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Human milk oligosaccharides (HMOs) and their effects on intestinal microorganisms

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2-AA	2-Aminobenzoic acid
2'-FL	2'-Fucosyllactose
3-FL	3-Fucosyllactose
3'-SL	3'-Sialyllactose
6'-SL	6'-Sialyllactose
ACN	Acetonitrile
DMSO	Dimethyl sulfoxide
FOS	Fructo-oligosaccharides
Fuc	Fucose
Gal	Galactose
Glc	Glucose
GlcNAc	N-acetylglucosamine
GOS	Galacto-oligosaccharides
HMO	Human milk oligosaccharides
HPAEC-PAD	High-pH anion-exchange chromatography with pulsed amperometric detection
HPLC	High Performance Liquid Chromatography
Lac	Lactose
LNB	Lacto-N-biose I
LNnT	Lacto-N-neotretraose
LDFT	Lacto-di-fucotetraose
LNDFH I	Lacto-N-difuco-hexaose I
LNDFH II	Lacto-N-difuco-hexaose II
LNFP I	Lacto-N-fucopentaose I
LNFP II	Lacto-N-fucopentaose II
LNFP III	Lacto-N-fucopentaose III
LNFP V	Lacto-N-fucopentaose V
LNT	Lacto-N-tetraose
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization — Time of Flight Mass Spectrometry
NeuAc	N-acetylneuraminic acid
OD	Optical density
RP-HPLC	Reversed-phase Chromatography

1. Introduction

1.1 HMOs-Human milk oligosaccharides

Human milk is considered to be the golden standard in infant nutrition. For many years, infant food companies have focused on strategies to imitate the composition of human milk with regard to protein classes, fatty acids and even various oligosaccharides. However, it has become obvious that the mere presence of a component in milk is not an indication for its biological activities. Therefore, today, the main focus is laid on the investigation of specific functions of individual milk components which is a prerequisite for the manufacturing of new products (Kunz *et al.* 1996, Kunz *et al.,* 2000).

Human milk oligosaccharides (HMOs) are thought to be beneficial for the human milk fed infant because of their anti-inflammatory, anti-infective and immune stimulating properties (Kunz *et al.*, 2000; Newburg *et al.*, 2005; Sharon and Ofek, 2000). The selective utilization of distinct HMOs as novel food ingredients, however, has been hampered mainly due to difficulties in obtaining or producing HMOs in larger amounts. Today, biotechnological tools are available or may be developed to produce sufficient amounts of individual components which can subsequently be investigated in functional studies.

1.1.1 History

As the golden standard for infant nutrition, human milk was studied by scientists and pediatricians for more than 100 years. Early at the end of 19th century, the infant's survival rate were only about 70% to 80% in their first year, people already realized that, compared with the 'bottle-fed' infants, the breast-fed infants had lower mortality and lower chance for the diarrhea infections and also some other diseases (Bode, 2012).

In 1886, Escherich, the leading microbiologist in pediatrics from Austria, had described that the physiology of infants' digestion had some relationship with the

intestinal microorganisms in infants' body (Escherich, 1886). Later in about 1900, Moro had further observation to indicate that both breast-feeding and intestinal bacteria are important to infants' health (Moro, 1990). In the same year, Tissier, from Paris, observed different fecal bacterial composition of breast-fed and bottle-fed infants (Tissier, 1990). Until then, people had first indicated that difference of bacterial composition in the infant's intestine is linked to human milk composition. It was also the first description of microorganisms and their importance for infants' health. But the specific components in human milk which affected the bacterial composition were still unknown.

More than 40 years later, about 1930, Michel Polonowski and Albert Lespagnol in Lille, France, first characterized the unknown carbohydrate fraction from human milk and named it as 'gynolactose', which was only weakly soluble in methanol. They worked for several years and found that this gynolactose consisted of various components, such as hexosamines, which contains essential component nitrogen. (Polonowski and Lespagnol, 1929; Polonowski and Lespagnol, 1930; Polonowski and Lespagnol, 1931; Polonowski and Lespagnol, 1933).

In 1954, Polonowski together with Montreuil introduced two-dimensional paper chromatography for the first time to separate the gynolactose into individual oligosaccharides, and identified the first two fucosyllactoses (2'-fucosyllactose and 3-fucosyllactose) in the fraction that had been unknown at that time (Polonowski and Montreuil, 1954). In the same time, the chemist Richard Kuhn and the pediatrician Paul György, did several researches on the connection between bacteria and 'gynolactose', and later proved that the 'bifidus factor' which was earlier reported by Schönfeld in 1926, contained oligosaccharides (Gauhe *et al.*, 1954; György *et al.*, 1954a; György *et al.*, 1954b; György *et al.*, 1954c; Rose *et al.*, 1954, Schönfeld, 1926).

In the following years, Jean Montreuil in France and Richard Kuhn in Germany both tried to describe the complex mixture in the 'gynolactose'. They specified more than 12 individual HMO structures, such as 2'- fucosyllactose, 3-fucosyllactose and

difucosyllactose, lacto-N-tetraose, lacto-N-fucopentaose I and II, difucosyllactose, and so on (Montreuil, 1960; Kuhn and Baer, 1956; Kuhn *et al.*, 1956, 1958, 1960; Kuhn and Gauhe, 1958, 1962). From about 1960s, Watkins investigated the blood group ABO and secretor system (Watkins, 1966). HMO, as the major components in human milk, contributed the structural characterization and blood group specificity. In 1967, Grollmann confirmed that in milk samples of non-secretor women, 2'-fucosyllactose can't be detected (Grollman and Ginsburg, 1967; Grollman *et al.*, 1969, 1970). From 1967, Akira Kobata and Victor Ginsburg developed a new method to determine the three different oligosaccharides pattern using only a small amount of milk. They have been able to show fourteen oligosaccharides at the same time. Since then, more individual HMO fractions were separated and characterized (Kobata, 2010; Kobata and Ginsburg, 1969, 1972a, b; Kobata *et al.*, 1969).

Later in 1983, Heinz Egge, one of the leading pioneers in HMO field, was one of the first researchers to apply the fast atom bombardment mass spectrometry (MS) to characterize HMOs (Egge *et al.*, 1983). Since then, a general method for mapping and sequencing of native and derivatized glycoconjugates was established.

In recent years, scientists further studied on nutritional and biological properties of HMOs. An analytical method was developed to separate and characterize neutral and acidic lactose-derived oligosaccharides without prior derivatization or reduction by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Kunz *et al.*, 1996a, b). There were also some researches on the biological functions of HMOs in both *vivo* study using ^{13}C-labeling method and *in vitro* study using cell culture method (Bode *et al.*, 2004a; Bode *et al.*, 2004b; Gnoth *et al.*, 2000; Gnoth *et al.*, 2001; Kuntz *et al.*, 2009; Kuntz *et al.*, 2008; Rudloff *et al.*, 2006; Rudloff *et al.*, 2012; Rudloff *et al.*, 1996; Rudloff *et al.*, 2002).

1.1.2 Structures

The core structure of human milk oligosaccharides contains three monomers: D-glucose (Glc), D-galactose (Gal), and N-acetylglucosamine (GlcNAc). With few exceptions, the lactose (Glc β1-4 Gal) is at the reducing end and the Gal residue

achieved an elongation with lacto-N-biose I (Gal β1-3 GlcNAc, LNB, type 1 chain) linked by β1-3 bond to the GlcNAc residue, or linked with N-acetyllactosamine (Gal β1- 4 GlcNAc, LacNAc, type 2 chain) by β1-3/6 bond to the GlcNAc residue. (Amano *et al.*, 2009; Kobata, 2010). With the elongation, totally thirteen core structures have been classified. There are also two main monomers which can further more modify the conformation of HMO structures: L-fucose (Fuc) and sialic acid [N-acetylneuraminic acid (NeuAc)]. Fuc can have attachment to any position of the core structure via α1–2/3/4 linkages and NeuAc can linked with β 2–3/6 respectively. Among the structures, the type 1 chain plays a predominant role and is the unique feature of HMOs compared with other mammals or anthropoids milk oligosaccharides. Figure 1-1 shows the structures of HMOs. Table 1-1 lists the core structure and the basic HMO structures and their common abbreviations.

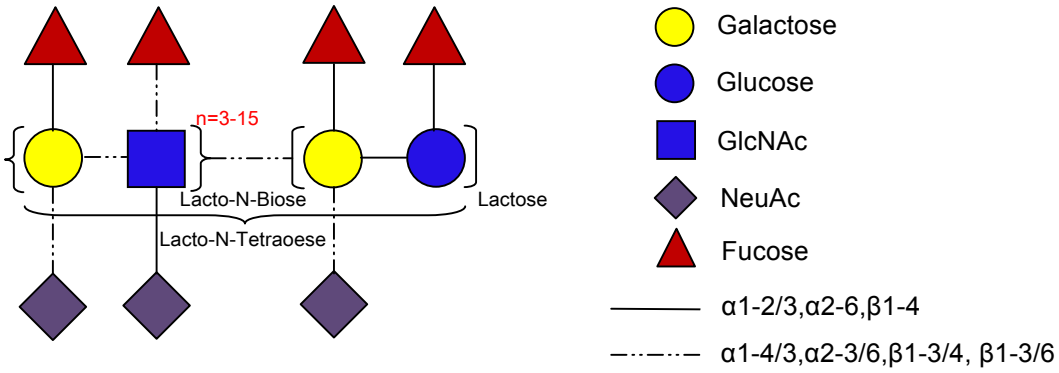


Figure 1-1 Structures of HMOs. [According to Sela and Mills, 2010, Zivkovic and Barile, 2011, with modifications]

Table 1-1 Core structure and the basic HMO structures and their common abbreviations (According to Kunz *et al.*, 2014, with modifications)

Component	Abbreviation	Structure
Lactose	Lac	Gal β1-4 Glc
Lacto-N-biose I (type 1 chain)	LNB	Gal β1-3 GlcNAc
N-acetyllactosamine (type 1 chain)	LacNAc	Gal β1-4 GlcNAc
2'-Fucosyl-lactose	2'-FL	Fuc α1-2 Gal β1-4 Glc
3 -Fucosyl-lactose	3 -FL	Gal β1-4 [Fuc α1-3] Glc
Lacto-N-tetraose	LNT	Gal β1-3 GlcNAc β1-3 Gal β1-4 Glc

Lacto-N-neotretraose	LNnT	Gal β1-4 GlcNAc β1-3 Gal β1-4 Glc
Lacto-difucosyl-tetraose	LDFT	Fuc α1-2 Gal β1-4 Glc [Fuc α1-3]
Lacto-N-fucopentaose I	LNFP I	Fuc α1-2 Gal β1-3 GlcNAc β1-3 Gal β1-4 Glc
Lacto-N-fucopentaose II	LNFP II	Gal β1-3 [Fuc α1-4] GlcNAc β1-3 Gal β1-4 Glc
Lacto-N-fucopentaose III	LNFP III	Gal β1-4 [Fuc α1-3] GlcNAc β1-3 Gal β1-4 Glc
Lacto-N-fucopentaose V	LNFP V	Gal β1-3 GlcNAc β1-3 Gal β1-4 [Fuc α1-3] Glc
Lacto-N-difuco-hexaose I	LNDFH I	Fuc α1-2 Gal β 1-3 [Fuc α1-4] GlcNAc β1-3 Gal β1-4 Glc
Lacto-N-difuco-hexaose II	LNDFH II	Gal β1-3 [Fuc α1-4] GlcNAc β1-3 Gal β 1-4 [Fuc α1-3] Glc
3'-Sialyllactose	3'-SL	NeuAc α2-3 Gal β1-4 Glc
6'-Sialyllactose	6'-SL	NeuAc α2-6 Gal β1-4 Glc
Sialyl-lacto-N-tetraose a	LST a	NeuAc α2-3 Gal β1-3 GlcNAc β1-3 Gal β1-4 Glc
Sialyl-lacto-N-tetraose b	LST b	Gal β 1-3 [NeuAc α2-6] GlcNAc β1-3 Gal β1-4 Glc
Sialyl-lacto-N-tetraose c	LST c	NeuAc α2-6 Gal β1-4 GlcNAc β1-3 Gal β1-4 Glc
Disialyl-lacto-N-tetraose	DS-LNT	NeuAc α2-3 Gal β1-3 [NeuAc α2-6] GlcNAc β1-3 Gal β1-4 Glc

1.1.3 Lewis blood type and secretor specific components

Until now, more than 100 HMOs have been identified and characterized (Kobata, 2010). The structure of HMOs from individuals differs due to the secretor (Se) and Lewis (Le) genes of the mother. From the secretor difference, it is divided into two kinds: Se and non-Se. Individuals with a Se gene loci encoding α1-2-fucoslyltransferase (FUT2) are classified as Secretors (Se). FUT2 can facilitate the addition of Fuc to terminal Gal in an α1-2 linkage. Those who lack a functional FUT2 enzyme are named as Non-secretors (non-Se). For the Lewis gene aspect, there are three different types: Le (a+b-), Le (a-b+) and Le (a-b-). Mothers who have an active

Le locus encoding α 1-3/4-fucosyltransferase (FUT3), are classified as Le positive. FUT3 can catalyzes the addition of Fuc to sub-terminal GlcNAc on type I chains in an α1-4 linkage. Individuals who can't express FUT3 are called Le negative. Based on different Se and Le genes expression, HMOs can be divided into four groups: Se+Le+, Se+Le−, Se−Le+ and Se−Le−. Figure 1-2 shows the HMO fucosylation of the four groups. Table 1-2 concludes the Fucosyltransterase and the proportion of the four groups; Table1-3 lists some typical HMO structure and their existence in the type of milk groups (Kumazaki and Yoshida, 1984; Thurl *et al.*, 2010; Viverge *et al.*, 1990; Xu *et al.*, 1996).

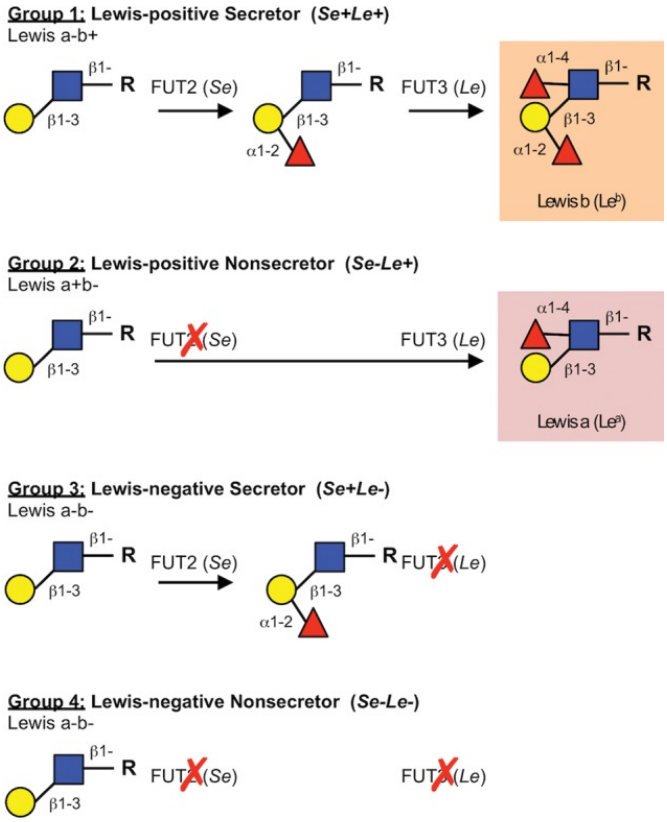


Figure 1-2 Secretor and Lewis blood group dependent HMO fucosylation. HMO fucosylation highly depends on a woman's Se and Le blood group status. If both FUT2 and FUT3 are expressed, milk contains HMO with Le b antigens. If only FUT3 is expressed, milk contains HMO with Le a antigens. If FUT3 is not expressed, HMOs contain neither Le a nor Le b antigens. (Bode, 2012)

Table 1-2 Fucosyltransferase and population of Lewis specific type (Kunz *et al.*, 2014)

Groups	Type	Fucosyltransferase	% Population
Se-Le+	Lewis a Nonsecretor	α1-4 Fucosyltransferase (FUT3)	20
Se+Le+	Lewis b Secretor	α1-2 Fucosyltransferase (FUT2) α1-4 Fucosyltransferase (FUT3)	70
Se+Le-	Lewis a^-b^- Secretor	α1-2 Fucosyltransferase (FUT2)	~10
Se-Le-	Lewis a^-b^- Nonsecretor	None	~10

Table 1-3 Lewis blood group and secretor specific HMO (Kunz *et al.*, 2014)

Name	Structure	Le(a^-b^+)	Le(a^+b^-)	Le(a^-b^-) Secretor	Le(a^-b^-) Non-secretor
2'-FL	Fuc α1-2 Gal β1-4 Glc	+	-	+	-
LDFT	Fuc α1-2 Gal β1-4 Glc[Fuc α1-3]	+	-	+	-
LNFP I	Fuc α1-2 Gal β1-3 GlcNAc β1-3 Gal β1-4 Glc	+	-	+	-
LNFP II	Gal β1-3 [Fuc α1-4] GlcNAc β1-3 Gal β1-4 Glc	+	+	-	-
LNDFH II	Gal β1-3 [Fuc α1-4] GlcNAc β1-3 Gal β 1-4 [Fuc α1-3] Glc	+	+	-	-

1.1.4 Concentrations

The concentration of HMOs varies widely during the lactation period decreasing from high amounts of up to 50 g / L in colostrum to about 10 – 15 g/L in mature milk (Kunz *et al.*, 1999; Thurl *et al.*, 2010; Urashima *et al.*, 2012). The individual difference can mainly be explained by the genetic variance of the donors who have different blood group specific components as well as a distinguished set of milk oligosaccharides. 2/3 of all milk oligosaccharides are neutral species while 1/3 are acidic components which may also be fucosylated. The neutral oligosaccharide fractions show a very distinct pattern ranging from about 100 (Pattern A), 150 (Pattern B) to about 50 (Pattern C) components (Kunz, *et al.* 1996a).

It is intriguing to speculate whether these obvious differences have an impact on the infants' health either immediately or in later life. This has not been investigated so far due to the difficulties to perform respective studies in humans.

A human milk fed infant receives a large amount (about 8-12 g) of HMOs per day based on an average drinking volume of 850 ml per day (Kunz *et al.*, 2000); including LNT, LNFP I and LNFP II, the uptake of which varies between 0.5 and 2 g per day (Rudloff *et al.*, 2006). Thus, a large amount of individual HMOs passes the gastrointestinal tract of infants with each feeding (Kuntz *et al.*, 2009; Kuntz *et al.*, 2008).

1.1.5 Biosynthesis

Many scientists and manufacturers have tried different methods to produce HMOs because of the potential effects of HMO on infant health. But until now, it is still difficulty to produce HMO in industry.

HMO has lactose (Gal β1-4 Glc) at the reducing end, and is elongated by mainly LNB via β 1-3 linkage or LacNAc via β 1-3/6 linkage. So the essential of HMO biosynthesis is trying to extent the lactose biosynthesis (Intanon *et al.*, 2014).

The most attractive method is *in vivo* approach, which supposes microbial fermentation to express specific enzyme gene. In 2001, Dumon used *E. coli* as an engineering bacteria to over-express the α-1,3-fucosyltransferase from *Helicobacter pylori*, and produce various fucosylated HMO when lactose added to the medium (Dumon *et al.*, 2001). Later in 2002, the group of Eric Samain tried to use a β-galactosidase-negative *E.coli* bacteria to over-express β-1, 3-N- acetylglucosaminyl -transferase gene from Neisseria meningitides (Priem *et al.*, 2002). In a recent study, Guo utilized *Pasteurella multocida* sialyltransferase to produce both 3'-sialyl-lactose and 6'- sialyl-lactose (Guo *et al.*, 2014).

However, due to the complex mechanism of the HMO biosynthesis, many technique keys remain unknown and the produce of HMO still need to be further researched on.

1.2 Intestinal microorganisms in infants

1.2.1 Intestinal microorganisms

Since there is no oxygen in the gut lumen, the strictly aerobic bacteria can't grow in the gut. The main types of intestinal microorganisms are strictly anaerobic bacteria and a small amount of facultative anaerobic bacteria. The facultative bacteria can perform either aerobic or anaerobic metabolism, such as *E. coli*, *Lactobacilli*, *Enterobacter, Streptococci* and S*taphylococci*, the strictly anaerobic bacteria can't utilize oxygen and lack the enzymes that detoxify oxygen, such as *Bifidobacteria*, *Bacteroides* and *Clostridium* (Hayashi *et al.*, 2002). Because of the growth condition of strictly anaerobic bacteria, even the air contact is minimized during the cultivation, there were still about half populations of bacteria that can't be cultivated. Among the not yet cultivated bacteria, about 70% are novel (Eckburg *et al.*, 2005).

1.2.2. Intestinal microorganisms in infants

1.2.2.1 Culture-based studies on bacteria in infants

Early in 1971, Mata and Urrutia studied the colonization of intestinal microorganisms in breast-fed infants. Later in 1980s, Rotimi and his research group researched on the bacterial flora in healthy neonates; Stark, Yoshioka and Benno compared the composition of intestinal microorganisms from breast-fed and formula-fed infants during their first year (Mata and Urrutia, 1971; Benno *et al.*, 1984; Rotimi and Duerden, 1981; Stark and Lee, 1982; Yoshioka *et al.*, 1983).

In 2008, Adlerberth summarized the facultative and anaerobic bacteria in infant within one week and 3 to 5 weeks, as well as the bacteria in adults. They included the colonization frequency and the mean log counts for the bacteria groups (Adlerberth and Wold, 2009). Table 1-4 summarized the gut bacteria in culture-based studies. The data from adults are for comparison.

Table 1-4 Colonization frequency (given in %) and mean log counts for various groups of gut bacteria in culture-based studies (Adlerberth and Wold, 2009)

	1week		3-5weeks		Adults	
	(%)	Counts in colonized (^{10}log)	(%)	Counts in colonized (^{10}log)	(%)	Counts in colonized (^{10}log)
Facultative bacteria						
Enterobacteria	87	6.2-9.4	99	6.1-9.6	98	8.7
E. coli	74	7.9-9.7	82	7.8-9.4	93	8.6
Other enterobacteria	16	8.7-10.0	42	7.7-8.7		
Enterococci	92	5.7-9.0	87	4.5-9.6		
E. faecalis			81	6.3-8.7	80	7.5
E. faecium			23	5.7-8.8	31	7.4
Staphylococci	49	5.0-5.1	60	4.7-10.1		
S. aureus	5	4.9-5.0	19	4.6-5.0	11	5.4
Coagulase -negative	21	7.0	48	5.2-5.9	31	7.4
Anaerobic bacteria						
Bacteroides	55	4.8-9.3	64	6.0-10.1	99	11.3
Veillonella	52		56	6.3-8.7	34	8.5
Bifidbacteria	77	6.2-10.2	69	4.3-11.3	74	10.2
Clostridia	51	3.1-7.2	60	3.0-8.1	100	9.8
Lactobacilli	35	4.4-7.0	35	5.0-9.1	78	9.6
Eubacterium	23	6.0-6.6	12	6.1-9.7	94	10.7
Gram-positive cocci	21	10.7	13	5.7-11.1	94	10.6

In Table 1-4, the proportion (%) of infants containing a particular group of bacteria and the faecal population counts in colonized infants (log CFU/g faeces) were given. Data were adapted and summarized from 18 references and the range indicated highest and lowest mean counts in the available studies. Data of adults were adapted from Finegold (1983), who reporting from 141 individuals. Counts in this study were calculated from dry faecal matter, which gives approximately one log unit higher values than counts per gram faeces (Adlerberth and Wold, 2009).

1.2.2.2 Factors influencing bacterial colonization pattern

The bacteria colonization may depend on the delivery mode (vaginal versus cesarean section) or feeding pattern. According to former studies, *E. coli* colonization in neonates was partly originated from the maternal faecal flora. *Klebsiella*, *Enterobacteria*, *Enterococci*, *C. difficile* and *Bifidobacteria* may spread between infants. For *Bifidobacteria*, the identified types have been found in both maternal and infant faecal microorganisms (Adlerberth *et al.*, 1999; Adlerberth *et al.*, 1998; Bettelheim and Lennox-King, 1976; Fryklund *et al.*, 1992; Martirosian *et al.*, 1995; Mitsuoka and Kaneuchi, 1977; Persson *et al.*, 1986; Singh *et al.*, 2005; Tannock *et al.*, 1990; Cheng *et al.*, 2013; Neu and Rushing, 2011; Putignani *et al.*, 2014; Salminen *et al.*, 2004).

The delivery mode is an important factor that influences the colonization pattern. There are two kinds of delivery mode, vaginal birth or caesarean section. Infant born by normal vaginal birth can contact with their mother's gut and vagina, so that they can acquire their mother's intestinal and vaginal microorganisms, such as *Bacteroides*, *Bifidobacteria* and *E. coli.* In contrast, infant born by caesarean section don't have the chance to contact their mother's intestinal and vaginal microorganisms and they couldn't acquire the *Bacteroides* and *Bifidobacteria* at the beginning (Adlerberth *et al.*, 1991; Adlerberth *et al.*, 2006; Adlerberth *et al.*, 2007; Hall *et al.*, 1990; Penders *et al.*, 2006; Neu and Rushing, 2011; Salminen *et al.*, 2004; Putignani *et al.*, 2014). Due to the reduced competition with *Bacteroides* and *Bifidobacteria*, the amount of other *Enterobacteriaceae*, such as *Klebsiella*, *Enterobacter* and *Clostridia* can increase.

The feeding pattern is another factor influencing the colonization pattern. Breast milk may contain kinds of bacteria. Martin *et al.* found that human milk is a source of lactic acid bacteria, such as *Lactobacillus gasseri* and *Enterococcus faecium* (Martin *et al.*, 2003). Kawada *et al.* investigated the transmission of *Staphylococcus aureus* between infants and their mothers through breast-feeding (Kawada *et al.*, 2003). It was thought that the feeding pattern influences the gut microorganisms and the breast-fed and bottle-fed infants would be different from the bacteria colonization

pattern. But recently, the formulas are more similar to breast milk, so that the differences of the gut bacteria between breast-feeding and formula-feeding infants are smaller. Table 1-5 summarized comparison the amount of gut bacteria of breastfed and formula fed infants before and after 1980 (Adlerberth and Wold, 2009).

Table 1-5 Results of studies comparing the gut bacteria of breast-fed and formula-fed infants before and after 1980 (According to Adlerberth and Wold, 2009). Numbers of positive results/total examinations are given.

	Number of studies					
Bacterial Groups	More in breastfed		No clear difference		Less in breastfed	
	Before 1980	After 1980	Before 1980	After 1980	Before 1980	After 1980
Bifidobacteria	5/6	7/27	1/6	20/27	0/6	0/27
Bacteroides	0/3	2/19	1/3	9/19	2/3	8/19
Clostridia	0/3	0/17	0/3	4/17	3/3	13/17
Lactobacilli	NT	2/16	NT	10/16	NT	4/16
Enterobacteriaceae	0/7	1/21	5/7	9/21	2/7	11/21
Enteroccocci	0/2	0/16	0/2	5/16	2/2	11/16
Staphylococci	NT	5/12	NT	7/12	NT	0/12

Note: NT means not tested.

From Table 1-5, the following points can be gathered. The results of *Bifidobacteria* before 1980 showed that the number was higher in breast-fed infant. After 1980, with the development of formula milk, most studies had no clear difference between breast-fed and formula-fed infants. No study showed less *Bifidobacteria* in breast-fed infants. For *Bacteroides* and *Clostridia*, *Enterobacteriaceae* and *Enteroccocci*, nearly no study indicated more bacteria in breast-fed infants. Data from *Staphylococci* revealed more bacteria in breastfed infants or no clear difference. Most studies of *Lactobacilli* found no difference in breast-fed or bottle-fed infants, and some studies actually report less *Lactobacilli* in breast infants. However, *L. rhamnosus* was observed more common in breast-fed infant, indicating that this particular species may be favored by breast-feeding (Adlerberth and Wold, 2009; Ahrne *et al.*, 2005).

1.3 Function of HMOs and influence on intestinal microorganisms

1.3 1 Biological functions of HMOs

1.3.1.1 Prebiotics effects

Many researches have indicated that HMOs showed the prebiotic effects because the interactions between HMOs and the infants' intestinal microbiota. In 1954, György *et al.* found that the probiotics, variant of *Lactobacillus bifidus* (later named as *Bifidobacterium bifidum*), need a special growth factor from human milk (György *et al.*, 1954c). But they didn't know which components were the growth factors. The growth factor was later proved to be HMOs by the following series of studies. In recent time, more studies focused on the interactions between HMOs and growth of probiotics. In 2007, LoCascio *et al.* researched on the glycol-profiling of the specific gut bacteria *Bifidobacterium longum biovar infantis*, and found that the bacteria has preferential consumption of certain short-chain HMOs secreted in early human lactation because it can express the enzymes to cleave the intact HMOs (LoCascio *et al.*, 2007). The Prebiotic effects of HMOs are shown in Figure 1-3 (Bode, 2009).

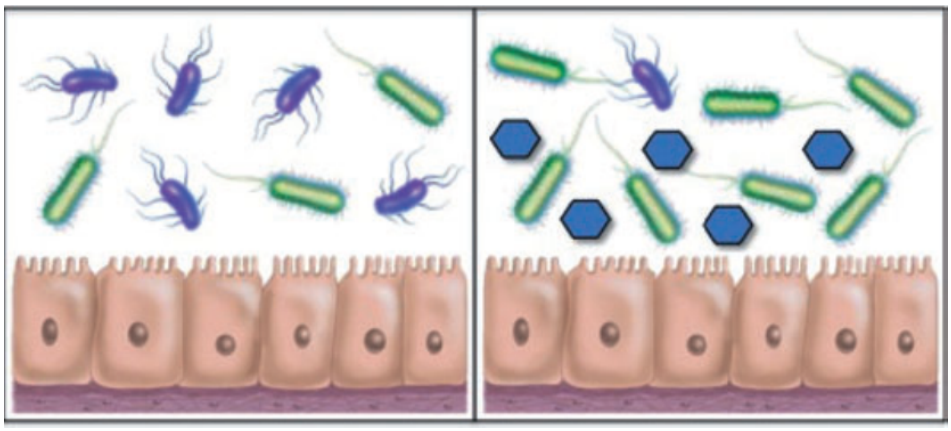


Figure 1-3 Prebiotic effects of HMOs. This simplified scheme shows that desired (green) and undesired (blue) bacteria have different capabilities of metabolizing HMOs. In the presence of HMOs (right), the desired bacteria metabolize HMOs and thrive while undesired bacteria cannot metabolize HMOs. In the absence of HMOs (left), both bacteria can grow. (According to Bode 2009)

1.3.1.2 Anti-adhesive and glycome-modifying effects

Most bacteria, both in commensals or pathogens format, can express glycan-binding proteins, which are named as lectins. Through lectins, the bacteria can bind to oligosaccharides on the host's epithelial cell surface. This ability can reveal the virulence of most bacteria, such *as Campylobacter jejuni, E. coli, Vibrio cholera* and *Salmonella* bacteria. The glycan-binding sometimes is determined by HMOs, because HMOs are structurally similar to some of the glycans binding to the intestinal epithelial cell surface. That means the HMOs can serve as soluble bacterial lectin ligand analogs, and can block the bacterial attachment. HMOs can also alter the glycan-related gene expression, modify the intestinal epithelial glycosylation machinery, therefore, the cell-surface glycome is changed and the bacteria has a barrier to attach, proliferate and colonize. Figure 1-4 shows the anti-adhesive and glycome-modifying effects of HMOs.

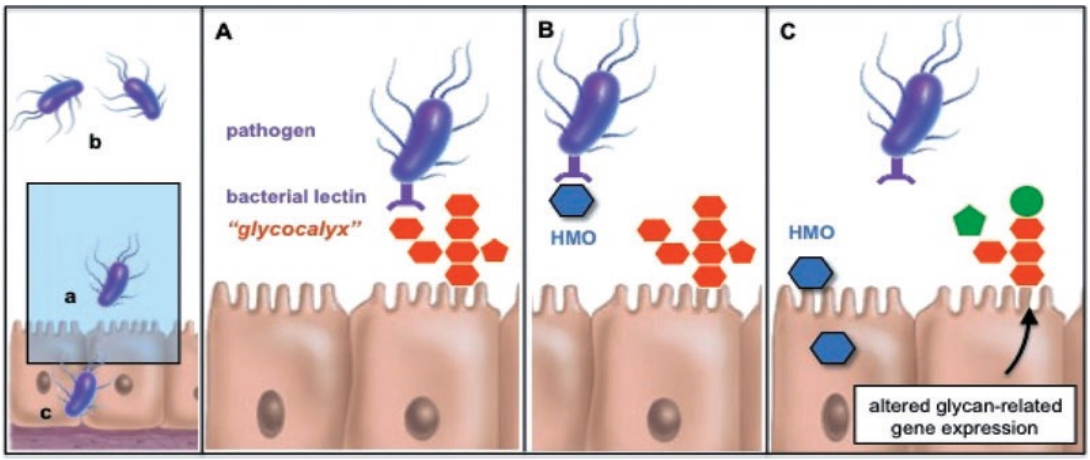


Figure 1-4 Anti-adhesive and glycome-modifying effects of HMOs. Most bacteria express glycan-binding proteins (lectins), that bind to glycans on the host's epithelial cell surface (A), which is essential for bacteria to attach (a) and to colonize the intestine (b). Some pathogens invade into the host (c). HMOs are structurally similar to glycans and can serve as bacterial lectin ligand analogs and block bacterial attachment (B). Human milk oligosaccharides (HMO) may also modify the cell-surface glycome ("glycocalyx") to impact bacterial attachment(C). (According to Bode 2009)

1.3.1.3 Systemic effects

Besides local functions, HMOs also may have some effects on systemic infectious, inflammatory and immune processes. First, in the immune system, HMO

can modify the protein-carbohydrate interactions through selectins, which have effects on the cell-cell interactions. E-selectin can adjust leukocyte deceleration (rolling) on endothelia cells, while P-selectin has effects on leukocyte and also helps the formation of platelet-neutrophil complexes (PNC). Some fucosylated and sialylated HMOs can resemble the binding determinants, like glycoconjugate ligands. The sialylated HMOs are found to have functions of reducing rolling on activated endothelial cells. Some complex HMOs can enhance the selectin-ligand binding; some can also inhibit the formation of PNC (Bode *et al.*, 2004a; Bode *et al.*, 2004b; Ley, 2003; Maaheimo *et al.*, 1995; Peters *et al.*, 1999; Rudloff *et al.*, 2002; Springer, 1994; Varki, 1997).

Based on these immunmodulatory effects, HMOs may be beneficial to breast-fed infants' immune system. For example, human milk can reduce the incidence for necrotizing enterocolitis (NEC) and other inflammatory diseases, the effective components in human milk were considered to be HMOs, but still to be proof (Bode *et al.*, 2004a; Bode *et al.*, 2004b; Maaheimo *et al.*, 1995; Rudloff *et al.*, 2002).

Second, HMOs also have some effects on immune system relating T-cells. The sialylated HMOs have influence on lymphocyte maturation and cord blood T-cells; it can increase the number of INFγ-producing CD3+CD4+ and CD3+CD8+ lymphocytes, IL13-producing CD3+CD8+ lymphocytes. In addition, some single HMO standard can also influence the peritoneal macrophages. For example, LNFP III and LNnT can inhibit naïve CD4+Tcell responses, and LNFP III itself can stimulate macrophage activity in vitro and can have benefit to the secretion of prostaglandin E2, IL-10 and TNF α (Atochina *et al.*, 2001; Atochina and Harn, 2005).

Third, HMOs may have effects on the brain glycoconjugate composition and functions. Early around 1980, researchers (Carlson and House, 1986; Witt *et al.*, 1979) studied the rat brain and found that Sialic acid and sialyllactose (SL) have an influence on brain sialic acid composition.

