8

Milk Oligosaccharides

T. Urashima, M. Kitaoka, S. Asakuma, and M. Messer

8.1. Introduction

Mammalian milk contains up to 10% carbohydrate, of which the disaccharide, lactose (Gal(β1-4)Glc), is usually a prominent component. Milk and colostrum also contain lesser amounts of other saccharides, referred to as milk oligosaccharides, nearly all of which have a lactose unit at their reducing end to which GlcNAc, Gal, Fuc and/or Neu5Ac or Neu5Gc residues can be attached (Jenness *et al*., 1964; Newburg and Neubauer, 1995; Boehm and Stahl, 2003; Urashima *et al*., 2001; Messer and Urashima, 2002). Pronounced heterogeneity as well as homology of milk oligosaccharide structures among different mammalian species has been documented (Urashima *et al*., 2001; Messer and Urashima, 2002).

It is well known that free lactose is a significant energy source for human infants, but the exact biological significance of the milk oligosaccharides remains to be clarified. The study of milk oligosaccharides was greatly stimulated by the discovery of a *Bifidobacterium* growth factor in human milk and colostrum, but there is still no unequivocal answer to the question of which oligosaccharides in human milk are responsible for the bifidus factor, even though it is acknowledged that the oligosaccharide fraction is responsible for promoting the growth of bifidobacteria within the infant colon.

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Another critical problem to be solved has been the metabolic fate of human milk oligosaccharides. Recently, it has been concluded that most of these oligosaccharides are neither digested nor absorbed within the infant small intestine (Engfer *et al.*, 2000) and that instead, they can act as prebiotics and soluble receptor analogues, inhibiting the attachment of pathogenic bacteria or viruses to receptors in the infant colon, as discussed below. There is evidence, however, that a minor proportion of human milk oligosaccharides can be absorbed intact (Gnoth *et al.*, 2001), acting as immunomodulators within the circulation, and that the sialic acid of sialyl milk oligosaccharides can be used as a precursor for the formation of brain gangliosides or sialoglycoproteins.

The problem of the *Bifidobacterium* growth factor is at present still open to speculation. The genome libraries of a few species of bifidobacteria that have become publicly available enable one to focus on a series of enzymes involved in the metabolism of carbohydrates by these bacteria. From this theoretical approach, using the DNA database, one can speculate on which oligosaccharide structure might be used for the metabolism of each strain of *Bifidobacterium*. Kitaoka *et al.* (2005) have recently hypothesized that lacto-*N*-biose I (Gal(β1-3)GlcNAc) is the genuine, i.e., specific, bifidus factor (see below).

In this chapter, we describe the structures of human and bovine milk oligosaccharides as well as those of some other species, discuss their biosynthesis, intestinal digestion/absorption and significance as prebiotics, soluble receptor analogues and immunomodulators, and describe the present and likely future industrial utilization of milk oligosaccharide-like materials.

8.2. The Chemical Structures of Human Milk Oligosaccharides: Analytical Methods

The structures of at least 93 oligosaccharides of human milk have been determined to date (Table 8.1), while mass spectra (MS) data have suggested the presence of almost 130 oligosaccharides in human milk or colostrum (Newburg and Neubauer, 1995). Moreover, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) analyses suggest that polysaccharides, consisting of more than 50 monosaccharide residues, as indicated by size exclusion chromatography, are also present in human milk. Therefore, considerably more than 130 different saccharides are probably present in human milk (Boehm and Stahl, 2003). Recently, as many as 200 different human milk oligosaccharides have been separated and studied by microfluidic HPLC – chip MS (Ninonuevo *et al.*, 2006).

It has been shown that human milk and colostrum are rich in oligosaccharides, in concentration as well as in variety, compared with the milk/colostrum of the cow and of many other species (Gopal and Gill, 2000). As

Table 8.1 The structures of human milk oligosaccharides

	Abbreviation	Oligosaccharide	Reference
1. Lactose series			
1	2'-FL	Fuc(α 1-2)Gal(β1-4)Glc	Kuhn *et al.* (1956b)
2	3-FL	Gal(β1-4)Glc | Fuc(α1-3)	Montreuil (1956)
3	DF-L	Fuc(α1-2)Gal(β1-4)Glc | Fuc(α1-3)	Kuhn and Gauhe (1958b)
4	LN triose II	GlcNAc(β 1-3)Gal(β 1-4)Glc	Dabrowski *et al.* (1983)
5	3'-GL	Gal(β 1-3)Gal(β 1-4)Glc	Donald and Feeney (1958)
6	4'-GL	Gal(β 1-4)Gal(β 1-4)Glc	Sugawara and ldota (1995)
7	6'-GL	Gal(β 1-6)Gal(β 1-4)Glc	Yamashita and Kobata (1974)
8	3'-SL	Neu5Ac(α 2-3)Gal(β 1-4)Glc	Kuhn and Brossmer (1959)
9	F-SL	Neu5Ac(α2-3)Gal(β1-4)Glc | Fuc(α1-3)	Gronberg *et al.* (1989)
2. Lacto-*N*-tetraose series			
1	LNT	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn and Baer (1956a)
2	LNFP I	Fuc(α 1-2)Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn *et al.* (1956c)
3	LNFP II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)	Kuhn *et al.* (1958a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
4	LNFP V	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-3)	Ginsberg *et al.* (1976)
5	LNDFH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-4)	Kuhn and Gauhe (1958b)
6	LNDFH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc \| \| Fuc(α1-4) Fuc(α1-3)	Kuhn and Gauhe (1960)
7	LSTa	Neu5Ac(α 2-3)Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn and Gauhe (1962)
8	LSTb	Neu5Ac(α2-6) \| Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Kuhn and Gauhe (1962)
9	F-LSTa	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-4)	Wieruszeski *et al.* (1985)
10	F-LSTb	Neu5Ac(α2-6) \| Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Wieruszeski *et al.* (1985)
11	DS-LNT	Neu5Ac(α2-6) \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Grimmonorez and Montreuil (1968)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
12	FDS-LNT I	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)	Kitagawa *et al.* (1991b)
13	FDS-LNT II	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)	Gronberg *et al.* (1990)
3. Lacto-*N*-neotetraose series			
1	LNnT	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn and Gauhe (1962)
2	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)	Kobata and Ginsburg (1969)
3	LSTc	Neu5Ac(α 2-6)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn and Gauhe (1962)
4	F-LSTc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)	Smith *et al.* (1987)
4. Lacto-*N*-hexaose series			
1	LNH	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)	Kobata and Ginsburg (1972a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
2	F-LNH I	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)	Yamashita *et al.* (1977a)
3	F-LNH II	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)	Dua *et al.* (1985)
4	DF-LNH	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3) | Fuc(α1-4)	Dua *et al.* (1985)
5	DF-LNHa	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)	Yamashita *et al.* (1977a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
6	TF-LNH	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) | Fuc(α1-4)	Sabharwal *et al.* (1988a)
7	S-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)	Kobata and Ginsburg (1972a)
8	FS-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)	Yamashita *et al.* (1977a)
9	FS-LNH I	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Gronberg *et al.* (1992)

(*Continued*)

Table 8.1 (*Continued*)

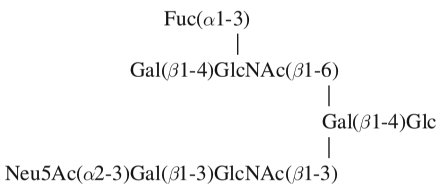
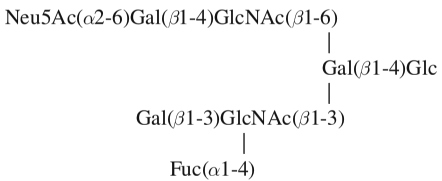
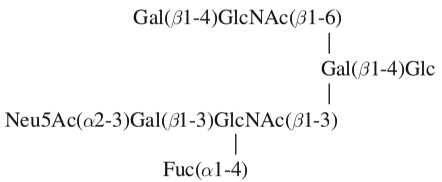
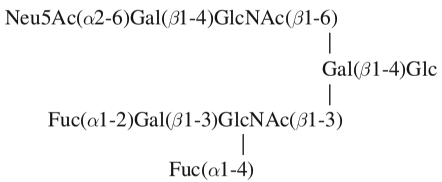
	Abbreviation	Oligosaccharide	Reference
10	FS-LNH II		Gronberg *et al.* (1992)
11	FS-LNH III		Gronberg *et al.* (1992)
12	FS-LNH IV		Kitagawa *et al.* (1989)
13	DFS-LNH I		Gronberg *et al.* (1992)

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Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
14	DFS-LNH II	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Fuc(α1-4)	Kitagawa *et al.* (1989)
15	DS-LNH I	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)	Kitagawa *et al.* (1991b)
16	DS-LNH II	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Kitagawa *et al.* (1991b)
17	FDS-LNH I	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Yamashita *et al.* (1976a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
18	FDS-LNH II	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Yamashita *et al.* (1976a)
19	FDS-LNH III	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Fuc(α1-4)	Kitagawa *et al.* (1989)
20	TS-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Fievre *et al.* (1991)
5. Lacto-*N*-neohexaose series			
1	LNnH	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-4)GlcNAc(β1-3)	Kobata and Ginsburg (1972b)

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Table 8.1 (*Continued*)

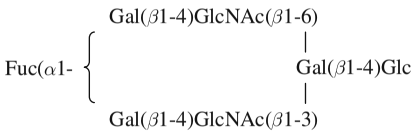
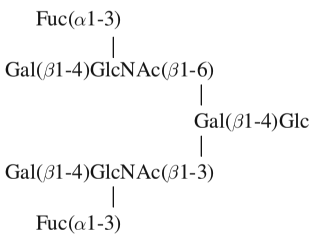
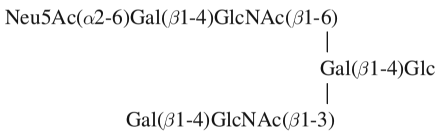
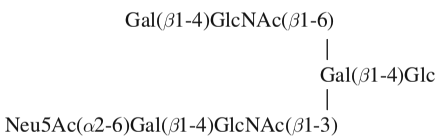
	Abbreviation	Oligosaccharide	Reference
2	F-LNnH		Kobata and Ginsburg (1972b)
3	DF-LNnH		Haeuw-Fievre *et al.* (1993)
4	S-LNnH I		Kobata and Ginsburg (1972b)
5	S-LNnH II		Tarrago et al. (1988)

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Table 8.1 (*Continued*)

