9.1–2665.3		0.0–1770.6	
LNFP III	52.3	8.9	71.6	17.7	73.5	16.2	71.9	22.0
Range	4.7–108.4		4.3–217.1		4.7–217.6		8.3–296.9	
LNFP II + LNDFH II	323.4	88.8	457.9	143.0	557.8	150.1	301.7	86.8
Range	17.5–1057.5		21.2–2292.0		33.8–2238.0		27.1–1095.3	
LNDFH I	144.6	29.9	225.6	62.3	156.5	37.0	90.4	25.3
Range	0.0–343.8		0.0–695.3		2.7–386.3		0.0–344.8	
Total†		2683.6		3426.8		2953.7		1414.0

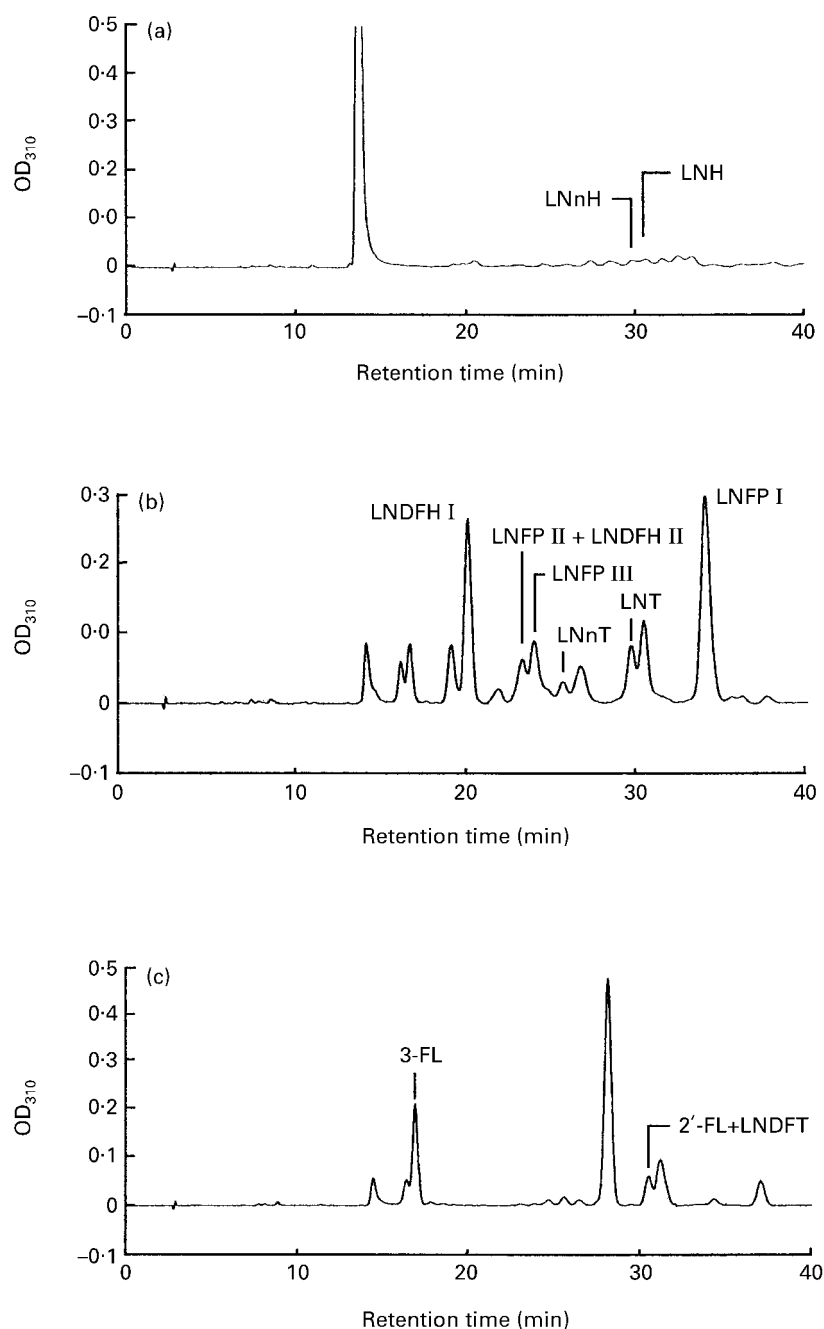
3-FL, 3-fucosyllactose; 2'-FL, 2'-fucosyllactose; LNDFT, lacto-N-difucotetraose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP, lacto-N-fucopentaose; LNDFH, lacto-N-difucohexaose.