1.3.2 Influence on epithelial cells and intestinal microorganisms

The intestinal colonization with balanced microorganisms is important for immune system (Kau *et al.*, 2011). There is an intensive interaction between intestinal microorganisms and immune system and the diversity and stability of gut microbiota is always important for health (Lozupone *et al.*, 2012; Maynard *et al.*, 2012). It has been known for a long time that HMOs has an important role on the intestinal colonization, but the specific functions were to be investigated. For many years, many researchers studied the functions of HMOs or the specific single HMO component on the epithelial cells or intestinal microorganisms.

Early in the 1950s, György found that oligosaccharides have effects on the growth of *Bifidobacterium bifidum subsp. Pensylvancium* that was isolated from infant faeces (György *et al.*, 1954c). This discovery leaded to a topic of research on the effects of oligosaccharides to the infant gut bacteria. In the next years, with the development of technology, scientists researched on the relationship between HMOs and intestinal cells or microorganisms in many aspects by different ways: in animals, *in vitro* and in humans. Until now, this topic is still of great scientific interest. In former studies, the research factor were HMO fractions or specific HMOs, such as neutral HMOs, Sialylated HMO, fucosylated HMO, or single HMO standard, such as 2'-FL, LNT, LNnT, 3'-SL and DS-LNT. Some studies were also about the HMO related monosaccharide, such as sialic acid, Fuc, Gal, Glc and GlcNAc, these components were also important for the study or comparison of HMOs. The investigations were with gut microbiota or cell culture lines (Coppa *et al.*, 2006; Jantscher-Krenn *et al.*, 2012a; Asakuma *et al.*, 2011; Angeloni *et al.*, 2005). The microorganisms were mainly *Bifidobacteria* in several subspecies, among which *B. infantis* was the most important in the early stage of infant. *L. acidophilus*, *L. lactis*, *E. coli*, *Staphylococcus, Bacillus, Streptococcus and Bacteroides* were also the studies because they were the important bacteria in infants' gut. For cell cultures, Caco-2 cells, HT-29 cells, Cord blood T cells were used for study. These studies found that HMOs influence intestinal microorganisms through the prebiotic effects and some kinds of intestinal

microorganisms can grow with HMOs as sole carbon substrates (Hunt *et al.*, 2012; LoCascio *et al.*, 2007; Marcobal *et al.*, 2010). And the effects on intestinal cell lines were as follows: Inhibition of adhesion of undesired bacteria; reduction of proliferation; alteration of cell dynamics; induction of differentiation and influence on apoptosis, and so on (Kuntz *et al.*, 2009; Kuntz *et al.*, 2008). Table 1-6 summarized the functions studies with single HMO and HMO fractions, the studies were performed *in vitro*, animals or humans.

Table 1-6 Examples for effect studies with single HMO and HMO fractions in vitro, animals or humans (According to Kunz *et al.*, 2014, with modifications)

Effect	Factor	Investigated in/with	Reference
Prebiotic effects; influence on different *Bifdobacteria*; description of special HMO using pathways	Neutral HMO	*B. infantis;* *B. bifidum, B. breve;* *B. longum*	(Asakuma *et al.*, 2011; Garrido *et al.*, 2012; Kitaoka, 2012; Sela *et al.*, 2008; Yoshida *et al.*, 2012)
Inhibition of adhesion of *E.coli*, *Vibrio cholerae*, and *Salmonella fyris* to Caco-2 cells	HMO	Caco-2 cells	(Coppa *et al.*, 2006)
Reduction of Entamoeba attachment and cytotoxicity	HMO with terminal Gal (e.g. LNT)	HT-29 cells	(Jantscher-Krenn *et al.*, 2012a)
Increase in INFγ-, IL-13-producing T cells	Sialylated HMO	Cord blood T cells	(Eiwegger *et al.*, 2004)
Influence on rolling and adhesion of human leukocytes	Sialylated and fucosylated HMO	High umbilical vein endothelial cells	(Bode *et al.*, 2004a)
Reduction of platelet neutrophil complex formation and neutrophile activation	Sialylated and fucosylated HMO	Ex vivo model with fresh human blood	(Bode *et al.*, 2004b)
Changes in cell surface glycosylation EPEC adhesion reduced	3'-SL	Human intestinal cell lines, gene expression	(Angeloni *et al.*, 2005)
Influence on brain sialic acid content	Sialic acid, sialyllactose	Rats	(Carlson and House, 1986; Wang, 2009; Witt *et al.*, 1979)
Cure of *H. pylori* infections (50%)	3'-SL	Rhesus monkeys	(Mysore *et al.*, 1999)

Effects depend on cell lines; inhibition/ reduction of proliferation; alteration of cell dynamics; induction of differentiation and/or influence on apoptosis	Neutral/sialylated HMO fraction or single HMO	Transformed and nontransformed intestinal cell lines (HT 29, Caco 2, HIEC)	(Kuntz *et al.*, 2009; Kuntz *et al.*, 2008)
Reduction of *S. pneumoniae* and *H. influenzae* adhesion	LNnT and sialylated LNnT	Rats	(IdanpaanHeikkila *et al.*, 1997)
Microbial composition influenced DSS-induced colitis reduced	3'-SL	Sialyltransferase-deficient mice	(Fuhrer *et al.*, 2010; Weiss and Hennet, 2012)
Necrotizing enterocolitis reduced	DS-LNT	Rats	(Jantscher-Krenn *et al.*, 2012b)
HMO consumption by *Bacteroides* via mucus utilizing pathways	HMO, LNnT,	Gnotobiotic mice	(Marcobal *et al.*, 2011)
Effects on SCFA and microbial modulation	HMO, LNnT	Pigs	(Li *et al.*, 2012)
No reduction of colonization of throat and nasopharynx with *S. pneumonia* or *H. pylori*; tendency of reduced 'abnormal' ears	LNnT supplemented formula	Infants	(Prieto, 2005)
No effects in acute otitis media	LNnT	Infants (placebo- controlled trial)	(Ukkonen *et al.*, 2000)
Reduction of diarrhea (e.g. *Campylobacter* diarrhea)	Association with total fucosylated oligosaccharides and 2'-FL in milk	Infants	(Morrow *et al.*, 2004; Ruiz-Palacios *et al.*, 2003)

Association with Crohn's disease	Nonsecretor status	Genotyping in young and adult individuals	(McGovern *et al.*, 2010)
Association with mortality, gram negative sepsis and necrotizing enterocolitis	Low or nonsecretor status	Preterm infants; secretor genotyping/ phenotyping	(Morrow *et al.*, 2011)
Consumption of HMOs by gut-related microorganisms	HMO	*Enterococcus, E. coli, Streptococcus, B. infantis, Bacteroides, Clostridium, L. lactis, L. acidophilus*	(Marcobal *et al.*, 2010)
Human milk glycomics and gut microbial genomics showed a correlation between HMO and gut microbiota	HMO	*Staphylococcus, Bacillus, Streptococcus, Bacteroides Bifidobacteria, et al.*	(De Leoz *et al.*, 2015)
Metabolism of Sialic acid by microbiota	Sialic acid	*C. sakazakii*	(Joseph *et al.*, 2013)
Consumption of HMOs by infant-gut associated *Bifidobacteria*	HMOs, Fuc, Gal, Glc, GlcNAc and single HMO	*B. infantis, B. bifidum, B. breve, B. longum*	(LoCascio *et al.*, 2007) (Asakuma *et al.*, 2011)
Molecular linkages between *Bifidobacteria* and HMOs	HMOs	*B. infantis*	(Sela and Mills, 2010)
HMOs promote the growth of gut microbiota	HMOs	*Staphylococcus epidermidis Staphylococcus aureus*	(Hunt *et al.*, 2012)

2. Objective

In former studies, the major research focus was on effects of the whole HMO fractions on intestinal microorganisms. So far, the influence of HMO fractions with specific Lewis blood type or differences between neutral and acidic HMO fractions has not been investigated yet.

The overall aim of this dissertation was to investigate the effects of HMOs on the growth of intestinal microorganisms. HMO fractions were isolated from human milk samples with known specific Lewis blood type (Lewis a, Lewis b and Lewis negative). The whole HMO fractions were separated into neutral and acidic fractions. We also used five single HMO standards for the microorganisms study, such as 2'-FL, 3-FL, LNT, LNnT and 6'-SL. To evaluate the results from HMOs, mono- and di- saccharides were used for the experiments (glucose, galactose and lactose), which are also an important carbon substrate for microorganisms'. According to previous studies and the existence of intestinal microorganisms in infants' gut, we selected seven kinds of microorganisms for the study: *Lactococcus lactis subsp. lactis, Lactobacillus acidophilus, Bifidobacterium longum subsp. infantis, Bifidobacterium longum subsp. longum, Bacteroides vulgatus, Cronobacter sakazakii, Akkermansia muciniphila.* The growth conditions for the microorganisms will be optimized using glucose as the sole carbon substrate. Then the glucose will be replaced by galactose, lactose, neutral and acidic HMO fractions, or single HMO standards. The growth curves will be used to determine growth, the carbon substrates in the medium will be labeled using 2-AA and analyzed by RP-HPLC, and the metabolic products will be analyzed by HPLC (analysis for fatty acid detection).

Different methods will be applied:

- to obtain growth curves after the application of various substrates to the culture medium;
- to determine utilized carbon substrates in the medium after labeling with 2-AA followed by RP-HPLC analysis;
- to analyze different metabolic products, such as various fatty acids.

3. Materials and methods

3.1 Materials

3.1.1 General laboratory equipment

Table 3-1 List of the general laboratory equipment and accessories

Equipment	Type	Company
Autoclave		Sanoclav
Centrifuge	Universal 32R	Hettich
Conical flask		Schott Duran
Deck glass	18*18 mm	Roth
Electronic Balance	BP4100S	Sartorius
Electronic Balance	R300S	Sartorius
Electronic Balance	SPD51	SCALTEC
Filter	0.45 µm, 25 mm	Macherey Nagel
Filter paper	0.45 µm 47 mm, PC55	Schleicher&Schuell
Filter paper	0.2 µm, 47 mm, RC58	Whatman
Incubator		WTC Binder
Injector	5 ml	BIBraun
Laboratory film	4IN*125FT	Parafilm
Lyophiliser	Christ ALPHA 1-4	SciQuip
Magnetic stirrer	MR3001	Heidolph
Measuring cylinder		Hirschmann EM Techcolor
Menzel Glass		Thermo Scientific
Microscope	Axiophot 2	ZEISS
Nitrogen generator		Whatman
Pump	Aircraft Type ZS 50 ARC	Hydro-Vacuum Aircraft
Pipet		Eppendorf
Pipet		Gilson Pipetman
Pipette		Assistant
PH meter		inoLab
Pump	P-500	Pharmacia
Pump	S1.5	Siemens
Refrigerator		Bosch, Liebherr, Siemens
Refrigerator	Minus 80	Nunc
Respirator	50 BE-EM	Visama Plus BIBraun MAGV Laborbedarf
Rotary evaporator		Buechi
Screw cap	1.0 mm 45S	chm
Select Plus		Unigloves

Spectrophotometer	Mode: GENESYS 10UV	ThermoSpectronic
Speed-Vac	plus SC100A	Savant
Vapornet	VN100	Savant
Stirrer	MR3001	Heidolph
Syringe filter	0.45 µm, 4 mm, Cellulose Acetate Membrane	Nalgene
Volumetric flask		Hirschmann EM Techcolor
Vortex mixer	M10 REAX top	Heidolph

3.1.2 Equipment for Chromatography

Table 3-2 List of Chromatography and accessories used in the study

Equipment	Type	Company
Dionex System	DP ICS-5000	Thermo Fisher Scientific
Dionex Autosampler	AS-AP dual channel	Thermo Fisher Scientific
Dionex Column	Carbopac PA-1 column (4 mm×250 mm)	Thermo Fisher Scientific
Dionex Column	Carbopac PA-1 guard column (2 mm × 50 mm)	Thermo Fisher Scientific
Dionex Column	Syncronis C18 column (250 ×4.6, 10 mm guard	Thermo Fisher Scientific
HPLC		Shimadzu
HPLC Column	RESOURECE Q; 6 ml	Amersham
HPLC Column Oven	CTO-10AC	Shimadzu
HPLC detector	SPD-10A	Shimadzu
HPLC fraction collector	FRC-10A	Shimadzu
HPLC pump	LC-10AS	Shimadzu
HPLC Solvent Delivery System	S1000	Sykam
MALDI target plate	AnchorChip TM 600	Bruker Daltonics
Mass spectra	Ultitraflex I instrument	Bruker Daltonics
Sephadex G25	TM 26/10 Desalting	HiPrep
Fraction collector	Frac-920	HiPrep

3.1.3 Reagents

Table 3-3 List of general information of reagents used in the study

Reagents	Chemical formula	Company	Molecular weight	Purity
2-Aminobenzoic acid (2-AA)				
6-aza-2- thiothymine (ATT)		Sigma Aldrich		
Acetic acid, Rotipuran	$C_2H_4O_2$	Roth	60.05	100%
Acetonitrile (ACN) Chromasolv Plus	C_2H_3N	Sigma-Aldrich	41.05	99.9%
Ammonium acetate	$C_2H_7NO_2$	Fluka	77.18	98.0%
Ammonium acetate	$C_2H_7NO_2$	Roth	77.08	99%
Ammonium acetate	CH_3COONH_4	Merck	77.08	98%
Bacto Peptone		BD		
Bacto Casitone		BD		
BBL Trypticase Soy		BD		
Brain Heart Infusion Broth		Fluka		
Caseinpeptone, Tryptone		OXOID		
Calcium chloride-2-hydrate	$CaCl_2 \cdot 2H_2O$	Riedel-deHaeen	147.02	99%
D (-)-Fructose	$C_6H_{12}O_6$	Merck	180.16	
D (+)-Galactose	$C_6H_{12}O_6$	Merck	180.16	
D (+)-Glucose	$C_6H_{12}O_6$	Roth	180.16	
D (+)-Glucose	$C_6H_{12}O_6$	Sigma	180.16	
D (+)-Lactose	$C_{12}H_{22}O_{11} \cdot H_2O$	Riedel-deHaeen	360.32	
Di-Ammonium hydrogen citrate	$C_6H_{14}N_2O_7$	Merck	226.19	
Difco Agar, Granulated		BD		
Dimethyl sulphoxide (DMSO)	C_2H_6OS	Sigma	78.13	
Di- Potassium hydrogen phosphate	K_2HPO_4	Merck	174.18	
Ethanol	C_2H_6O	AppliChem	46.07	99.2%
Isomaltotriose, DP3 Supelco				
Lactose milk sugar, Monohydrate	$C_{12}H_{22}O_{11}\,H_2O$	Sigma	360.3	

L-cysteine hydrochloride Monohydrate	L-cysteine-HCl· H_2O	Sigma-Aldrich	175.63	98%
Magnesium Sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	Roth	246.48	99%
Manganese-sulfate monohydrate	$MnSO_4 \cdot H_2O$	Roth	169.02	99%
Meat Extract		Fluka		
Potassium chloride	KCl	Roth	74.56	99.5%
Potassium dihydrogen phosphate	KH_2PO_4	Roth	136.09	99%
Resazurin		Roth		
Sodium Acetate	$C_2H_3NaO_2$	Roth	82.03	
Sodium chloride	NaCl	Roth	58.44	
Sodium hydroxide	NaOH	Riedel-deHaeen	40.00	98.5%
Sodium hydrogen carbonate	$NaHCO_3$	Fluka	84.01	99.7%
Sodium citrate tribasic dehydrate	$C_6H_5NaO_7$ $2H_2O$	Sigma-Aldrich	294.10	99.0%
Sodium cyanoborohydride	CH_3BNNa	Sigma-Aldrich		95%
Sodium dihydrogen phosphate	NaH_2PO_4 $2H_2O$	Merck	156.01	98%
Sodium sulfate	Na_2SO_3	Merck	126.04	96%
Soluble Starch	$(C_6H_{10}O_5)_n$	Roth	$(162.14)_n$	
Tween 80		Merck		
Water, Rotisolv Ultra LC-MS	H_2O	Roth		
Water, Aqua ad iniectabilia	H_2O	Baxter		
Yeast Extract		Merck		

3.1.4 HMO fractions and standard

Neutral and acidic HMO fractions were isolated from human milk samples with known Lewis specific blood type modified according to Kunz *et al.* 1996 (Kunz *et al.*, 1996a).

Human milk samples were from six mothers who were at the first weeks of lactation. Among the six human milk samples, three samples were from mothers with Lewis a blood type, labeled as: LeA1, LeA2 and LeA3; two samples were from mothers

with Lewis b blood type, labeled as LeB1and LeB2; one sample was from mother with Lewis negative blood type, labeled as Le0.

HMO single standards used in the study were as follows: 2´-FL, 3-FL, LNT, LNnT, 6´-SL were from Glycom (Copenhagen, Denmark), LDFT and LNFP V were from Elicityl (Crolles, France), LNFP I, LNFP II, LNFP III, LNDFH I, LNDFH II were from Dextra laboratories (West Berkshire, United Kingdom).

3.1.5 Microorganisms

Table 3-4 General information of bacteria, including their source and medium for cultivation

Name of bacteria	Abbreviation	Bacteria No.	Medium
Lactococcus lactis subsp. lactis	*L. lactis*	DSMZ 20481	MRS medium
Lactobacillus acidophilus	*L. acidophilus*	DSMZ 20079	MRS medium
Bifidobacterium longum subsp. infantis	*B. infantis*	DSMZ 20088	*Bifidobacterium* medium
Bifidobacterium longum subsp. longum	*B. longum*	DSMZ 20219	*Bifidobacterium* medium
Bacteroides vulgatus	*B. vulgatus*	DSMZ 1447	Modified Reinforced Clostridial Broth
Cronobacter sakazakii	*C. sakazakii*	DSMZ 4485	MRS medium
Akkermansia muciniphila	*A. muciniphila*	DSMZ 22959	Modified Reinforced Clostridial Broth

3.1.6 Mediums

MRS medium (g/L):

Casein peptone (tryptic digest) 10, Meat extracts 10, Yeast extracts 5, Glucose 20, Tween80 1, K_2HPO_4 2, Na-acetate 5, $(NH_4)_2$citrate 2, $MgSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot H_2O$ 0.05.

MRS medium with L-cysteine (g/L):

Casein peptone (tryptic digest) 10, Meat extracts 10, Yeast extracts 5, Glucose 20, Tween80 1, K_2HPO_4 2, Na-acetate 5, $(NH_4)_2$citrate 2, $MgSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot H_2O$ 0.05, L-cysteine-HCl$\cdot H_2O$ 2.

Bifidobacterium medium:

Casein peptone (tryptic digest) 10.00 g, Yeast extract 5.00 g, Meat extract 5.00 g, Bacto Soytone 5.00 g, Glucose 10.00 g, K_2HPO_4 2.00 g, $MgSO_4 \cdot 7H_2O$ 0.20 g, $MnSO_4 \cdot H_2O$ 0.05 g, Tween80 1 ml, NaCl 5.00 g, L-cysteine-HCl·H_2O 0.50 g, salt solution (see below) 40.00 ml, Resazurin (25 mg/100 ml) 4.00 ml, distilled water 950.00 ml. pH 6.8.

Salt solution: $CaCl_2 \cdot 2\ H_2O$ 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.50 g, K_2HPO_4 1.00 g, KH_2PO_4 1.00 g, $NaHCO_3$ 10.00 g, NaCl 2.00 g, distilled water 1000.00 ml.

Modified Reinforced Clostridial Broth:

Peptone 10.0 g, Meat Extract 10.0 g, Yeast Extract 3.0 g, Glucose 5.0 g, NaCl 5.0 g Soluble Starch 1.0 g, L-cysteine-HCl 0.5 g, Sodium Acetate 3.0 g, Resazurin (0.025%) 4.0 ml, distilled water 1000.0 ml. pH 6.8.

3.2 Methods

3.2.1 Isolation and separation of HMO fractions

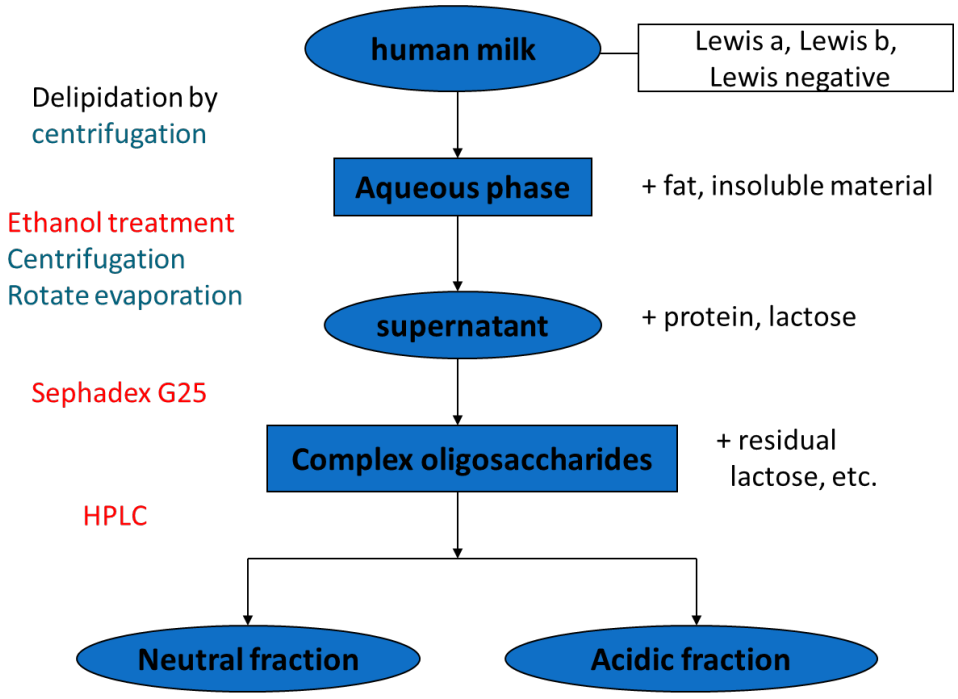


Figure 3-1 Isolation of neutral and acidic HMO fractions from human milk with known Lewis specificity (Lewis a, Lewis b and Lewis negative) (Kunz *et al.*, 1996a).

As shown in Figure 3-1, neutral and acidic HMO fractions were isolated from human milk samples step by step. Human milk samples with known Lewis specificity were obtained from the mothers who were at the first stage of their breast-feeding. The

volume for isolation was 50 ml or 100 ml. The samples were stored at -20°C before isolation. Milk samples were melted in 37°C water bath and centrifuged at 4676 g for 20 min at 4°C with centrifuge (Universal 32R, Hettich). The supernatant was filtrated by means of quartz wool and the aqueous phase was taken for the next step. The volume of the supernatant was determined. Then, double volume ethanol was added to the supernatant. Mixed sample was put on the ice overnight or at least 4 hours to remove the protein. After that, the sample was centrifuged and put on a rotate evaporation (Buechi, Essen, Germany) to remove the ethanol. With the above steps, most lipid and protein were removed. The following step of lactose removal was achieved by the Sephadex G25 gel filtration chromatography (HiPrep). The Sephadex G25 system was equipped with the column (HiPrep 26/10 Desalting) filled with Sephadex G-25-Fine, cross-linked dextran. The equipment was set up with 60 ml/h flow rate of pure water (Baxter) as solvent, with the injection volume of 5 ml. The detector was refractometer. The fractions were collected (Fraction collector, Frac-920, Amersham Biosciences, Sweden) from 1st to 60th tubes. Some fractions were analyzed by HPAEC-PAD to check the start of the fractions containing lactose. After this step, the complex HMO fractions contained neutral and acidic fractions were obtained.

To separate the neutral and acidic HMO fractions from the whole HMO fractions, Normal Phase Anion Exchange Chromatography (Shimadzu) was utilized. The HPLC was equipped with a column called RESOURCE Q with the oven temperature 25°C, 6 ml (Amersham Biosciences, Sweden) with the detector SPD-10A (wavelength: 214 nm, lamp: D2) and the pump LC-10AS. The solvent contained solvent A and B: solvent A was water and solvent B was 0.6 M NaCl. The flow of the solvent was 2.00 ml/min and the gradient was: from 0 to 7.5 min, 100% A; from 7.5 to 52 min, B was increased to 100% with set points of 55% at time 50 min; in the next 3 min, B decreased to 50% and stayed the same for 5 min; in the last 5 min, the solvent returned to 100% A. The fractions were collected by Fraction collector FRC-10A (Shimadzu), from 1st to 130th fractions (1 ml). For the neutral HMO fractions, fractions were collected from 2 to 12 min and were lyophilized directly. For the acidic HMO fractions, fractions were collected from 15 min to 65 min (end of collection) and lyophilized. Then the dried acidic fractions were diluted with water and subjected to gel filtration by Sephadex G25 chromatography to remove the salt. The fraction after desalting was also analyzed by HPAEC-PAD. The sample was lyophilized and the acidic HMO fractions were obtained.

3.2.2 Analysis of HMO fractions and HMO standards

After the isolation, neutral and acidic HMO fractions were identified by MALDI-TOF-MS to get the main components of each HMO fraction. For further analysis to quantify the HMO fractions, HPAEC-PAD was used. To analyze the HMO fractions in the microorganism cultivation study, 2-AA was used for labeling and RP-HPLC was used for analysis. The general information of these analytical methods was as following:

MALDI-TOF-MS: Matrix Assisted Laser Desorption Ionization — Time of Flight-Mass Spectrometry. Mass spectra were recorded using an Ultraflex I instrument (Bruker Daltonics, Bremen, Germany), equipped with a nitrogen laser emitting at 337 nm; a dextran hydrolysate was used to calibrate the equipment and the analytical method according to Blank *et al.* 2011. Hundred single-laser pulse spectra were summed up for each mass spectrum. For analysis, a volume of 0.5 µl of 6-aza-2-thiothymine (ATT, Sigma Aldrich, St. Louis, USA) matrix (35 mM in 25 mM aqueous ammonium bicarbonate) solution was deposited on a MALDI AnchorChipTM 600 target plate (Bruker Daltonics) and mixed with 0.5 µl of the HMO sample. The matrix-sample spots were dried under a stream of cold air. Oligosaccharides profiles were acquired in positive-ion reflectron mode over a mass range of m/z 360–3000 according to Dotz *et al.* 2014. Data acquisition and analysis were performed by flexControl and flexAnalysis 3.0 software (Bruker DaltonicsGermany), as well as GlycoWorkbench software tools, which have been developed as part of the EUROCarbDB project.

HPAEC-PAD: High-pH anion-exchange chromatography with pulsed amperometric detection. HPAEC was performed on a Dionex ICS 5000 system (Thermo Fisher Scientific GmbH, Dreieich, Germany), equipped with a Dionex Carbopac PA-1 column (4 mm × 250 mm, temperature controlled at 30°C) in combination with a Carbopac PA-1 guard column (2 mm × 50 mm). Samples (15-30 µl) were injected by means of a Dionex AS-AP dual channel autosampler. The solvents contained solvent A and B: Solvent A was 100 mM sodium hydroxide (NaOH); Solvent B was 250 mM sodium acetate in 100 mM NaOH. The solvent flow was 0.5 ml/min and the gradient was: from 0 to 15 min, 100% A; from 15 to 83 min, B was increased to 100% with set points of 20% B at time 40 min and 60% B at 70 min; after 4 min of 100% B, the solvent was returned to 100% A within 2 min, followed by an 16 min equilibration.

RP-HPLC: Reversed-phase chromatography includes any chromatographic method that uses a hydrophobic stationary phase. RP-HPLC refers to liquid chromatography (Molnár and Horváth, 1976). RP-HPLC was performed on a Dionex ICS 5000 system (Thermo Fisher Scientific GmbH, Dreieich, Germany), equipped with a Thermo Scientific Syncronis C18 column (250 ×4.6, 10 mm guard, temperature controlled at 30°C) Samples (10-25 µl) were injected by means of a Dionex AS-AP dual channel autosampler. Buffer A was 150mM Na-Citrate, with 5% acetonitrile (ACN), pH 4.5; buffer B was 50 mM NH_4-acetate, with 7.5% ACN, pH 4.0; LCMS-grade ACN was used as solvent C. The 0.5 ml/min solvent flow was isocratic (50% A, 50% B) for 75 minutes, then the solvent was gradually changed during 25 min to (40% A, 40% B, 20% C) and kept constant for 5 min. During the next 30 min initial conditions were gradually restored, followed by a 5 min equilibration (Leo *et al.*, 2009).

HPLC-Analysis for fatty acid detection: An ion exclusion chromatography was performed on a HPLC System S1000 Solvent Delivery System (Sykam, Eresing, Germany) with an oven (70°C). The column was CarboSep H Plus (7.8 mm x 300 mm, Transgenomic, Omaha, USA). Samples were filtrated by Whatman Spartan 30/20.2 RC cellulose filters (Whatmann, Dassel, Germany) and the volume of 50 µl sample was injected by antosampler (S5200, Sykam, Eresing, Germany). The detector for the system was RI 2000-Detector (Schambeck SFD GmbH, Bad Honnef, Germany) operating at 40°C. The solvent was 5 mM H_2SO_4, and the flow rate was 0.3 ml/min. In standards mixture (1 mM), the following substances were added: Citrate, Malate, Succinate, Lactate, Formate, Acetate, Propionate, Butyrate, Valerate and Carbonate.

3.2.3 Methods for preparation of mediums/buffers

3.2.3.1 MRS medium (DSMZ medium 11)

All the ingredients were dissolved one after the other in distilled water. The pH was adjusted to 6.0 to 6.5. Then the mixture was divided into 200 ml bottles. Then the media in the bottle was autoclaved at 121°C for 30 min.

For pre-cultivation of bacteria, all the ingredients were dissolved. For the growth study of different saccharides, all the ingredients were dissolved except glucose. Sterilized glucose, galactose, lactose or HMO fractions were added right before inoculation.

3.2.3.2 *Bifidobacterium* medium

All the ingredients were dissolved one after the other except L-cysteine-HCl in distilled water. Then the mixture was autoclaved at 121°C for 40min. Then the medium was cooled to 98°C within the autoclave and was then cooled down under oxygen free gas (mixture of CO_2 and N_2) to avoid redissolving of oxygen. Sterilized L-cysteine-HCl was added into the medium and the pH was adjusted to 6.8, using 8 N NaOH. Then the medium was dispensed into 50 ml bottles under the same gas phase.

3.2.3.3 Modified Reinforced Clostridial Broth

All the ingredients were dissolved one after the other except L-cysteine-HCl in distilled water. The pH was adjusted to 6.8. Then the medium was autoclaved at 121°C for 40 min. Afterwards, the medium was brought at 98°C and further cooled down under oxygen free gas (mixture of CO_2 and N_2). Finally sterilized L-cysteine-HCl was added into the medium and the medium was dispensed the mixture into 50 ml bottles under the same gas phase.

3.2.3.4 Buffer for RP-HPLC

Buffer A (1L): 150 mM Na-Citrate, with 5% ACN, pH 4.5.
Buffer B (1L): 50 mM NH_4-Acetate, with 7.5% ACN, pH 4.0.
Steps: 1. Weigh exact amount of solid chemicals: Na-Citrate: 44.11 g, NH_4-acetate: 3.85 g.

2. Melt the chemicals with about 200 ml water.
3. Filtrate the solvent with the 0.45 µm, 47 mm filter paper.
4. Add the exact volume of ACN: for buffer A: 50 ml, for buffer B: 75 ml.
5. Add some water to the solvent to about 900 ml.
6. Adjust the pH using 37% HCl to the expected pH.

3.2.4 Cultivation of intestinal microorganism

3.2.4.1 Activation

The steps for activation of the bacteria from the 'Freeze Dried' bacteria in the glass tube are as follows:

Steps: 1. Prepare the medium for the bacteria.

2. Open glass tube after outside disinfection with ethanol under sterilized condition, and take out the tube with dry bacteria inside.

3. Add 1 ml medium to the bacteria tube, mix with pipette, flush the tube with nitrogen if the bacteria is strict anaerobic.

4. Inoculate the 1 ml bacteria to the bottle with medium and cultivate in the culture room with proper temperature (37°C).

3.2.4.2 Pre-cultivation

Each microoganism was cultivated starting with 50 ml or 100 ml medium. After reaching the highest density, new inoculation was made using a reduced medium volume with steps of 50 ml, 20 ml, 5 ml, and finally 2 ml or 1 ml for growth study with HMOs. In each new step, 10% from the original bacteria culture were inoculated.

3.2.4.3 Cultivate with glucose, galactose and lactose

L. lactis and *L. acidophilus* were grown on MRS medium (without glucose) to which different carbon substrates have been added. No carbon substrate was added to analysis growth on MRS medium (without glucose) as a growth control. Glucose was added in different concentration of 1 mM, 2 mM, 5 mM, and 10 mM, galactose was studied on different concentration of 1 mM, 2 mM, 5 mM, and 10 mM and lactose was added in different concentration of 1 mM, 2 mM, 5 mM, and 10 mM. The cultivation volume was 5 ml. Each concentration was performed in triplicate.

3.2.4.4 Cultivate with HMO fractions and HMO standard

All the seven microorganisms were cultivated with no carbon substrate (growth control), 1 mM HMO fractions (neutral or acidic) or 1 mM single HMO standard. HMO fractions included: LeA1, LeA2, LeA3, LeB1, LeB2, and Le0. Each HMO fraction contained neutral and acidic fractions. Single HMO standard included: 2'-FL, 3-FL, LNT, LNnT, 6'-SL. Each carbon substrate was performed in triplicate.

3.2.4.5 Growth curves and growth rate calculation

Growth curves were measured by OD_{500nm} value using spectrophotometer. All samples (70 µl) from culture were measured in triplicate plus a blank every 1 or 2 h in the study with glucose, galactose and lactose. In HMO studies, all samples from culture were measured in triplicate plus a blank every 8 to 10 h.

3.2.4.6 Microscope

For routine investigation under the microscope (Zeiss microscope, Axiophot 2), normal carrier glass was used with the magnification 1000 fold.

3.2.4.7 Storage

For short storage, the liquid culture was stored at 4°C or the culture was frozen at -20°C. For long storage, agar dilution series were stored at 4°C after colonies have been grown.

3.2.4.8 Isolation or purification

To isolate the bacteria from contamination, or purify bacteria, the agar dilution series had been performed. The steps are as follows:

1. Wash the agar three times and prepare the agar tubes (3 ml of 3% agar in a 25 ml glass tube) before the bacteria isolation.

2. Warm the medium in the 60°C water bath; boil 7 to 8 agar tubes until melted.

3. Change stopper of the agar tubes and added 6 ml medium to each agar tube, and then put the tubes in the 45°C water bath.

4. Put one drop of liquid culture of the bacterium to the first agar tube, mix and then pour out about 1-2 ml from the first agar tube to the second tube, and so on, until the last tube.

5. Put the tube directly in the ice to cool down, and flush the tubes with anaerobic gas (nitrogen/carbon dioxide).

6. Incubate the tubes with bacteria in the culture room with 37°C, wait one or two weeks to get the single colony in the last two or three tubes.

7. Pick a single colony and inoculate either the next agar dilution series or liquid medium to get a pure bacteria.

3.2.5 2-AA labeling and analysis by RP-HPLC

3.2.5.1 Glycan labeling and 2-AA labeling

Glycans may be derivatized to introduce a chromophore or fluorophore, facilitating detection after chromatographic or electrophoretic separation.

Derivatization can also be applied to link charged or hydrophobic groups at the reducing end to enhance glycan separation and mass-spectrometric detection.

2-AA labeling: 2-Aminobenzoic acid labeling via reductive amination (Figure3-2).

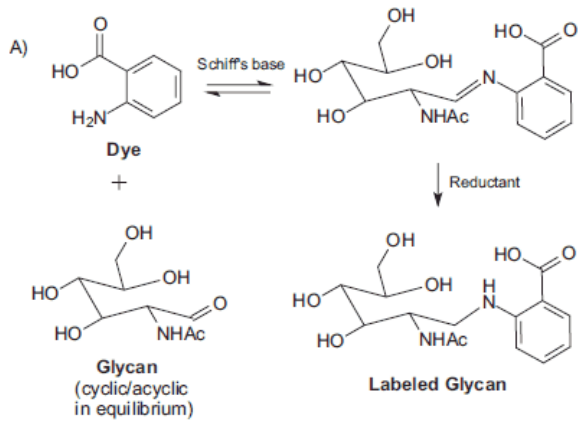


Figure 3-2 Labeling of 2-Aminobenyoic acid (2-AA) via reductive amination (Ruhaak *et al.*, 2010).