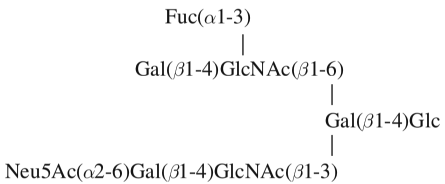
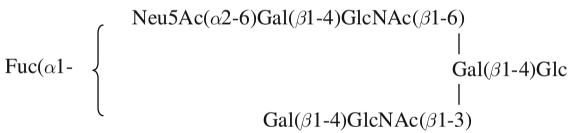
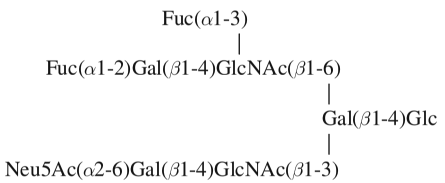
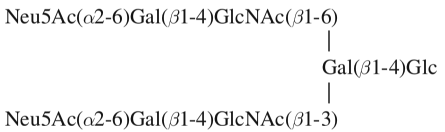
	Abbreviation	Oligosaccharide	Reference
6	FS-LNnH I		Gronberg *et al.* (1989)
7	FS-LNnH II		Kobata and Ginsburg (1972b)
8	DFS-LNnH		Gronberg *et al.* (1992)
9	DS-LNnH		Gronberg *et al.* (1992)

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Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
10	FDS-LNnH	Neu5Ac(α2-3/6)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3/6)Gal(β1-4)GlcNAc(β1-3) \| Fuc(α1-3)	Yamashita *et al.*(1976a)
6. *para*-Lacto-*N*-hexaose series			
1	F-paraLNH I	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-3)	Sabharwal *et al.* (1988b)
2	F-paraLNH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-4)	Bruntz *et al.* (1988)
3	DF-paraLNH	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| \| Fuc(α1-4) Fuc(α1-3)	Yamashita *et al.* (1977b)
4	TF-paraLNH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| \| Fuc(α1-4) Fuc(α1-3)	Strecker *et al.* (1988)
5	TF-paraLNH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| \| \| Fuc(α1-4) Fuc(α1-3) Fuc(α1-3)	Bruntz *et al.* (1988)
6	DF-paraLNH sulfate I Fuc (α1-2)	6S | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-3)	Guerardel *et al.* (1999)

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Table 8.1 (*Continued*)

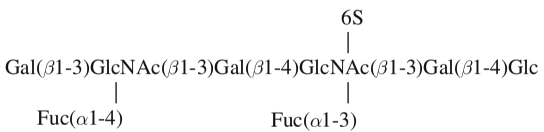
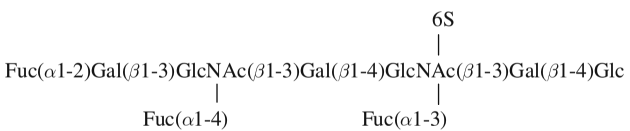
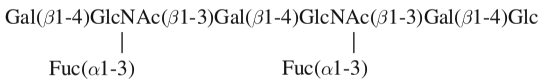
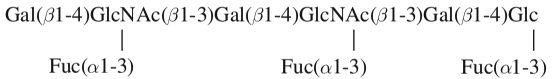
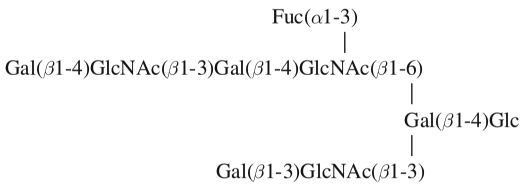
	Abbreviation	Oligosaccharide	Reference
7	DF-paraLNH sulfate II		Gruerardel *et al.* (1999)
8	TF-paraLNH sulfate		Gruerardel *et al.* (1999)
7.*Para*-Lacto-*N*-neohexaose series			
1	DF-paraLNnH		Yamashita *et al.* (1977b)
2	TF-paraLNnH		Bruntz *et al.* (1988)
8. Lacto-*N*-octaose series			
1	F-LNO		Yamashita *et al.* (1976b)

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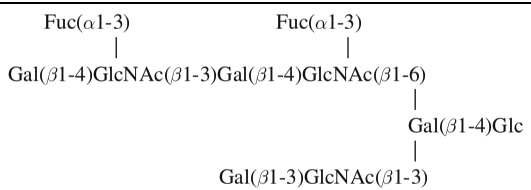
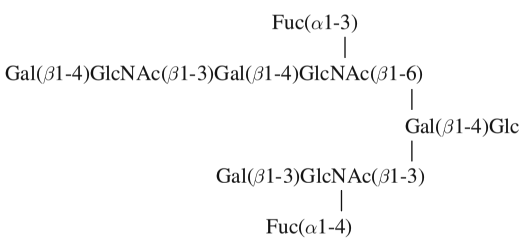
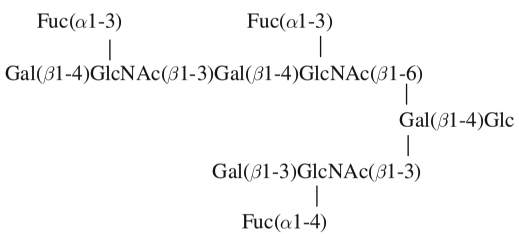
Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
2	DF-LNO I		Tachibana *et al.* (1978)
3	DF-LNO II		Tachibana *et al.* (1978)
4	TF-LNO		Tachibana *et al.* (1978)

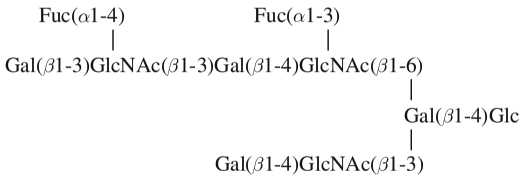
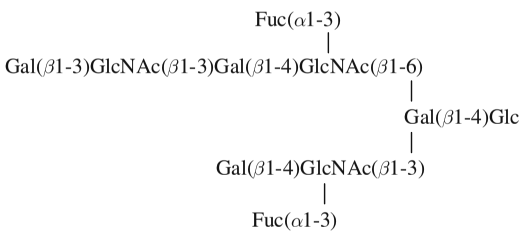
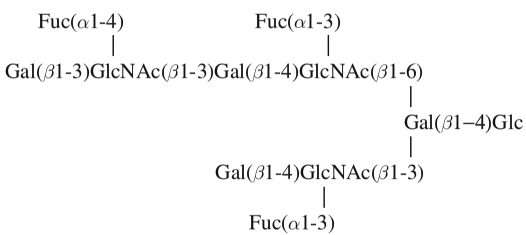
(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
5	FS-LNO	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(β2-3)Gal(β1-3)GlcNAc(β1-3) \| Fuc(α1-4)	Kitagawa *et al.* (1993)
6	DFS-LNO	Fuc(α1-3) \| Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) \| Fuc(α1-4)	Kitagawa *et al.* (1991a)
9. Lacto-*N*-neooctaose series			
1	F-LNnO	Fuc(α1-3) \| Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-4)GlcNAc(β1-3)	Yamashita *et al.* (1976b)

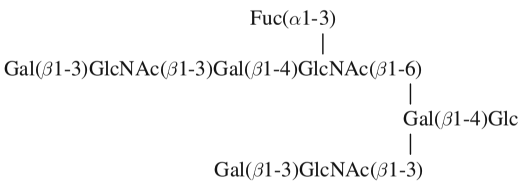
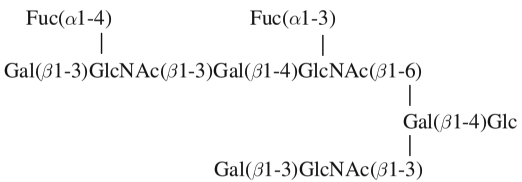
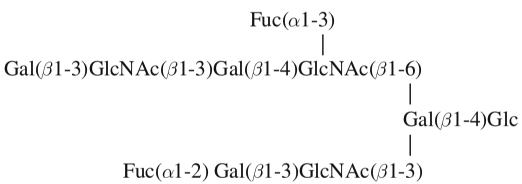
(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
2	DF-LNnO I		Tachibana *et al.* (1978)
3	DF-LNnO II		Tachibana *et al.* (1978)
4	TF-LNnO		Tachibana *et al.* (1978)

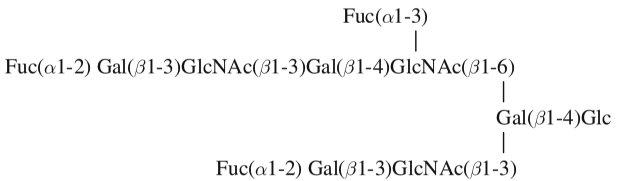
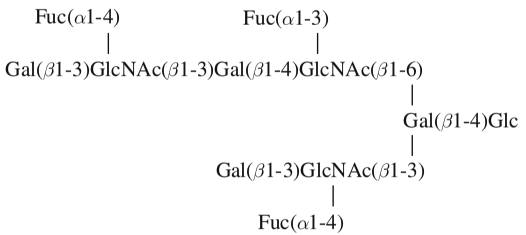
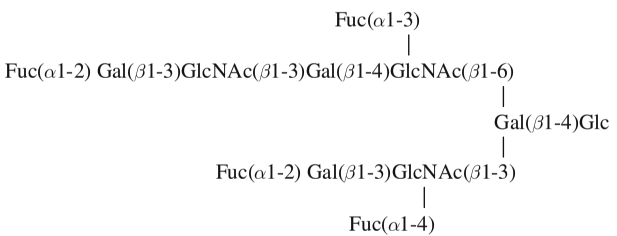
(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
10. iso-Lacto-*N*-octaose series			
1	F-isoLNO		Kogclberg *et al.* (2004)
2	DF-isoLNO I		Strecker *et al.* (1989)
3	DF-isoLNO II		Strecker *et al.* (1989)

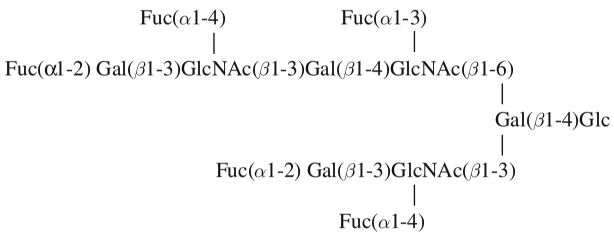
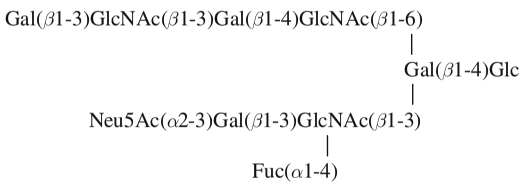
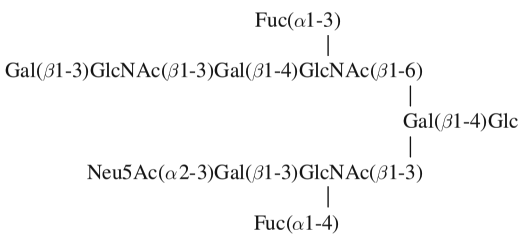
(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
4	TF-isoLNO I		Strecker *et al.* (1991)
5	TF-isoLNO II		Kogelberg *et al.* (2009)
6	TetraF-isoLNO		Hacuw-Fievre *et al.* (1993)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
7	PF-isoLNO		Haeuw-Fievre *et al.* (1993)
8	FS-isoLNO		Kitagawa *et al.* (1991a)
9	DFS-isoLNO I		Kitagawa *et al.* (1991a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
10	DFS-isoLNO II	Fuc(α1-4) \| Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) \| Fuc(α1-4)	Kitagawa *et al.* (1991a)
11	TFS-isoLNO	Fuc(α1-3) \| Fuc(α1-2) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) \| Fuc(α1-4)	Kitagawa et al. (1993)
12. *para*-Lacto-*N*-octaose series, Lacto-*N*-decaose series and others			
1	Tetra F-paraLNO	Fuc(α1-2)Gal(β1-3) GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)- \| Fuc(α1-4) \| Fuc(α1-3) –Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-3)	Haeuw-Fievre *et al.* (1993)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
2	F-LND	Fuc(α1-3) \| Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc NAc(β1-6) \| \| Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc \| Gal(β1-3)GlcNAc(β1-3)	Chai *et al.* (2005)
3		Fuc(α1-4) \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc	Kitagawa *et al.* (1990)
4		Fuc(α1-4) \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal	Kitagawa *et al.* (1990)
5		Fuc(α1-3) \| Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3)Gal(β1-3)	Gronberg *et al.*(1992)

described below, these oligosaccharides are considered to play a significant role in the protection of human neonates against infection by pathogenic microorganisms. It is likely that, because of their immaturity, human neonates are relatively susceptible to infection by pathogenic microorganisms; the presence of significant amounts and a large variety of milk oligosaccharides may therefore be advantageous insofar as they supplement the other, known, anti-infection properties of the milk/colostrum. In this connection, it is notable that the milk of species, such as monotremes, marsupials and a few species of eutherians, including Ursidae, that produce very altricial neonates, also contain relatively high concentrations of oligosaccharides, with lesser amounts of free lactose (Messer and Urashima, 2002).