\* For details of procedures see p. 62.

† The sum of the above oligosaccharides quantified in human milk in the present study.

## Neutral oligosaccharides in human milk

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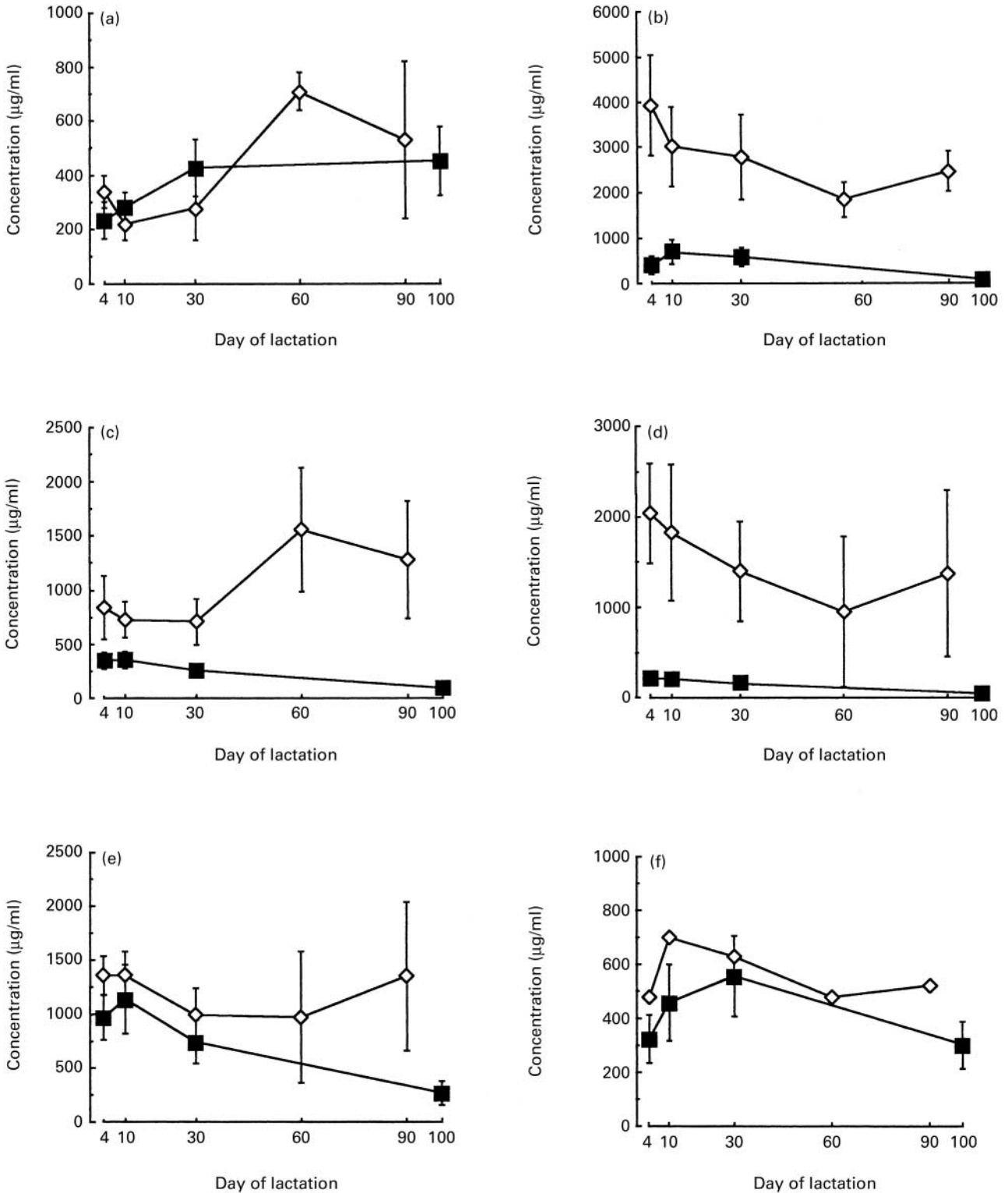
**Fig. 2.** High-performance liquid chromatogram of the derivatives with pyridylation of (a), peak 3; (b), peak 4; (c), peak 5 separated from human milk at 4 d postpartum. OD<sub>310</sub>, optical density at 310 nm; LNnH, lacto-N-neohexaose; LNnH, lacto-N-hexaose; LNDFH, lacto-N-difucohexaose; LNFP, lacto-N-fucopentaose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; 3-FL, 3-fucosyllactose; 2'-FL, 2'-fucosyllactose; LNDFT, lacto-N-difucotetraose.