During cultivation of bacteria with HMO fractions, samples (50 µl) for the RP-HPLC were taken at 0 h and at the end (24 h, 40 h or 48 h). Filtrated the samples with sterilized filter and the samples were dealt with 2-AA labeling for the analysis.

3.2.5.2 Protocol for 2-AA labeling

This protocol was according to previous studies (Bigge *et al.,* 1995; Ruhaak *et al.*, 2008) with modifications.

A. Preparation of fresh labeling solutions, described as following:

1. Add 150 µl glacial acetic acid to 500 µl DMSO and mix well.
2. Add 100 µl of this mixture to 4.8 mg (0.35 M) 2-AA labeling dye (2-Aminobenzoic acid, Sigma A-1506) and mix until dissolved.
3. Add total dye solution to 6.3 mg (1 M) reluctant $NaCNBH_3$ (Sodium cyanoborohydride, Fluka 71435) and mix well.
4. Add 5ul of labeling reagent to each dried samples, put lid on and mix.
5. Incubate 2-3 h at 65°C, vortex every 30 min, centrifuge after each vortexing step.
6. After incubation, centrifuge shortly and let it cool down to room temperature.
7. Add 100 µl water to transfer to C18 column (C18 Sep-Pak light, Water WAT 023501.130 mg).

B. Removal of excess of labeling reagents by Sep-Pak C18 column, the steps are as following:

1. Before use of Sep-Pak C18, wash with 1 ml MeOH and 2 ml H_2O.
2. Add sample in max volume 500 µl on C18 column.
3. Wash the column with 2 ml H_2O.
4. Elute with 1 ml 50% MeOH and collect in bottle.
5. Dry with N_2 or by Speed-vac.

3.2.5.3 Analyze the substrates from cultivation by RP-HPLC.

After 2-AA labeling of samples from cultivation experiment, the samples were dried. 100 µl water was added to each bottle and 70 µl was taken for analysis by RP-HPLC. The method for RP-HPLC was described in 3.2.2.

3.2.6 Analysis of metabolic products

During cultivation of bacteria with HMO fractions, samples (70 µl) for analyze the metabolic products were taken at 0 h, 15 h and at the end (24 h, 40 h or 48 h). The samples were stored in -20°C. Before analysis by HPLC, the samples were taken out of the freezer, thawed and immediately centrifuged (11430 g, 5 min). The supernatant was put into vials for HPLC. The inject volume for the HPLC was 50 µl. Peaks were

identified based on the retention time and in comparison with the standards. Table 3-5 shows the measured areas of different concentrations of various standards. Figure 3-3 and 3-4 show the calibration of standards.

Table 3-5 Measured peak areas (means) of different concentrations of various standards

µM	200	400	600	800	1000
Glucose	36,18	73,095	109,71	148,27	190,05
Lactate	8,01	16,48	29,56	38,665	49,38
Formate	6,19	9,81	14,875	20,525	27,08
Acetate	4,01	9,355	13,3	19,515	28,15
Propionate	0	1,925	6,65	8,805	11,115
Ethanol	0	0	0	1,32	1,75

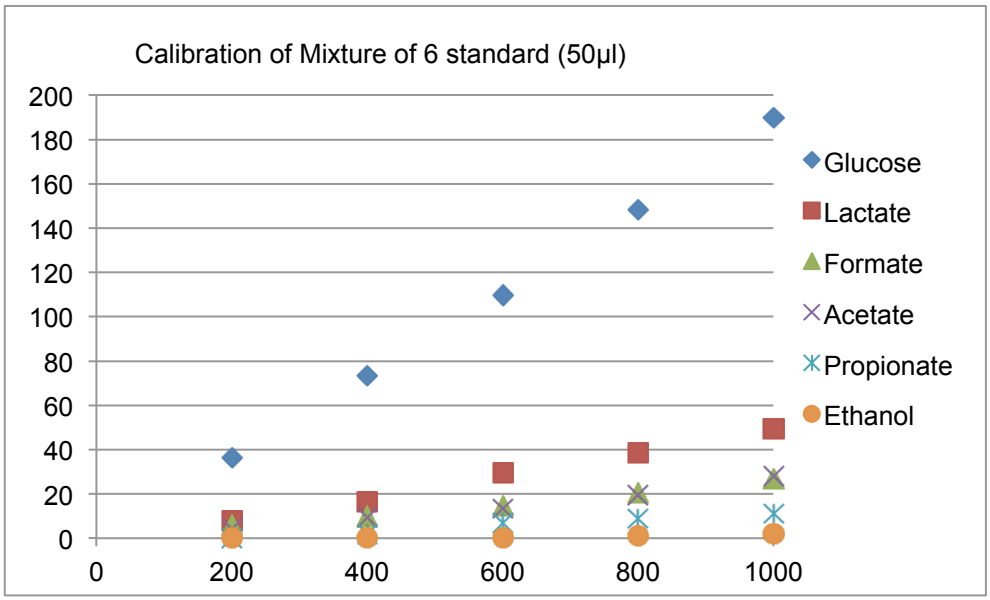


Figure 3-3 Calibration of mixture of six standard. The standards were as follows: glucose, lactate, formate, acetate, propionate and ethanol.

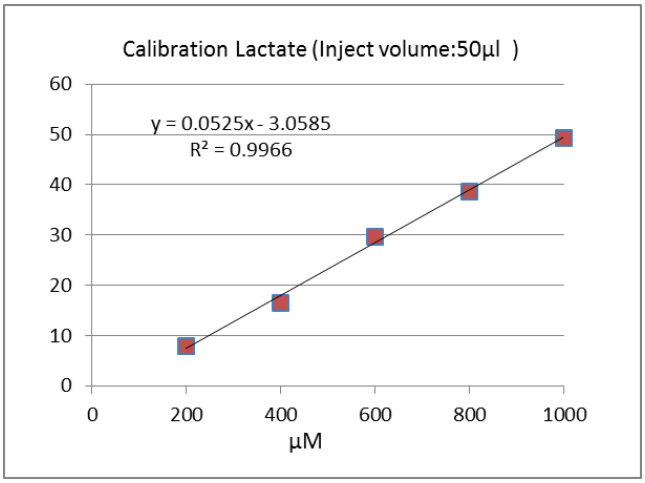


Figure 3-4 Calibration of standard lactate. The linear equation (y) and coefficient of delimitation (R^2) were shown.

4 Results

4.1 Isolation and separation of HMO fractions from human milk

4.1.1 Results from Sephadex G25 gel filtration

Human milk samples were delipidated by centrifugation and treated by ethanol to remove proteins. Then, the supernatant was subjected to Sephadex G25 gel filtration chromatography to remove the most part of lactose in the human milk samples. From the chromatogram of the milk carbohydrate fractions, the elution of lactose can be identified. Fractions with lactose were discarded. The fraction with lactose start point was taken for analysis using HPAEC-PAD to check the amount of lactose in the fraction. Figure 4-1 shows the HPAEC-PAD chromatogram of the specific fraction from Sephadex G25 gel filtration. As can be seen in Figure 4-1, this fraction contains little lactose (retention time would be 20.7 min).

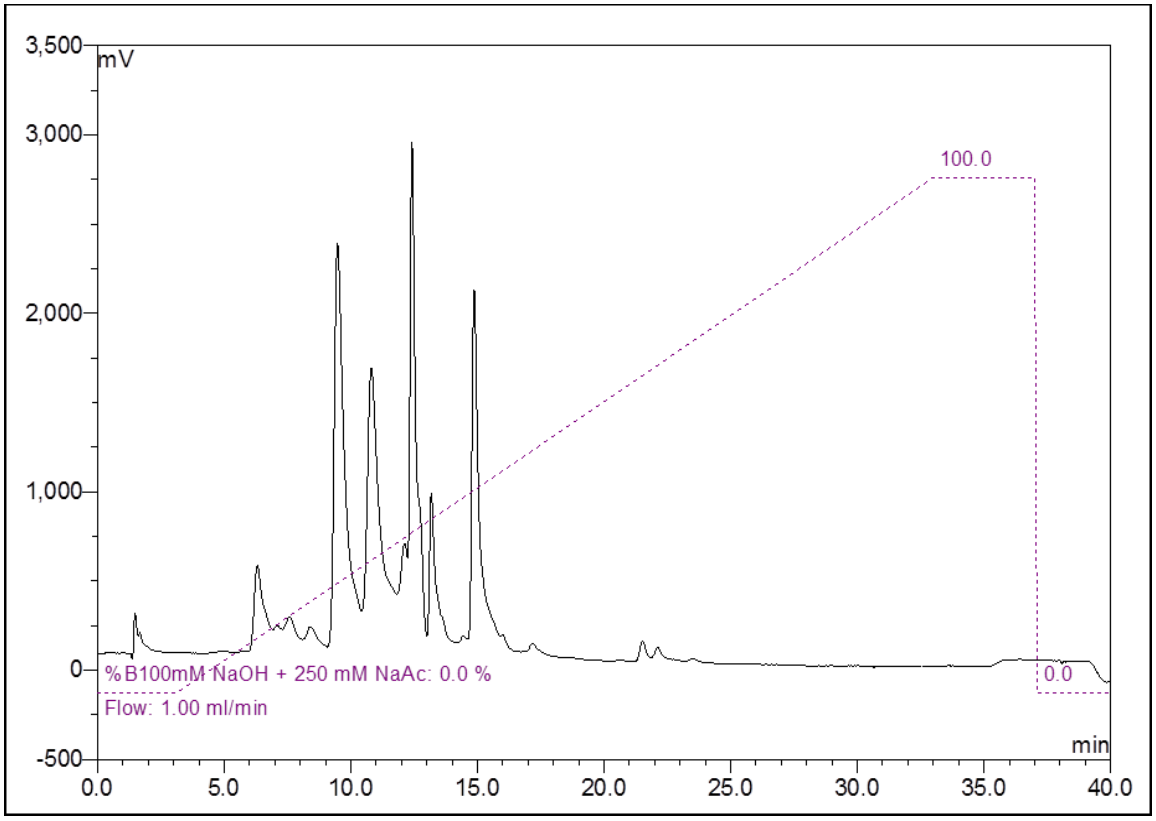


Figure 4-1 HPAEC-PAD chromatogram of a Sephadex G25 fraction eluting from the column immediately before the lactose peak. The flow rate was 1.00 ml/min; the eluent was 100 mM NaOH plus 250 mM NaAc.

4.1.2 Separation of complex HMO fractions into neutral and acidic fractions using Normal Phase Anion Exchange Chromatography

After Sephadex G25 gel filtration chromatography, most part of lactose was discarded and the complex HMO fractions were obtained. To separate neutral and acidic HMO fractions, the lactose-free carbohydrate fraction was subjected to Normal Phase Anion Exchange Chromatography according to Kunz *et al.* 1996. During the separation, the neutral HMO fractions were eluted within 12 min and the acidic HMO fractions from 12 to 60 min using 250 mM NaAc in buffer B.

4.1.3 Desalting of acidic HMO fractions

After Normal Phase Anion Exchange Chromatography, the neutral and acidic HMO fractions (the latter containing large amounts of salt) were collected. For the neutral HMO fractions, lyophilization was used to dry the samples. The acidic fractions with salt were subjected to Sephadex G25 gel filtration chromatography again to remove the salt. From the chromatography, the peak of salt was acquired and the fraction immediately before salt peak was taken for analysis using HPAEC-PAD to check the amount of salt in the fraction. Figure 4-2 shows the HPAEC-PAD chromatogram of the specific fraction from Sephadex G25 gel filtration for desalting.

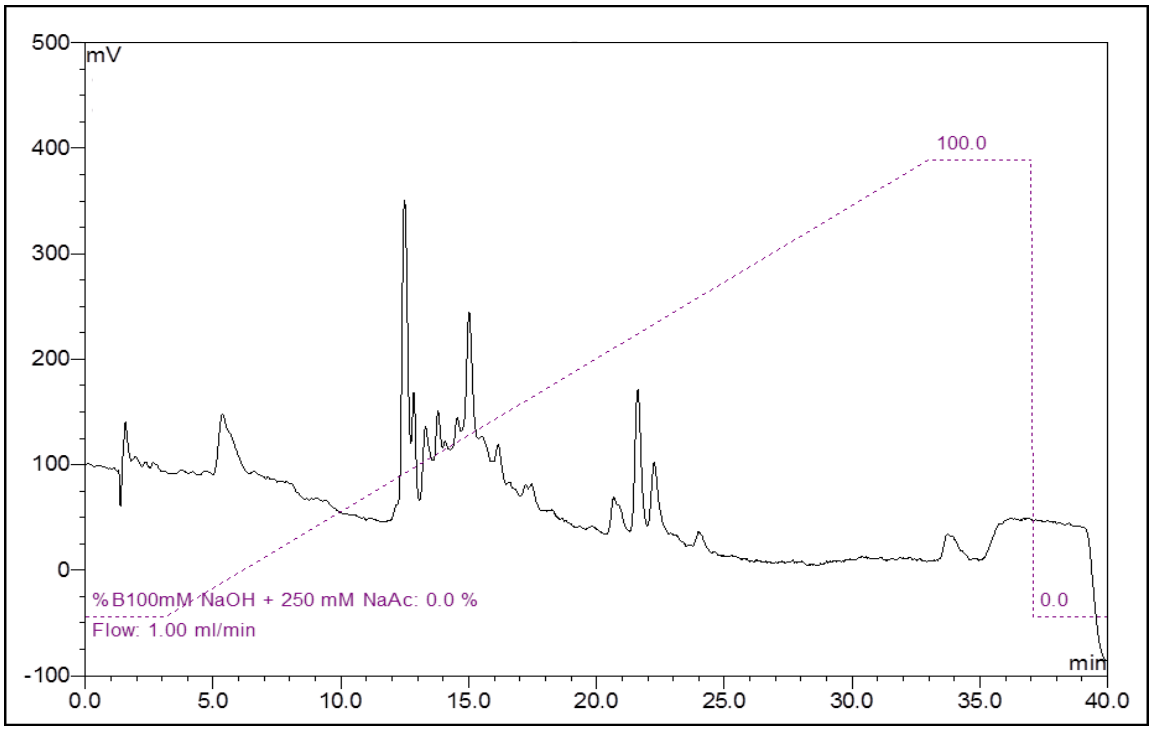


Figure 4-2 HPAEC-PAD chromatogram of a Sephadex G25 fraction eluting from the column immediately before the salt peak. The flow rate was 1.00 ml/min; the eluent was 100 mM NaOH plus 250 mM NaAc.

4.1.4 Neutral and acidic HMO fractions

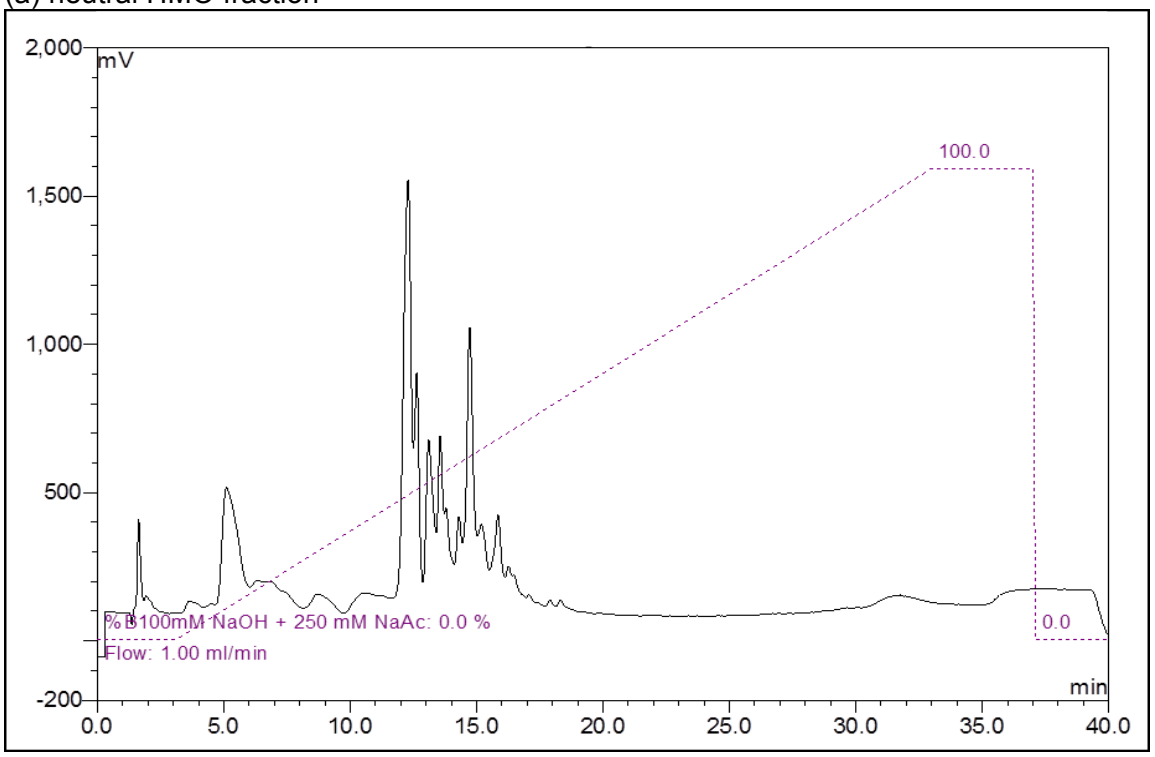
After desalting by Sephadex G25 gel filtration, the acidic HMO fractions were obtained. Lyophilization was used to dry the samples and the samples were weighed separately. The amounts of each fraction are shown in Table 4-1. The neutral and acidic HMO fractions were analyzed using HPAEC-PAD, the chromatograms are shown in Figure 4-3. Then, the samples were dissolved with pure water with final concentration of 10 mg/ml for analysis and for growth studies with microorganisms.

Table 4-1 Amount of neutral and acidic HMO fractions isolated from human milk samples

Human milk sample	Lewis Specificity	Volume of milk sample (ml)	Amount of neutral HMO fractions (mg)	Amount of acidic HMO fractions (mg)
LeA1	Lewis a	50	80	70
LeA2	Lewis a	100	274	290
LeA3	Lewis a	100	170	110
LeB1	Lewis b	100	230	210
LeB2	Lewis b	50	110	100
Le0	Lewis 0	100	230	160

As shown in the Table 4-1, there are in total seven human milk samples in which three samples are from the mothers with Lewis A blood specificity, two samples are from mothers with Lewis B blood specificity and one sample was from the mother with Lewis negative blood specificity. The volume of the human milk samples and the amount of neutral and acidic HMO fractions isolated from the human milk samples are listed. The starting volume of the milk samples was 50 ml or 100 ml. The amount of neutral HMO fractions arranged from 80 mg to 274 mg, and the amount of acidic HMO fractions were from 70 mg to 290 mg.

(a) neutral HMO fraction



(b) acidic HMO fraction

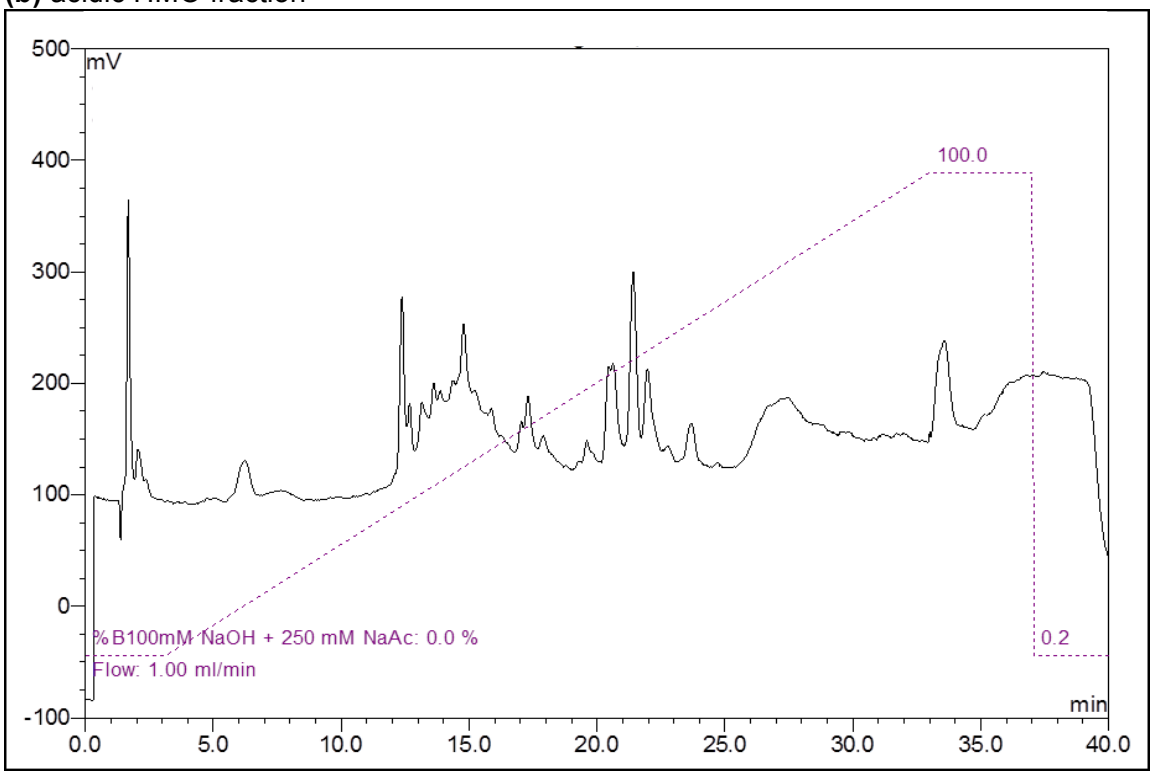


Figure 4-3 HPAEC-PAD chromatogram of (a) a neutral HMO fraction and (b) an acidic HMO fraction.

4.2 MALDI-TOF-MS of HMO fractions

All the neutral HMO fractions as well as neutral single HMO standards were analyzed by MALDI-TOF-MS. For further analysis, the samples were chemically modified with 2-Aminobenzoic acid (2-AA) after the growth study with various microorganisms. To check this labeling method as well as to compare the samples before and after labeling, the MALDI-TOF-MS was also utilized. The profiles of oligosaccharides were acquired in positive – ion reflectron mode over a mass range of m/z 360-3000. Table 4-2 lists the range of m/z and the correspondent chemical composition. Figure 4-4 shows the MALDI-TOF-MS spectra of HMO fractions before or after 2-AA labeling.

Table 4-2 The range of m/z and the correspondent chemical composition of the detected HMO

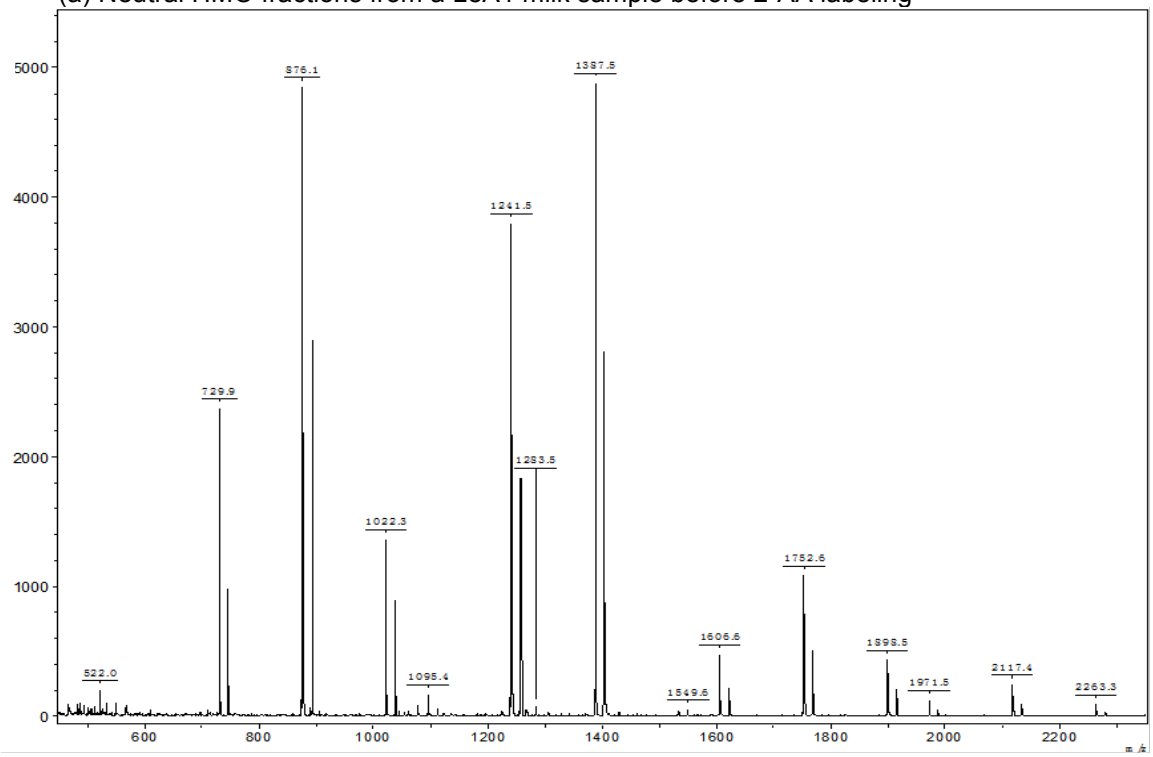
m/z [M+Na]+	Chemical composition	m/z [M+Na]+	Chemical composition
511	2´-FL Hex2Fuc1	**1387**	dFLNH Hex4HexNAc2Fuc2
657	dFL Hex2Fuc2	**1533**	tFLNH Hex4HexNAc2Fuc3
730	LNT/LNnT Hex3HexNAc1	**1606**	Hex5HexNAc3Fuc1
876	LNFP Hex3HexNAc1Fuc1	**1752**	Hex5HexNAc3Fuc2
1022	dFLNT Hex3HexNAc1Fuc2	**1825**	Hex6HexNAc4
1095	LNH Hex4HexNAc2	**1898**	Hex5HexNAc3Fuc3
1241	FLNH Hex4HexNAc2Fuc1		

From Table 4-2, the m/z values of the detected components are described. The main components have the m/z range from 511 to 1898. From the m/z, the chemical composition can be acquired. The Hex-term means Hexose-unit, Fuc represent Fucose-unit, HexNAc means N-Acetylglucosamin-unit. 2'-FL is also Hex2Fuc1 because

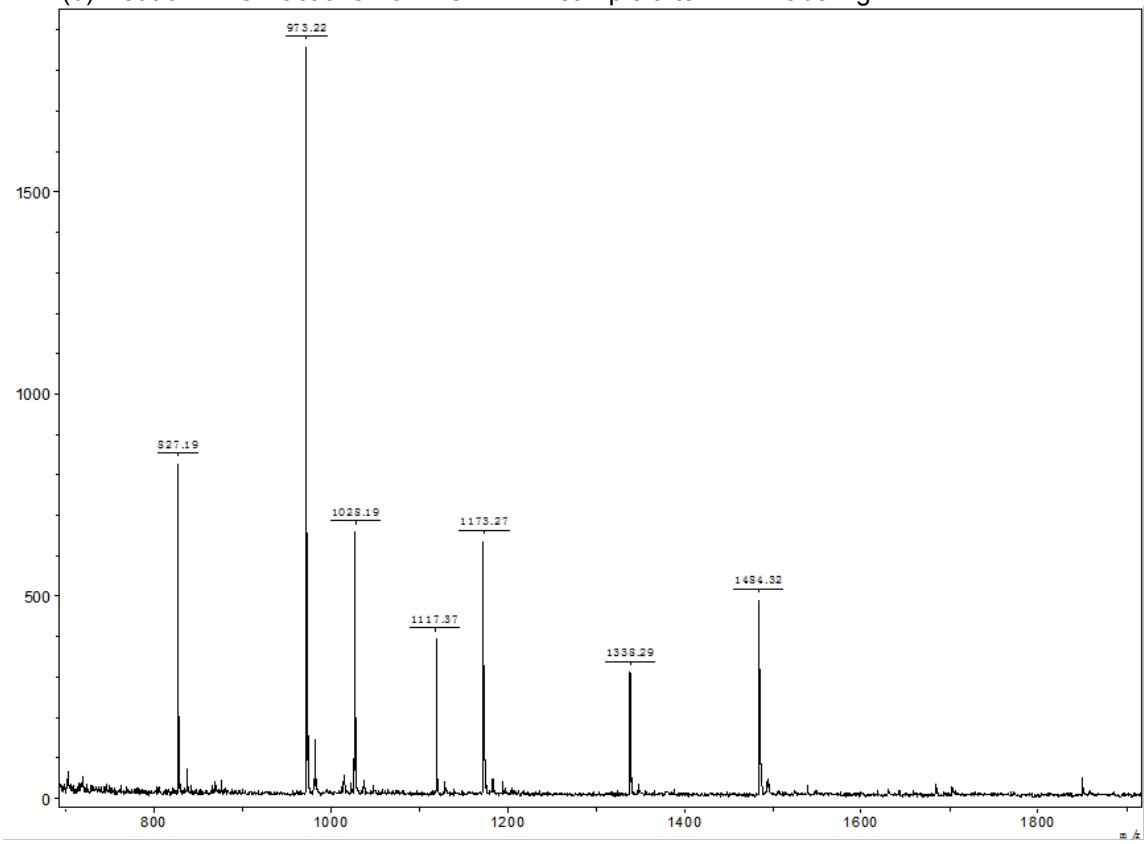
it constitutes of two Hexose-units and one Fucose-unit. LNT and LNnT are also Hex3HexNAc because they built with three Hexose-units and one N-Acetylglucosamin -unit. LNFP is also Hex3HexNAc1Fuc1 because it constitutes of three Hexose-units, one N-Acetylglucosamin-unit and one Fucose-unit.

Due to 2-AA labeling the m/z-values differ from unlabeled samples, the spectra after 2-AA labeling showed the m/z value that is 97 higher than the original m/z value in the table. For example, the m/z of LNT/LNnT is 730 before labeling, and after 2-AA labeling, the value increase to 827.

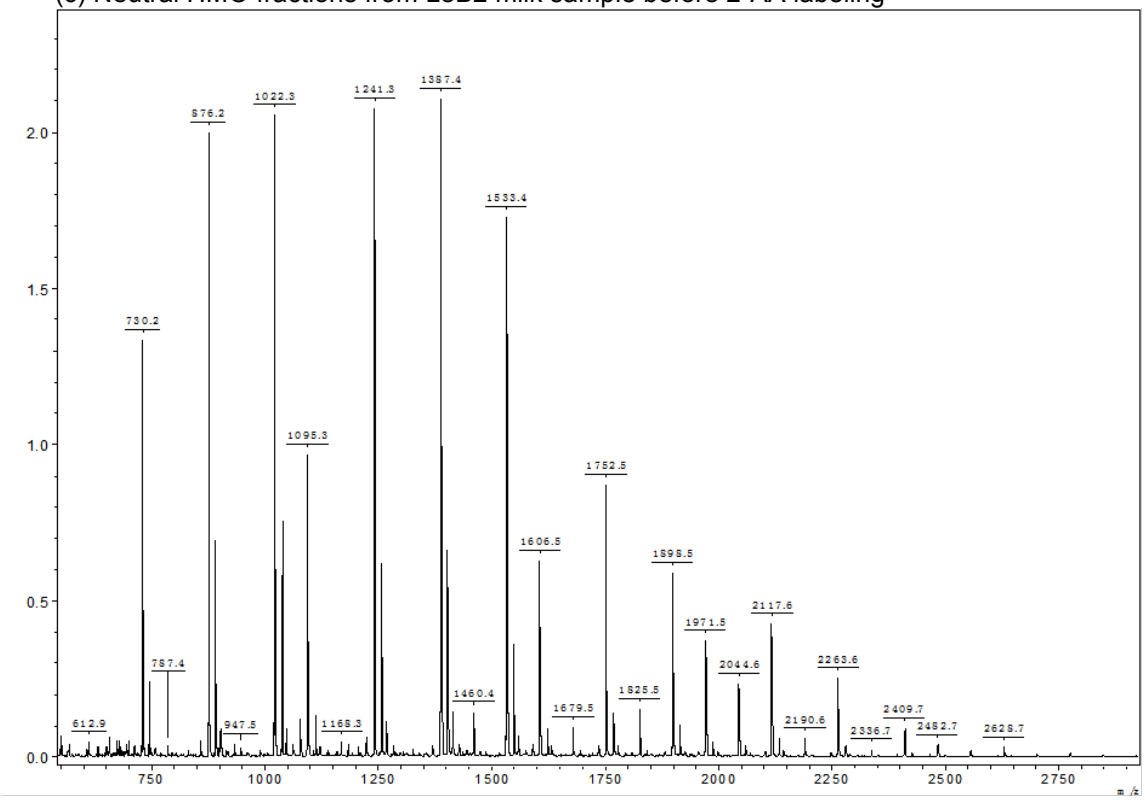
(a) Neutral HMO fractions from a LeA1 milk sample before 2-AA labeling



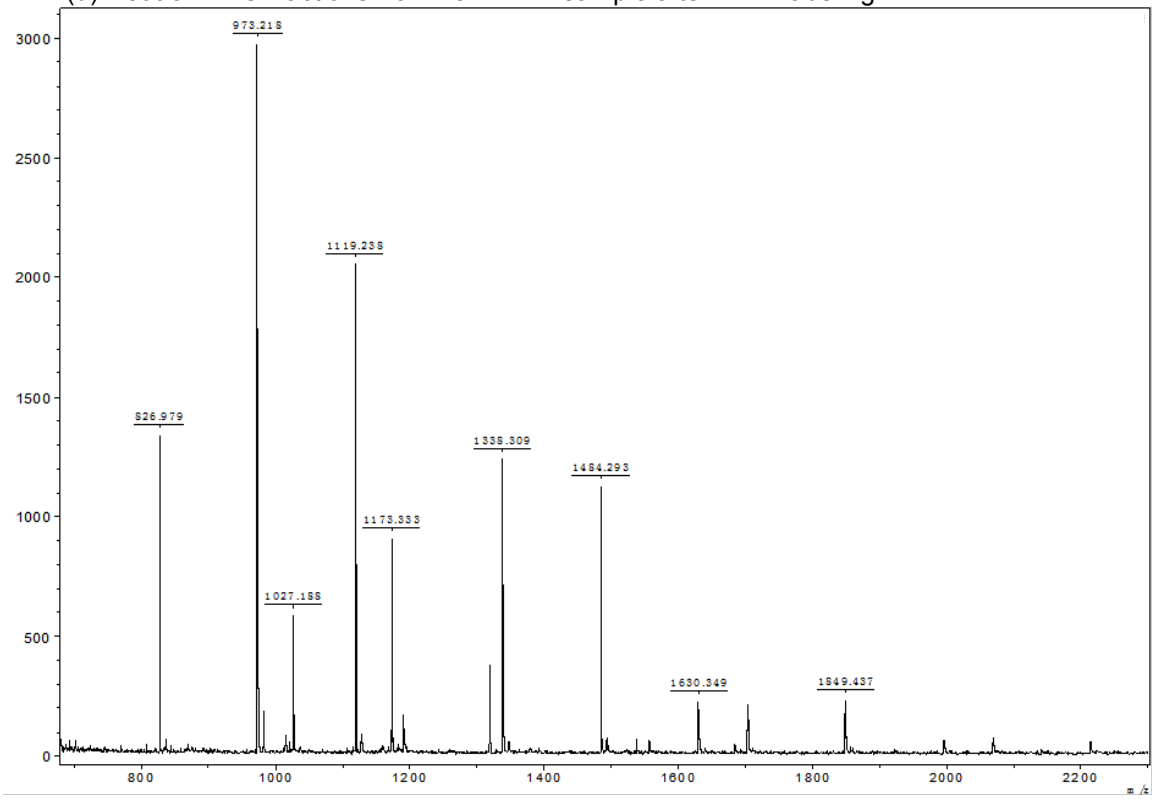
(b) Neutral HMO fractions from LeA1 milk sample after 2-AA labeling