The 93 human milk oligosaccharides, the structures of which have been determined to date, can be grouped into 12 series based on their core units as in Table 8.2 (Haeuw-Fievre *et al.*, 1993). The many variations of the oligosaccharides are constructed by the addition of a Neu5Acα 2-3/2-6 residue to Gal or GlcNAc, and of Fucα1-2/1-3/1-4 to Gal, GlcNAc or a reducing Glc of the core units.

Table 8.2 The 12 core structures of human milk oligosaccharides

	Abbreviation	Oligosaccharide
1	Lactose	Gal(β 1-4)Glc
2	Lacto-*N*-tetraose	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc
3	Lacto-*N*-neotetraose	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
4	Lacto-*N*-hexaose	Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-3)GlcNAc(β1-3)
5	Lacto-*N*-neohexaose	Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-4)GlcNAc(β1-3)
6	*para*-Lacto-*N*-hexaose	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
7	*para*-Lacto-*N*-neohexaose	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
8	Lacto-*N*-octaose	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-3)GlcNAc(β1-3)

(*Continued*)

Table 8.2 (*Continued*)

	Abbreviation	Oligosaccharide
9	Lacto-*N*-neooctaose	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-4)GlcNAc(β1-3)
10	Iso-Lacto-*N*-octaose	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)
11	*para*-Lacto-*N*-octaose	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
12	Lacto-*N*-decaose	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)GlcNAc(β1-6) | | Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)

The main structural features of human milk oligosaccharides are the presence of oligosaccharides containing the type I unit (Gal(β1-3)GlcNAc), as well as those containing the type II unit (Gal(β1-4)GlcNAc), and that oligosaccharides containing the type I predominate over those containing the type II unit. The milk oligosaccharides of other species investigated to date mostly have the type II but not the type I unit. The many varieties of oligosaccharides in human milk and colostrum are produced by the addition of Neu5Ac and/or Fuc residues to these two units.

Isolation of the milk oligosaccharides has more recently been accomplished by normal-phase or reverse-phase high-performance liquid chromatography (HPLC) or by high pH anion exchange chromatography with pulsed amperometric detection (HPEAC-PAD). It is noteworthy, for example, that Gronberg *et al.* (1989, 1990, 1992) isolated several novel minor oligosaccharides by HPLC using triethylamine as an ion-pair reagent. On the other hand, the selective separation of specific epitopes has been carried out by affinity chromatography using a monoclonal antibody or lectin. For example, Kitagawa *et al.* (1988, 1989, 1990, 1991a,b, 1993) selectively separated oligosaccharides containing sialyl Le^a, a tumor-related carbohydrate epitope, by affinity column chromatography in which MSW113, the monoclonal antibody specific for this unit, was bound to Protein A-Sepharose GL4B as a ligand: the oligosaccharides containing sialyl Le^a were adsorbed by the column and were then eluted by raising the pH of the eluent.

At present, oligosaccharide structures are usually characterized by resonance assignments of the reporter groups in one-dimensional ^{1}H-NMR, of each chemical shift in one-dimensional ^{13}C-NMR and two-dimensional NMR such as ^{1}H–^{13}C correlated spectroscopy (^{1}H–^{13}C COSY), heteronuclear single quantum coherence experiments (HSQC), ^{1}H–^{1}H homonuclear Hartmann–Hahn experiments (^{1}H–^{1}H HOHAHA), heteronuclear multiple bond correlation experiments (HMBC), with the aid of MALDI-TOFMS or fast atom bombardment mass experiments spectrometry (FAB-MS) analysis.

Further detailed information on structural and technological aspects of human and bovine milk oligosaccharides can be obtained from a recent review by Mehra and Kelly (2006).

8.3. Human Milk Oligosaccharides: Quantitative Aspects

Milk oligosaccharides can be quantified using reverse-phase or normal-phase HPLC subsequent to pre- or post-column labeling techniques. Derivatizations are often performed by condensation of 2-aminopyridine, 2-aminobenzamide, 2-aminobenzoic acid or 1-phenyl-3-methyl-5-pyrazolone to the reducing end of the sugar aldehyde (Hase *et al*., 1978; Honda *et al*., 1989; Bigge *et al*., 1995; Fun *et al*., 1995; Tokugawa *et al*., 1996; Fu and Zopf, 1999; Sumiyoshi *et al*., 2003a; Sumiyoshi *et al*., 2003b). Sialyl oligosaccharides can be quantified by capillary electrophoresis, with detection at 205 nm (Bao *et al*., 2007).

Mature human milk and colostrum contain 12–13 and 22–24 g/L of oligosaccharides, respectively (Newburg and Neubauer, 1995). Oligosaccharides constitute the third quantitatively largest component, after lactose and lipids, of the dry matter of human milk.

The concentration of neutral oligosaccharides in human milk is greater than that of acidic oligosaccharides. The neutral fraction contains many fucosyl oligosaccharides. For example, Thurl *et al*. (1996) showed that human milk contains significant amounts of 2′-fucosyllactose (2′-FL: Fuc(α1-2)Gal(β1-4)Glc), lacto-*N*-fucopentaose I (LNFP I: Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc), lacto-*N*-difucohexaose I (LNDFH I: Fuc(α1-2)Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)Glc) and lacto-*N*-tetraose (LNT: Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc).

Representative acidic oligosaccharides of human milk are sialyl lacto-*N*-neotetraose c (LST c: Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc), disialyl lacto-*N*-tetraose (DSLNT: Neu5Ac(α2-3)Gal(β1-3)[Neu5Ac(α2-6)]Gal(β1-4)Glc), 3′-*N*-acetylneuraminyllactose (3′-SL: Neu5Ac(α2-3)Gal(β1-4)Glc) and 6′-*N*-acetylneuraminyllactose (6′-SL: Neu5Ac(α2-6) Gal(β1-4)Glc) (Thurl *et al*., 1996; Shen *et al*., 2000). Bao *et al*., (2007) stated that the most prominent acidic oligosaccharide in the milk/colostrum is DSLNT, whereas Asakuma *et al*. (2007) found that in colostrum it is LST c.

Changes in the concentrations of the following saccharides during the course of lactation may be crucial with respect to the biological significance of human milk/colostrum for infants: 2′-FL, 3-fucosyllactose (3-FL: Gal(β1-4)[Fuc(α1-3)]Glc), LNT, lacto-*N*-neotetraose (LNnT: Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc), LNFP I, lacto-*N*-fucopentaose II (LNFP II: Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Glc(β1-4)Glc), lacto-*N*-fucopentaose III (LNFP III: Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc), LNDFH I, lacto-*N*-difucohexaose II (LNDFH II: Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)Glc], lacto-*N*-hexaose (LNH: Gal(β1-3)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc), lacto-*N*-neohexaose (LNnH: Gal(β1-4)GlcNAc (β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc), 6′-SL, 3′-SL, sialyl lacto-*N*-tetraose a (LST a: Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc), sialyl lacto-*N*-tetraose b (LST b: Gal(β1-3)[Neu5Ac(α2-6)]GlcNAc(β1-3)Gal(β1-4)Glc), LST c and DSLNT (Coppa *et al*., 1999; Chaturdevi *et al*., 2001).

The concentrations of oligosaccharides in milk or colostrum, as well as changes in their concentrations during the course of lactation, are likely to be significant with respect to their ability to act as anti-infection agents and to stimulate the growth of bifidobacteria in the infant's colon, as described below.

It is of interest that among mammals whose milk oligosaccharides have been investigated, humans are the only species in which oligosaccharides containing lacto-*N*-biose I units dominate over those containing *N*-acetyllactosamine (Gal(β1-4)GlcNAc) units (Urashima *et al*., 2001). The high expression of lacto-*N*-biose I-containing oligosaccharides in human lactating mammary glands, relative to that of other milk oligosaccharides, may be the key to the acquisition of a bifidus flora in the colon of the human infant.

8.4. Biosynthesis of Milk Oligosaccharides

As most milk oligosaccharides, including those of humans, contain a lactose unit at their reducing end, it is generally considered that they are synthesized by the action of a variety of glycosyltransferases acting on free lactose as the acceptor. Lactose is synthesized within lactating mammary glands from UDP-Gal (donor) and glucose (acceptor) by a transgalactosylation catalyzed by lactose synthase, an enzyme that is a complex of a β-4-galactosyltransferase I and α-lactalbumin. Other tissues do not contain α-lactalbumin but do contain β-4-galactosyltransferase which transfers galactose from UDP-Gal to non-reducing GlcNAc residues in glycoconjugates to synthesize *N*-acetyllactosamine (Gal(β1-4)GlcNAc) units. In lactating mammary glands, the presence of α-lactalbumin changes the preferred acceptor of β-4-galactosyltransferase from GlcNAc to glucose (Rajput *et al*., 1996). Thus, the expression of α-lactalbumin in the lactating mammary gland is the key to the presence of lactose in milk.

Since lactose is an obligatory acceptor for the mammary glycosyltransferases, α-lactalbumin is also essential for the presence of milk oligosaccharides. The exact number of glycosyltransferases involved in the biosynthesis of the oligosaccharides is still uncertain. These enzymes are known to be very specific, their specificity being directed toward both the type of linkage and the acceptor molecule. For example, in addition to the above-mentioned β-galactosyltransferase, there are probably at least two other human mammary β-galactosyltransferases, the actions of which are independent of α-lactalbumin, that catalyze the synthesis of Gal(β1-3)GlcNAc-R and Gal(β1-4)GlcNAc-R structures such as those shown in Table 8.1. Although human milk contains traces of three different galactosyllactoses, it is possible that these trisaccharides are formed by the transferase actions of β-galactosidase rather than by three specific galactosyltransferases. Judging from the variety of structures among the human milk oligosaccharides (Table 8.1) one can assume that the human mammary gland probably contains at least three β-*N*-acetylglucosaminyltransferases, three α-fucosyltransferases and two sialyltransferases.