present study increased from 2683.6 mg/l at 4 d postpartum to 3426.8 mg/l at 10 d postpartum, and then decreased to 1414.0 mg/l at 100 d postpartum. However, the concentration of individual oligosaccharides varied greatly among the donors and some oligosaccharides were not detected in specific lactation periods of some donors.

No relationship was observed between oligosaccharide content and the ABO blood group of the donors in the present study.

## Discussion

These findings provide precise information about the concentration and pattern of change of individual neutral oligosaccharides in human milk. Large differences were observed between the data obtained in the present study and those reported by Coppa *et al.* (1999) for Italian women (Fig. 3). Coppa *et al.* (1999) may have overestimated the amounts because of overlapping of the



**Fig. 3.** Changes in concentration of major oligosaccharides in human milk during the course of lactation. (■), Data from present study (Japanese women); (◇), data from Coppa *et al.* (1999) for Italian women. (a) 3-FL; (b) 2'-FL + LNDFT; (c) LNT; (d) LNnT; (e) LNFPI; (f) LNFPII + LNDFHII.

peaks corresponding to several oligosaccharides. However, the differences could also be due to the small sample sizes in both studies and the natural variability among women. It is well known that the oligosaccharide content of milk varies according to blood groups Lewis a, b, or x and

their different secretory status (Thurl *et al.* 1997). Oligosaccharide content in milk has also been suggested to vary according to racial group as shown by Erney *et al.* (2000). Methodological issues may also explain the differences between the two studies. We used HPLC analysis

after pyridylamination of the fractions separated by gel filtration of the milk carbohydrates, whereas Coppa *et al.* (1999) used high pH anion exchange chromatography of the fractions separated directly from the milk by centrifugation and filtration with a 0.22  $\mu\text{m}$  membrane. At the present time, it is not known whether one method is more accurate than the other.

In particular, the core oligosaccharides such as LNT, LNnT, LNH and LNnH in the present study were significantly lower than those reported by Coppa *et al.* (1999). It is possible that there is lower activity of the enzymes  $\beta$  3 or  $\beta$  6 N-acetylglucosaminyltransferase, which are involved in the biosynthesis of those oligosaccharides, in the lactating mammary glands of Japanese women.

In the present study, the contents of  $\alpha$  1-2 fucosyloligosaccharides increased from 4 to 10 d postpartum, and that of  $\alpha$  1-3/4 fucosyloligosaccharides increased from 4 to 30 d postpartum. This suggests that  $\alpha$  1-2 and  $\alpha$  1-3/4 fucosyltransferase activities are high in early lactation. The content of  $\alpha$  1-2 fucosyloligosaccharides started to decrease from 10 d postpartum, suggesting that  $\alpha$  1-2 fucosyltransferase activity decreased at this stage. On the other hand, the contents of  $\alpha$  1-3/4 fucosyloligosaccharides decreased from 30 to 100 d postpartum, suggesting that  $\alpha$  1-3/4 fucosyltransferase activity decreased from 30 to 100 d postpartum. These data also suggest that total fucosyltransferase activity is high in early lactation and decreases from 30 to 100 d postpartum. Wiederschain & Newburg (1995) reported that fucosyltransferase activity in milk is maximal in colostrum, and then decreases temporarily, followed by an increase until 30 d postpartum. In the study of Coppa *et al.* (1999) the contents of 2'-FL + LNDFI and LNFI were maximal at 4 d postpartum, whereas the content of LNFI + LNDFI was maximal at 10 d postpartum. The content of 3-FL was maximal at 60 d postpartum and then decreased. The difference in milk fucosyloligosaccharides contents between our data and Coppa *et al.* (1999) may be caused by differences in fucosyltransferase activities in the lactating mammary glands of Japanese *v.* Italian women.