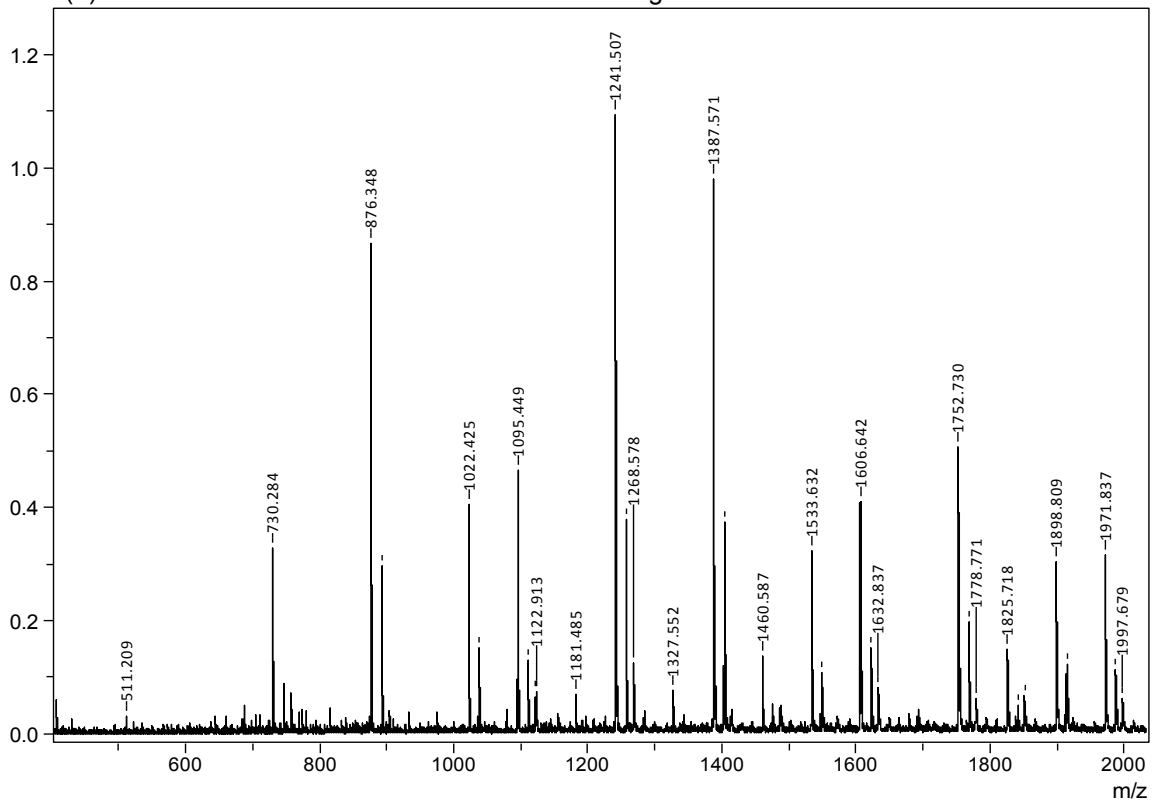
(c) Neutral HMO fractions from LeB2 milk sample before 2-AA labeling



(d) Neutral HMO fractions from LeB2 milk sample after 2-AA labeling



(e) Neutral HMO fractions from Le0 before labeling



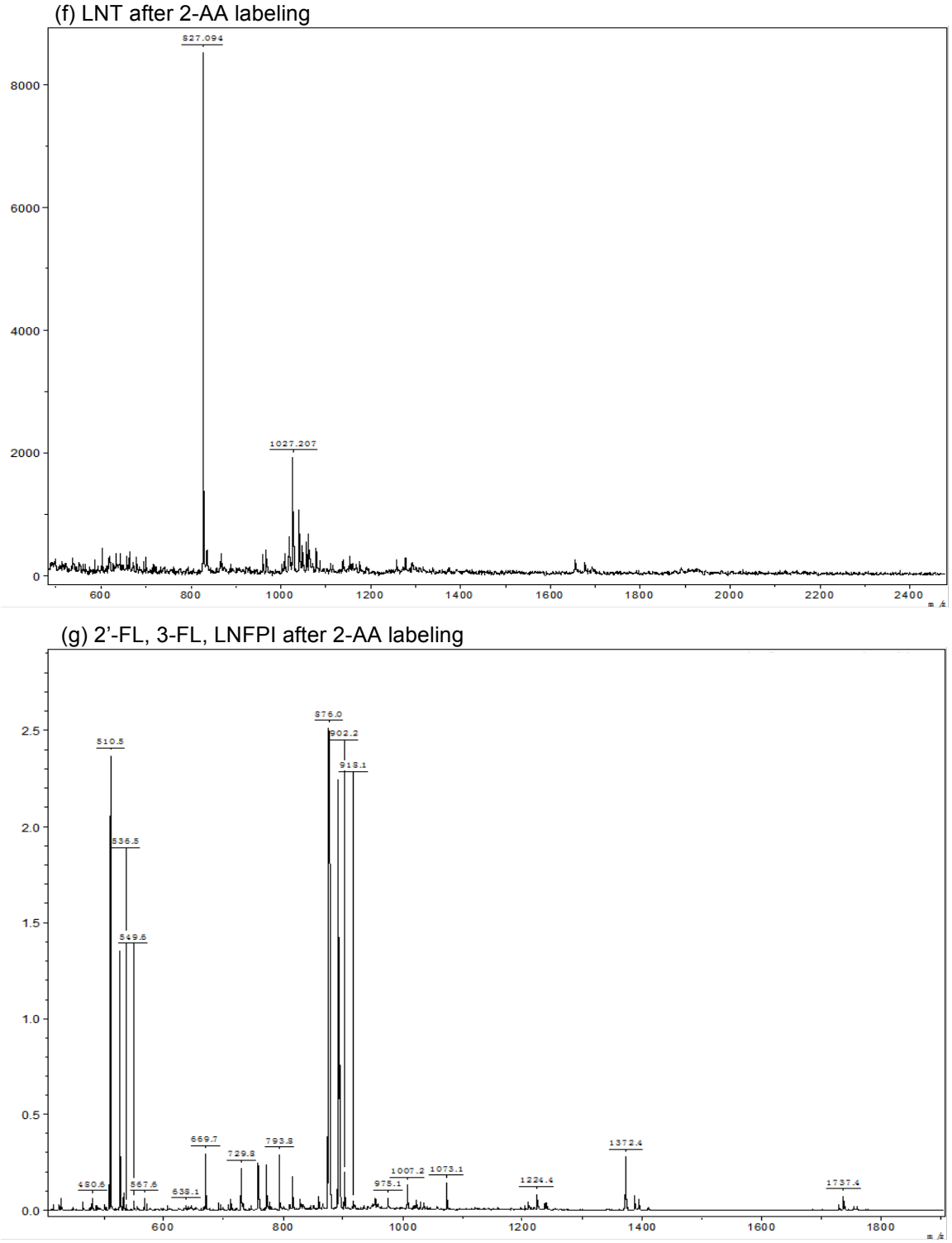


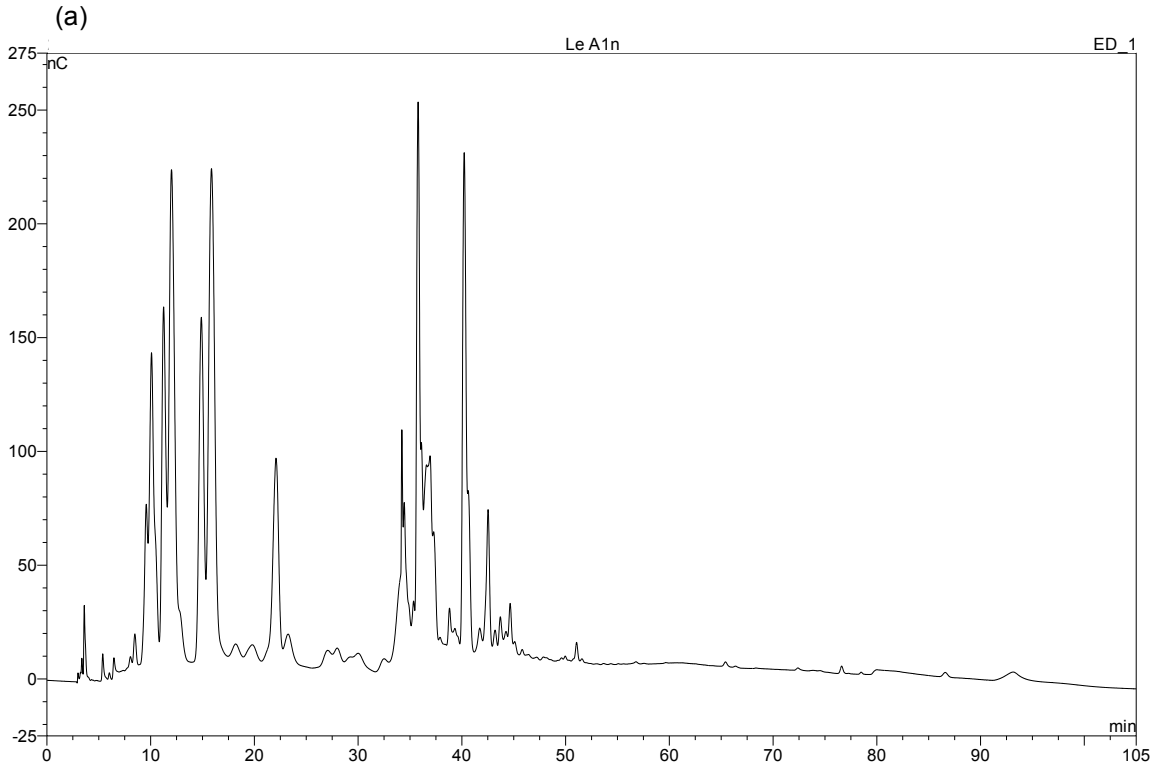
Figure 4-4 MALDI-TOF-MS spectra of HMO fractions before or after 2-AA labeling. (a) Neutral HMO fractions from a milk sample LeA1 before 2-AA labeling. (b) Neutral HMO fractions from milk sample LeA1. (c) Neutral HMO fractions from LeB2 milk sample. (d) Neutral HMO fractions from LeB2 milk sample. (e) Neutral HMO fractions from Le0. (f) Single HMO standards of LNT (g) and 2'-FL, 3-FL, LNFPI.

4.3 Analysis of HMO fractions by HPAEC-PAD

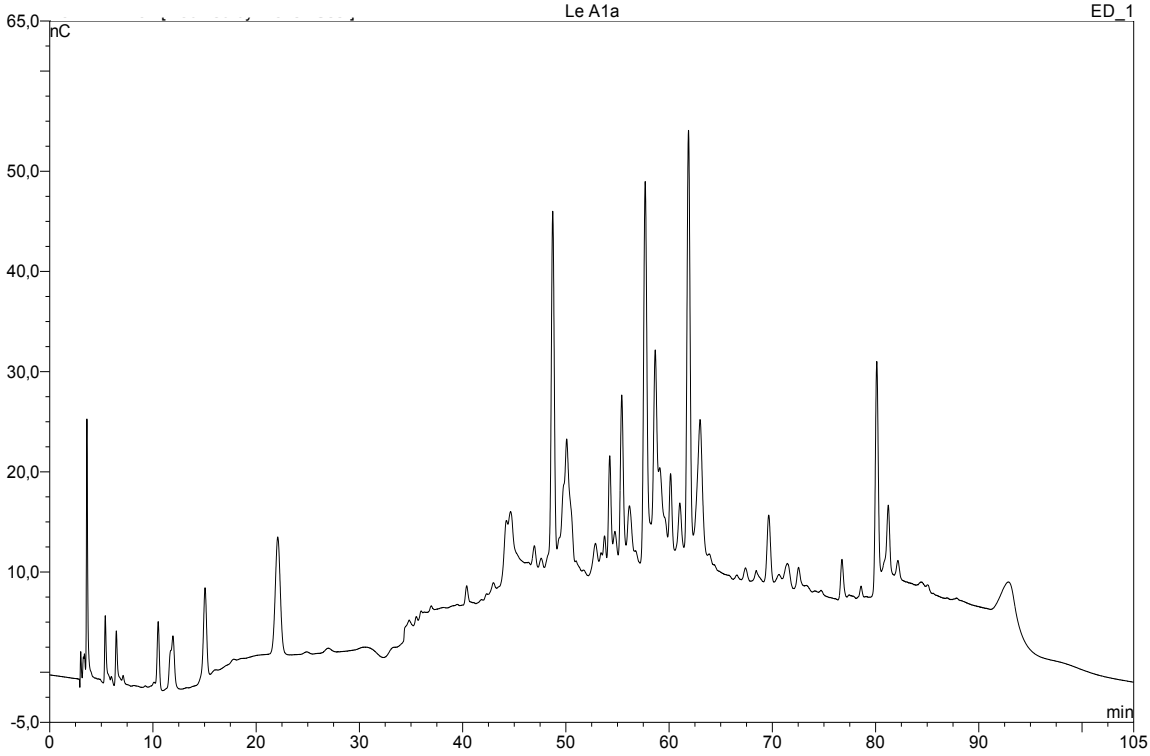
After the analysis by MALDI-TOF-MS, all the neutral and acidic HMO fractions were analyzed by HPAEC-PAD. Table 4-3 shows the retention time of single HMO standard. Figure 4-5 shows the chromatograms from three neutral HMO fractions and one acidic HMO fraction.

Table 4-3 Retention time of single HMO standard for HPAEC-PAD

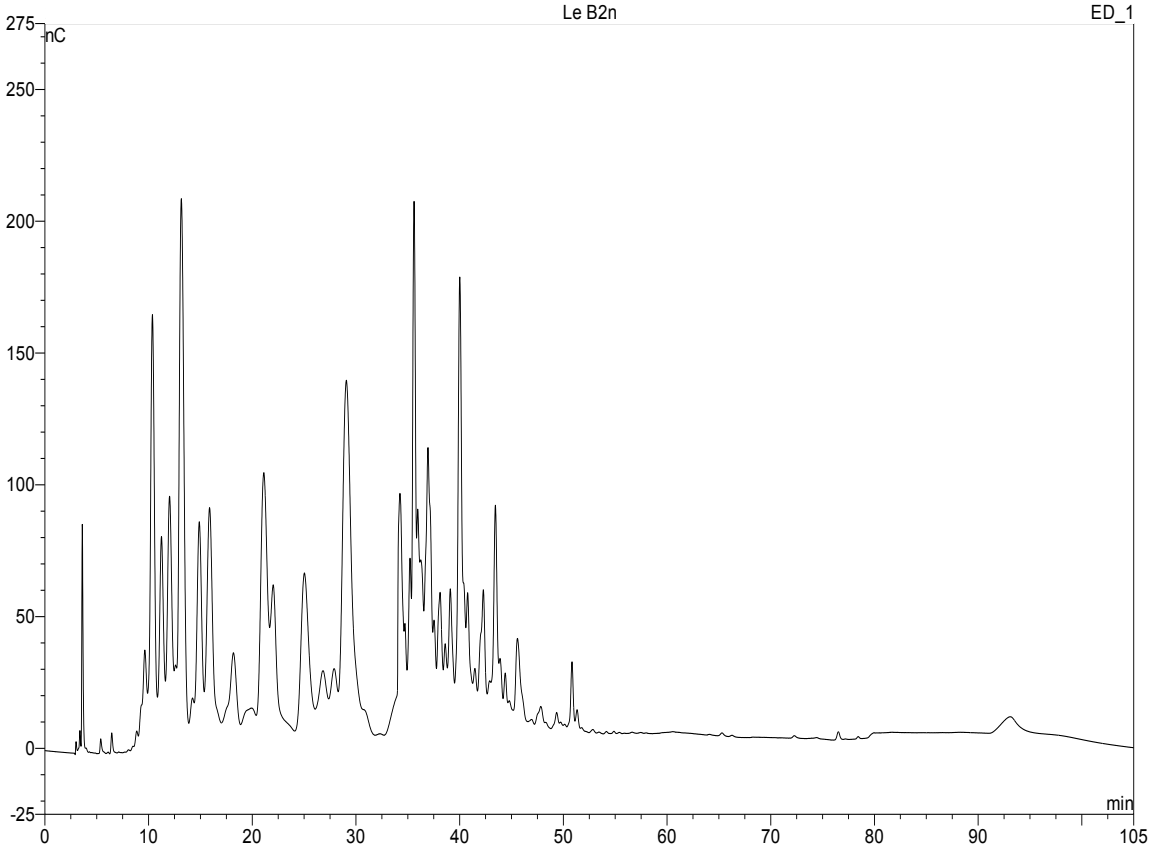
HMO	Retention time (min)	HMO	Retention time (min)
LNDFH II	10	**LNFP I**	31,1
LNDFH I	13,9	**LNT**	41,2
3-FL	14,2	**Sial-acid**	45,3
LNFP III	16	**LST c**	57,0
LNFP II	16,6	**6'-SL**	58,0
LDFT	18,5	**3'-SL**	59,2
Lactose	20,7	**LST a**	63
LNnT	34,9	**LST b**	65
LNFP V	25	**DS-LNT**	81
2'-FL	26,4		



(b)


Le A1a
ED_1
nC
65,0
50,0
40,0
30,0
20,0
10,0
-5,0
min
0
10
20
30
40
50
60
70
80
90
105

(c)


Le B2n
ED_1
nC
275
250
200
150
100
50
0
-25
min
0
10
20
30
40
50
60
70
80
90
105

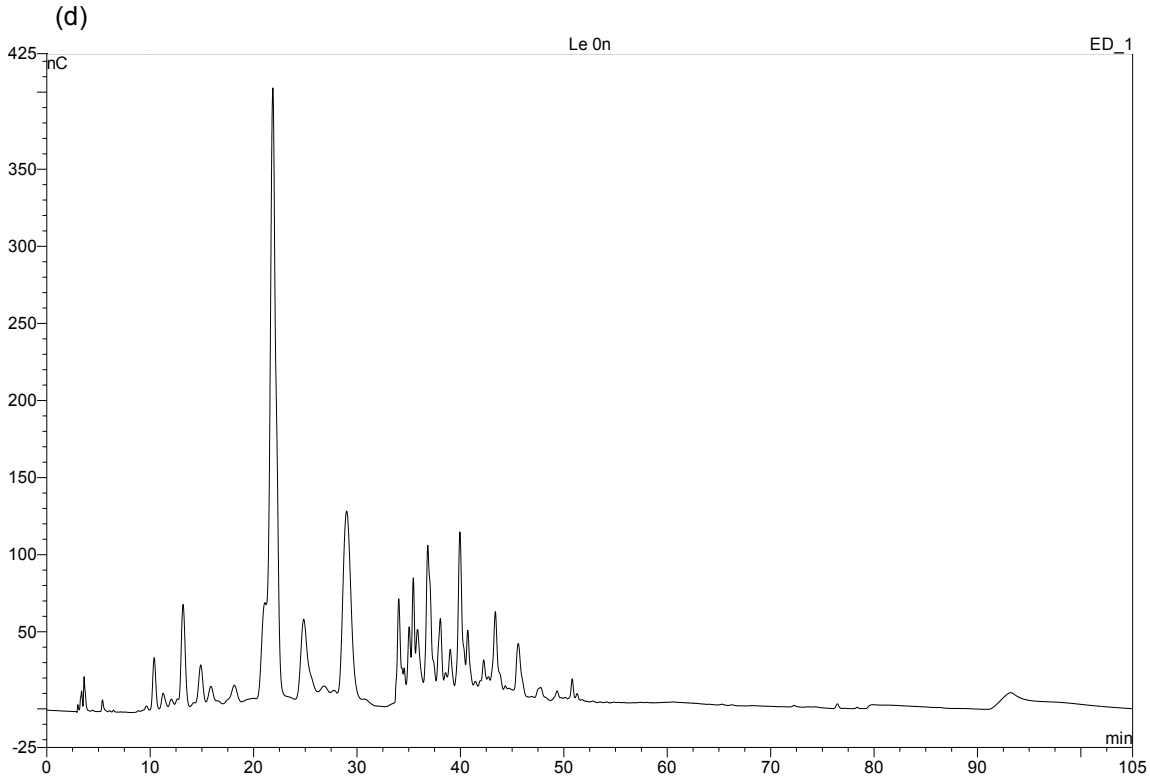


Figure 4-5 Chromatograms of the HMO fractions from samples by HPAEC-PAD. (a) neutral HMO fraction from milk sample LeA1. (b) acidic HMO fraction from milk sample LeA1. (c) neutral HMO fractions from milk sample LeB2. (d) neutral HMO fractions from milk sample Le0.

4.4 Growth study of microorganisms on glucose, galactose and lactose

To investigate the effect of different concentrations of glucose, galactose and lactose on the microorganisms, *Lactococcus lactis subsp. lactis* and *Lactobacillus acidophilus* were studied. The medium for *Lactococcus lactis subsp. lactis* was MRS medium (without glucose), and different concentrations of glucose, galactose or lactose were added later. The medium for *Lactobacillus acidophilus* was MRS medium (without glucose) with L-cysteine, and different concentrations of glucose, galactose or lactose were added later.

Figure 4-6 shows the growth curves of *L. acidophilus* at various concentrations (0 mM, 1 mM, and 2 mM) of glucose, galactose or lactose. Figure 4-7 shows the growth curves of *L. lactis* at various concentrations (0 mM, 1 mM, 2 mM and 5 mM) of glucose, galactose or lactose.

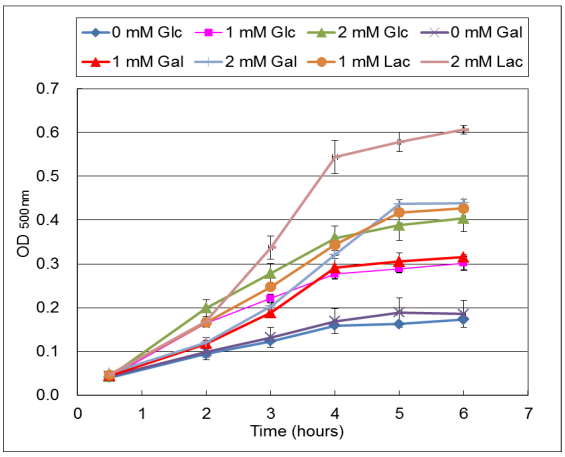


Figure 4-6 Growth curves of *L. acidophilus* grown in MRS medium (without glucose) on various concentrations of glucose, galactose or lactose

The growth curves showed that the OD_{500nm} of *L. acidophilus* could reach about 0.2 without any carbon substrate added. When 1 mM glucose or galactose added, the OD_{500nm} increased to 0.3. When the concentration increased to 2 mM, the OD_{500nm} continued to increase to 0.4. When 1 mM lactose added, the OD_{500nm} was similar to 2 mM glucose or galactose added. When the concentration of lactose increased to 2 mM, the OD_{500nm} could reach as high as 0.6. The growth curves showed that *L. acidophilus* could grow on glucose, galactose and lactose.

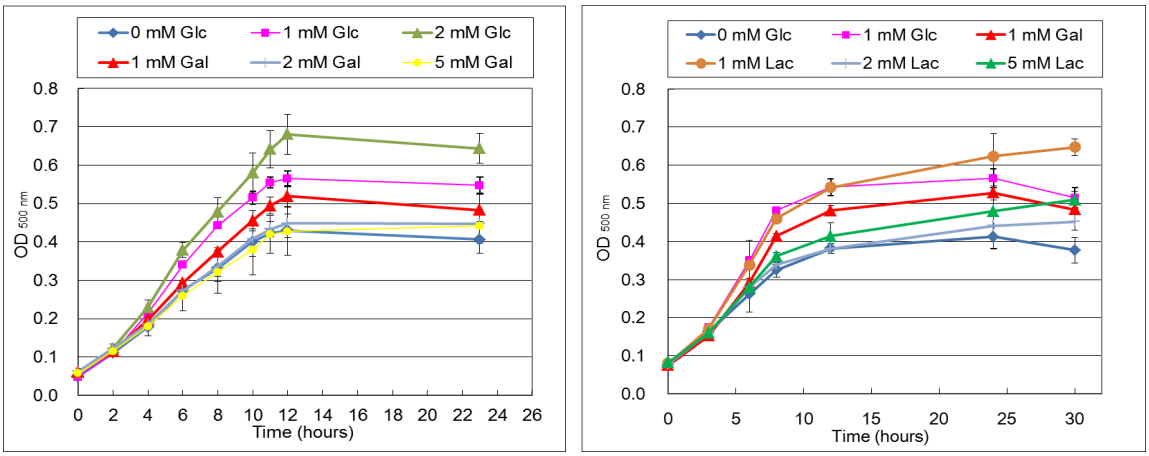


Figure 4-7 Growth curves of *L. lactis* grown in MRS medium (without glucose) on various concentrations of glucose, galactose (left) or lactose (right).

The growth curves showed that the OD_{500nm} of *L. lactis* could reach about 0.4 without any carbon substrate added. When 1 mM glucose or galactose added, the OD_{500nm} increased to 0.5 to 0.6. When the concentration increased to 2 mM, the OD_{500nm} of glucose continued to increase to about 0.7, while the OD_{500nm} of galactose decreased

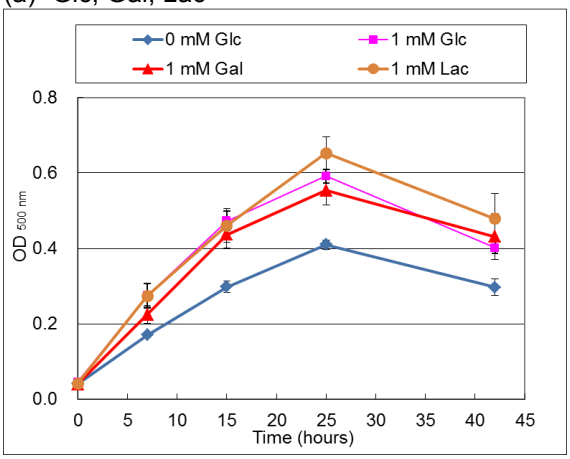
back to 0.4. When the concentration of galactose increased to 5 mM, the OD_{500nm} stayed the same as 2 mM. When 1 mM lactose added, the OD_{500nm} could reach about 0.65. When the concentration increased to 2 mM, the OD_{500nm} decreased back to about 0.45. When increased to 5 mM, the OD_{500nm} increased to 0.5, but still lower than with 1 mM glucose, galactose or lactose. The results showed that *L. lactis* could grow on glucose, low concentration of galactose or lactose.

4.5 Growth study of microorganisms on HMO fractions and HMO standard

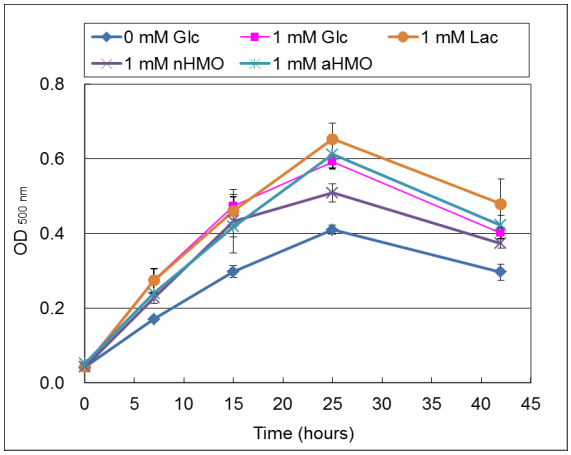
4.5.1 *Lactococcus lactis subsp. lactis*

To investigate the effect of HMOs on the growth of *Lactococcus lactis subsp. lactis*, 1 mM HMOs (neutral and acidic HMOs, as well as five single HMO standards) were used as sole carbon substrate in MRS medium, replacing glucose. Other carbon substrates, including glucose, galactose and lactose with the same concentration were also analyzed for comparison. Medium without carbon substrate was used as control. Figure 4-8 shows the growth curves of *L. lactis* on different carbon substrates.

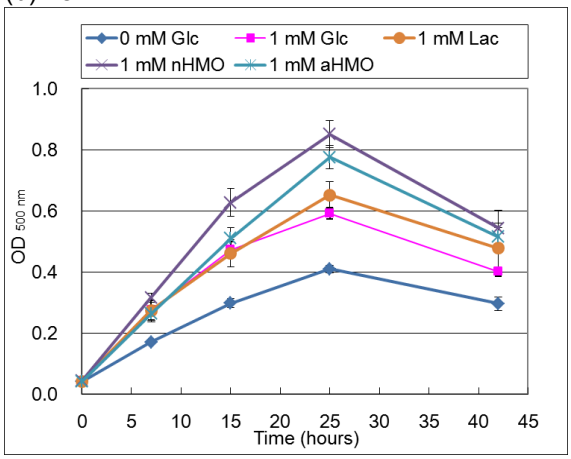
(a) Glc, Gal, Lac



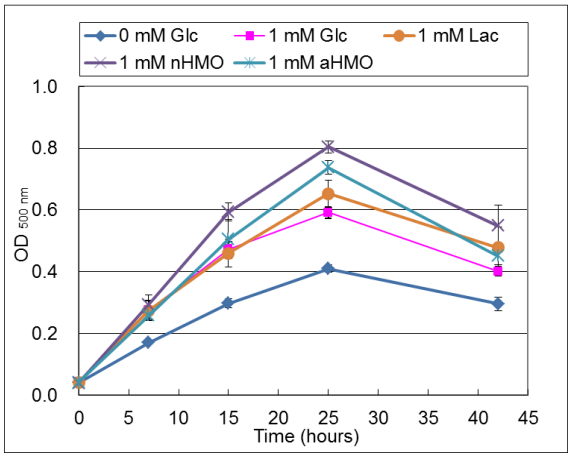
LeA2



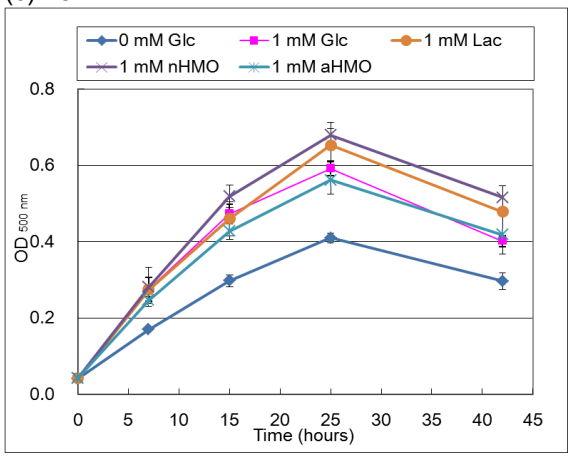
(b) LeA1



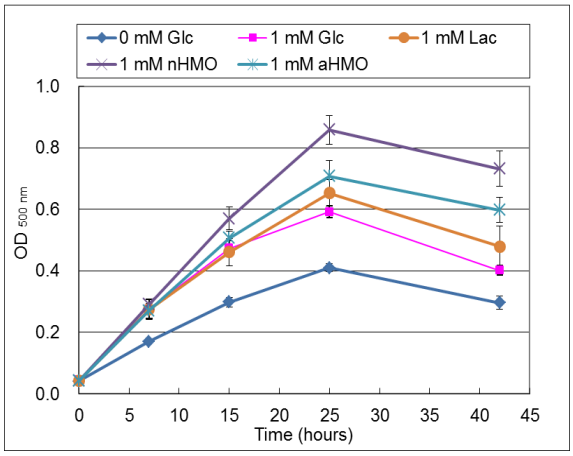
LeA3



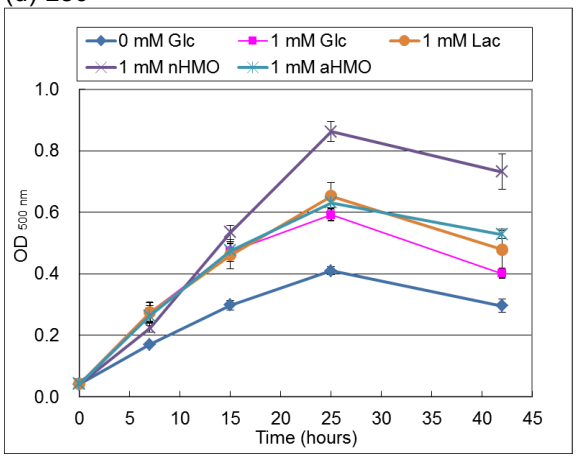
(c) LeB1



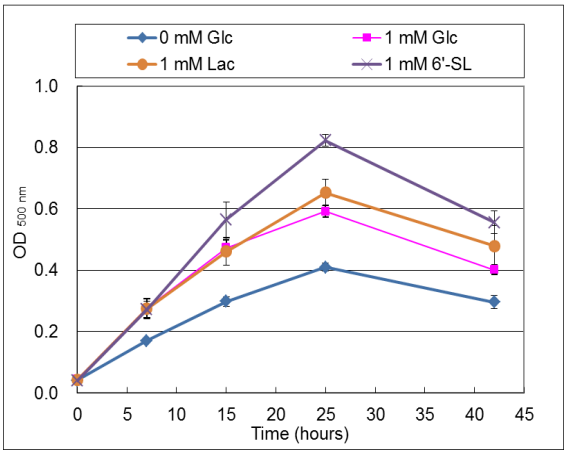
LeB2



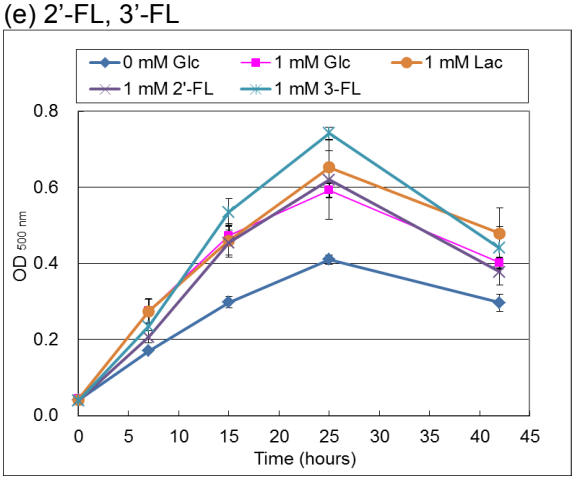
(d) Le0



6'-SL



(e) 2'-FL, 3'-FL



LNT, LNnT

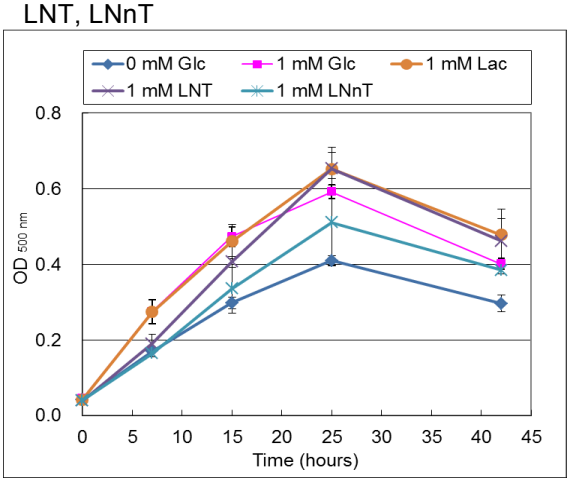


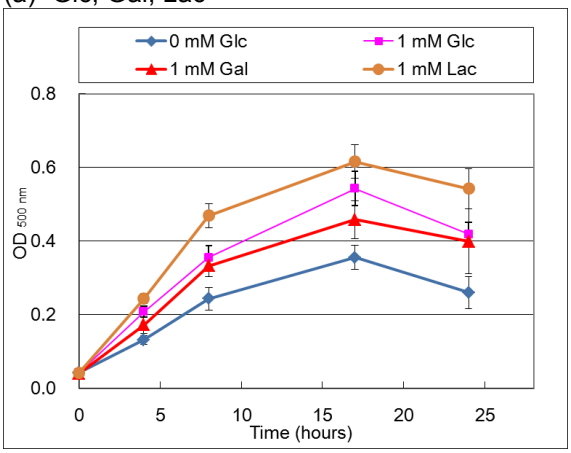
Figure 4-8 Growth curves of *L. lactis* grown in MRS medium (without glucose) on different carbon substrate. (a) Glucose, galactose, and lactose (left), as well as neutral and acidic HMO fractions from LeA2 (right). (b) Neutral and acidic HMO fractions from LeA1 (left) and LeA3 (right). (c) Neutral and acidic HMO fractions from LeB1 (left) and LeB2 (right). (d) Neutral and acidic HMO fractions from Le0 (left) and single HMO standard 6'-SL (right). (e) Single HMO standard 2'-FL, 3-FL (left) and LNT, LNnT (right).