Additional glycosyltransferases are found in lactating mammary glands of non-human species. An α-galactosyltransferase that synthesizes α-3′-galactosyllactose (isoglobotriose), a trisaccharide present in bovine, ovine and caprine colostrum and the milk or colostrum of several other species but not in human milk or colostrum (Urashima *et al*., 2001). The synthesis of α-4′-galactosyllactose (globotriose), which has been found in colostrum of the bottlenosed dolphin (Uemura *et al*., 2005), is presumably catalyzed by a different α-galactosyltransferase. Lactating mammary glands of the tammar wallaby contain a very active β-galactosyltransferase that is involved in the synthesis of a series of β(1-3)-linked galactosyllactoses that are unique to the milk of marsupials (Messer and Nicholas, 1991). The mammary glands of this species also contain an unusual β-*N*-acetylglucosaminyltransferase that attaches a *N*-acetylglucosaminyl residue to the trisaccharide β-3′-galactosyllactose and to the tetrasaccharide β-3′, 3″-digalactosyllactose (Urashima *et al*., 1992).

Although the milk/colostrum of most mammalian species contains lactose as the dominant saccharide, constituting more than 80% of the carbohydrate, milk oligosaccharides are found at higher concentrations than lactose in the milk of monotremes, marsupials and a few species of eutherians such as bears, giant panda, mink and white-nosed coati (Urashima *et al*., 2001; Messer and Urashima, 2002). It has been suggested that the ratio of oligosaccharides to lactose in milk is based on the ratio of expression of the glycosyltransferases to α-lactalbumin within the lactating mammary gland (Messer and Urashima, 2002). In those species whose mammary glands are characterized by a low expression of α-lactalbumin, the biosynthesis of lactose is likely to be relatively slow; therefore, their mammary glycosyltransferases would tend to utilize almost all the available free lactose for the synthesis of oligosaccharides. In

the milk of such species, e.g., monotremes, very little lactose would accumulate and the ratio of milk oligosaccharides to lactose would be relatively high.

8.5. Gastrointestinal Digestion and Absorption of Milk Oligosaccharides

When infants consume milk, the free lactose contained therein is split into galactose and glucose by intestinal lactase (neutral β-galactosidase, lactose-phlorizin hydrolase), an enzyme that is located in the membrane of the microvilli of the brush border of the small intestine. The two monosaccharides are transported into the enterocytes by a specific mechanism, whereupon the glucose enters the circulation and is used as an energy source while most of the galactose is converted to glucose in the liver, to be used as an energy source as well.

Much less is known about the exact fate of human milk oligosaccharides. These are resistant to enzymatic hydrolysis by the intestinal lactase of the brush border (Engfer *et al.*, 2000) and there is evidence that the major part survives passage through the small intestine and enters the colon where they are fermentated by colonic bacteria (Brand-Miller *et al.*, 1998; Newburg, 2000). Evidently, the brush border of the small intestine does not contain enzymes, such as sialidase, fucosidase or *N*-acetylglucosaminidase, that can remove sialic acid, fucose or *N*-acetylglucosamine residues, respectively, from the lactose units of the milk oligosaccharides. A small fraction of human milk oligosaccharides is absorbed intact, perhaps by receptor-mediated endocytosis (Gnoth *et al.*, 2001), some of which are excreted in the urine. It is unclear what proportion and exactly which of the ingested milk oligosaccharides are absorbed, but there is evidence suggesting that circulating oligosaccharides may have immunological effects on endothelial cells (Rudloff *et al.*, 1996).

There has been interest to investigate whether the sialic acid of sialyl milk oligosaccharides can be absorbed and utilized as precursors for the biosynthesis of brain gangliosides and sialoglycoproteins. Rat milk contains significant amounts of sialyllactose (Kuhn, 1972) which can be hydrolyzed to sialic acid and lactose by a very active small intestinal neuraminidase that is present in suckling rats. Since this enzyme has a low pH optimum and is absent from the brush border, it is probably of lysosomal, i.e., intracellular origin (Dickson and Messer, 1978). An intracellular location for this neuraminidase implies that the ingested sialyllactose has to be transferred into the enterocytes before it can be digested within lysosomes or supranuclear vacuoles; the most likely mechanism for this transfer is pinocytosis or endocytosis. It has been found that when adult rats were fed sialyllactose, there was an increase in brain ganglioside, including GM3, content and an improvement in the ability to learn to swim (Sakai *et al.*, 2006). It has also been reported that the amount of sialic acid bound to brain

gangliosides and sialoglycoproteins is significantly higher in breast-fed than in formula -fed infants; this may be due to a difference between human milk and infant formula with respect to their content of sialic acid (Wang *et al*., 2003). When piglets were supplemented with increasing amounts of sialic acid as casein glycomacropeptides for 35 days, there were proportionate increases in protein-bound sialic acid concentrations in the frontal cortex and improvements in learning (Wang *et al*., 2007). These observations support the notion that the sialic acid of milk oligosaccharides and also of sialoglycopeptides can be absorbed and utilized despite the fact that most milk oligosaccharides are considered to be indigestible within the small intestine.

It would be of interest to investigate whether, and to what extent, other monosaccharides, such as fucose, that are constituents of the neutral milk oligosaccharides, can similarly be absorbed and used as biosynthetic precursors. Rudloff *et al*. (2006) recently fed ^{13}C galactose to breast-feeding women and determined the amount of ^{13}C incorporated into lactose and both neutral and acidic oligosaccharides, notably LNT and fucosyl LNT. Incorporation of ^{13}C was also observed in the fraction containing difucosyl LNT, fucosyl LNH and difucosyl LNH (Rudloff *et al*., 2006). These results should make it possible to feed ^{13}C-enriched milk oligosaccharides to infants, thus facilitating studies on their metabolic fate.

8.6. *Bifidobacterium* Growth Stimulation by Human Milk Oligosaccharides

As noted above, although a small fraction of human milk oligosaccharides is known to be absorbed intact, it is now generally accepted that the major part survives passage through the small intestine and enters the colon. At this location, they are believed to act as prebiotics, stimulating the growth of bifidobacteria, and as soluble receptor analogues that inhibit the attachment of pathogenic microorganisms to the colonic epithelial cells. *Bifidobacterium longum* ssp. *infantis* has recently been shown to ferment purified human milk oligosaccharides *in vitro* as a sole carbon source, whereas another gut commensal, *Lactobacillus gasseri*, did not; this supports the hypothesis that human milk oligosaccharides selectively amplify specific bacterial populations in the infant intestine (Ward *et al*., 2006; Ninonuevo *et al*., 2007). There is evidence that the metabolic activity of the bifidobacteria reduces the colonic pH, which has the additional effect of inhibiting the proliferation of pathogenic organisms such as *Shigella flexneri* and *Escherichia coli* (Gopal and Gill, 2000). It has recently been shown that this strain is able to grow on human milk oligosaccharides as the only carbon source (Ward *et al*., 2007). The degradation of these oligosaccharides was studied during the growth of *B. longum* ssp. *infantis*

ATCC 15697 and it was found that tri- to hepta-saccharides were completely degraded during the growth over 25 and 50 h (LoCascio *et al.*, 2007).

Intestinal colonization by bifidobacteria is especially important to the health of infants because there is evidence that it prevents infection by some pathogenic organisms and reduces the incidence of diarrhea (Bezkorovainy, 1989). In breast-fed infants, bifidobacteria usually dominate the intestinal flora within 1 week after birth, constituting 95–99.9% of the bacterial population over time (Rotimi and Duerden, 1981; Benno and Mitsuoka, 1986). By contrast, intestinal colonization by bifidobacteria is not as rapid or as predominant in bottle-fed infants, who, prior to the early 20th Century, often experienced infection by pathogenic bacteria (Bezkorovainy, 1989). To improve the growth of intestinal bifidobacteria, saccharides such as lactulose (Petuely, 1957) have been used as supplements to formula milk, resulting in bottle-fed infants being healthier. However, the intestinal flora of bottle-fed infants consists of about 90% bifidobacteria and 10% Enterobacteriaceae; this ratio is smaller than that in breast-fed infants (Benno *et al.*, 1984).

Growth factors for bifidobacteria in human milk, so-called bifidus factors, have been investigated for many years. It was initially thought that, in this regard, nitrogen-containing sugars represented the main difference between breast milk and formula, but this was shown to be due to the use of a strain of *Bifidobacterium bifidum* that requires GlcNAc for growth (Gyorgy *et al.*, 1954; Veerkamp, 1969). Further studies revealed that oligosaccharides in human milk are candidate bifidus factors (Bezkorovainy, 1989), but a specific oligosaccharide responsible for stimulating the growth of bifidobacteria was not identified, mainly because of the complexity caused by the fact that human milk contains more than 100 kinds of oligosaccharides. Recently, however, Kitaoka *et al.* (2005) presented a new hypothesis based on a novel metabolic pathway for galactose in bifidobacteria, which proposed that Gal(β1-3)GlcNAc (lacto-*N*-biose I, LNB) structures, which are found in type I human milk oligosaccharides, act as specific bifidus factors.

Derensy-Dron *et al.* (1999) reported the presence of an enzyme in cell-free extracts of *B. bifidum* that reversibly converted LNB to α- D-galactopyranose-1-phosphate (Gal-1-P) and GlcNAc. This enzyme, which also converts Gal(β1-3)GalNAc (galacto-*N*-biose, GNB) to Gal-1-P and GalNAc, was named β-1,3-galactosyl-*N*-acetylhexosamine phosphorylase (EC 2.4.1.211) (Derensy-Dron *et al.*, 1999). Subsequently, a shorter name, lacto-*N*-biose phosphorylase (LNBP), was used for this enzyme (Kitaoka *et al.*, 2005). Derensy-Dron *et al.* (1999) assumed that the enzyme played a role in the metabolism of mucin sugars by bifidobacteria during colonization of the intestine. This assumption has been supported by the presence of a bifidobacterial endo-α-*N*-acetylgalactosaminidase that hydrolyzes the linkage between galacto-*N*-bioside and serine or threonine in *O*-linked glycoproteins of mucins (Fujita *et al.*, 2005).

Kitaoka *et al*. (2005) purified LNBP from a cell-free extract of *B. bifidum* and found that its partial amino acid sequence was homologous with that of the BL1641 protein of *Bifidobacterium longum* ssp. *longum* NCC2705, the complete genomic sequence of which was available (Schell *et al*., 2002). A homologous gene was then cloned from the type strain of *B. longum* ssp. *longum* JCM1217 and the expressed protein showed LNBP activity. The LNBP showed no significant identity with any other proteins of known functions. Thus, LNBP appears to belong to a new family of enzymes.