Recently, Chaturvedi *et al.* (2001) determined the level of individual oligosaccharides in the milk of Mexican women. The  $\alpha$  1-3/4 fucosyloligosaccharide contents were similar to those of our study, whereas  $\alpha$  1-2 fucosyloligosaccharide content was higher, increasing from 7 to 14 weeks postpartum. Again, we do not know whether these differences are determined by ethnicity, Lewis blood group status, methodology or the small numbers of mothers. Only large-scale studies of lactating women in different populations using uniform methods will answer these questions.

Although we determined the content of the representative neutral oligosaccharides, human milk contains more than 100 different oligosaccharides (Newburg & Neubauer, 1995). Many oligosaccharides have large molecular weights. Unfortunately at present we cannot determine the content of individual higher oligosaccharides with any technique. The difference between the combined total of the oligosaccharides determined in the present study and the total oligosaccharide content of human milk reported in the literature (12–14 g/l in mature milk

and 20–24 g/l in colostrum) reflects the large quantities of acidic and large-molecular-weight oligosaccharides in human milk.

It has been postulated that some of the human milk oligosaccharides act as analogues or homologues to host cell surface receptors for pathogens. LNT or LNnT inhibit attachment of *Streptococcus pneumoniae* (Andersson *et al.* 1986) to pharyngeal or buccal epithelial cells, and fucosyloligosaccharides inhibit the adhesion of enteropathogenic *Escherichia coli* (Cravioto *et al.* 1991; Cervantes *et al.* 1995) or heat-stable enterotoxin of *E. coli* (Cervantes *et al.* 1995; Newburg *et al.* 1999) to intestinal cells. Our data, showing that neutral oligosaccharide content is very high in colostrum and gradually declines as lactation proceeds, supports the hypothesis that oligosaccharides function as anti-infective factors in newborn infants whose immune systems are not mature.

Many of the milk oligosaccharides are growth factors for the intestinal micro-organisms Bifidobacterium, among the most concentrated being LNT and LNFI (György & Rose, 1955). The high content of oligosaccharides in colostrum would be favourable for establishment of the Bifidus flora in the newborn colon. The oligosaccharide concentrations in the colostrums of preterm milk have been found to be significantly higher than full-term colostrum (Coppa *et al.* 1997), suggesting that the oligosaccharides act as anti-pathogenic factors and Bifidobacterium growth factors for the preterm babies, too.

Lactating mothers produce about 600–900 ml milk/d through the first year after delivery (Neville, 1995), and their babies take a few grams of oligosaccharides/d. On the other hand, the content of oligosaccharides in infant formula is low because bovine milk, the raw material for infant formula, has a significantly lower oligosaccharides content than human milk (Neeser *et al.* 1991). Since the composition of infant formulas is modelled on that of human milk, consideration should be given to the addition of human milk oligosaccharides, the third largest solute in human milk, to infant formula. Attempts to synthesise LNT, LNnT (Murata *et al.* 1999a) and fucosyloligosaccharides (Murata *et al.* 1999b; Farkas *et al.* 2000) have been made by transglycosylation with glycosyltransferase or glycosidase specifically for the purpose of addition to infant formula. Our data provide a basis for estimating the amounts that might be considered appropriate.

## References

- Andersson B, Porras O, Hanson LÅ, Lagergård T & Svanborg-Edén C (1986) Inhibition of attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by human milk and receptor oligosaccharides. *Journal of Infectious Disease* **153**, 232–237.
- Cervantes LE, Newburg DS & Ruiz-Palacios GM (1995)  $\alpha$  1-2 fucosylated chains (H-2 and Lewis b) are the main human milk receptor analogs for *Campylobacter*. *Pediatric Research* **37**, 171A.
- Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK & Newburg DS (2001) Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology* **11**, 365–372.