The growth curves show that *L. lactis* could reach maximum OD_{500nm} in about 25 hours. The maximum OD_{500nm} without carbon substrate was about 0.4. When 1 mM glucose or galactose was added, the growth increased and the maximum OD_{500nm} could reach about 0.5 to 0.6. With 1 mM lactose, the maximum OD_{500nm} was higher than 0.6. When 1 mM neutral or acidic HMOs added, the growth with neutral HMOs was better than the growth with lactose, the maximum OD_{500nm} with acidic HMOs were lower than with neutral HMOs except the case with LeA2. The growth with five single HMO standards showed that the maximum OD_{500nm} with 6'-SL and 3-FL were higher than that with lactose, LNT showed a similar OD_{500nm} with lactose, while the maximum OD_{500nm} with 2'-FL and LNnT were lower.

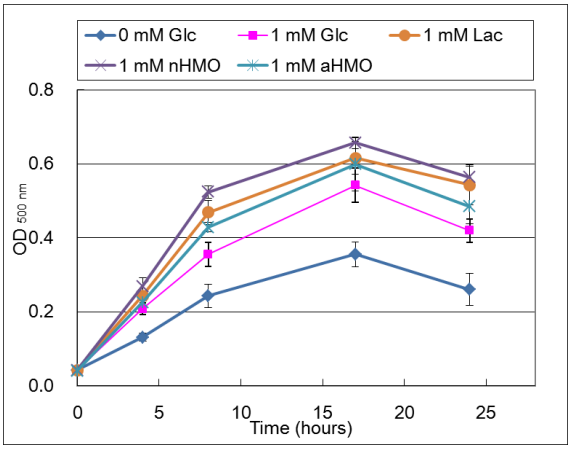
4.5.2 *Lactobacillus acidophilus*

To investigate the effect of HMOs on the growth of *Lactobacillus acidophilus*, similar experiments were done according to *L. lactis* with the modification of adding L-cysteine in the MRS medium. Figure 4-9 shows the growth curves of *L. acidophilus* on different carbon substrates.

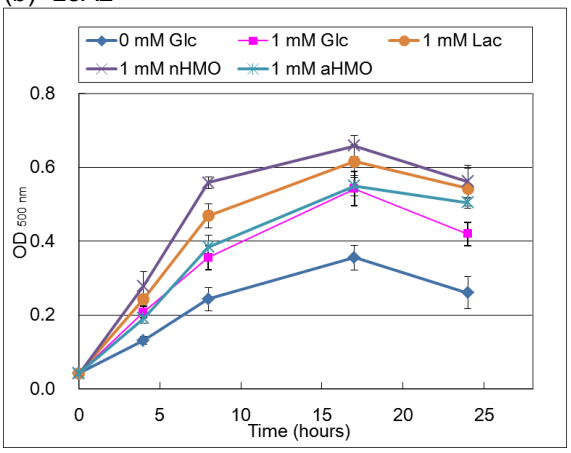
(a) Glc, Gal, Lac



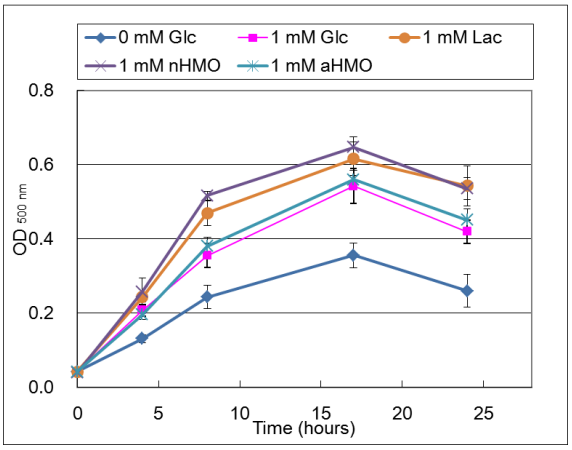
LeA1



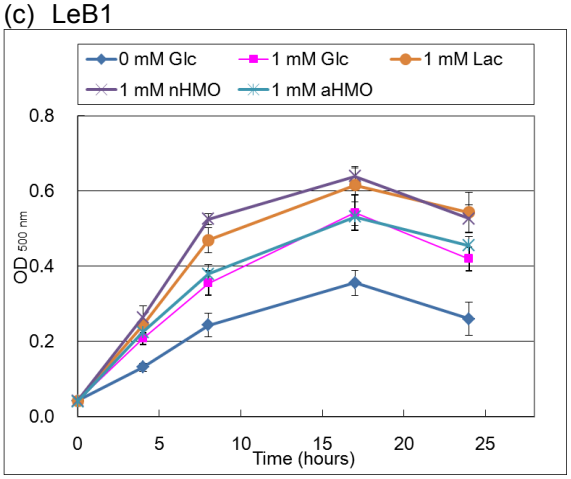
(b) LeA2



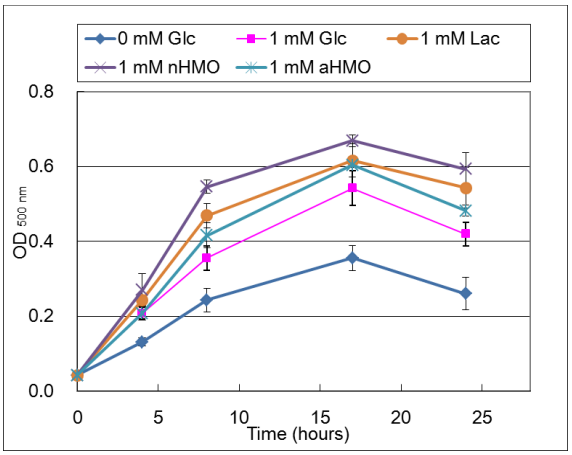
LeA3



(c) LeB1



LeB2



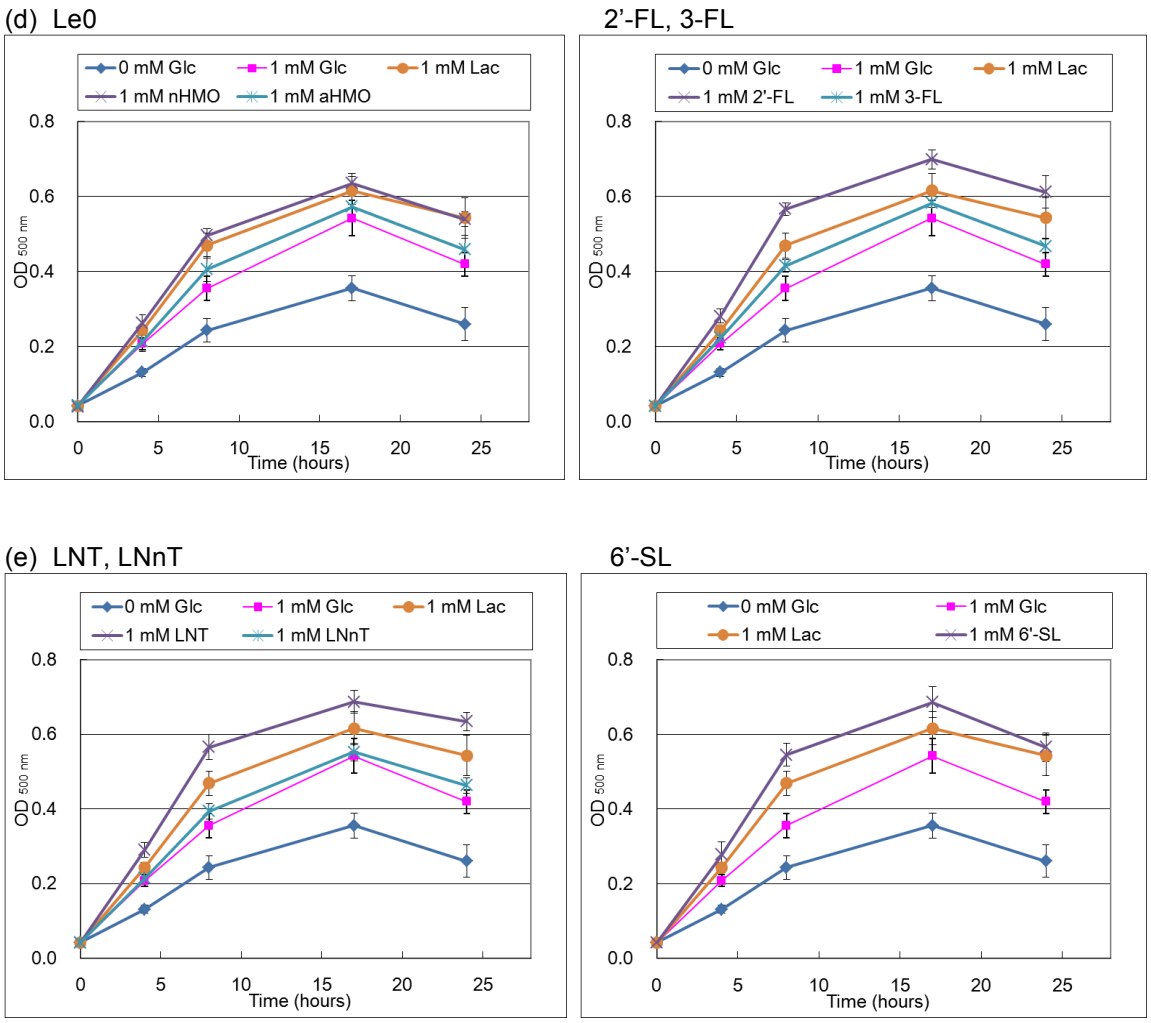


Figure 4-9 Growth curves of *L. acidophilus* grown in MRS medium with L-Cysteine (without glucose) on different carbon substrates. (a) Glucose, galactose, and lactose (left), as well as neutral and acidic HMO fractions from LeA1 (right). (b) Neutral and acidic HMO fractions from LeA2 (left) and LeA3 (right). (c) Neutral and acidic HMO fractions from LeB1 (left) and LeB2 (right). (d) Neutral and acidic HMO fractions from Le0 (left) and single HMO standard 2'-FL, 3-FL (right). (e) Single HMO standard LNT, LNnT (left) and 6'-SL (right).

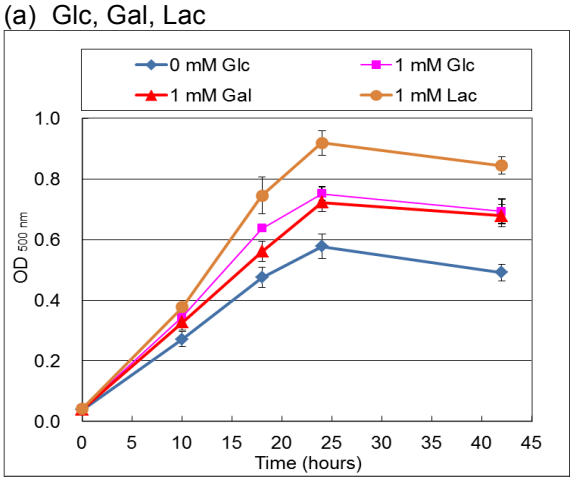
The growth curves showed that *L. acidophilus* could reach maximum OD_{500nm} in about 17 hours. The maximum OD_{500nm} without carbon substrate was about 0.35. When 1 mM glucose and galactose added, the growth was increased; the maximum OD_{500nm} could reach about 0.45 with galactose and about 0.1 higher than that with glucose. With 1 mM lactose, the maximum OD_{500nm} was about 0.6. When 1 mM neutral or acidic HMOs added, the growths with neutral HMOs were all better than the growth with lactose, the maximum OD_{500nm} with acidic HMOs were lower than with neutral HMOs. The growth with five single HMO standards showed that the maximum OD_{500nm} with 2'-FL, LNT and

6'-SL were higher than lactose, while the maximum OD_{500nm} with 3-FL and LNnT were lower than lactose, but higher than glucose.

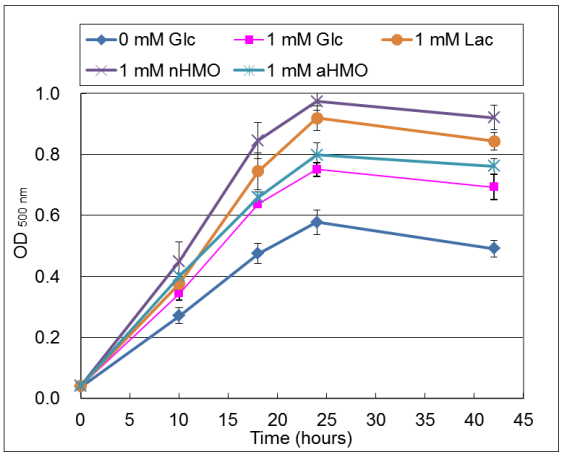
4.5.3 *Bifidobacterium longum subsp. infantis*

To investigate the effect of HMOs on the growth of *Bifidobacterium longum subsp. infantis*, similar experiments were done according to *L. lactis* with the modification of changing the MRS medium to *Bifidobacterium* medium. Figure 4-10 shows the growth curves of *B. infantis* on different carbon substrates.

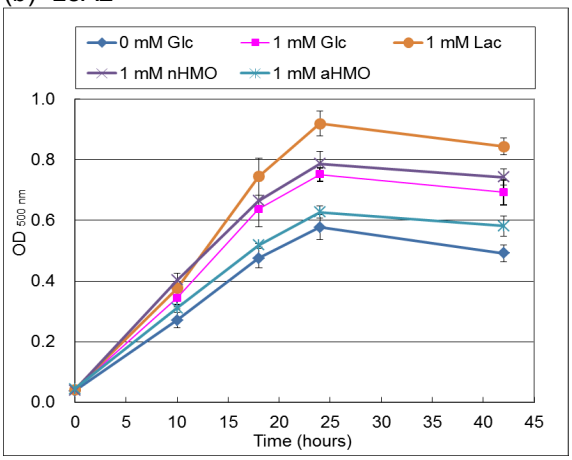
(a) Glc, Gal, Lac



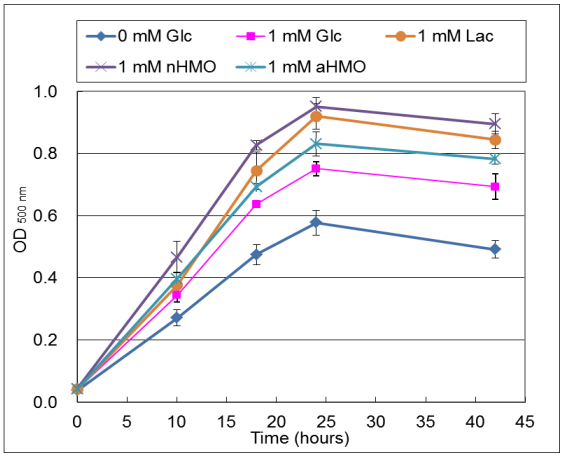
LeA1



(b) LeA2



LeA3



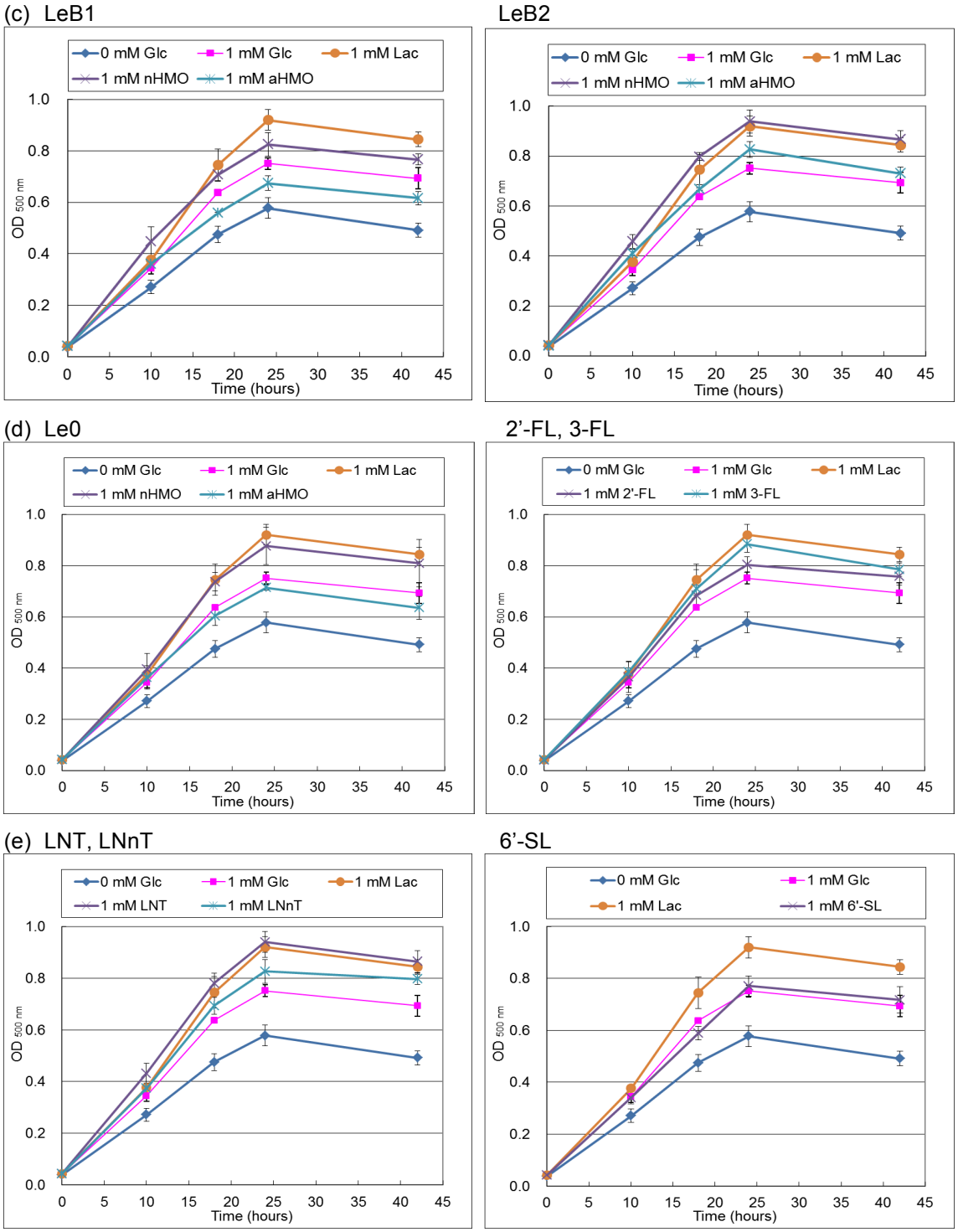


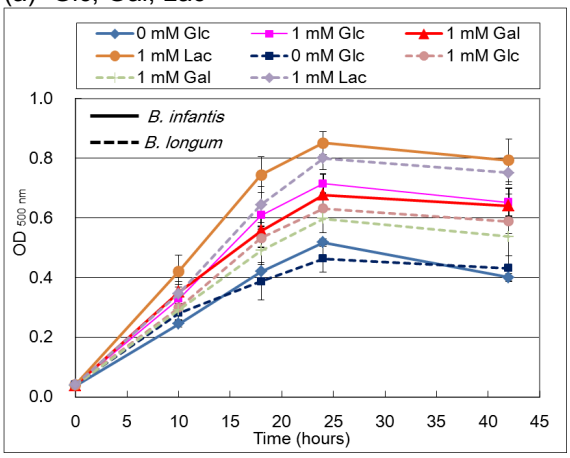
Figure 4-10 Growth curves of *B. infantis* grown in *Bifidobacterium* medium (without glucose) on different carbon substrates. (a) Glucose, galactose, and lactose (left), as well as neutral and acidic HMO fractions from LeA1 (right). (b) Neutral and acidic HMO fractions from LeA2 (left) and LeA3 (right). (c) Neutral and acidic HMO fractions from LeB1 (left) and LeB2 (right). (d) Neutral and acidic HMO fractions from Le0 (left) and single HMO standard 2'-FL, 3-FL (right). (e) Single HMO standard LNT, LNnT (left) and 6'-SL (right).

The growth curves showed that *B. infantis* could reach maximum OD_{500nm} in about 24 hours. The maximum OD_{500nm} without carbon substrate was about 0.6. When 1 mM glucose or galactose was added, the growth rate was increased; the maximum OD_{500nm} reached between 0.7 and 0.8. With 1 mM lactose, the maximum OD_{500nm} was higher, reached about 0.9. When 1 mM neutral or acidic HMOs added, the growth with six neutral HMOs were all better than the growth with glucose, in which three of neutral HMOs reach higher OD_{500nm} than with lactose, one of neutral HMOs reach the similar OD_{500nm} as with lactose. The maximum OD_{500nm} with acidic HMOs was lower than with neutral HMOs. The growth with five single HMO standards showed that the maximum OD_{500nm} with LNT was higher than lactose, while the maximum OD_{500nm} with 2'-FL, 3-FL and LNnT were between glucose and lactose. The maximum OD_{500nm} with 6'-SL was similar to with glucose.

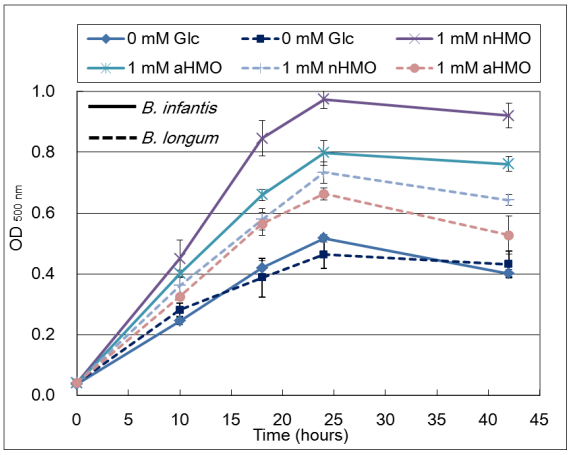
4.5.4 *Bifidobacterium longum subsp. infantis vs. Bifidobacterium longum subsp. longum*

To compare the effect of HMOs on the growth of *Bifidobacterium longum subsp. infantis* and *Bifidobacterium longum subsp. longum*, the same experiments were done according to *B. infantis* together with *B. longum*. Figure 4-11 shows the growth curves of *B. infantis* vs. *B. longum* on different carbohydrate substrates.

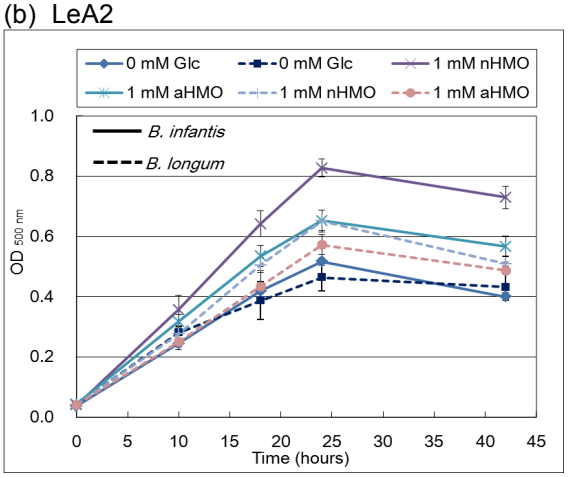
(a) Glc, Gal, Lac



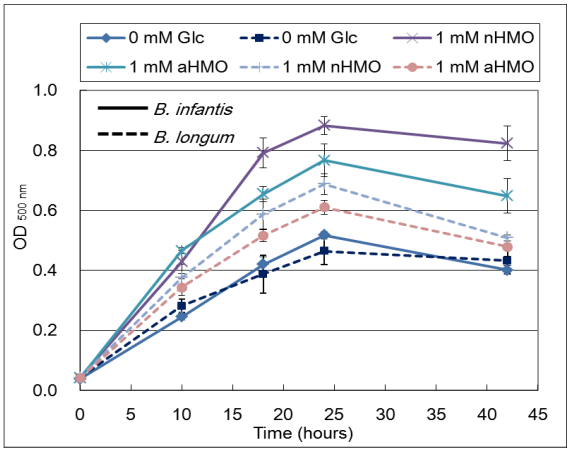
LeA1



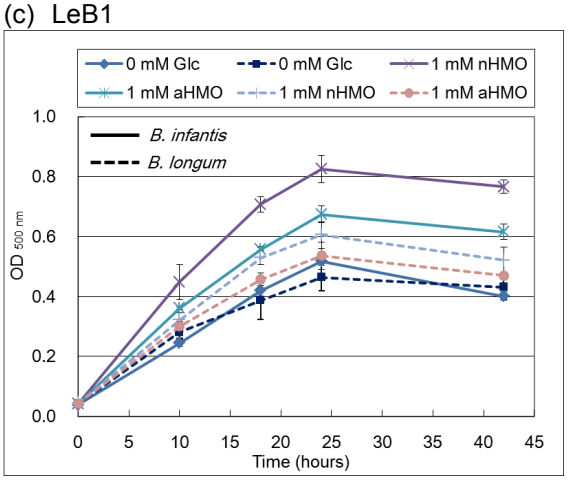
(b) LeA2



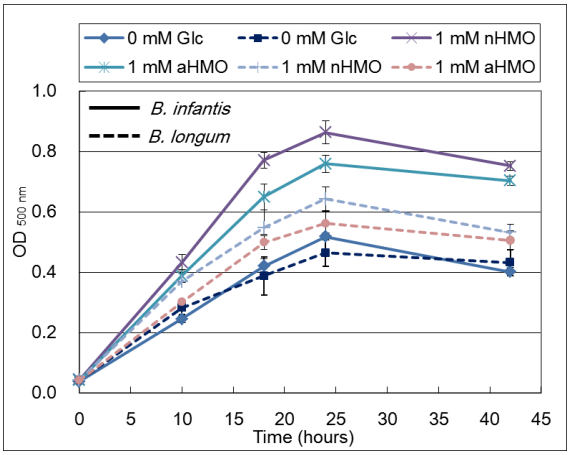
LeA3



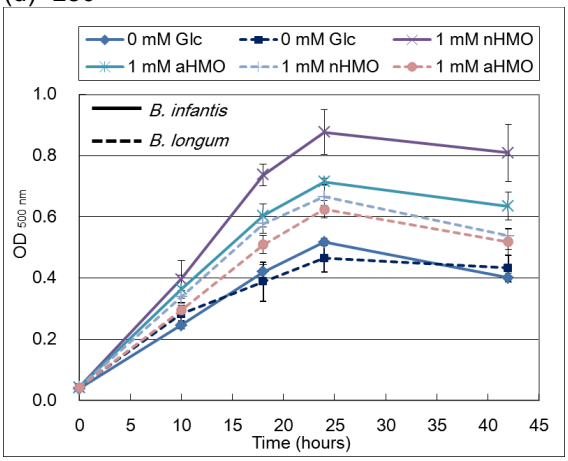
(c) LeB1



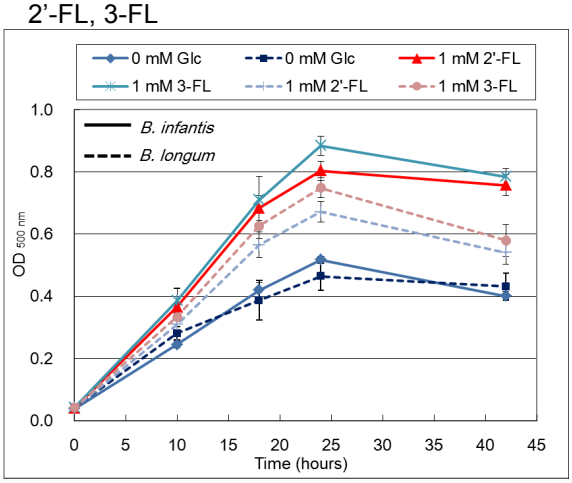
LeB2



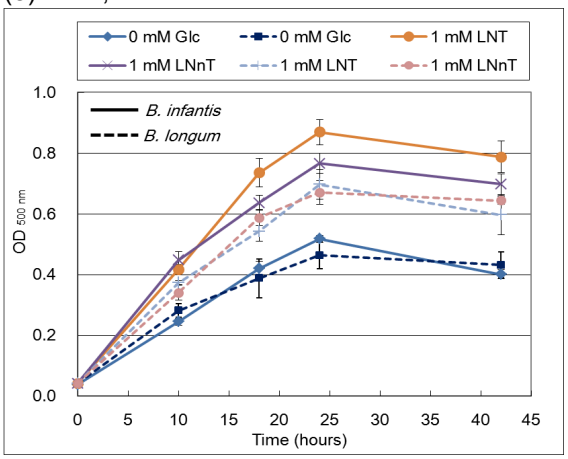
(d) Le0



2'-FL, 3-FL



(e) LNT, LNnT



6'-SL

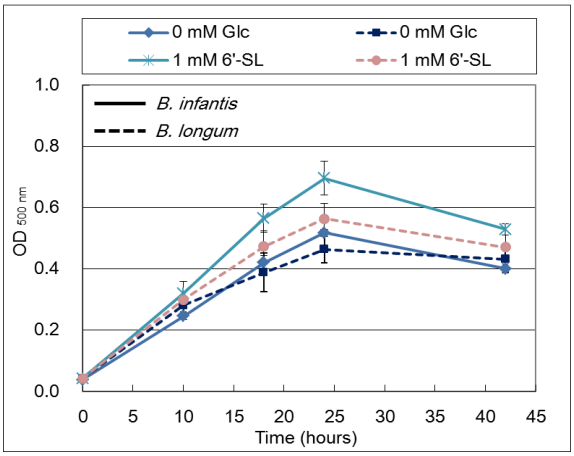


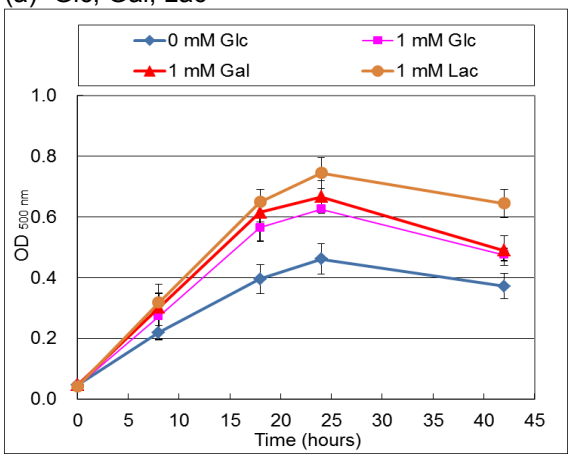
Figure 4-11 Growth curves of *B. infantis* and *B. longum* grown in *Bifidobacterium* medium (without glucose) on different carbon substrates. (a) Glucose, galactose, and lactose (left), as well as neutral and acidic HMO fractions from LeA1 (right). (b) Neutral and acidic HMO fractions from LeA2 (left) and LeA3 (right). (c) Neutral and acidic HMO fractions from LeB1 (left) and LeB2 (right). (d) Neutral and acidic HMO fractions from Le0 (left) and single HMO standard 2'-FL, 3-FL (right). (e) Single HMO standard LNT, LNnT (left) and 6'-SL (right).

The growth curves showed that *B. infantis* and *B. longum* could both reach the maximum OD_{500nm} in about 25 hours. The growth of *B. infantis* was better than that of *B. longum* on all the carbon substrates. The maximum OD_{500nm} without carbon substrate of the two bacteria were similar, between 0.4 and 0.5. When 1 mM glucose or galactose was added, the growth rates both bacteria were increased; the maximum OD_{500nm} of *B. infantis* could reach around 0.7; while the maximum OD_{500nm} of *B. longum* could reach about 0.6. With 1 mM lactose, the maximum OD_{500nm} of *B. infantis* was higher, reached about 0.85, while the maximum OD_{500nm} of *B. longum* was about 0.8. When 1 mM neutral or acidic HMOs was added, both bacteria showed that the growths with neutral HMOs were better than with acidic HMOs. The maximum OD_{500nm} with acidic HMOs for both bacteria were lower than with neutral HMOs, but higher than without carbon substrate. The growths with five single HMO standards showed that the maximum OD_{500nm} with four neutral standards were higher than that with the acidic standard 6'-SL. The results with 2'-FL and 3-FL showed that the maximum OD_{500nm} with *B. infantis* were more than 0.1 higher for both substrates than with *B. longum*. The OD_{500nm} with 3-FL with both bacteria was about 0.1 higher than with 2'-FL. The study with LNT and LNnT showed that for *B. infantis*, the maximum OD_{500nm} with LNT was about 0.1 higher than with LNnT, while for *B. longum*, LNT showed a similar OD_{500nm} as LNnT.

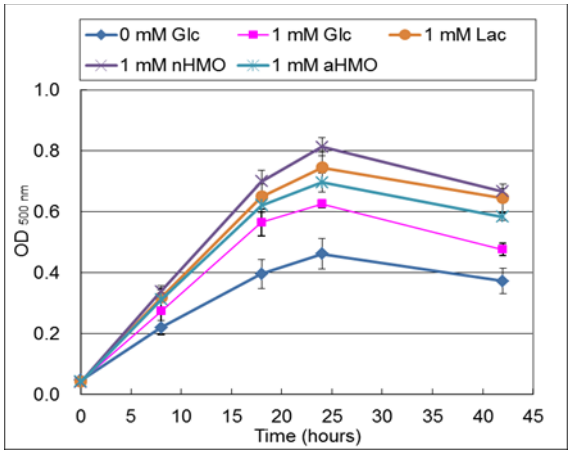
4.5.5 *Bacteroides vulgatus*

To investigate the effect of HMOs on the growth of *Bacteroides vulgatus*, similar experiments were done according to *L. lactis* with the modification of changing the MRS medium to Modified Reinforced Clostridial Broth. Figure 4-12 shows the growth curves of *B. vulgatus* on different carbon substrates.