The LNBP gene, BL 1641, seems to be located in a gene cluster in the *B. longum* genome (Schell *et al*., 2002) as shown in Figure 8.1. The members of the cluster were annotated as follows: BL1638-1640, component proteins of ATP-binding cassette (ABC)-type sugar transporter; BL1642, mucin desulfatase; BL1643, galactose-1-phosphate uridylyltransferase (EC 2.7.7.10, GalT); BL1644, UDP-glucose 4-epimerase (EC 5.1.3.2, GalE). Judging from the members of the cluster, LNBP seems to be related to the metabolism of mucin sugars because GNB, one of the substrates of LNBP, is the core structure of mucin type I sugars. The proteins coded by BL1643 and BL1644 genes are well-known members of the Leloir pathway for galactose metabolism. In this pathway, Gal-1-P formed by a galactokinase (EC 2.7.1.6, GalK) is converted to α-glucose-1-phosphate by the action of GalT and GalE, to enter the glycolytic pathway. The bifidobacterial gene cluster does not include GalK; this is consistent with the fact that Gal-1-P is formed directly by LNBP, without

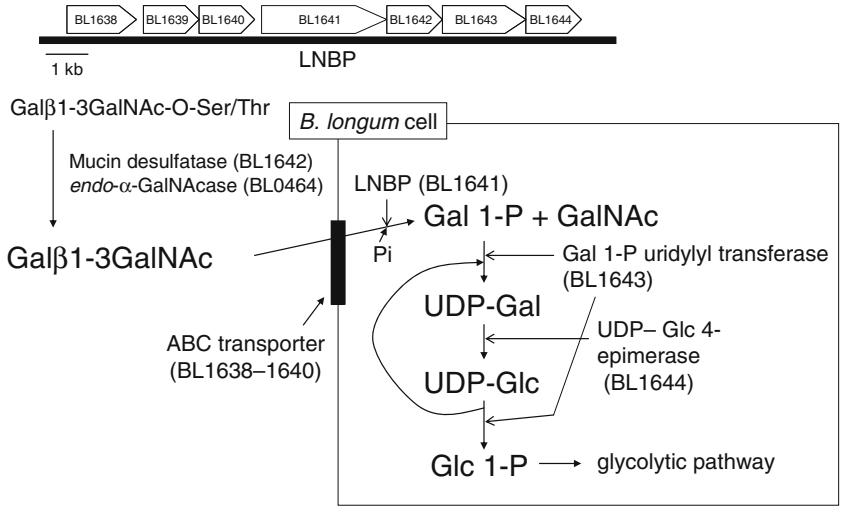


Figure 8.1. The gene cluster of *Bifidobacterium longum* ssp. *longum* containing the lacto-*N*-biose phosphorylase gene. Modified Figs. 2 and 3 of Kitaoka *et al*. (2005).

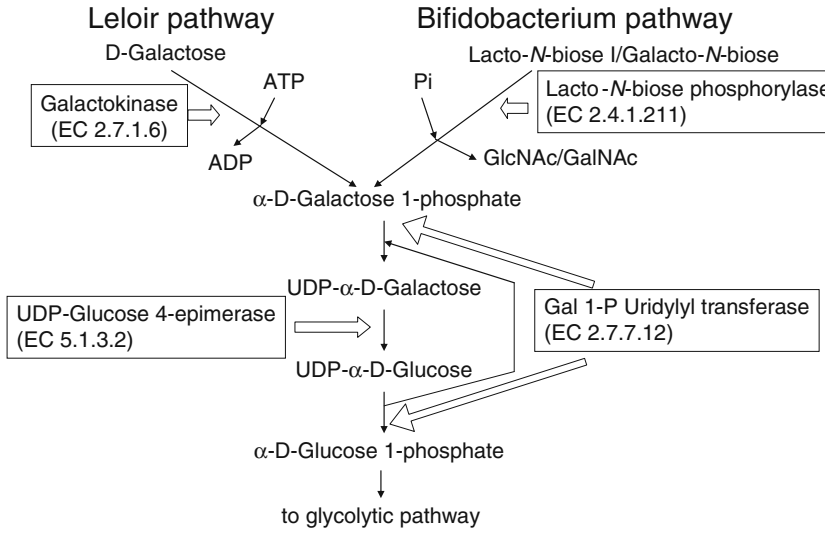


Figure 8.2. Comparison of the novel *Bifidobacterium* pathway with the Leloir pathway.

the consumption of ATP (Figure 8.2). On the other hand, it is suggested that N-acetylglucosamine is converted to N-acetylglucosamine-1-phosphate by a novel enzyme, N-acetylhexosamine 1-kinase, and then to UDP-N-acetylglucosamine by UDP glucose hexose 1-phosphate uridyltransferase, finally entering to metabolic pathway of amino sugars (Nishimoto and Kitaoka, 2007a).

Two types of galactose metabolism are known (de Vos and Vaughan, 1994). One is the Leloir pathway and the other is initiated by the phosphoenol pyruvate-dependent phosphotransferase system (PTS) transporter, in which lactose is transported into the cell across its membrane by a lactose-specific PTS transporter forming lactose-6-phosphate. *Bifidobacterium longum* ssp. *longum* does not possess the PTS pathway as evidenced by its genomic sequence (Schell *et al*., 2002; Kitaoka *et al*., 2005). A GalK gene is found in the *B. longum* genome (BL1210) accompanied by a GalT gene (BL1211), but no GalE gene is found near these genes. *Bifidobacterium longum* ssp. *longum* possesses a different GalE gene (BL1671), which is not accompanied by genes for enzymes related to the Leloir pathway. Based on the above observations, it can be hypothesized that the gene cluster containing the LNBP gene encodes the major metabolic pathway for galactose in *B. longum* ssp. *longum* and that the substrate of LNBP plays an important role for this organism. Since LNBP has been found in several strains of *B. longum* ssp. *longum* and *B. bifidum*, this pathway might be present in many bifidobacteria. LNB might also be an important sugar source for bifidobacteria. In light of the above, Kitaoka *et al.*

(2005) formulated the hypothesis that the LNB structure which occurs at the non-reducing end of several human milk oligosaccharides is a specific bifidus factor (Figure 8.3). Since LNB-containing oligosaccharides predominate over those containing *N*-acetyllactosamine in human milk (see above), this LNB hypothesis seems attractive.

It should be noted that free LNB is not found among the human milk oligosaccharides. For the hypothesis to be valid, bifidobacteria would have to have enzymes that liberate LNB from human milk oligosaccharides, since LNBP cannot act on β-glycosides of LNB (Derensy-Dron *et al.*, 1999; Kitaoka *et al.*, 2005). Considering the fact that lacto-*N*-tetraose and lacto-*N*-fucopentaose I are the major components of human milk oligosaccharides along with 2′-FL, lacto-*N*-biosidase and α-fucosidase enzymes would be required for the entry of these oligosaccharides into the LNB pathway (Figure 8.3). The gene encoding an α-fucosidase that hydrolyzes lacto-*N*-fucopentaose I and 2′-FL has been already cloned from *B. bifidum* (Katayama *et al.*, 2004). This activity was found in the culture supernatant of *B. bifidum* but not of *B. breve* or *B. longum*. As a lacto-*N*-biosidase that hydrolyzes lacto-*N*-tetraose into lacto-*N*-biose I and lactose has recently been cloned from *B. bifidum* JCM1254

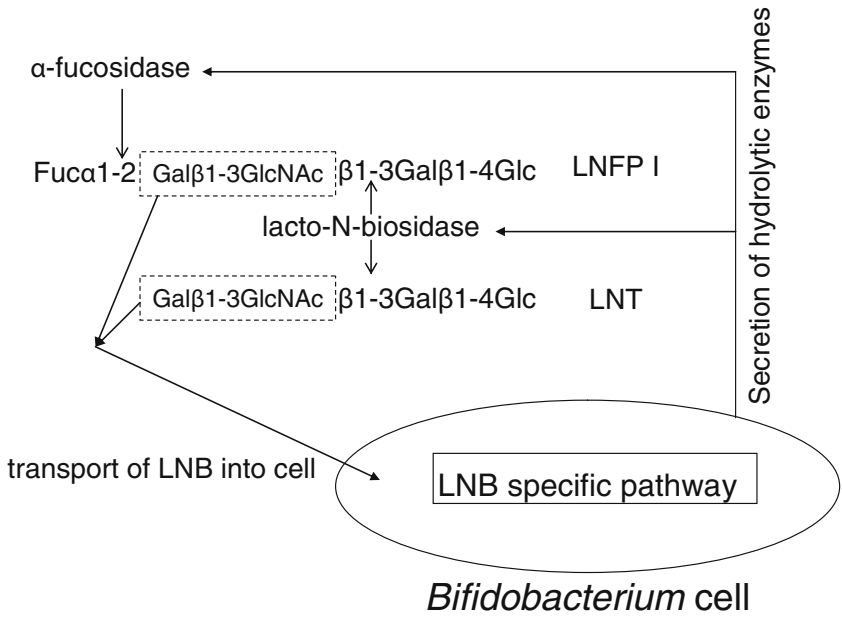


Figure 8.3. The lacto-*N*-biose hypothesis. *Bifidobacteria* secrete hydrolytic enzymes that act on milk oligosaccharides to form lacto-*N*-biose, which is selectively transported into bifidobacterial cells to be metabolized by lacto-*N*-biose phosphorylase.

(Wada *et al.*, 2008), the finding of this enzyme activity, as well as of α-fucosidase, should mean that *B. bifidum* and possibly other bifidobacterial strains can colonize the colon of breast-fed infants. In addition, an ABC type transporter, which delivers LNB through the cell membrane, has been purified from *B. longum* JCM1217 and crystallized (Wada *et al.*, 2007).

To validate the LNB hypothesis, it may be necessary to develop a practical method for the production of LNB on a large scale. Should LNB become easily available, the enduring enigma of the bifidus factor of human milk may be solved. Recently Nishimoto and Kitaoka (2007b) succeeded in the production of this disaccharide on a large scale using four different enzymes that were incubated in a medium containing sucrose, GlcNAc, phosphate, UDP-Glc and $MgCl_2$.

Changes in the concentrations of specific colostrum oligosaccharides may be significant with respect to the development of the colonic flora of breast-fed infants. The concentrations in colostrum of LNDFH I and LNFP I, which contain the lacto-*N*-biose I unit, have been found to be maximal at 1.9 and 2.1 g/L, respectively, on day 2 of lactation, while that of LNT, which also contains this unit, is higher on days 2 and 3 than at the beginning of lactation (Asakuma *et al.*, 2008). The relatively high concentrations of these oligosaccharides and the increase in LNT content during the early lactation period may significantly affect the formation of the colonic bifidus flora. The decrease in 2′-FL and increase in LNT during the first 3 days of lactation may mean that at the beginning of lactation the anti-infection properties of milk oligosaccharides are based mainly on their action as receptor analogues, inhibiting the adhesion of pathogens; thereafter, these properties may depend mainly on their prebiotic effect on the formation of the bifidus flora.

8.7. Milk Oligosaccharides as Anti-Pathogenic Agents

Pathogenic bacteria and viruses, to begin their infection, need to attach to the colonic mucosa, which they do by adhering to specific carbohydrate structures of glycoconjugates on the surface of the colonic epithelial cells. Because many milk oligosaccharides contain structural units that are homologous to these carbohydrate structures, it has been suggested that they act as soluble receptor analogues, inhibiting the adhesion of the pathogens, thus preventing infection.