- Chaturvedi P, Warren CD, Ruiz-Palacios GM, Pickering LK & Newburg DS (1997) Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives. *Analytical Biochemistry* **251**, 89–97.
- Coppa GV, Bruni S, Zampini L, Galeazzi T, Capretti R, Facinelli B & Gabrielli O (2000) Human milk oligosaccharides inhibit the adhesion of *Listeria monocytogenes*, *Escherichia coli*, *Vibrio cholerae* and *Salmonella typhi*. In *20th International Carbohydrate Symposium*, Hamburg, 27 August–1 September 2000. Abstract, International Carbohydrate Organization.
- Coppa GV, Gabrielli O, George P, Catassi C, Montanari MP, Varaldo PE & Nichols BL (1990) Preliminary study of breast-feeding and bacterial adhesion to uroepithelial cells. *Lancet* **335**, 569–571.
- Coppa GV, Pierani P, Zampini L, Carloni I & Gabrielli O (1999) Oligosaccharides in human milk during different phases of lactation. *Acta Paediatrica* **430**, Suppl., 89–94.
- Coppa GV, Pierani P, Zampini L, Gabrielli O, Carlucci A, Catassi C & Giorgi PL (1997) Lactose, oligosaccharide and monosaccharide content of milk from mothers delivering preterm newborns over the first month of lactation. *Minerva Pediatrica* **49**, 471–475.
- Cravioto A, Tello A, Villafán H, Ruiz J, Vedovo S & Nesser JR (1991) Inhibition of localized adhesion of enteropathogenic *Escherichia coli* to Hep-2 cells by immunoglobulin and oligosaccharide fractions of human colostrums and breast milk. *Journal of Infectious Disease* **163**, 1247–1255.
- Dai D, Nanthkumar NN, Newburg DS & Walker WA (1990) Role of oligosaccharides and glycoconjugates in intestinal host defense. *Journal of Pediatric Gastroenterology and Nutrition* **30**, s23–s33.
- Dubois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.
- Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O’Ryan ML, Ruiz-Palacios G, Hilty MD, Pickering LK & Prieto PA (2000) Variability of human milk neutral oligosaccharides in a diverse population. *Journal of Pediatric Gastroenterology and Nutrition* **30**, 181–192.
- Farkas E, Thiem J & Ajisaka K (2000) Enzymatic synthesis of fucose-containing disaccharides employing the partially purified  $\alpha$ -L-fucosidase from *Penicillium multicolor*. *Carbohydrate Research* **328**, 293–299.
- György P, Norris RF & Rose CS (1954) Bifidus factor. I. A variant of *Lactobacillus bifidus* requiring a special growth factor. *Archives of Biochemistry and Biophysics* **48**, 193–201.
- György P & Rose CS (1955) Microbiological Studies on Growth Factor for *L. bifidus* var. *pennsylvanicus*. *Proceedings of the Society for Experimental Biology and Medicine* **90**, 219–223.
- Hase S (1994) High-performance liquid chromatography of pyridylaminated saccharides. *Methods in Enzymology* **230**, 225–237.
- Idota T, Kawakami H, Murakami Y & Sugawara M (1995) Inhibition of cholera toxin by human milk fractions and sialyllactose. *Bioscience, Biotechnology, and Biochemistry* **59**, 417–419.
- Jourdan GW, Dean L & Roseman S (1971) The sialic acids XI. A periodate-resorcinol method for quantitative estimation of free sialic acids and their glycosides. *Journal of Biological Chemistry* **246**, 430–435.
- Kimura K, Matsumoto K, Ishihara C, Harada K & Miyagi A (1995) Structure determination of galacto-oligosaccharides by pyridylamination and NMR spectroscopy. *Carbohydrate Research* **270**, 33–42.
- Kimura K, Watanabe Y, Matsumoto K & Miyagi A (1997) Studies on the natural galacto-oligosaccharides in commercial cow’s milk. *YAKULT Institute Report* **17**, 1–7.
- Kondo A, Suzuki J, Kuraya N, Hase S, Kato I & Ikenaka T (1990) Improved method for fluorescence labeling of sugar chains with sialic acid residues. *Agricultural and Biological Chemistry* **54**, 2169–2170.
- Kunz C & Rudloff S (1993) Biological functions of oligosaccharides in human milk. *Acta Paediatrica* **82**, 903–912.
- McVeagh P & Miller JB (1997) Human milk oligosaccharides: only the breast. *Journal of Paediatrics and Child Health* **33**, 281–286.
- Miller JB & McVeagh P (1999) Human milk oligosaccharides: 130 reasons to breast-feed. *British Journal of Nutrition* **82**, 333–335.