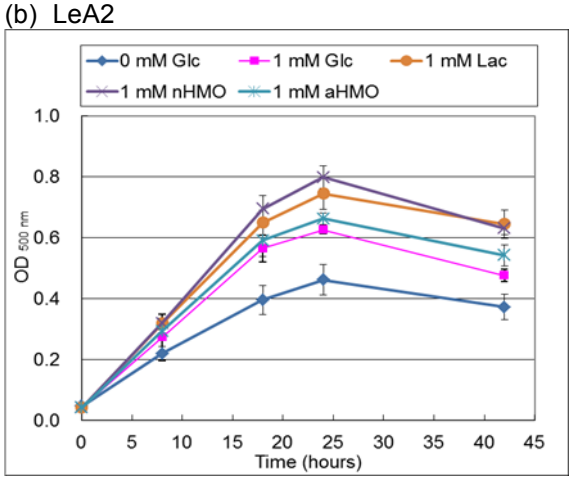
(a) Glc, Gal, Lac



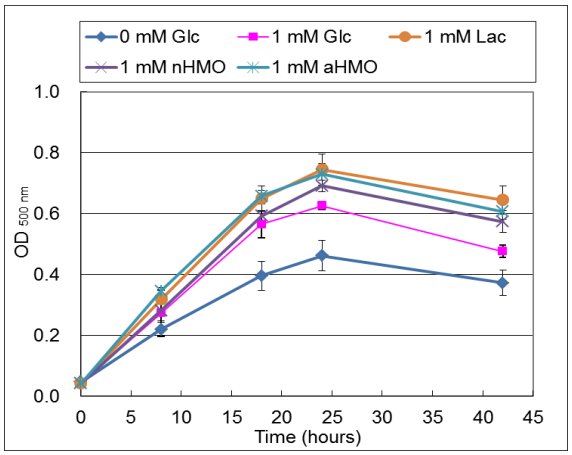
LeA1



(b) LeA2



LeA3



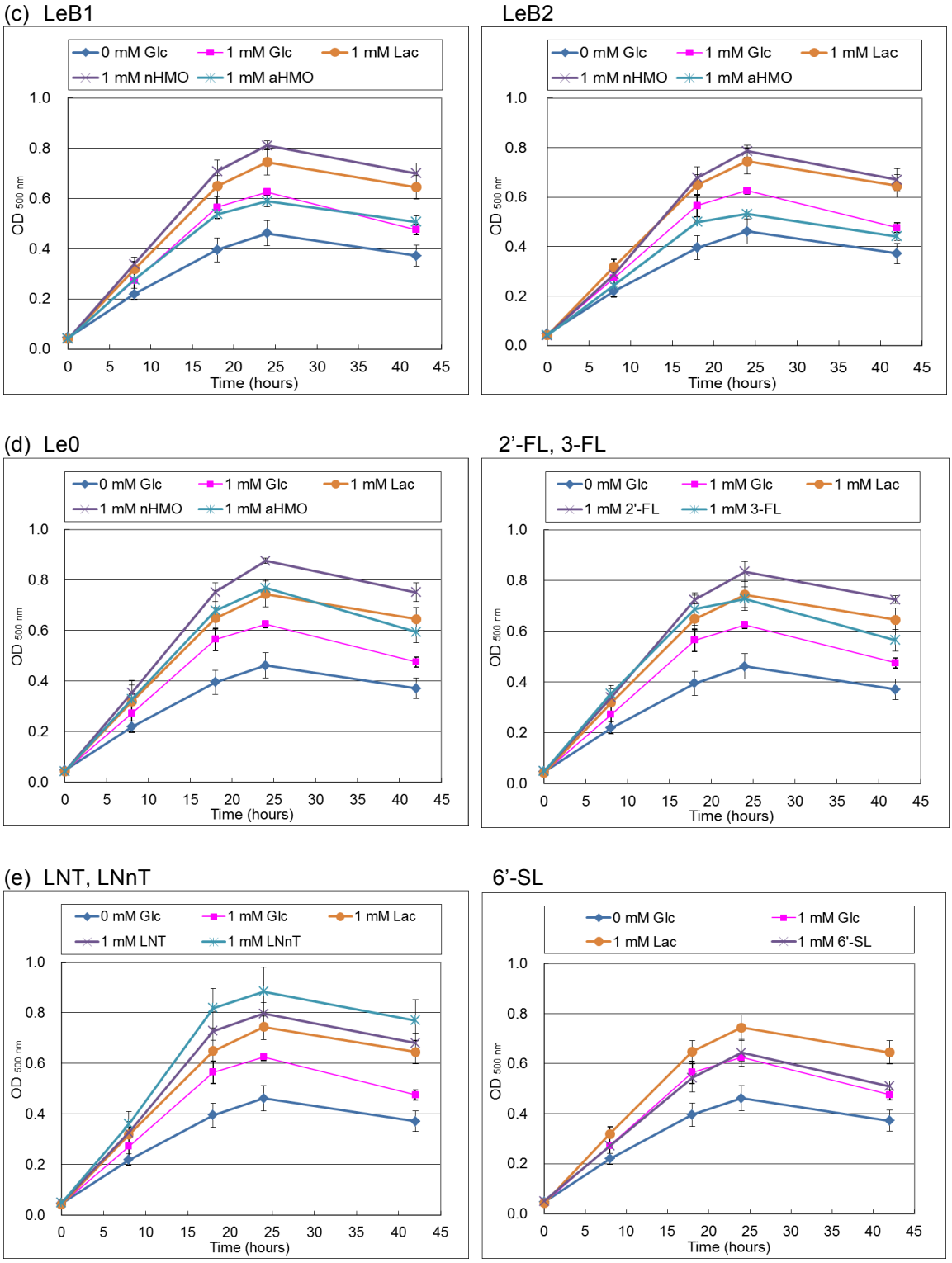


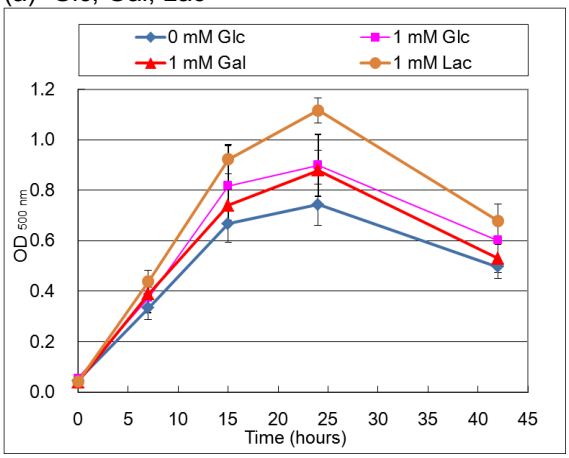
Figure 4-12 Growth curves of *B. vulgatus* grown in Modified Reinforced Clostridial Broth (without glucose) on different carbon substrates. (a) Glucose, galactose, and lactose (left), as well as neutral and acidic HMO fractions from LeA1 (right). (b) Neutral and acidic HMO fractions from LeA2 (left) and LeA3 (right). (c) Neutral and acidic HMO fractions from LeB1 (left) and LeB2 (right). (d) Neutral and acidic HMO fractions from Le0 (left) and single HMO standard 2'-FL, 3-FL (right). (e) Single HMO standard LNT, LNnT (left) and 6'-SL (right).

The growth curves showed that *B. vulgatus* could reach maximum OD_{500nm} in about 24 hours. The maximum OD_{500nm} without substrate was about 0.45. When 1 mM glucose and galactose added, the growth was increased; the maximum OD_{500nm} could reach 0.6 to 0.7. With 1 mM lactose, the maximum OD_{500nm} was higher, reached about 0.75. When 1 mM neutral or acidic HMOs added, the growth with neutral HMOs was better than the growth with lactose in most cases. The maximum OD_{500nm} with acidic HMOs were lower than with neutral HMOs except the case with LeA3.The growth with five single HMO standards showed that the maximum OD_{500nm} with 2'-FL, LNT and LNnT were higher than lactose, while the maximum OD_{500nm} with 3-FL was similar to with lactose. The maximum OD_{500nm} with 6'-SL was similar to that with glucose.

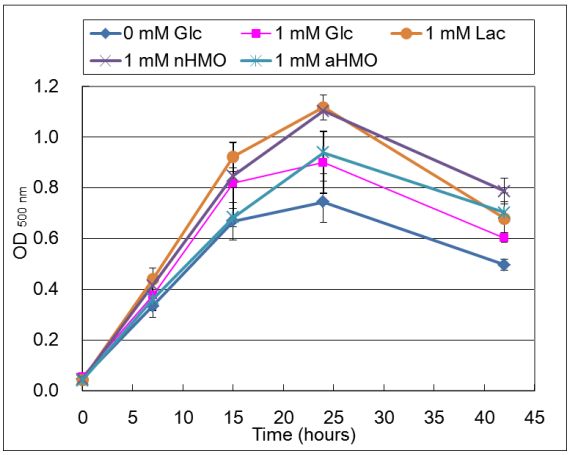
4.5.6 *Cronobacter sakazakii*

To investigate the effect of HMOs on the growth of *Cronobacter sakazakii*, similar experiments were done according to *L. lactis* with MRS medium. Figure 4-13 shows the growth curves of *C. sakazakii* on different carbon substrates.

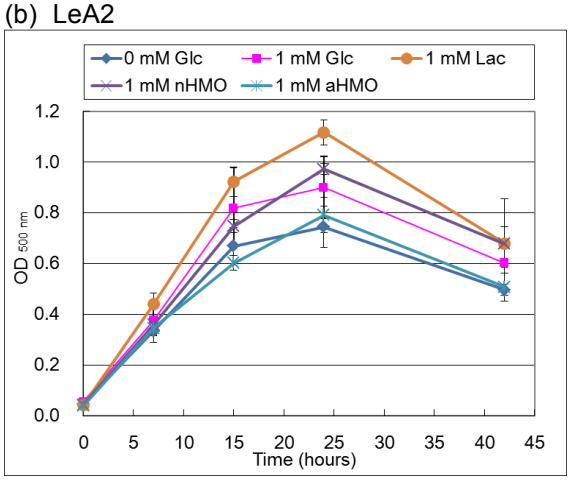
(a) Glc, Gal, Lac



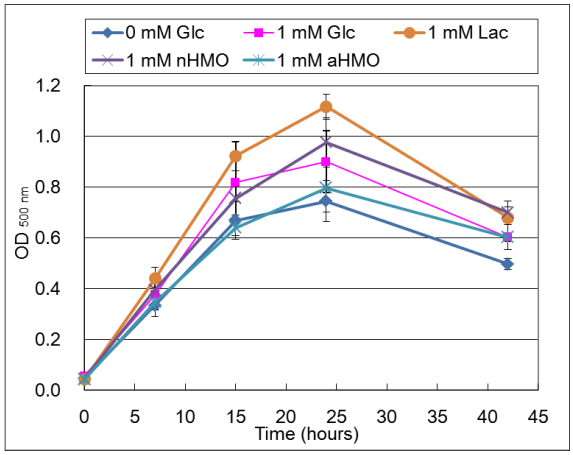
LeA1



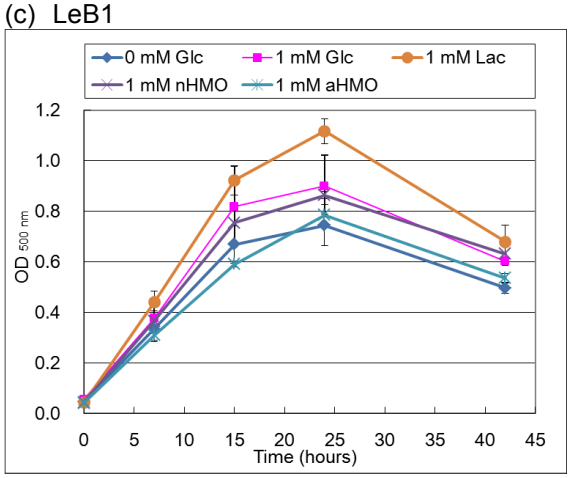
(b) LeA2



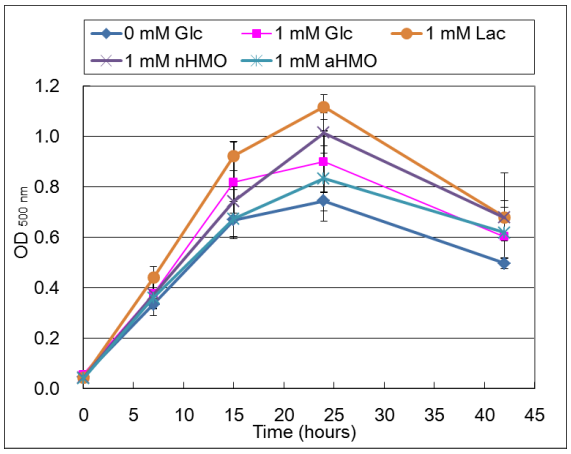
LeA3



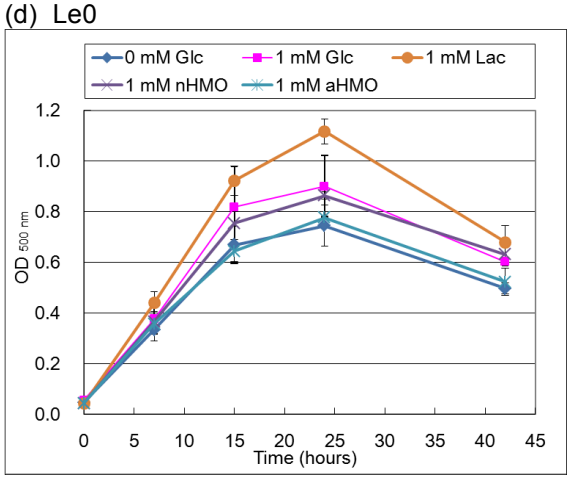
(c) LeB1



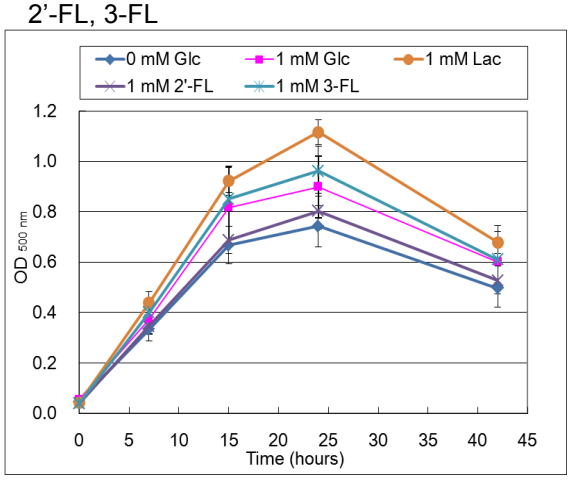
LeB2



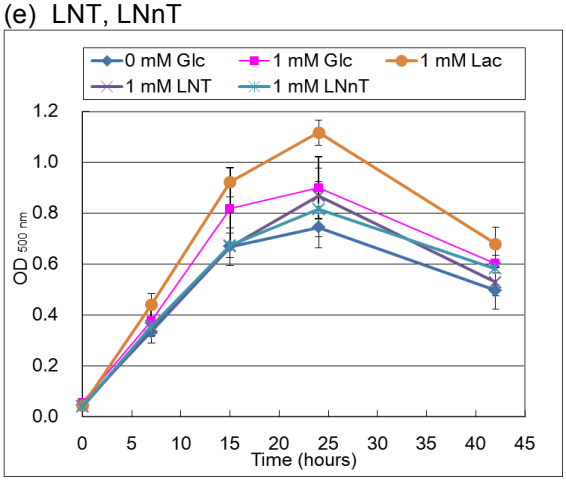
(d) Le0



2'-FL, 3-FL



(e) LNT, LNnT



6'-SL

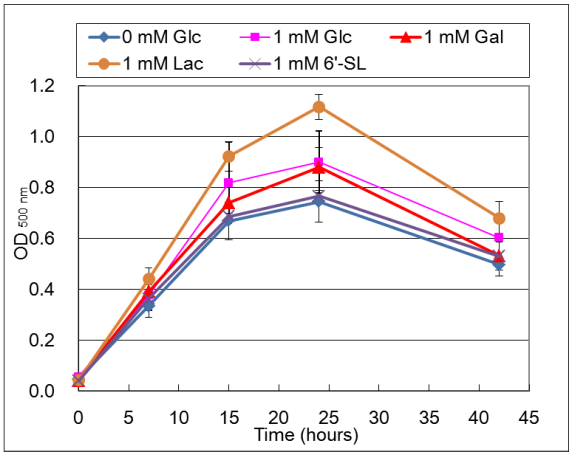


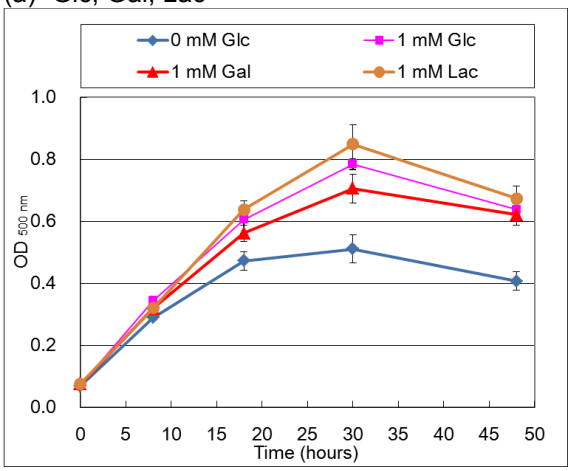
Figure 4-13 Growth curves of *C. sakazakii* grown in MRS medium (without glucose) on different carbon substrates. (a) Glucose, galactose, and lactose (left), as well as neutral and acidic HMO fractions from LeA1 (right). (b) Neutral and acidic HMO fractions from LeA2 (left) and LeA3 (right). (c) Neutral and acidic HMO fractions from LeB1 (left) and LeB2 (right). (d) Neutral and acidic HMO fractions from Le0 (left) and single HMO standard 2'-FL, 3-FL (right). (e) Single HMO standard LNT, LNnT (left) and 6'-SL (right).

The growth curves showed that *C. sakazakii* could reach maximum OD_{500nm} in about 24 hours. The maximum OD_{500nm} without carbon substrate was about 0.75. When 1 mM glucose and galactose added, the growth rate was increased; the maximum OD_{500nm} could reach 0.8 to 0.9. With 1 mM lactose, the maximum OD_{500nm} was higher, reached about 1.1. When 1 mM neutral HMOs added, the growth with six neutral HMOs were all better than the growth without glucose, in which one of neutral HMOs reached the OD_{500nm} similar to with lactose, three of neutral HMOs reached the OD_{500nm} between with lactose and with glucose, OD_{500nm} of the rest two neutral HMOs were lower than with glucose. The maximum OD_{500nm} with 1 mM acidic HMOs were all lower than with neutral HMOs, and the OD_{500nm} were mostly lower than with glucose except the case with mother 2 from Lewis a specificity. The growth with five single HMO standards showed that the maximum OD_{500nm} with 3'-FL was higher than with glucose, the maximum OD_{500nm} with 2'-FL, LNT, LNnT and 6'-SL were all a little bit lower than with glucose. The maximum OD_{500nm} with 6'-SL was similar to without glucose.

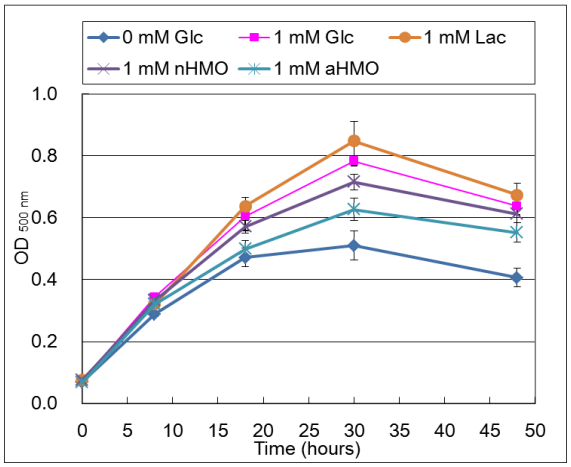
4.5.7 *Akkermansia muciniphila*

To investigate the effect of HMOs on the growth of *Akkermansia muciniphila*, similar experiments were done according to *L. lactis* with the modification of changing the MRS medium to Modified Reinforced Clostridial Broth. Figure 4-14 shows the growth curves of *A. muciniphila* on different carbon substrates.

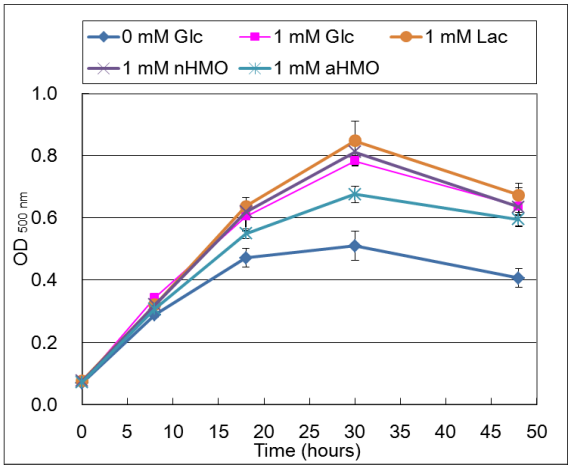
(a) Glc, Gal, Lac



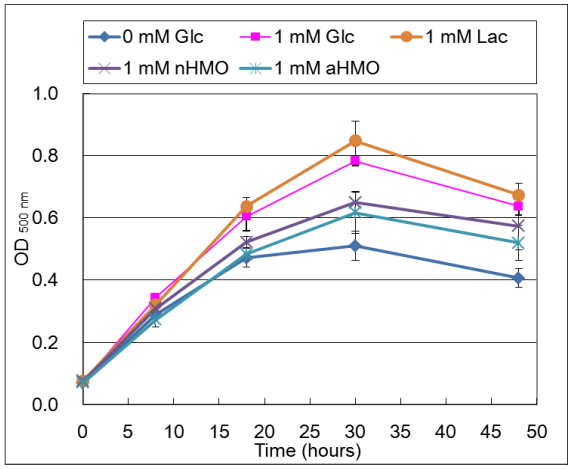
LeA1



(b) LeA2



LeA3



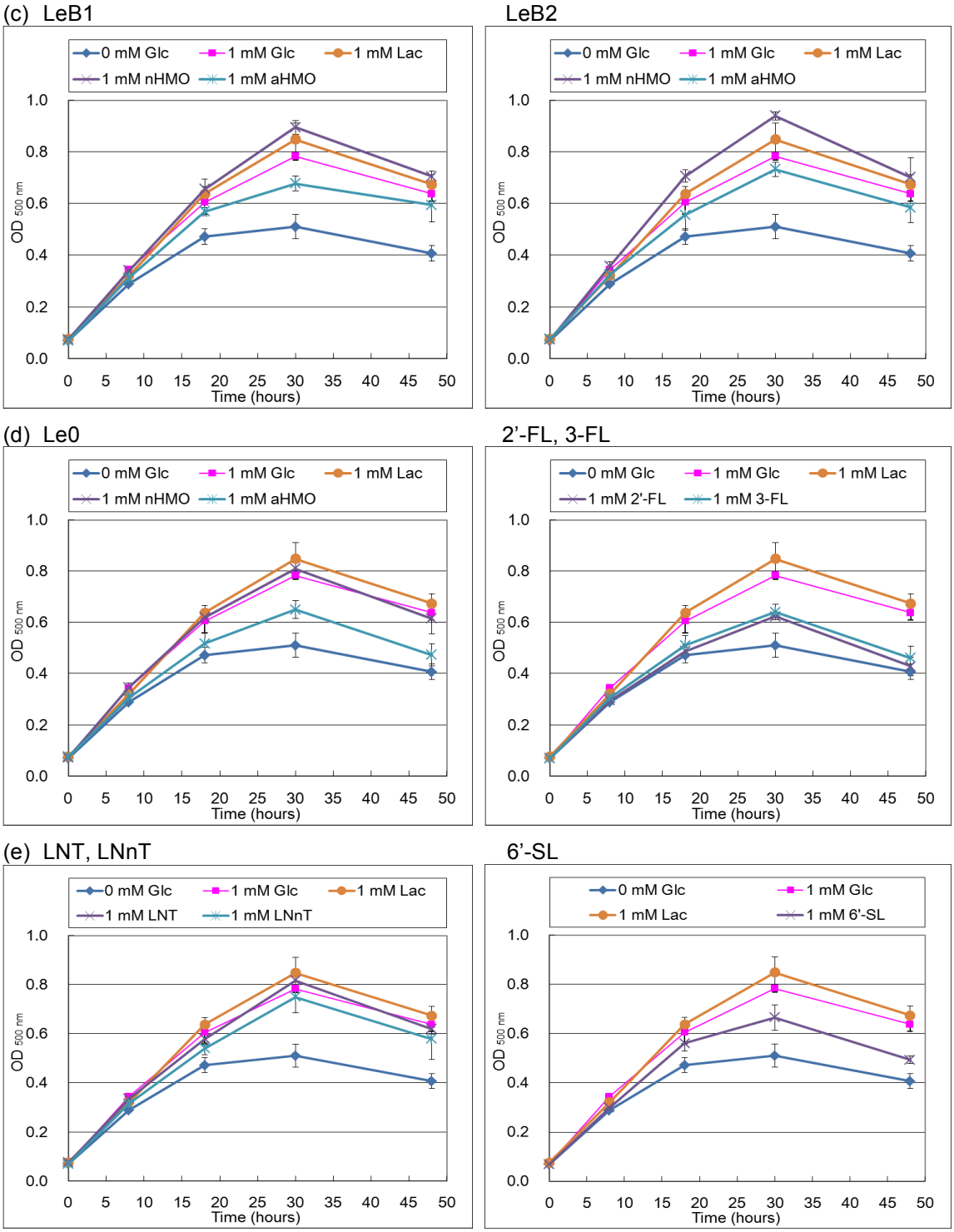


Figure 4-14 Growth curves of *A. muciniphila* grown in Modified Reinforced Clostridial Broth (without glucose) on different carbon substrates. (a) Glucose, galactose, and lactose (left), as well as neutral and acidic HMO fractions from LeA1 (right). (b) Neutral and acidic HMO fractions from LeA2 (left) and LeA3 (right). (c) Neutral and acidic HMO fractions from LeB1 (left) and LeB2 (right). (d) Neutral and acidic HMO fractions from Le0 (left) and single HMO standard 2'-FL, 3-FL (right). (e) Single HMO standard LNT, LNnT (left) and 6'-SL (right).

The growth curves showed that *A. muciniphila* could reach maximum OD_{500nm} in about 30 hours. The maximum OD_{500nm} without carbon substrate was about 0.5. When 1 mM glucose and galactose added, the growth rate was increased; the maximum OD_{500nm} of glucose can reach about 0.8, while the maximum OD_{500nm} of galactose was about 0.7. With 1 mM lactose, the maximum OD_{500nm} was higher, reached about 0.85. When 1 mM neutral HMOs added, the growth with six neutral HMOs were all better than the growth without glucose, in which two of neutral HMOs reached the OD_{500nm} higher than with lactose, two of neutral HMOs reached the OD_{500nm} similar to with glucose, OD_{500nm} of the rest two neutral HMOs were lower than with glucose. The maximum OD_{500nm} with 1 mM acidic HMOs were all lower than with neutral HMOs, and the OD_{500nm} were all lower than with glucose. The growth with five single HMO standards showed that the maximum OD_{500nm} with 2'-FL, 3-FL and 6'-SL were lower than with glucose, among which 6'-SL is the highest. The maximum OD_{500nm} with LNT, LNnT were similar to with glucose. The growth with LNT was better than with LNnT.

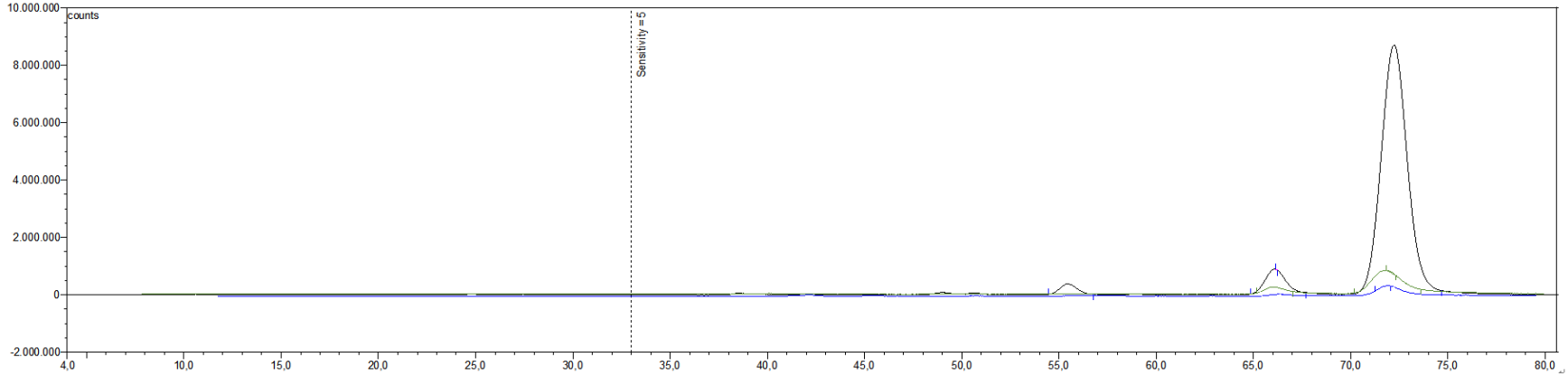
4.6 Analysis of substrates in the medium before and after cultivations

During the cultivation of each microorganism with different carbon substrates, 50 µl was taken from each sample at the beginning (0 h) and at the end (after 24 to 40 h depending on different bacteria). All the samples were labeled with 2-AA and analyzed via RP-HPLC. Table 4-4 shows the retention time of single HMO standards as well as the internal standard IS3 and 2-AA by RP-HPLC. Figure 4-15 shows the chromatogram of carbon substrate in the medium before and after cultivations by *B. infantis* and *B. longum*. Figure 4-16 shows the chromatogram of carbon substrate in the medium before and after cultivation by *B. vulgatus*.

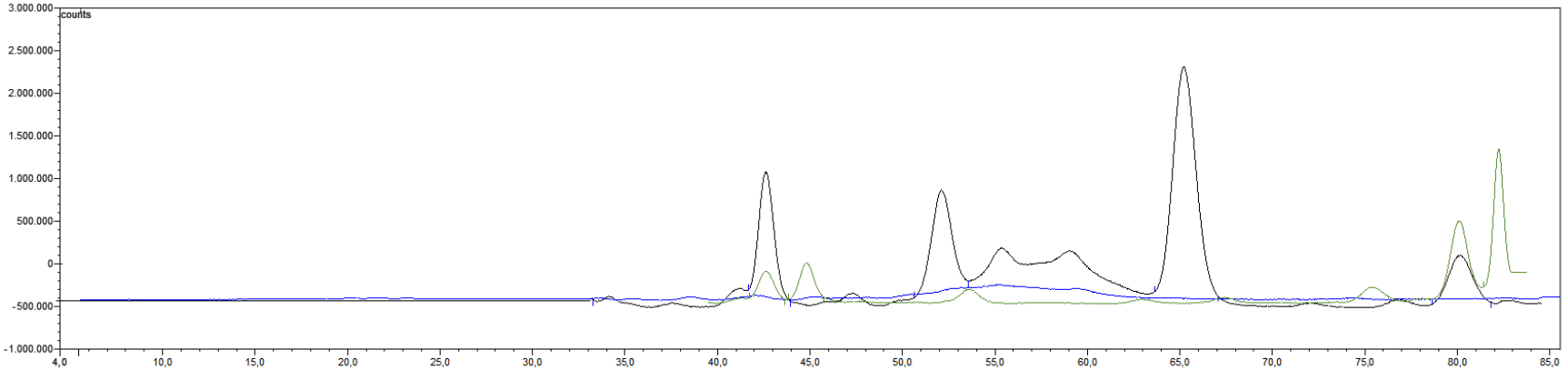
Table 4-4 Retention time of single HMO standard by RP-HPLC after 2-AA labelling

HMO	Retention time (min)	HMO	Retention time (min)
6'-SL	31,0	**IS3**	64,0
3-FL	46,4	**LNT**	67,0
LNFP II	54,8	**Lactose**	72,5
LNFP III	55,1	**2'-FL**	87,6
LNnT	60,7	**2-AA**	94,4
LDFT	61,0		
LNFP I	62,0		