The following anti-adhesion phenomena have been observed for human or other milk oligosaccharides or glycoconjugates. A trisaccharide unit, Gal(β1-4)GlcNAc(β1-3)Gal(β1-, which is found in lacto-*N*-neotetraose, etc., inhibits the adherence of *Streptococcus pneumoniae* to buccal epithelial cells (Andersson *et al.*, 1986). The bacteria that are inhibited from binding to enterocytes by fucosylated oligosaccharides include *Campylobacter jejuni*,

strains of *E. coli* and their heat-stable toxin, and enteropathogenic *E. coli* (Newburg *et al.*, 1990; Cravioto *et al.*, 1991; Cervantes *et al.*, 1995).

The observation that 2′-FL inhibits the binding of *C. jejuni* to H(O) antigen (Fuc(α1-2)Gal(β1-4)GlcNAc) in the infant colon is noteworthy (Ruiz-Palacios *et al.*, 2003), because this trisaccharide is the most abundant oligosaccharide in human milk (Thurl *et al.*, 1996). Intestinal infection by *C. jejuni* is one of the most common causes of diarrhea worldwide (Ruiz-Palacios, 1997). An inverse correlation has been observed between the concentration of 2′-FL in breast milk and the frequency of diarrhea in breast-fed infants, supporting the view that 2′-FL reduces the pathogenicity of *C. jejuni* (Morrow *et al.*, 2004). Asakuma *et al.* (2008) recently observed that the concentration of 2′-FL in human colostrum was 2.5 g/L at the start of lactation and decreased during the 2 subsequent days, suggesting that inhibition by 2′-FL of the adhesion of *C. jejuni* to the colonic mucosa is most significant immediately after birth. However, a recent study found that the concentration of 2′-FL in the milk of Italian and Burkinabe women was 1.0 and 1.8 g/L, respectively, on the first day of lactation, and 4.2 and 8.4 g/L on the third day (Musumeci *et al.*, 2006).

Sialylated oligosaccharides, at physiologic concentrations, strongly inhibit the binding of influenza A virus and S-fimbriated enteropathogenic *E. coli* to their respective host target cells (Zopf and Roth, 1996). It is recognized that sialyl Le^x (Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc) or Le^b (Fuc(α1-2)Gal(β1-3)[Fuc(α1-4)]GlcNAc) epitopes have affinity for a lectin, a carbohydrate-binding protein, that is found on the surface of *Helicobacter pylori*, a Gram-negative bacterium, the main host of which is man. It resides in the gastric mucosa and adheres to the epithelial cells lining the stomach. Some 50% of the world population is infected by this organism, with a higher incidence in developing countries. *Helicobacter pylori* is associated with the development of peptic ulcers, mucosa-associated lymphoid-tissue (MALT) lymphoma and gastric adenocarcinoma (Blaser, 1996; Crespo and Suh, 2001).

The binding of *H. pylori* to various carbohydrate structures is mediated by two adhesins, Bab A and Sab A, which are expressed on its surface. As Bab A and Sab A recognize and bind Le^b and sialyl Le^x, respectively, it is likely that oligosaccharides or glycoconjugates containing Le^b or sialyl Le^x inhibit the attachment of *H. pylori* to gastric epithelial cells, thus preventing its colonization within the stomach. Recently, an adhesion assay was used to investigate the capacity of pig milk to inhibit *H. pylori* binding to a neoglycoprotein that has Le^b or sialyl-di-Le^xunits conjugated to human serum albumin. α1,3/4Fucosyltransferase transgenic FVB/N mice, known to express Le^b and sialyl Le^x in their gastric epithelium, were colonized by *H. pylori* and subsequently treated with porcine milk or water. The expression of the Le^b and sialyl Le^x carbohydrate epitopes on pig milk proteins was breed- and individual-specific and correlated with the ability of porcine milk to inhibit *H. pylori* adhesion to the gastric

mucosa (Gusteffsson *et al*., 2006). As human milk oligosaccharides such as LNDFH I or 3-fucosyl-3′-*N*-acetylneuraminyllactose (Neu5Ac(α2-3) Gal(β1-4) [Fuc(α1-3)]Glc) contain Le^b or sialyl Le^x, it seems likely that these effects may be achieved also by human milk oligosaccharides.

Other researchers have reported on the interaction of *H. pylori* with sialylated glycans. The preferred interaction is with α3-linked sialic acid; glycans having α6-linked Neu5Ac are non-binding. For example, 50% inhibition by *H. pylori* of hemagglutination of human erythrocytes was observed at a low concentration of some sialylated saccharides. The data show that S-3-PG (Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer), Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)GlcNAc, 3′-*N*-acetylneuraminyl-*N*-acetyllactosamine (Neu5Ac(α2-3)Gal(β1-4)GlcNAc) as well as 3-*N*-acetylneuraminyl-lacto-*N*-neotetraose (Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc) all bound to *H. pylori* CCUG17874 at similar strength. 3′-SL also bound to this organism but its binding ability was somewhat weaker than that of the above saccharides. It has also been reported that LST a, a human milk oligosaccharide, was able to bind to another strain, *H. pylori* J99 (Johansson *et al*., 2005).

The binding of 3′-SL to *H. pylori* CCUG17874 is noteworthy because this saccharide is found in human milk and bovine colostrum. Asakuma *et al*. (2007) found that at the start of lactation the concentration of 3′-SL in human colostrum is 360 mg/L, similar to that of 6′-SL. However, the concentration of 3′-SL decreased during the 2 subsequent days of lactation, whereas that of 6′-SL did not. This suggests that, very early in lactation, 3′-SL may be more significant in the prevention of transmission of *H. pylori* from mother to infant than later on.

Recent studies on the ability of various fractions of human milk oligosaccharides to inhibit the adhesion of three intestinal microorganisms (enteropathogenic *E. coli* serotype O119, *Vibrio cholerae* and *Salmonella fyris*) to differentiated Caco-2 cells have shown that the acidic fraction had an anti-adhesive effect on all three pathogenic strains. The neutral high molecular weight fraction significantly inhibited the adhesion of *E. coli* O119 and *V. cholerae*, but not that of *S. fyris*; the neutral low molecular weight fraction was effective toward *E. coli* O119 and *S. fyris* but not *V. cholerae* (Coppa *et al*., 2006). This demonstrated that human milk oligosaccharides inhibit the adhesion to epithelial cells not only of common pathogens such as *E. coli* but also of other aggressive bacteria such as *V. cholerae* and *S. fyris*. Thus, oligosaccharides may be important factors in human milk that defend against acute diarrhea in breast-fed infants.

It is thought that adhesion of *Neisseria meningitidis*, a human-specific pathogen causing meningitis and septicemia, is mediated by type IV pili (Hakkarainen *et al*., 2005). A microtiter well pili-binding assay was used to investigate the binding of type IV pili isolated from *N. meningitidis* to different glycoproteins. Inhibition of pili binding to bovine thyroglobulin and human salivary agglutinin by fractionated human and bovine milk

oligosaccharides was demonstrated. The binding of *Neisseria pili* to bovine thyroglobulin was most effective and was clearly inhibited by neutral human or acidic bovine milk oligosaccharides at concentrations of 1–2 g/L, suggesting that these fractions had the potential ability to inhibit the attachment of this bacterium to the colonic mucosa (Hakkarainen *et al*., 2005).

There is evidence that oligosaccharides from milks other than human can act as receptor analogues, inhibiting the adhesion of pathogenic microorganisms. Fractions containing milk oligosaccharides, in the form of supernatants that had been separated from colostrum and from transitional, mature and late lactation milk of Spanish brown cows by ethanol precipitation and subsequent centrifugation, were used to investigate the inhibition of hemagglutination by seven enterotoxigenic *E. coli* strains (K99, FK, F41, F17, B16, B23 and B64). These strains had been isolated from diarrheal calves. The fractions from the transitional and late lactation milk inhibited hemagglutination by all of these strains, whereas those from colostrum and late lactation milk produced weaker inhibition (Martin *et al*., 2002). It was assumed that this inhibition was due to 3′-SL, 6′-SL, 6′-*N*-acetylneuraminyl-*N*-acetyllactosamine (6′-SLN: Neu5Ac(α2-6)Gal(β1-4)GlcNAc) and disialyl lactose (DSL: Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc). The fractions from transitional and mature milk, in which the ratio of 6′-SL to 3′-SL was higher than in the fractions from colostrum and late lactation milk, had a stronger effect than the others.

It has been suggested that Neu5Gc(α2-3)Gal(β1-4)Glc, which is found in ovine (Nakamura *et al*., 1998) and caprine (Urashima *et al*., 1997) colostrum, inhibits the attachment of enterotoxigenic *E. coli* K99 to the infant's colon, Neu5Gc(α2-3)Gal being the receptor recognized by *E. coli* K99 adhesins (Kyogashima *et al*., 1989). Globotriose (Gal(α1-4)Gal(β1-4)Glc), which is present in bottle-nosed dolphin colostrum (Uemura *et al*., 2005), is suggested as a possible inhibitor of the binding of Shigella toxin and Shiga-like toxin produced by pathogenic *E. coli* (*Lindberg et al*., 1987; Samael *et al*., 1990). Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc, which has been found in the milk of the white-nosed coati (Urashima *et al*., 1999) and mink (Urashima *et al*., 2005), has been suggested to be a possible inhibitor of the binding of toxin A produced by *Clostridium difficile* (Clark *et al*., 1987).

8.8. Immuno-Modulating Effect of Milk Oligosaccharides

Although direct detection of human milk oligosaccharides in the blood of infants has not yet been reported (Bode, 2006), it nevertheless seems very likely that small amounts of intact human milk oligosaccharides are normally absorbed from the gastrointestinal tract, and that they are transported into the blood, on the basis of their observed urinary excretion (Rudloff *et al*.,

1996; Obermeier *et al*., 1999). It follows that they may alter protein–carbohydrate interactions also at a systemic level. For example, recent studies suggest that human milk oligosaccharides interfere with the adhesion of neutrophils to vascular endothelial cells (Klein *et al*., 2000) and platelets (Bode *et al*., 2004). These effects appear to be based on the structural resemblance of some human milk oligosaccharides to the glycoprotein ligands of selectins. Selectins are trans-membrane proteins that are involved in cell–cell interactions in the immune system. P-selectin mediates leukocyte deceleration (rolling) on activated endothelial cells and initiates leukocyte extravasation at sites of inflammation. P-selectin is also involved in the formation of platelet–neutrophil complexes (PNC), a sub-population of highly activated neutrophils primed for adhesion, phagocytosis and enhanced production of reactive oxygen species. Recent studies suggest that oligosaccharides containing sialyl Le^x or its stereoisomer sialyl Le^a, which resemble the P-selectin ligand, inhibit the binding of selectin ligands to the surface of endothelial cells and platelets; this interferes with the formation of PNC, the effect of which is anti-inflammatory. The following oligosaccharide fractions were tested *in vitro* to establish whether they reduce leukocyte deceleration on U937 cells, which express the P-selectin ligand: total human milk oligosaccharides, neutral oligosaccharides, total acidic oligosaccharides, neutral oligosaccharides with a polymerization degree of 4, fucosylated oligosaccharides and disialyl lacto-*N*-tetraose. The acidic oligosaccharides fraction produced a slight but definite reduction of P-selectin ligand binding, similar to that of standard sialyl Le^x, whereas the total neutral oligosaccharides and neutral fucosylated oligosaccharides fractions did not (Schumacher *et al*., 2006). These results support the notion of anti-inflammatory effects of acidic human milk oligosaccharides.