- Murata T, Inukai T, Suzuki M, Yamagishi M & Usui T (1999a) Facile enzymatic conversion of lactose into lacto-N-tetraose and lacto-N-neotetraose. *Glycoconjugate Journal* **16**, 189–195.
- Murata T, Morimoto S, Zeng X, Watanabe S & Usui T (1999b) Enzymatic synthesis of  $\alpha$ -L-fucosyl-N-acetylglucosamines and 3’-O- $\alpha$ -L-fucosyllactose utilizing  $\alpha$ -L-fucosidases. *Carbohydrate Research* **320**, 192–199.
- Nakhla T, Fu D, Zoph D, Brodsky NL & Hurt H (1999) Neutral oligosaccharide content of preterm human milk. *British Journal of Nutrition* **82**, 361–367.
- Neesser JR, Golliard M & Vedovo SD (1991) Quantitative determination of complex carbohydrates in bovine milk and in milk-based infant formulas. *Journal of Dairy Science* **74**, 2860–2871.
- Neville MC (1995) Volume of Caloric Density of Human Milk. In *Handbook of Milk Composition*, pp. 99–113 [RG Jensen, editor]. San Diego, CA: Academic Press.
- Newburg DS (1996) Oligosaccharides and glycoconjugates in human milk: Their role in host defense. *Journal of Mammary Gland Biology and Neoplasia* **1**, 271–283.
- Newburg DS (1997) Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria? *Journal of Nutrition* **127**, Suppl. 5, 980s–984s.
- Newburg DS (1999) Human milk glycoconjugates that inhibit pathogens. *Current Medicinal Chemistry* **6**, 117–127.
- Newburg DS (2000) Oligosaccharides in human milk and bacterial colonization. *Journal of Pediatric Gastroenterology and Nutrition* **30**, s8–s17.
- Newburg DS & Neubauer SH (1995) Carbohydrates in milks: Analysis, Quantities, and Significance. In *Handbook of Milk Composition*, pp. 273–349 [RG Jensen, editor]. San Diego, CA: Academic Press
- Newburg DS, Pickering LK, McCluer RH & Cleary TG (1999) Fucosylated oligosaccharides of human milk protect suckling mice from heat-stable enterotoxin of *Escherichia coli*. *Journal of Infectious Disease* **162**, 1075–1080.
- Portelli J, Gordon A & May JT (1998) Effect of human milk sialyllactose on Cytomegalovirus (letter). *European Journal of Clinical Microbiology and Infectious Diseases* **17**, 66.
- Saito T, Tsuji T, Kitazawa H, Kawai Y & Itoh T (1998) Application of two-dimensional mapping for an Analysis of Galactosyllactoses in yogurt. *Bioscience, Biotechnology, and Biochemistry* **62**, 1445–1447.
- Stahl B, Thurl S, Zeng J, Karas M, Hillenkamp F, Steup M & Sawatzki G (1994) Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Analytical Biochemistry* **223**, 218–226.
- Suzuki J, Kondo A, Kato I, Hase S & Ikenaka T (1991) Analysis by high-performance anion-exchange chromatography of component sugar as their fluorescent pyridylamino derivatives. *Agricultural and Biological Chemistry* **55**, 283–284.
- Takemoto H, Hase S & Ikenaka T (1985) Microquantitative analysis of neutral and amino sugars as fluorescent pyridyl-amino derivatives by high-performance liquid chromatography. *Analytical Biochemistry* **145**, 245–250.



- Thurl S, Henker J, Siegel M, Tovar K & Sawatzki G (1997) Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconjugate Journal* **14**, 795–799.
- Watkins WM (1996) *Glycoproteins: Their Composition, Structure and Function*, pp. 462–512 [A Gottschalk, editor]. Amsterdam: Elsevier.
- Wiederschain GY & Newburg DS (1995) Human milk Fucosyltransferase and  $\alpha$ -L-fucosidase activities change during the course of lactation. *Journal of Nutritional Biochemistry* **6**, 582–587.
- Yanagida K, Natsuka S & Hase S (1999) A pyridylation method aimed at automatic oligosaccharide analysis of N-linked sugar chains. *Analytical Biochemistry* **274**, 229–234.
- Yasuno S, Kokubo K & Kamei M (1999) New method for determining the sugar composition of glycoproteins, glycolipids, and oligosaccharides by high-performance liquid chromatography. *Bioscience, Biotechnology, and Biochemistry* **63**, 1353–1359.
- Yoshioka H, Iseki K & Fujita K (1983) Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics* **72**, 317–321.
- Zopf D & Roth S (1996) Oligosaccharide anti-infective agents. *Lancet* **347**, 1017–1021.