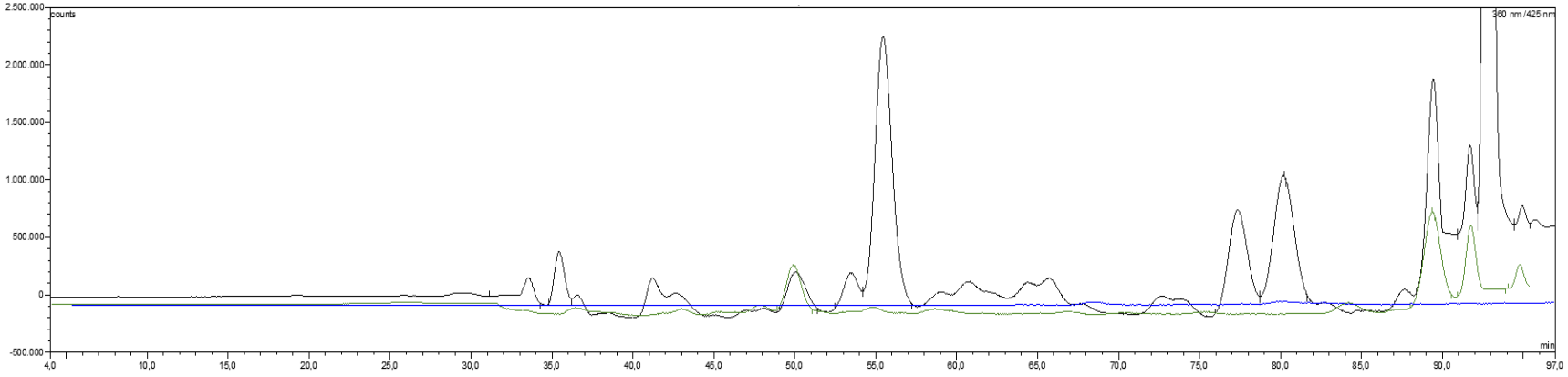
(a) lactose



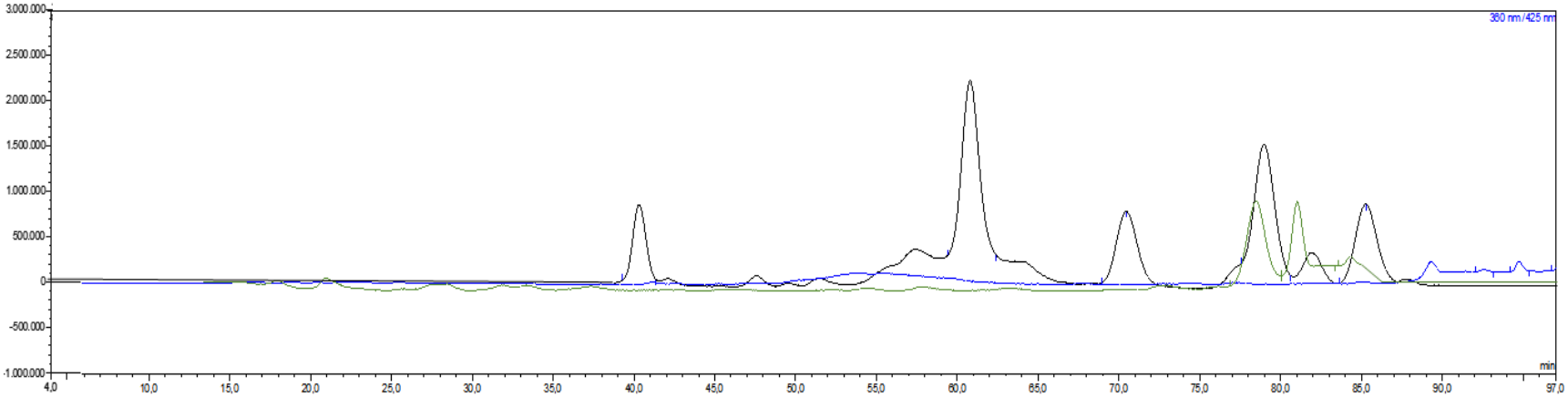
(b) LeA3n



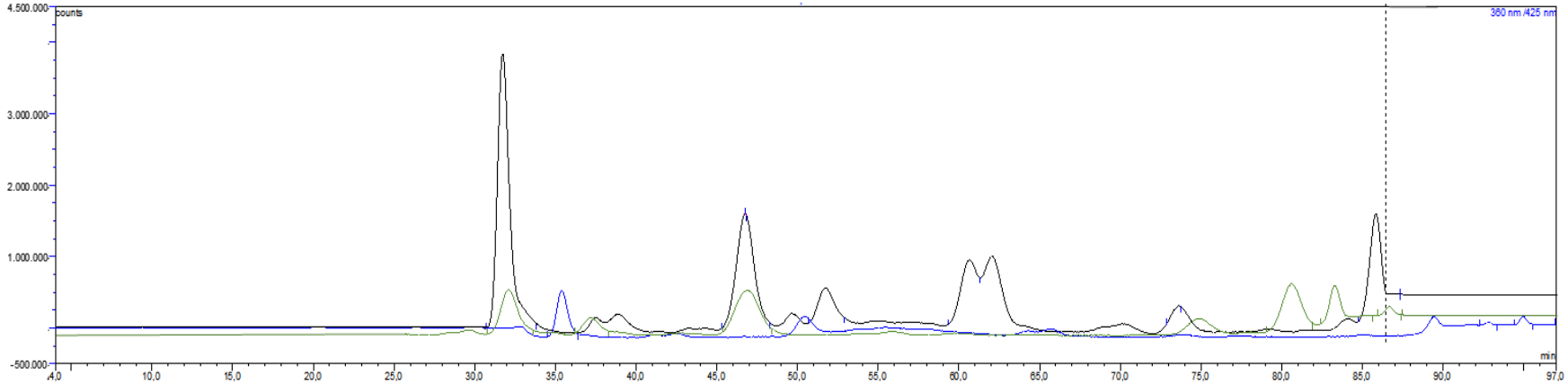
(c) LeA3a



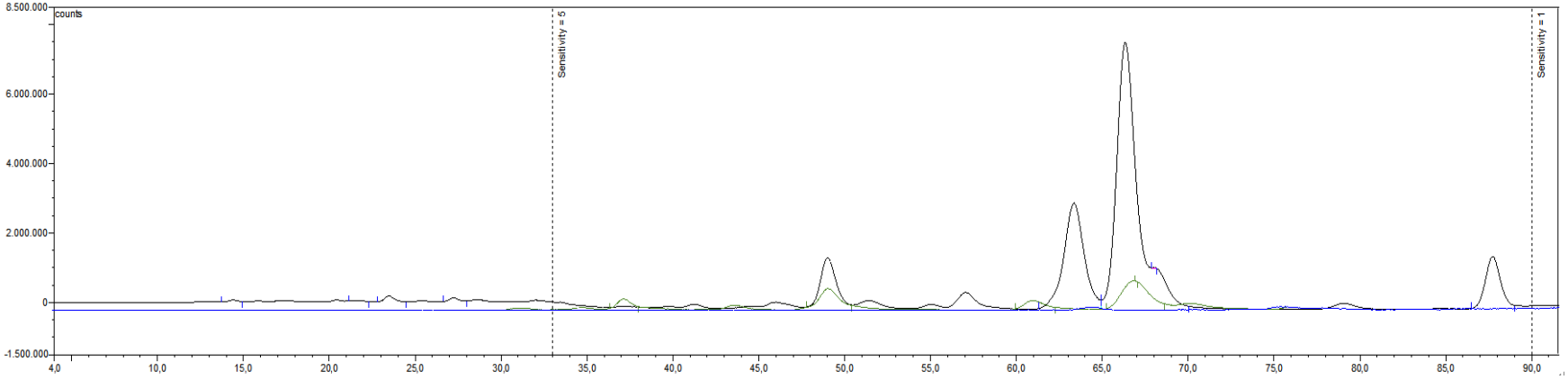
(d) LeB2n



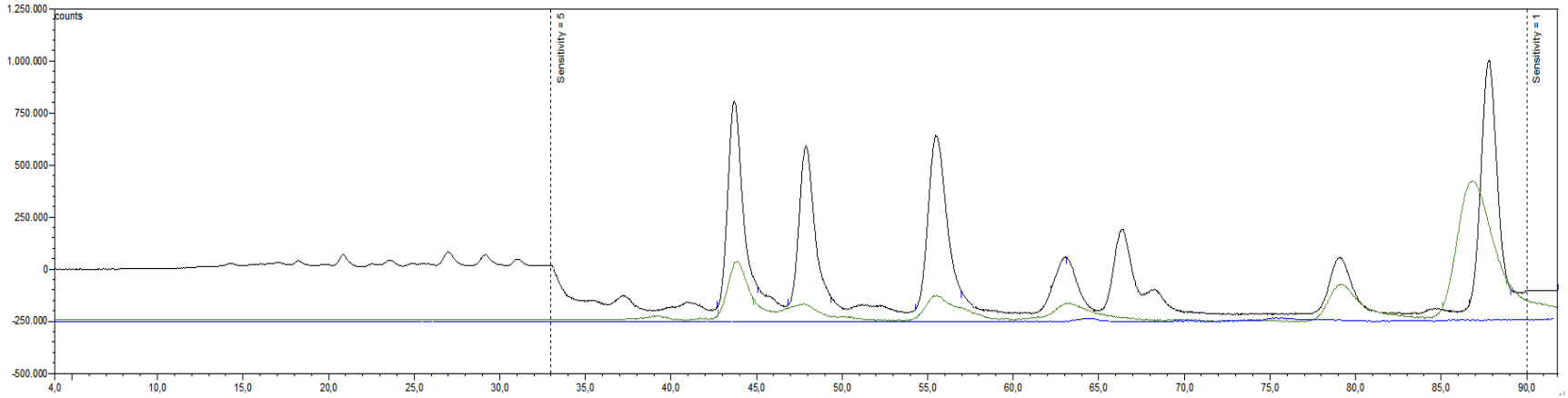
(e) LeB2a



(f) Le0n



(g) Le0a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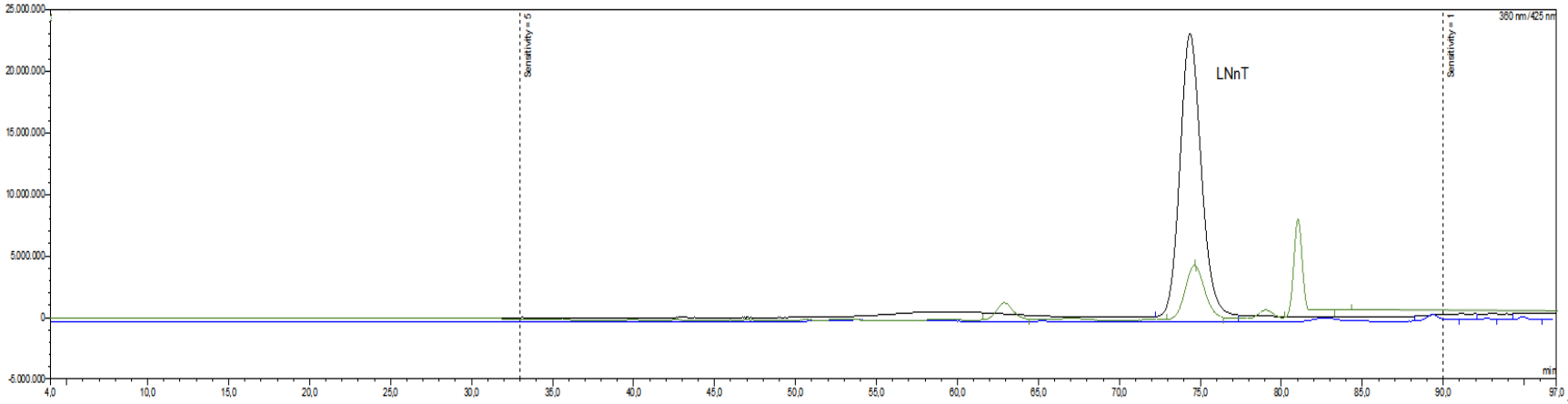
(h) LNnT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Sensitivity = 1
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min

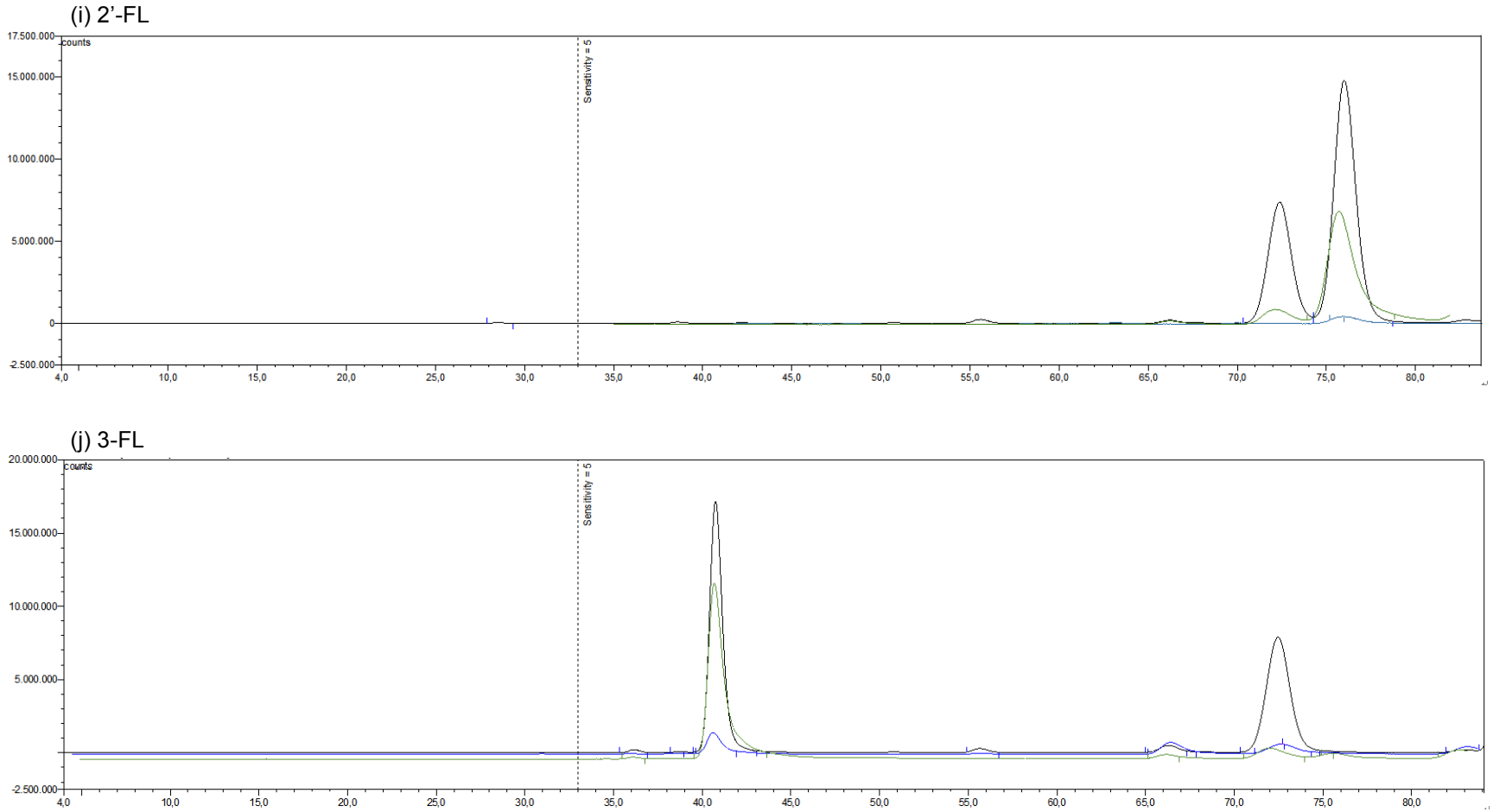
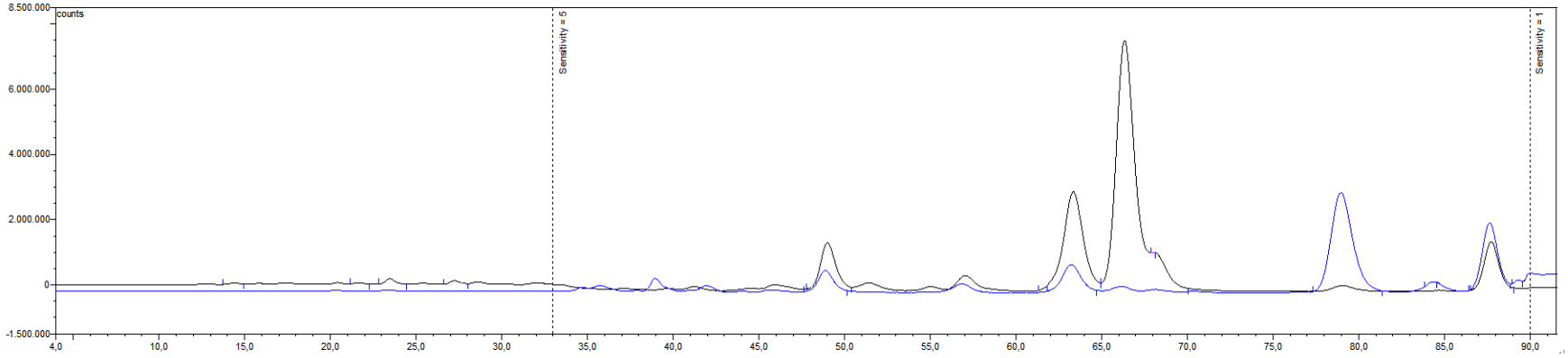


Figure 4-15 RP-HPLC Chromatograms of different carbon substrates in the medium before (black) and after cultivation by *B. infantis* (blue) and *B. longum* (green). The carbon substrates were as follows: (a) lactose; (b) neutral HMO fractions from LeA3; (c) acidic HMO fractions from LeA3; (d) neutral HMO fractions from LeB2; (e) acidic HMO fractions from LeB2; (f) neutral HMO fractions from Le0; (g) acidic HMO fractions from Le0; (h) single standard LNnT; (i) single standard 2'-FL; (j) single standard 3-FL.

The chromatograms from Figure 4-15 show the amount of different carbon substrates before and after cultivation of microorganisms. The results from lactose (a) showed that lactose could be nearly totally metabolized by *B. infantis*, while the amount of lactose after metabolizing by *B. longum* was higher. In the chromatogram of neutral HMO fractions from LeA3 (b), there were eight main peaks, which represented the components. After cultivation, both bacteria metabolized the components from four peaks. For the other four peaks, *B. infantis* could also metabolize them, while *B. longum* could only metabolize two of them to some extent and could not metabolized the other two. For *B. longum*, there were two new peaks, which may derive from semi-metabolized products during the cultivation. The results of the chromatogram of acidic HMO fractions from LeA3 (c) showed the similar results to neutral HMO fractions. From a total of more than 10 peaks, *B. infantis* could metabolize almost all the components, while *B. longum* could not metabolize the components from four peaks. From the results of neutral HMO fractions of LeB2 (d), *B. infantis* could also metabolize all the components, while *B. longum* could not metabolized three peaks of components. From the results of acidic HMO fractions of LeB2 (e), *B. infantis* could metabolize most of the components, except components from two small peaks. *B. longum* could not metabolize four peaks of components and produced two new peaks of components during cultivation. In Figure 4-15 (f) and (g), the results from neutral and acidic HMO fractions of Lewis negative samples metabolized by both microorganisms were shown. *B. infantis* could metabolize both neutral and acidic HMO fractions. *B. longum* could metabolize most of the neutral HMO fractions, only two peaks were not totally degraded. And *B. longum* could metabolize the acidic HMO fractions to some extent for most of the peaks of components. The results from single standard LNnT (h) showed that *B. infantis* could metabolize LNnT, while *B. longum* could only metabolize the components to some extent and the new peak showed the products during cultivation. In Figure 4-15 (i) and (j), the results from single standards 2'-FL and 3-FL were shown. Both standards contained two peaks, in which one of the peaks (72min) stayed the same in both chromatograms. From the chromatograms, similar results could be obtained. *B. infantis* could metabolize both 2'-FL and 3-FL, while *B. longum* could only metabolize the two single standards to some extent.

(a) Le0n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35,0
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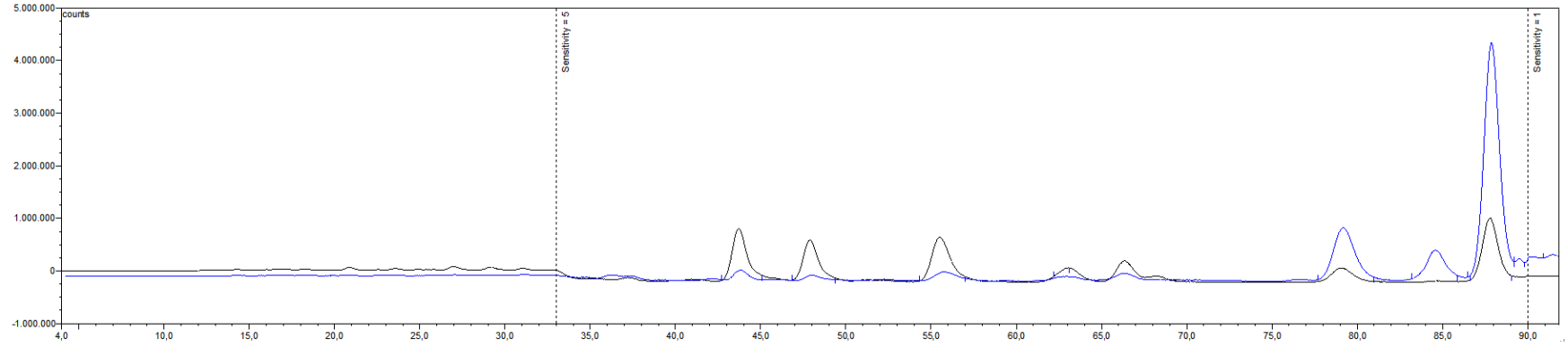
90,0

(b) Le0a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1.000.000
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-1.000.000
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85,0
90,0

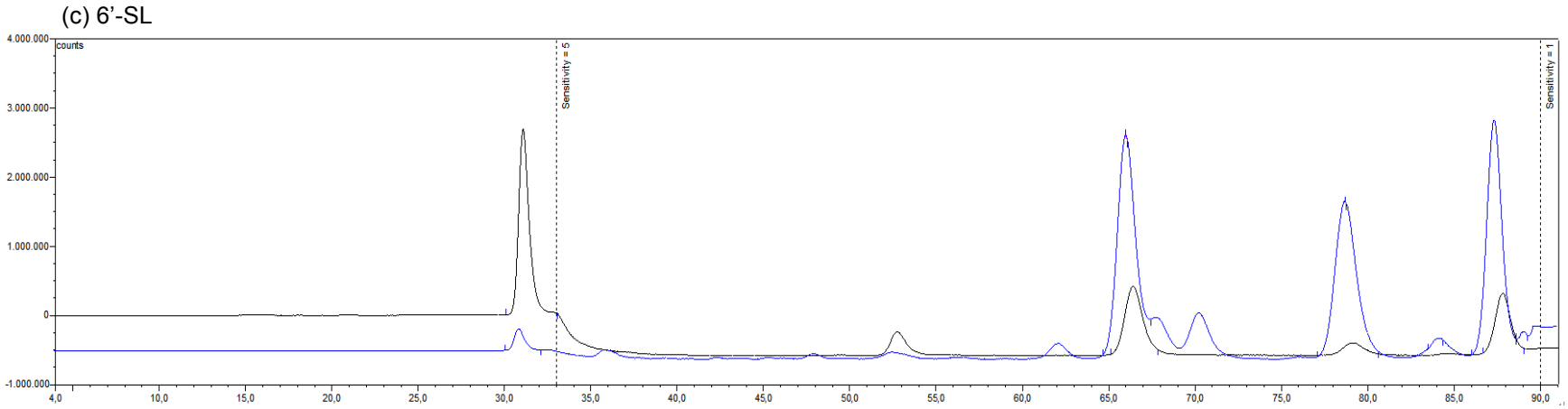


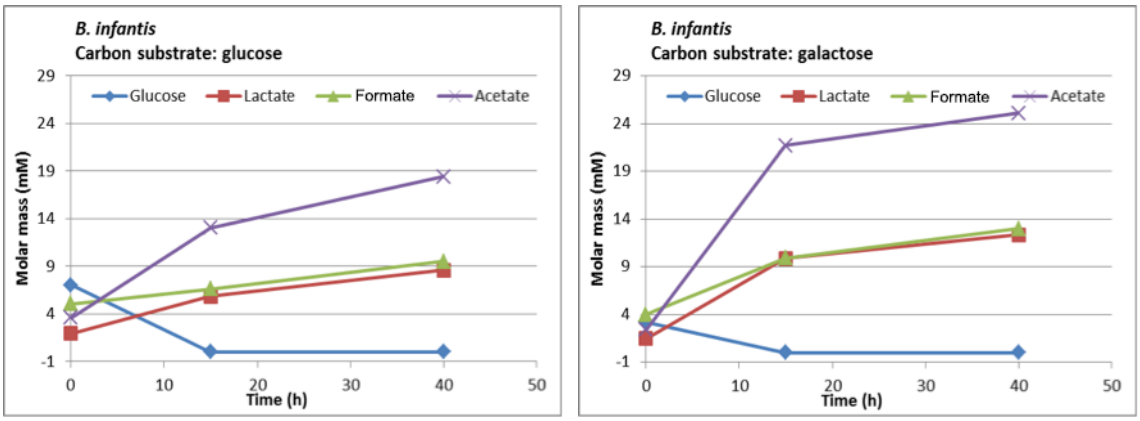
Figure 4-16 RP-HPLC Chromatograms of different carbon substrates in the medium before (black) and after cultivation by *B. vulgatus* (blue). The carbon substrates were as follows: (a) neutral HMO fractions from Le0; (b) acidic HMO fractions from Le0; (c) single standard 6'-SL.

The chromatograms from Figure 4-16(a) showed that after cultivation, most of carbon substrates of neutral HMO fractions from Lewis negative could be metabolized by *B. vulgatus*, and during the cultivation, a new peak of components was produced. In Figure 4-16(b), the chromatograms showed that most components of acidic HMO fractions from Lewis negative could be metabolized by *B. vulgatus*, and in the meanwhile, two peaks of components were increased and there was also one new peak appeared. For the single standard 6'-SL, the peak of 6'-SL was largely decreased, but there were some peaks increased after the cultivation.

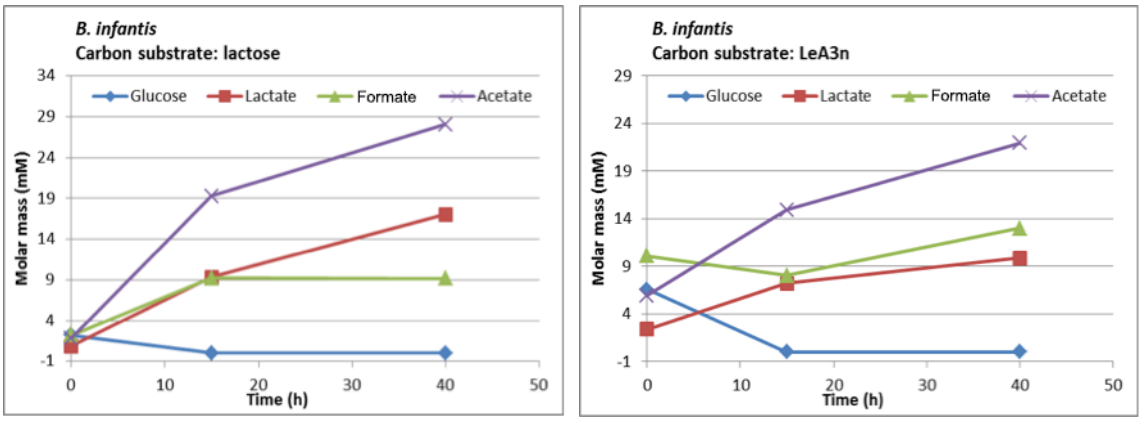
4.7 Analysis of metabolic products of cultivation

To investigate the metabolic products of cultivation, the samples were taken for HPLC at the 0 h, 15 h and 40 h from cultivation. The amount of glucose, lactate, formate and acetate were quantified by HPLC analysis. Figure 4-17 shows the amount of glucose and metabolic products from the culture of *B. infantis* on different carbon substrates, such as glucose, galactose, lactose, neutral and acidic HMO fractions, as well as single HMO standards. Figure 4-18 shows the amount of glucose and metabolic products from the culture of *B. longum* on different carbon substrate, such as glucose, galactose, lactose, neutral and acidic HMO fractions and single HMO standard.

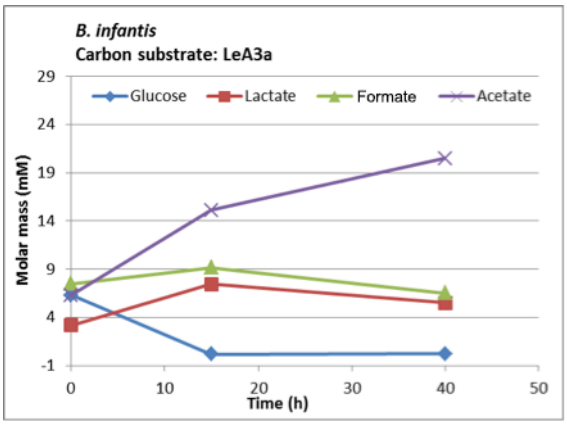
(a)

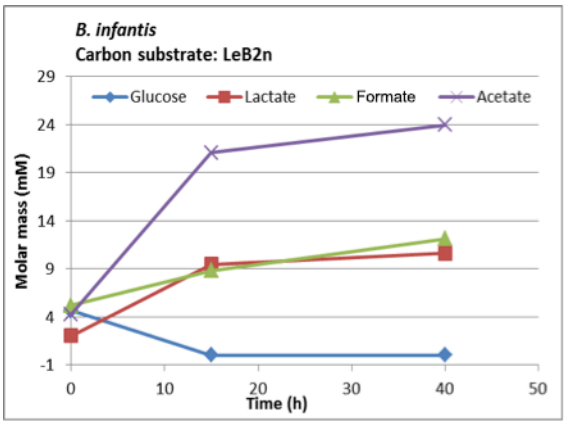


(b)

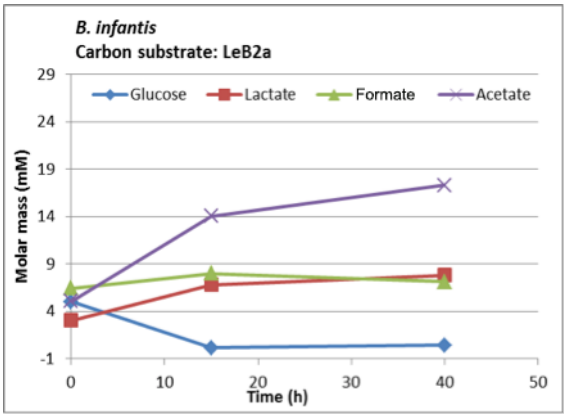


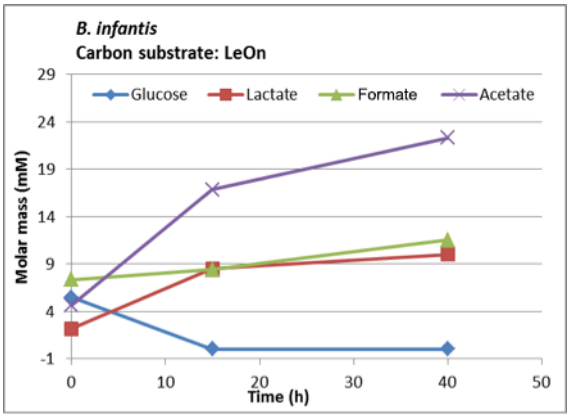
(c)


B. infantis
Carbon substrate: LeA3a
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

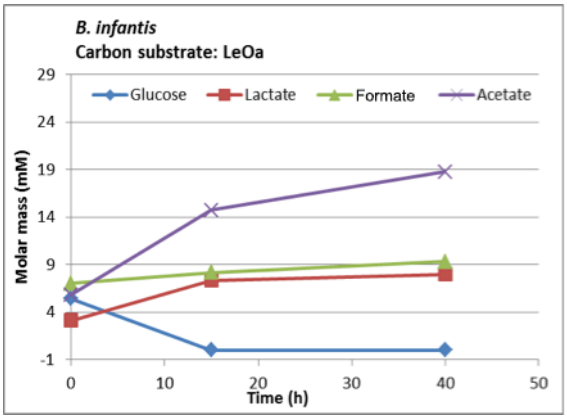

B. infantis
Carbon substrate: LeB2n
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

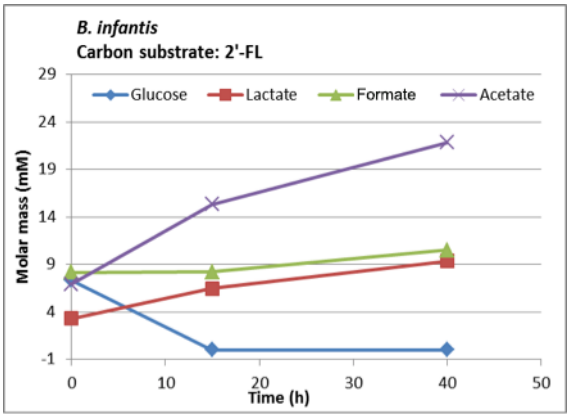
(d)


B. infantis
Carbon substrate: LeB2a
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

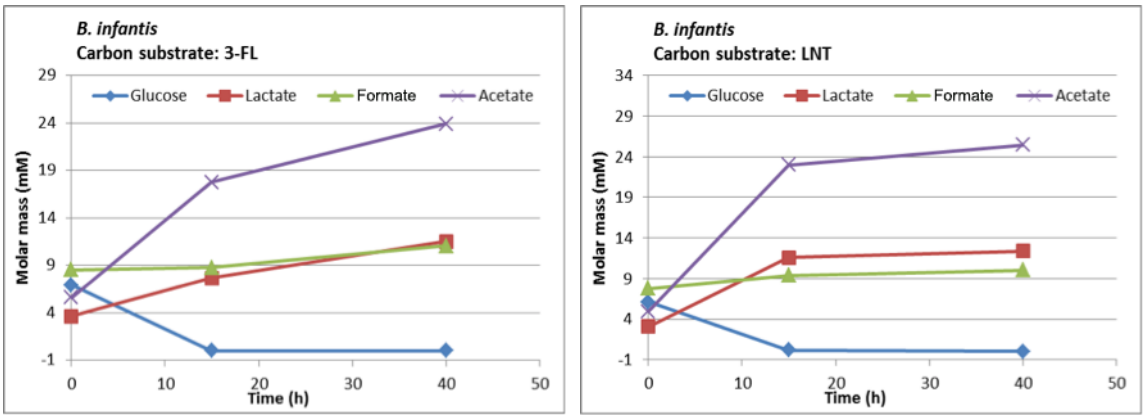

B. infantis
Carbon substrate: LeOn
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

(e)


B. infantis
Carbon substrate: LeOa
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)


B. infantis
Carbon substrate: 2'-FL
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

(f)



(g)

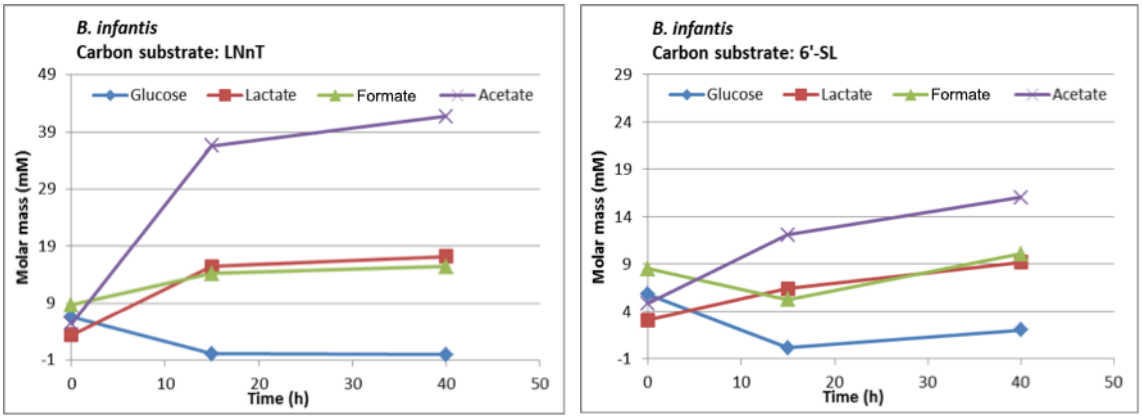
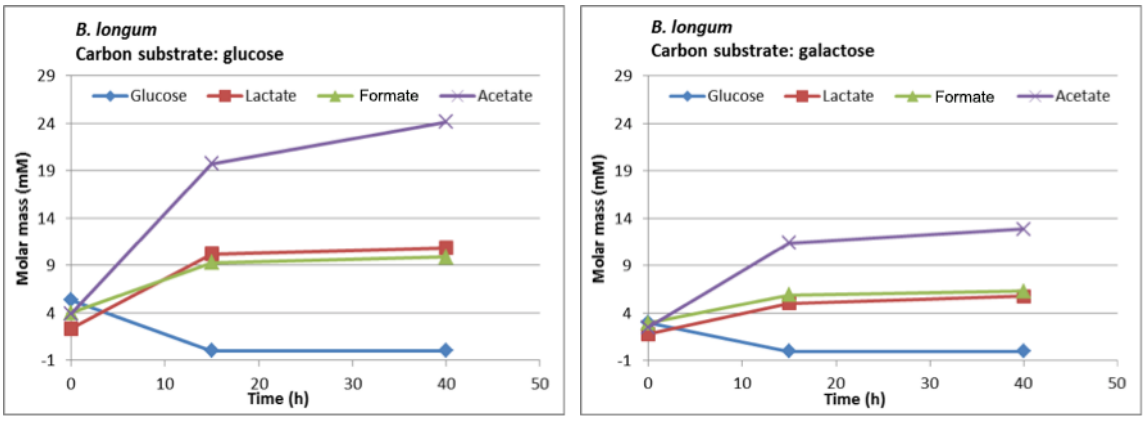


Figure 4-17 Analysis of metabolic products from *B. infantis* grown in *Bifidobacterium* medium on different carbon substrates. (a) Glucose (left) and galactose (right). (b) Lactose (left) and the neutral HMO fractions from LeA3 (right). (c) Acidic HMO fractions from LeA3 (left) and neutral HMO fractions from LeB2 (right). (d) Acidic HMO fractions from LeA2 (left) and neutral HMO fractions from Le0 (right). (e) Acidic HMO fraction from Le0 (left) and HMO standard 2'-FL (right). (f) HMO standard 3-FL (left) and HMO standard LNT (right). (g) HMO standard LNnT (left) and 6'-SL (right).

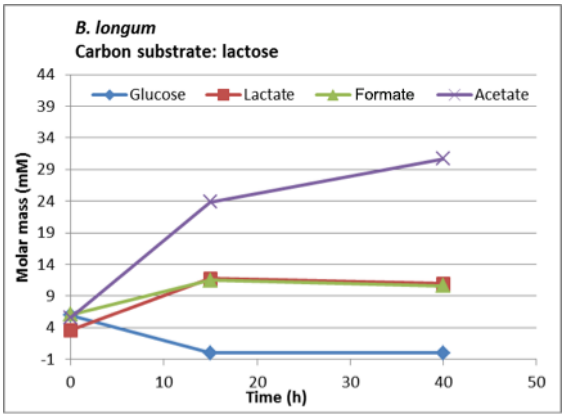
The chromatograms above from HPLC showed that *B. infantis* could metabolize the carbon substrates and produce mainly lactic acid, acetic acid, and some formic acid. The amount of glucose showed that the initial amount of glucose were from 3 to 7 mM, and glucose can be metabolized within 15 hours, and for most carbon substrates, the amount of glucose stayed at 0 mM until the end, except the case for 6'-SL, the glucose increased to 2 mM after 15hours. For the product lactate, the initial amount was between 3 and 4 mM, and in the first 15hours, it showed an increase for all the carbon substrates. After 15 hours, most carbon substrates showed a

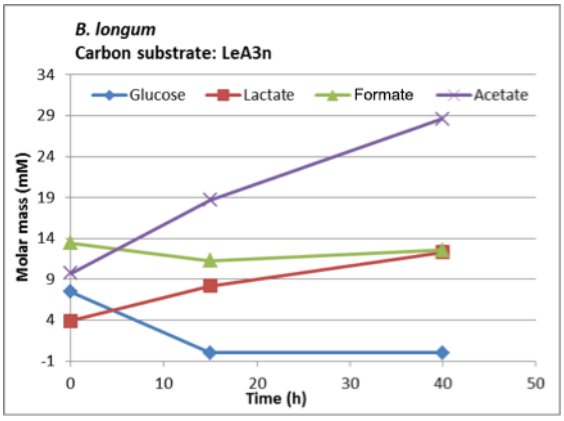
continuously increase by a lower increase rate. The exception occurred for the acidic HMO fractions from LeA3, the lactate was decreased. The amount of lactate in the end was mostly around 9 mM, and ranged up to about 19 mM. For the product formate, the initial amount was from 3 to 10 mM. About half of the carbon substrates showed an increase in the first 15 hours, two of the substrates showed a decrease, and the rest showed nearly the same. After 15 hours, most carbon substrates stayed the same and only four of them showed an increase, the rest stayed almost the same. The amount of formate in the end was around 9 mM. For the product acetate, the initial amount was from 3 to 8 mM. The amount of acetate showed an increase for all the carbon substrates in the first 15hours. For most carbon substrates, the amount of acetate reached to 14 to 20 mM. Only one of the carbon substrates was lower: the amount of 6'-SL was 12 mM and two of them were higher than 20 mM: the amount from LNT was about 23 mM and the amount from LNnT was about 37 mM. After 15 hours, the amount showed also a continuously increase for all the carbon substrate, but with a lower increase rate. The final amount of acetate was from 19 to 25 mM, with the exception of 6'-SL and LNnT. The amount from 6'-SL was 16 mM and the amount from LNnT was 25 mM.