However, Klein *et al*. (2000) showed that, *in vitro*, the neutral milk oligosaccharide fraction can inhibit the binding of neutrophils to TNF-stimulated endothelium and that the whole milk oligosaccharides fraction enhanced the formation of platelet–neutrophil complexes (Klein *et al*., 2000).

It has been reported that the incidence of necrotizing enterocolitis, a condition which is considered to be an exaggerated immune response, is about 85% lower in breast-fed than in formula-fed infants. This is consistent with an anti-inflammatory effect of absorbed human milk oligosaccharides (Lucas and Cole, 1990).

Another potential human milk oligosaccharide target could be DC-SIGN (dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin). This is expressed on dendritic cells (DC) in the intestine and other tissues and is involved in the capture of different pathogens, including HIV-1, hepatitis C, cytomegalovirus, Dengue virus, *Mycobacterium* and *Candida albicans*. Unidentified components in human milk bind to DC-SIGN and inhibit HIV-1 transfer to CD4+ T lymphocytes. DC-SIGN has high affinity

for Le^x, which is a unit contained in some human milk oligosaccharides, suggesting that the unknown inhibitory milk components could be milk oligosaccharides. The putative complex oligosaccharides with multiple Le^x determinants may inhibit DC-SIGN-mediated interactions similar to the multivalent binding hypothesis for selectins (Bode, 2006; Naaling, 2005).

The question of whether human milk oligosaccharides influence cytokine production and activation of cord blood T cells has recently been investigated (Eiwegger *et al.*, 2004). Cord blood mononuclear cells from randomly chosen healthy newborns were co-cultured for 20 days with acidic or neutral oligosaccharides, and intracellular cytokine production and surface marker expression of T cells were studied using flow cytometry. The authors used concentrations of oligosaccharides (neutral human milk oligosaccharides, 10 μg/ml; acidic human milk oligosaccharides, 1 μg/ml) that were considered by them to mimic physiologic conditions, although these concentrations are considerably lower than the calculated values of 100–200 mg/L for circulating human milk oligosaccharides mentioned by Bode (2006). The acidic, but not the neutral oligosaccharide fraction, increased the percentage of interferon-γ-producing CD3+CD4+ and CD3+CD8+ cells, of IL-13 production in CD3+CD8+ cells and significantly elevated CD25+ expression in CD3+CD4+ cells. These results showed that human milk oligosaccharides affect cytokine production and activation of cord blood-derived T cells *in vitro*. Oligosaccharides and, in particular, acidic milk oligosaccharides may therefore influence lymphocyte maturation in breast-fed newborns. The authors concluded that human milk oligosaccharides can modulate the immune system of the maturing infant.

A recent study on rats showed that goat milk oligosaccharides have an anti-inflammatory effect in the colon (Daddaowa *et al.*, 2006). In this study, colitis was induced by the hapten, trinitrobenzenesulfonic acid (TNBS). The experimental rats (OS) were fed a diet containing 500 mg/kg per day of goat milk oligosaccharides, from 2 days prior to the induction until day 6, after which all the rats were killed, the entire colon was removed, opened and scored for visible damage and then divided into several pieces for biochemical determinations. When the OS rats were compared with control rats in which colitis had been induced by TNBS but that had not been treated with oligosaccharides, it was found that the OS rats showed decreased anorexia, reduced loss of body weight, reduced bowel wall thickening and less necrosis of the colon. Biochemically, the colon of the rats had lower levels of inducible oxide nitric synthase (iNOS), cyclooxygenase 2 (COX2), interleukin-1β and mucin 3, as well as increased trefoil factor 3. These results showed that goat milk oligosaccharides are anti-inflammatory when administered as a pretreatment in the TNBS model of rat colitis, most likely due to their action as prebiotics resulting in favorable changes in the colonic bacterial flora. Since TNBS-induced colitis is widely used as a preclinical model of

inflammatory bowel disease in humans, it was suggested that goat milk oligosaccharides may be useful in the management of this disease.

Another study was performed to evaluate the effect of oligosaccharides from goat milk in a rat model of dextran sodium sulfate (DSS)-induced colitis (Lara-Villoslada *et al.*, 2006). DSS treatment produced a decrease in body weight that was not observed in rats fed the goat milk oligosaccharides. DSS also caused an acute colonic inflammatory process that was weaker in rats fed the goat milk oligosaccharides, as shown by colon myeloperoxidase activity, as well as clinical symptoms measured by a scoring system. The rats that had been fed goat milk oligosaccharides also showed less severe lesions. It was concluded that the goat milk oligosaccharides reduced intestinal inflammation and contributed to the recovery of damaged colonic mucosa.

8.9. Chemical Structures and Features of Bovine Milk Oligosaccharides: Milk Oligosaccharides of Other Domestic Farm Animals

The structures of 11 acidic and 10 neutral bovine milk oligosaccharides that have been discovered to date are shown in Table 8.3 (Gopal and Gill, 2000; Urashima *et al.*, 2001; Nakamura and Urashima, 2004). Most of these oligosaccharides had been isolated from colostrum (Gopal and Gill, 2000). Bovine colostrum contains more than 1 g/L of oligosaccharides (Nakamura *et al.*, 2003), the majority of which are acidic, whereas the mature milk contains only trace amounts (Gopal and Gill, 2000). The low concentration of oligosaccharides in mature bovine milk makes it difficult to use such milk in the production of human infant formulae designed to provide prebiotics and receptor analogues (see above). This means that certain chemically produced oligosaccharides that are not normally found in milk, but which have functions similar to those of human milk oligosaccharides, could with advantage be incorporated into infant formulae. Recently, 39 oligosaccharides have been found in bovine colostrum by employing microchip liquid chromatography separation and high-performance mass spectroscopy including Fourier transform ion cyclotron resonance (FTICR) and time-of- flight (TOF) analysis. The presence of LNnT, LNnH, lacto-N-novopentaose I (Gal(β 1-3)[Gal(β 1-4)GlcNAc(β 1-6)]Gal(β 1-4)Glc) and their N-acetylneuraminyl or N-glycolylneuraminyl derivatives was suggested by this method (Tao, *et al.*, 2008).

Most of the oligosaccharide fraction of bovine colostrum consists of 3′-SL, 6′-SL, 6′-SLN and DSL, with 3′-SL constituting 70% of the total oligosaccharide content. Changes in the levels of 3′-SL, 6′-SL and 6′-SLN in Holstein colostrum *pre-partum* to 1 week *post-partum* are shown in Figure 8.4. The levels were maximal immediately after parturition, rapidly

Table 8.3 The structures of bovine milk oligosaccharides

	Abbreviation	Oligosaccharide	Reference
1		GalNAc(β 1-4)Glc	Saito *et al.* (1984)
2	LacNAc	Gal(β 1-4)GlcNAc	Saito *et al.* (1984)
3	F-LacNAc	Gal(β1-4)GlcNAc \| Fuc(α1-3)	Saito *et al.* (1984)
4	α3'GalNAcL	GalNAc(α 1-3)Gal(β1-4)Glc	Urashima *et al*. (1991)
5	α3'-GL	Gal(α 1-3)Gal(β 1-4)Glc	Urashima *et al*. (1991)
6	3'-GL	Gal(β 1-3)Gal(β 1-4)Glc	Saito *et al.* (1987)
7	4'-GL	Gal(β 1-4)Gal(β 1-4)Glc	Kimura *et al.* (1997)
8	6'-GL	Gal(β 1-6)Gal(β 1-4)Glc	Saito *et al.* (1987)
9	3'-GalNAcL	GalNAc(β 1-3)Gal(β1-4)Glc	Watanabe *et al.* (2006)
10	novo LNPI	Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-3)	Urashima *et al.* (1991)
11		Gal(β 1-4)Glc-3'-PO_4	Cumar *et al.* (1965)
12		Neu5Ac(α 2-3)Gal	Kuhn and Gauhe. (1965)
13	3'-SL	Neu5Ac(α 2-3)Gal(β 1-4)Glc	Schneir and Rafelson (1966)
14	6'-SL	Neu5Ac(α 2-6)Gal(β 1-4)Glc	Kuhn and Gauhe. (1965)
15	3'-Neu5GcL	Neu5Gc(α 2-3)Gal(β 1-4)Glc	Kuhn and Gauhe. (1965)
16	6'-Neu5GcL	Neu5Gc(α 2-6)Gal(β 1-4)Glc	Veh *et al.* (1981)
17	6'-SLacNAc	Neu5Ac(α 2-6)Gal(β 1-4)GlcNAc	Kuhn and Gauhe. (1965)
18	6'-Neu5GcLacNAc	Neu5Gc(α 2-6)Gal(β 1-4)GlcNAc	Veh *et al.* (1981)
19		Neu5Ac(α 2-3)Gall-3Gall-4Glc	Parkkinen and Jinne (1987)
20	DSL	Neu5Ac(2-8)Neu5Ac(α 2-3) Gal(β1-4)Glc	Kuhn and Gauhe. (1965)
21	6'-SLacNAc-1-phosphate	Neu5Ac(α 2-6)Gal(β1-4)GlcNAc-1-PO_4	Parkkinen and Jinne (1987)
22	6'-SLacNAc-6-phosphate	Neu5Ac(α 2-6)Gal(β1-4)GlcNAc-6-PO_4	Parkkinen and Jinne (1987)

decreasing by 48 h *post-partum* (Nakamura *et al*., 2003). In another study, the concentrations of 3′-SL, 6′-SL, 6′-SLN and DSL were found to be 681, 243, 239 and 201 mg/L, respectively, in Holstein colostrum and 867, 136, 220 and 283 mg/L, respectively, in Jersey colostrum immediately after parturition (McJarrow and van Amelsfort-Schoonbeek, 2004).

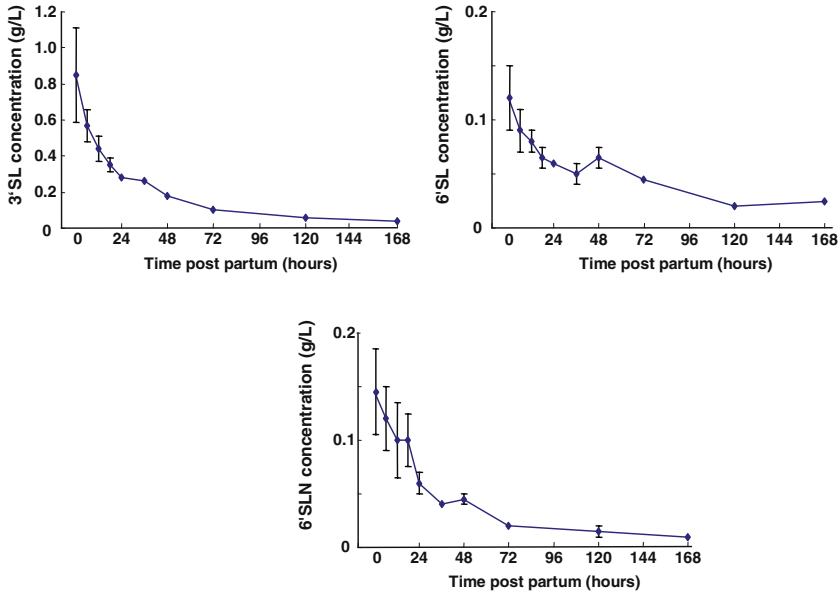


Figure 8.4. Changes in the concentrations of 3′-SL, 6′-SL and 6′-SLN in Holstein bovine colostrum during early lactation. Values are indicated as means ± SD ($n = 4$). Reproduced from Nakamura *et al.* (2003) with permission.

Among the neutral oligosaccharides, the following are characteristic of bovine colostrum, insofar as they have not been found in the milk or colostrum of other mammals: GalNAc(β1-3)Gal(β1-4)Glc (Watanabe *et al.*, 2006), GalNAc(α1-3)Gal(β1-4)Glc and GalNAc(β1-4)Glc. It is noteworthy that both α(1-3)- and β(1-3)-linked galactosyllactose and *N*-acetylgalactosaminyllactose have been found among the bovine oligosaccharides

As shown in Table 8.3, some bovine milk oligosaccharides have a *N*-acetyllactosamine (Gal(β1-4)GlcNAc) unit at their reducing end, in contrast to human milk oligosaccharides, almost all of which have a lactose residue in that position. The core units of most bovine milk oligosaccharides are lactose or *N*-acetyllactosamine, unlike human milk oligosaccharides the core units of which are LNT, LNnT, LNH and LNnH, etc. Both the variety and the concentration of fucosylated oligosaccharides in bovine colostrum and milk are very low; this is in contrast to human milk oligosaccharides, many of which are fucosylated.

It can be expected that milk oligosaccharides of other domestic farm animals, such as goats and sheep, will also be used as biofunctional materials. The content of milk oligosaccharides in goat milk is 0.25–0.30 g/L; this is higher than that of bovine (0.03–0.06 g/L) or ovine (0.02–0.04 g/L) milk. In addition, the variety of oligosaccharides in goat milk is greater than that in bovine or ovine

milk, as shown by the profiles of HPEAC analysis (Martinez-Ferez *et al.*, 2006; Mehra and Kelly, 2006). Colostrum from Japanese Saanen breed goats contains more 6′-SL than 3′-SL; it also contains 6′-*N*-glycolylneuraminyllactose, 6′-SLN, Gal(α1-3)Gal(β1-4)Glc, Gal(β1-3) Gal(β1-4)Glc, Gal(β1-6)Gal(β1-4)Glc and 2′-FL (Urashima *et al.*, 1994; Urashima *et al.*, 1997). Another study has shown that mature milk from Spanish goats contains 6′-SL, 3′-SL, DSL, *N*-glycolylneuraminyllactose, 3′-galactosyllactose, *N*-acetylglucosaminyllactose, LNH and additional high molecular oligosaccharides, as demonstrated by analysis with FAB-MS (Martinez-Ferez *et al.*, 2006). Ovine colostrum contains more 3′-*N*-glycolylneuraminyllactose than 3′-SL and 6′-SL (Nakamura *et al.*, 1998) and, notably, contains Neu5Gc in preference to Neu5Ac.

8.10. Milk Oligosaccharides of Other Mammals

Milk oligosaccharides of the following species, other than human and cow, have been studied and characterized (see also Urashima *et al.*, 2001); brown capuchin, buffalo, horse, goat, sheep, Ezo brown bear, Japanese black bear, polar bear, white-nosed coati, elephant, rat, dog, beluga, Minke whale, giant panda, crabeater seal, hooded seal, bearded seal, harbor seal, mink, bottlenose dolphin, echidna, platypus, and tammar wallaby (Urashima *et al.*, 2001). These oligosaccharides contain mainly lactose, lacto-*N*-neotetraose, lacto-*N*-neohexaose or para-lacto-N-neohexaose as core units, and there are species-specific features of the structures with respect to the presence or absence of Lewis x (Gal(β1-4)[Fuc(α1-3)]GlcNAc), H antigen (Fuc(α1-2)Gal), A antigen (GalNAc(α1-3)[Fuc(α1-2)]Gal), B antigen (Gal(α1-3)[Fuc(α1-2)]Gal) and α-Gal epitope (Gal(α1-3)Gal(β1-4)GlcNAc). As described above, the milk or colostrum oligosaccharides of non-human mammals either contain only the type II (Gal(β1-4)GlcNAc) but not the type I (Gal(β1-3)GlcNAc) unit, or else the former saccharides dominate over the latter.

The tammar wallaby is unique insofar as the neutral milk oligosaccharides of this species, and probably of most or all other marsupials, consist of at least two series, a major series, which can be described as $[\text{Gal}(\beta1\text{-}3)]_{n=1\sim5}$ Gal(β1-4)Glc, and a minor one, the members of which contain a branched unit of GlcNAc(β1-6) (Messer *et al.*, 1980, 1982; Collins *et al.*, 1981; Bradbury *et al.*, 1983). Of the major series, only the trisaccharide Gal(β1-3)Gal(β1-4)Glc has been found also in the milk/colostrum of some eutherian species, but higher members have not been detected (Urashima *et al.*, 2001; Messer and Urashima, 2002). It is notable that no fucosyl oligosaccharides have so far been detected in the milk of any marsupial (Messer and Urashima, 2002). In this respect the milk oligosaccharides of marsupials differ from those of both eutherians and monotremes since the major neutral milk oligosaccharides of the echidna and

platypus (monotremes) are fucosyllactose and difucosyllactose, respectively (Messer and Urashima, 2002). Furthermore, the higher milk oligosaccharides of the platypus contain lacto-*N*-neotetraose or lacto-*N*-neohexaose as core units (Amano *et al.*, 1985), which is a feature that is shared by many eutherian milk oligosaccharides. In these respects, also, eutherian milk oligosaccharides are more similar to those of monotremes than to those of marsupials.

The milk of all three infraclasses of mammals (eutherians, marsupials and monotremes) contains acidic (sialyl) in addition to neutral oligosaccharides (Urashima *et al.*, 2001). Milk of the echidna, a monotreme, uniquely contains, as its major oligosaccharide, a sialyllactose in which the sialic acid residue has a 4-*O*-acetyl substituent (Kamerling *et al.*, 1982).

It is worth noting that oligosaccharides dominate over free lactose in the milk of monotremes, marsupials and a few species of eutherians such as the Canoidea (other than the dog, *Canis familiaris*); the biological significance of this phenomenon, which appears to be found mainly in species whose neonates are altricial, is open to speculation (Messer and Urashima, 2002).

8.11. Future Aspects of Milk Oligosaccharides

In the above discussion, the main focus has been on the chemical structures and biological significance of oligosaccharides of human milk and colostrum, rather than of other species. Among more than 100 human milk oligosaccharides, each oligosaccharide may have its own specific role as a prebiotic and/or receptor analogue for different pathogenic microorganisms. Based on these functions, the possible utilization of artificial human milk oligosaccharide-like components on an industrial scale will be discussed below.

2′-FL is significant in the prevention of diarrhea caused by *C. jejuni* (Ruiz-Palacios *et al.*, 2003; Morrow *et al.*, 2004). As bovine milk does not contain this saccharide, there is a need for the development of techniques for its preparation and also incorporation into infant formulae produced from this milk. Murata *et al.* (1999a) prepared Fuc(α1-2)Gal(β1-4)GlcNAc, a saccharide that is similar to 2′-FL, using *p*-nitrophenyl-α-L-fucopyranoside as a donor and *N*-acetyllactosamine as an acceptor, by reverse hydrolysis with fucosidase from porcine liver. However, the yields were low and Fuc(α1-3) Gal(β1-4)GlcNAc and Fuc(α1-6)Gal(β1-4)GlcNAc were formed as undesirable by-products. It may be that one could use a different, more suitable, fucosidase that would give a higher yield of 2′-FL, and one could develop a method that uses fucose instead of *p*-nitrophenyl-α-L-fucopyranoside as a donor and lactose as an acceptor.

As shown by Kitaoka *et al.* (2005), LNT has the most potential as a candidate for prebiotics. As this saccharide has not been detected in bovine milk, there is a need for development of a method for its preparation, so that it

can be incorporated into infant formulae. Murata *et al*. (1999b) prepared lacto-*N*-triose II (GlcNAc(β1-3)Gal(β1-4)Glc) by the action of β-3-*N*-acetylglucosaminyltransferase from bovine serum, using lactose as the acceptor and UDP-GlcNAc as the donor. They then prepared LNT by reverse hydrolysis using β-galactosidase from recombinant *Bacillus circulans* ATCC31882, with lacto-*N*-triose II as the acceptor and lactose as a donor. However, bovine serum β-3-*N*-acetylglucosaminyltransferase is not available on an industrial scale, and the cost of UDP-GlcNAc is still very high. Because the LNB structure in LNT is considered to act as a specific bifidus factor in human milk (Kitaoka *et al*., 2005), LNB is also a possible candidate as a prebiotic. LNB, a disaccharide, should be much easier to produce than LNT, a tetrasaccharide. Nishimoto and Kitaoka (2007b) succeeded in the mass production of LNB using LNBP.

It can be expected that, in future, milk oligosaccharides will be isolated from the colostrum or milk of cows, or of other domestic farm animals, on an industrial scale for use in the production of infant formulae. Bovine colostrum contains more than 1 g/L of sialyl oligosaccharides, of which 3′-SL constitutes 70% (Nakamura *et al*., 2003). As noted above, there is evidence that 3′-SL prevents the adhesion of *H. pylori* to the gastric mucosa (Karlsson, 1998; Mysore, 1999; Sharon and Ofek, 2000). It can therefore be expected that 3′-SL isolated from bovine colostrum will be incorporated into infant formulae and biofunctional foods to prevent this adhesion.

Furthermore, the oral administration of goat milk oligosaccharides may be useful as a treatment for inflammatory bowel disease (Daddaowa *et al*., 2006; Lara-Villoslada *et al*., 2006) and it is possible that oligosaccharides from goat milk or colostrum or bovine colostrum will be used for this purpose. It has been noted, however, that the presence of Neu5Gc in caprine and ovine milk (see above) and the milk of other non-humans may be a significant drawback, since this type of sialic acid is not normally found in human milk oligosaccharides. There appears to be evidence that circulating anti-Neu5Gc-antibodies can be found in humans, probably as a result of dietary ingestion of Neu5Gc (Bode, 2006).

Nevertheless, oligosaccharides isolated from milk or colostrum of domestic farm animals, as well as milk oligosaccharide-like components prepared by synthetic methods, the functions of which are similar to those of milk oligosaccharides, can in future be expected to be used in industry as biofunctional materials.

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