(a)

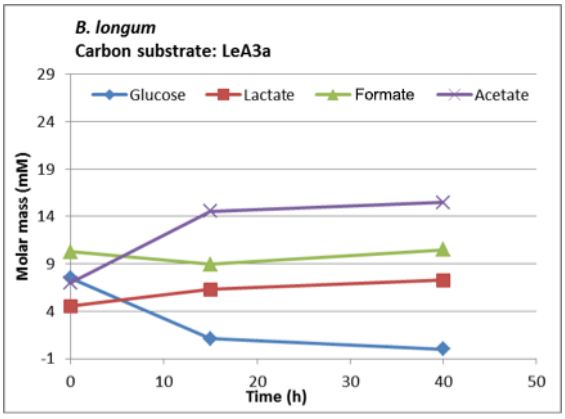


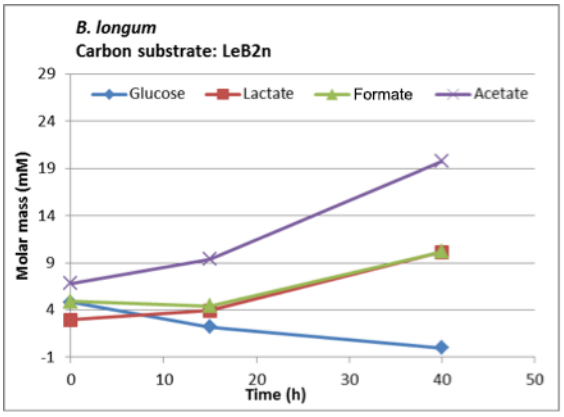
(b)


B. longum
Carbon substrate: lactose
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

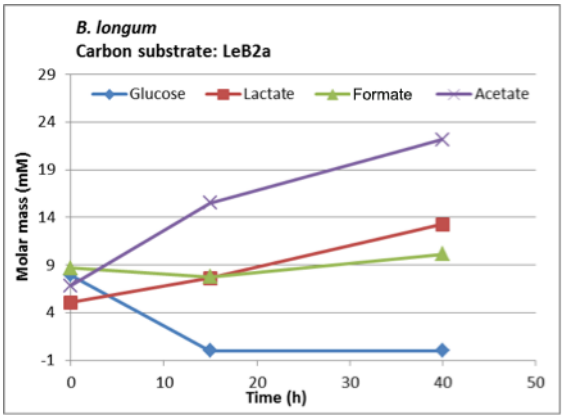

B. longum
Carbon substrate: LeA3n
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

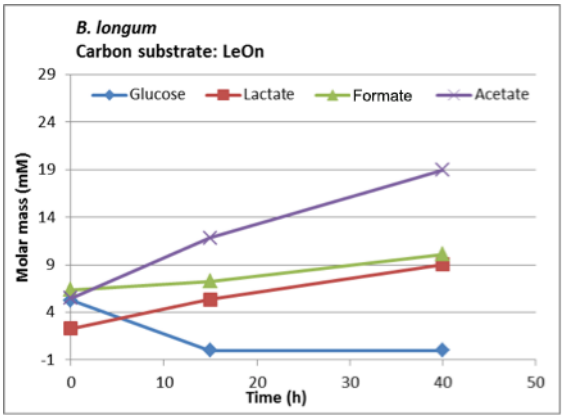
(c)


B. longum
Carbon substrate: LeA3a
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

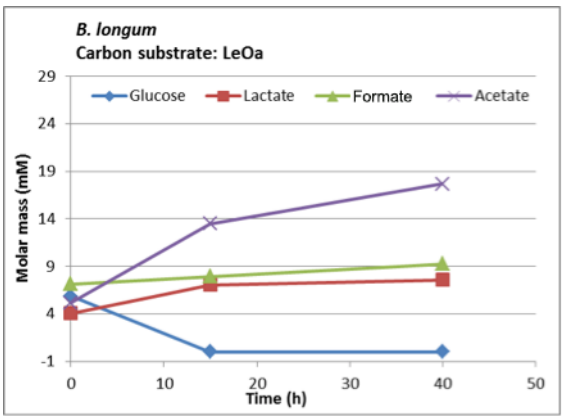

B. longum
Carbon substrate: LeB2n
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

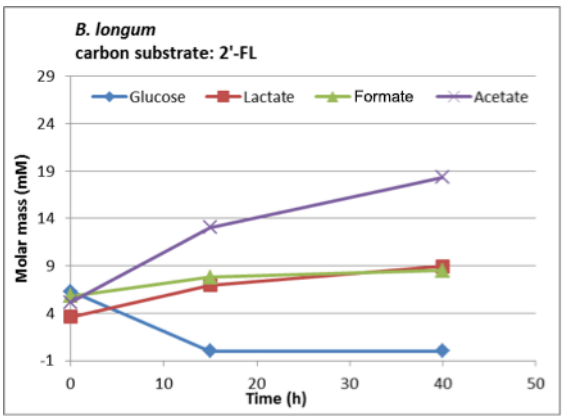
(d)


B. longum
Carbon substrate: LeB2a
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

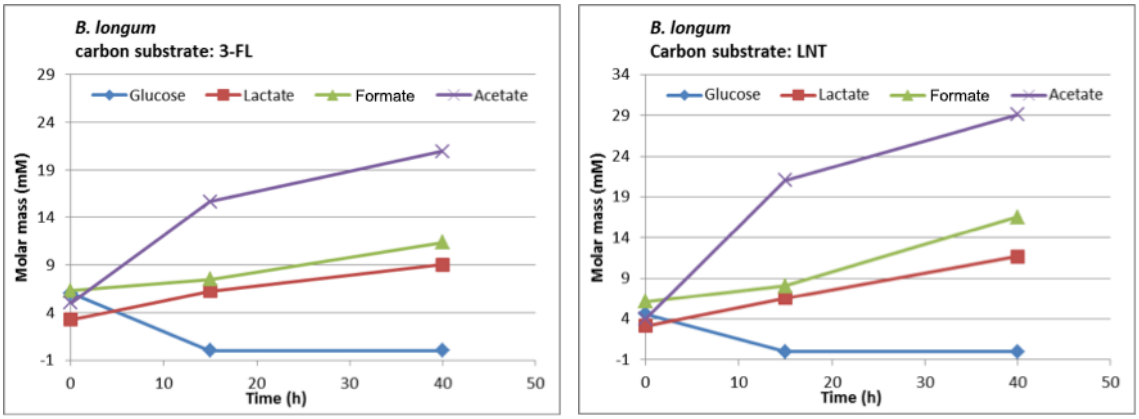

B. longum
Carbon substrate: LeOn
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

(e)


B. longum
Carbon substrate: LeOa
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)


B. longum
carbon substrate: 2'-FL
Glucose
Lactate
Formate
Acetate
Molar mass (mM)
Time (h)

(f)



(g)

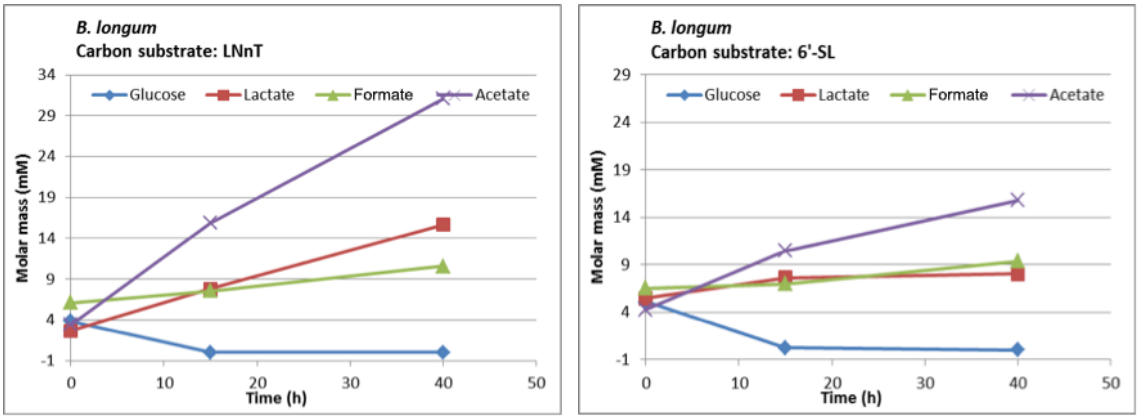


Figure 4-18 Analysis of metabolic products from *B. longum* grown in *Bifidobacterium* medium on different carbon substrates. (a) Glucose (left) and galactose (right). (b) Lactose (left) and neutral HMO fractions from LeA3 (right). (c) Acidic HMO fractions from LeA3 (left) and neutral HMO fractions from LeB2 (right). (d) Acidic HMO fractions from LeA2 (left) and neutral HMO fractions from Le0 (right). (e) Acidic HMO fraction from Le0 (left) and HMO standard 2'-FL (right). (f) HMO standard 3-FL (left) and HMO standard LNT (right). (g) HMO standard LNnT(left) and 6'-SL (right).

The chromatograms from HPLC showed that *B. longum* could also metabolic the carbon substrates and produce mainly lactic acid, acetic acid, and some formic acid. The amount of glucose showed that the initial amount of glucose were also from 3 to 8 mM. Glucose can be metabolized within 15 hours for most of the carbon substrates and stayed at 0 mM until the end. The exception occurred for the acidic HMO fraction from LeA3 and the neutral HMO fraction from LeB2, glucose was not metabolized within 15 hours, but the amount of glucose was also 0 mM in the end. For the product lactate, the initial amount was between 3 and 5 mM, and in the first 15 hours, it showed an increase for all the carbon substrates, increased by 2 to 5 mM.

After 15 hours, nine of the carbon substrates showed a continuously increase, in which one of carbon substrates increased by a higher increase rate, two of carbon substrates showed a lower increase rate, and the rest increase by the same increase rate. One of the carbon substrates showed a decrease after 15 hours and the rest stay the same after 15 hours. The amount of lactate in the end ranged from 5 to 15 mM. For the product formate, the initial amount was mostly around 5 mM, with three exceptions. Most of the carbon substrates showed an increase in the first 15 hours, three of the substrates showed a decrease, and the rest showed nearly the same. After 15 hours, seven of the carbon substrates showed a continuously increase and the rest stayed almost the same amount. The amount of formate in the end was around 10 mM, except the amount from LNT, about 17 mM. For the product acetate, the initial amount was from 4 to 10 mM. The amount of acetate showed an increase for all the carbon substrate in the first 15hours, the same as *B. infantis*. For most carbon substrates, the amount of acetate reached to 13 to 16 mM. Only three of the carbon substrates were around 10 mM: the amount of galactose, the neutral HMO fraction from LeB2 and the single standard 6'-SL. Three of them were about 20 mM: the amount of glucose, the neutral HMO fraction from LeA3 and the single standard LNT. The highest one was from lactose, reached up to 24 mM. After 15 hours, the amount showed also a continuously increase for all the carbon substrates, in which one of them showed a higher increase rate, three of them showed the same increase rate, and the rest showed a lower increase rate. The final amount of acetate ranged from 13 to 32 mM, in which galactose was about 13 mM, was the lowest, glucose was about 24 mM. Four of them were around 30 mM: lactose, neutral HMO fractions from LeA3, the single standard LNT and LNnT. The rest were around 19 mM, from 16 mM to 22 mM.

5 Discussion

5.1 Isolation of HMO fractions from human milk

Neutral and acidic HMO fractions were isolated from six human milk samples. In principle, from the whole HMO fractions, about 2/3 of the whole HMO fractions are neutral, about 1/3 are acidic. As shown in Table 4-1, the amounts of neutral HMO fractions from 50 ml human milk was 80 or 110 mg; the amounts of neutral HMO fractions from 100 ml human milk were from 170 to 274 mg, which were double values from 50 ml human milk, with some fractions higher. In principle, the amounts of acidic HMO fractions would be half of the neutral HMO fractions. But the values from HMO fractions were higher than half neutral fractions, and for LeA2, the amount of acidic fractions were higher than neutral fractions. The reasons for higher neutral and acidic fractions could be the following: first, the procedure of isolation from human milk were complex, there might be still some other components left in the neutral HMO fractions, such as lactose; second, the desalting of acidic HMO fractions were not that accurate, so that there were still some salts left; third, the acidic HMO fractions contained not only acidic HMOs, but also some other acidic fractions. The results indicated that the purity of the isolated HMO fractions were not as high as the single HMO standards, it could also explain the later results in microorganisms study.

5.2 Growth study of microorganisms on glucose, galactose and lactose

The growth study of *L. acidophilus* on glucose, galactose and lactose showed that the highest OD_{500nm} without carbon substrate was about 0.2. The value with 1 mM glucose or galactose was about 0.3, the difference from the value without carbon substrate was about 0.1. The values with 2 mM glucose, 2 mM galactose or 1 mM lactose were about 0.4, the difference from the value without carbon substrate was about 0.2. Thus, when the amount of glucose or galactose was doubled, the difference was also doubled. The value with 1 mM lactose was similar to 2 mM glucose or galactose. The reason is that 1 mol lactose can be hydrolyzed into 1 mol glucose and 1 mol galactose. The value with 2 mM lactose was about 0.6, the

difference from the value without carbon substrate was about 0.4, the difference was also doubled when the amount of lactose was doubled.

The growth study of *L. lactis* on glucose, galactose and lactose showed that the highest OD_{500nm} without carbon substrate was about 0.4. The value with 1 mM glucose was about 0.55 and the value with 1 mM galactose was about 0.5. The reason could be the structure difference of glucose and galactose, *L. lactis* couldn't utilize the galactose as well as glucose. The highest OD_{500nm} with 2 mM glucose was about 0.7; the difference from the value without carbon substrate was doubled compared with 1 mM glucose. The value with 2 mM galactose was reduced to about 0.45, and the value with 5 mM galactose was similar to 2 mM galactose. The results showed that the high concentration of galactose could inhibit the growth of *L. lactis*. The reason may be that the galactose had the feedback inhibition. The highest OD_{500nm} with 1 mM lactose was about 0.65, the value with 2 mM lactose was about 0.45 and the value with 5 mM lactose was about 0.5. The reason could be that when the concentration of lactose was 2 mM, the inhibit effect of galactose was dominant. When the concentration of lactose increased to 5 mM, there may be more glucose hydrolyzed and the growth increased a little bit.

5.3 Growth study of microorganisms on HMO fractions and HMO standard

5.3.1 *Lactococcus lactis subsp. lactis*

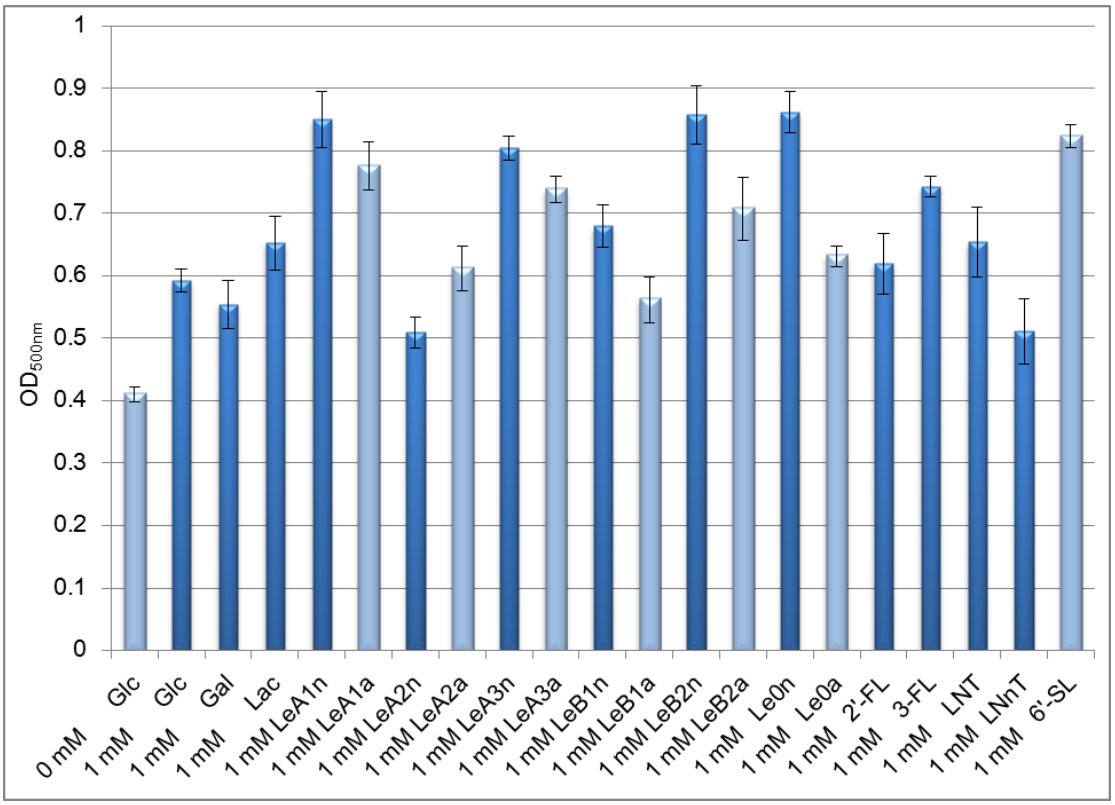


Figure 5-1(a) Comparison of the highest OD_{500nm} (Mean ± SD, n=3) of *L. lactis* grown in MRS medium (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-1(a), the highest OD_{500nm} from different substrate utilized by *L. lactis* can be compared. The OD_{500nm} without carbon substrate was the lowest. The highest OD_{500nm} with the two kinds of mono-saccharide, glucose and galactose, were higher, because glucose and galactose can supply carbon substrate to the bacteria. The OD_{500nm} of lactose, which belongs to di-saccharide, was even higher, because 1 mol lactose contains 1 mol glucose and 1mol galactose. The HMO fractions are the mixture of oligosaccharides with different DP (the degree of polymerization, DP ≥3), the highest OD_{500nm} of the six kinds of neutral HMO fractions were mostly higher than

the OD_{500nm} from lactose with the exception of LeA2n. The highest OD_{500nm} for the acidic HMO fractions showed general lower values compared with the corresponding neutral fractions. The reason may be the different components from neutral and acidic HMO fractions, the neutral HMO fractions have a similar pH values to the medium (pH 6.0 to 6.5), while the acidic HMO fractions have lower pH, which may decrease the pH value of the medium and thereby affect to low pH for optimal growth of bacteria. Another possible reason was that there may be some other component in the acidic HMO fractions, it is not pure acidic HMO, but contained some other acidic substrates, like some salts and so on. The salt may be inhibitory for the growth. The highest OD_{500nm} from the five single HMO standards were similar to lactose, in which the OD_{500nm} from LNnT was lower and the OD_{500nm} from 6'-SL was higher. The 2'-FL and 3-FL are the tri-saccharides, and the LNT and LNnT are tetra-saccharides. In principle, the tri-saccharides contain three monosaccharides, but *L. lactis* may not contain all the enzymes to hydrolyze tri-saccharides to the monosaccharide. For the tetra-saccharides, the reason was the same. The reason why the LNnT was lower could be that the LNnT is the new pattern of LNT, which may contains different bond from LNT, and then the *L. lactis* may utilize LNT better than LNnT. From the comparison of the HMO fractions with Lewis a, Lewis b and Lewis 0 specificity, there was no distinct difference between the Lewis patterns. The highest OD_{500nm} of the HMO fractions from LeA1, LeA3, LeB2 and Le0 were similar. The bacteria didn't grow well on HMO fractions from LeA2 and LeB1, especially the neutral fractions from LeA2, even lower than the acidic fractions from LeA2. The reason may be that the number of samples for each Lewis group was too small to represent a group of fractions. Another reason could be that the individual differences from each milk sample since, the donate mothers were distinct from each other.

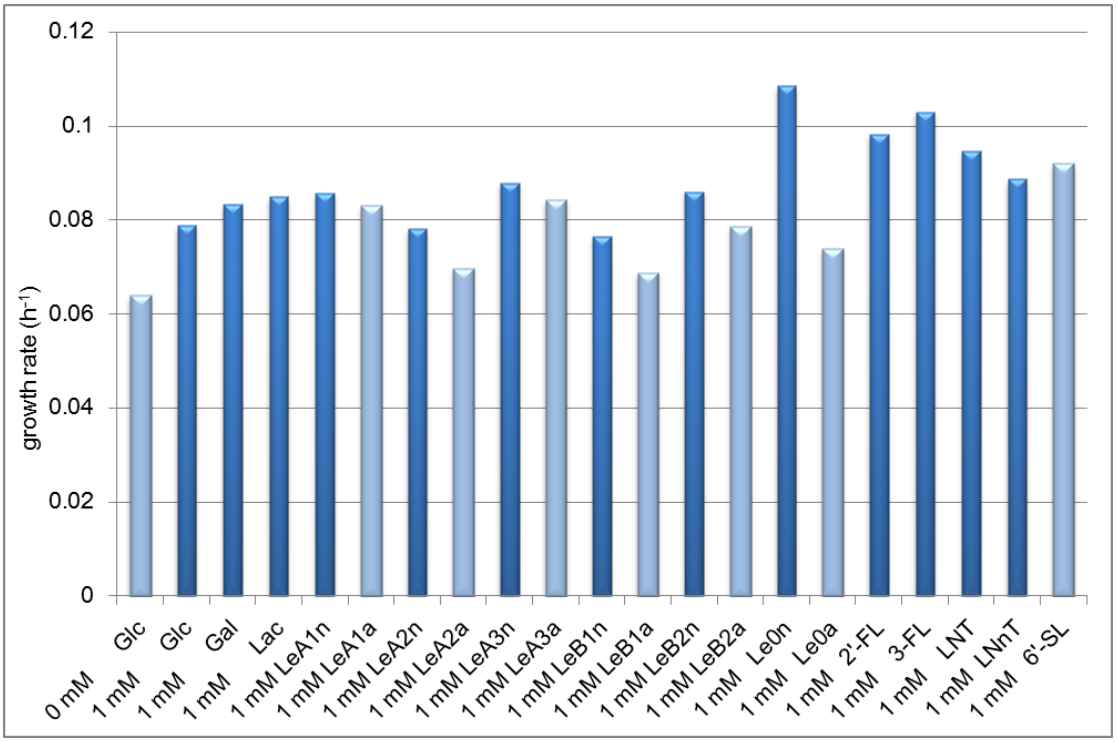


Figure 5-1(b) Comparison of the growth rate (h^{-1}) from *L. lactis* grown in MRS medium (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

The growth rate, which reflects the growth speed of the microorganisms, is another important factor to describe growth of the microorganisms. It describes the increase of OD_{500nm} per time unit. From the Figure 5-1(b), the growth rate from *L. lactis* grown on different substrates can be compared. Without any carbon substrate, the growth rate was about 0.06, was also the lowest compared with other substrates. The reason is the limited amount of carbon substrate. The growth rates from HMO fractions were mostly from 0.07 to 0.09, except the growth rate from the Le0n sample, which reached to about 0.11. The growth rates from the five single HMO standards were almost higher than those from the HMO fractions. The reason may be related to the purity of the HMOs, the single HMO standards were with higher purity compared with the HMO fractions.

From the results combined with the highest OD_{500nm} and the growth rate, the following conclusions can be drawn: lactose, neutral fractions from LeA1, neutral and

acidic fraction from LeA3, neutral fractions from LeB2, 3-FL, LNT and 6'-SL were substrates which seem to have beneficial effect on the growth of *L. lactis*.

5.3.2 *Lactobacillus acidophilus*

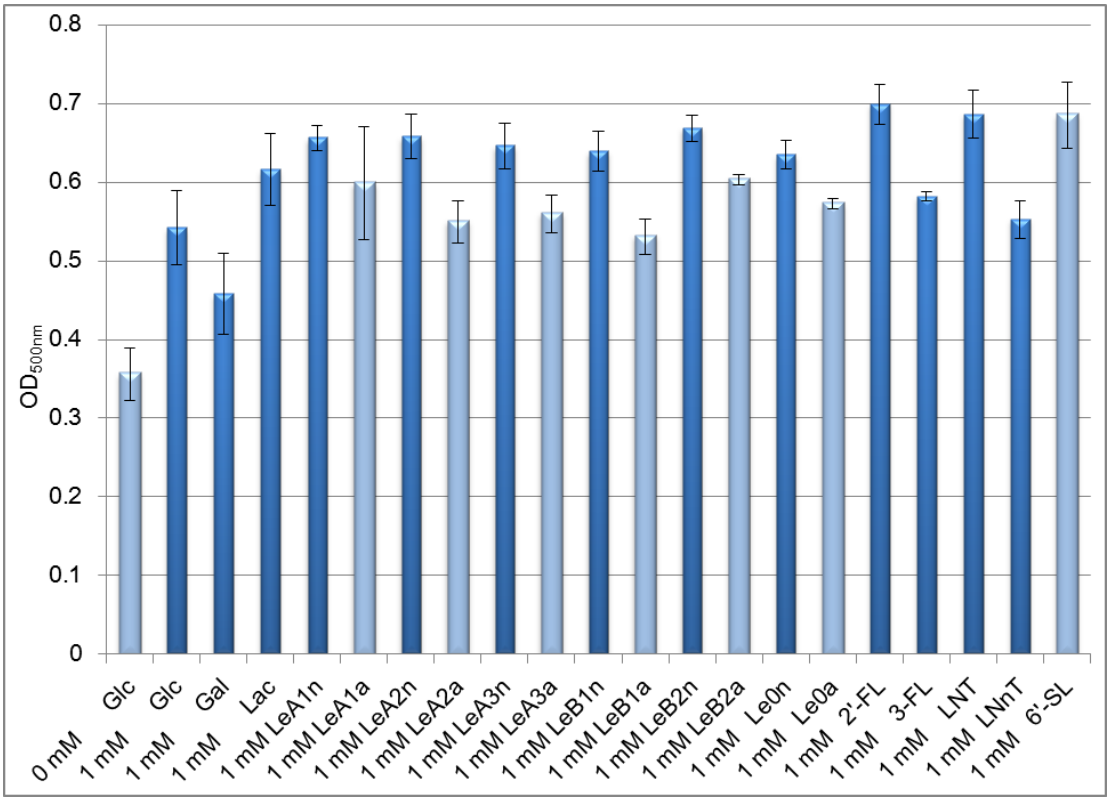


Figure 5-2(a) Comparison of the highest OD_{500nm} (Mean ± SD, n=3) of *L. acidophilus* grown in MRS medium plus L-cysteine (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-2(a), the highest OD_{500nm} from different substrates utilized by *L. acidophilus* can be compared. The highest OD_{500nm} without carbon substrate, glucose, galactose and lactose were similar to *L. lactis*. The highest OD_{500nm} of the five kinds of neutral HMO fractions were all higher than the values from lactose and the corresponding acidic HMO fractions. The highest OD_{500nm} from the single HMO standard 2'-FL, LNT and 6'-SL showed the highest OD_{500nm} compared with all the other substrates. The highest OD_{500nm} of single HMO standards 3-FL and LNnT were

between glucose and lactose. The reason could be the structure difference from 2'-FL and 3-FL, LNT and LNnT. The bacteria may secrete the enzyme that can hydrolyze the chemical bonds in 2'-FL and LNT, but fewer bonds in 3-FL and LNT can be hydrolyzed. From the comparison of the HMO fractions with different Lewis specificity, the values from the six HMO fractions were all similar. Not only on distinct from different Lewis pattern, but also not distinct from different HMO fractions with the same Lewis specificity.

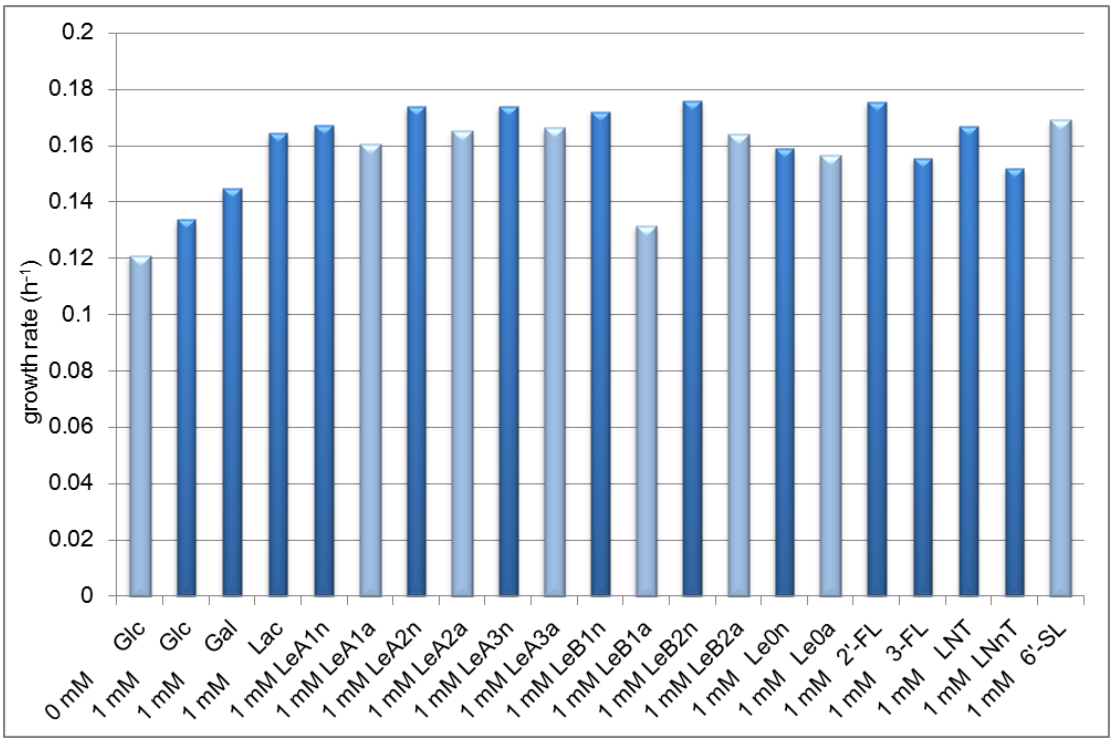


Figure 5-2(b) Comparison of the growth rate (h^{-1}) from *L. acidophilus* grown in MRS medium plus L-cysteine (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-2(b), the growth rate from *L. acidophilus* grown on different substrates can be compared. Without any carbon substrate, the growth rate is about 0.12, was also the lowest compared with other substrates. The growth rates of glucose and galactose were around 0.14, and the growth rate of lactose was about

0.16. The growth rates from HMO fractions were mostly higher than glucose and galactose, between 0.16 and 0.18, except the growth rate from LeB1a sample, which was about 0.13. The growth rates from neutral fractions were all higher than the corresponding acidic fractions. The growth rates from the five single HMO standards had the similar values from HMO fractions.

From the results combined with the highest OD_{500nm} and the growth rate, the following conclusions can be drawn: lactose, neutral fractions from LeA1, LeA2, LeA3, LeB1, LeB2, acidic fraction from LeA1, LeB2 and single standard 2'-FL, 3-FL and 6'-SL were substrates which seem to have beneficial effect on the growth of *L. acidophilus*.

5.3.3 *Bifidobacterium longum subsp. infantis*

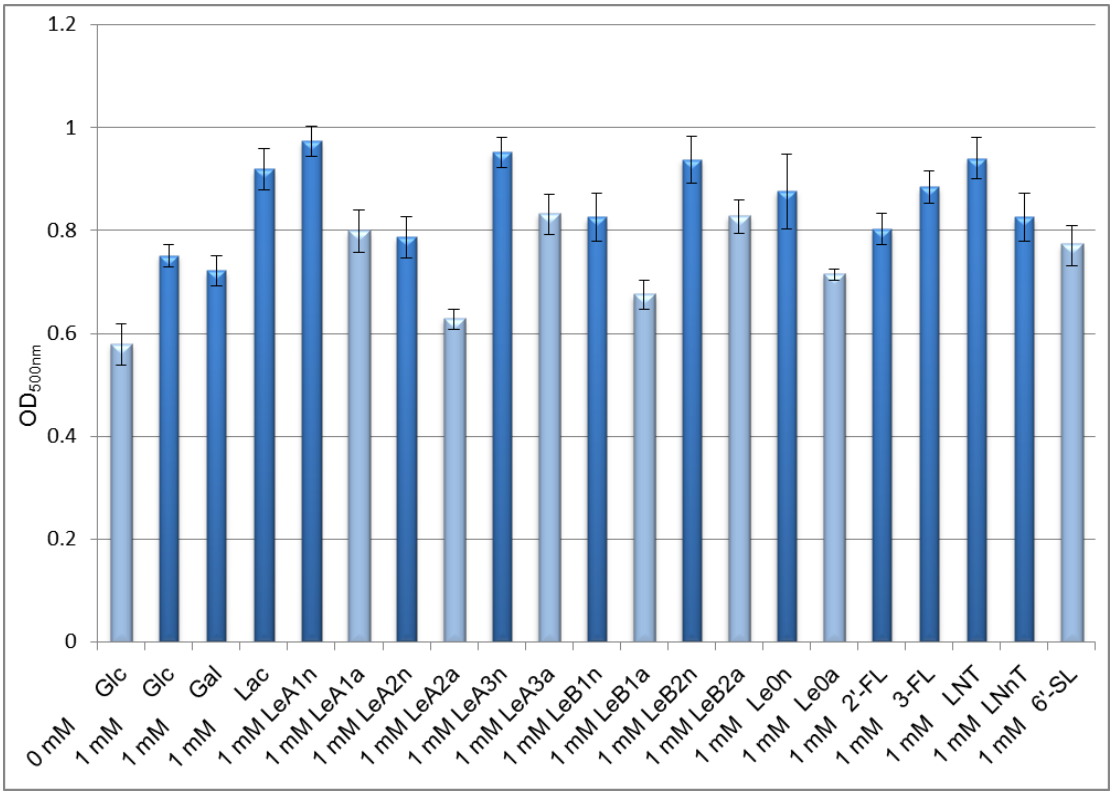


Figure 5-3(a) Comparison of the highest OD_{500nm} (Mean ± SD, n=3) of *B. infantis* grown in *Bifidobacterium* medium (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-3(a), the highest OD_{500nm} from different substrates utilized by *B. infantis* can be compared. The highest OD_{500nm} of the six kinds of neutral HMO fractions were all higher than that of glucose, in which three of them were higher than that of lactose. The highest OD_{500nm} from neutral HMO fractions were between 0.8 and 0.9, the values from the acidic HMO fractions were between 0.6 and 0.8. The highest OD_{500nm} of the five single HMO standards showed that they were all higher than those of glucose and galactose, but only the OD_{500nm} of LNT was higher than lactose. The highest OD_{500nm} of 6'-SL was the lowest among the five. The reason could be that it is acidic HMO standard. From the comparison of the HMO fractions with different Lewis specificity, there were no difference from Lewis a, Lewis b and Lewis 0 pattern. The results seem to be different from individual HMO samples, but not depending on the Lewis blood type.

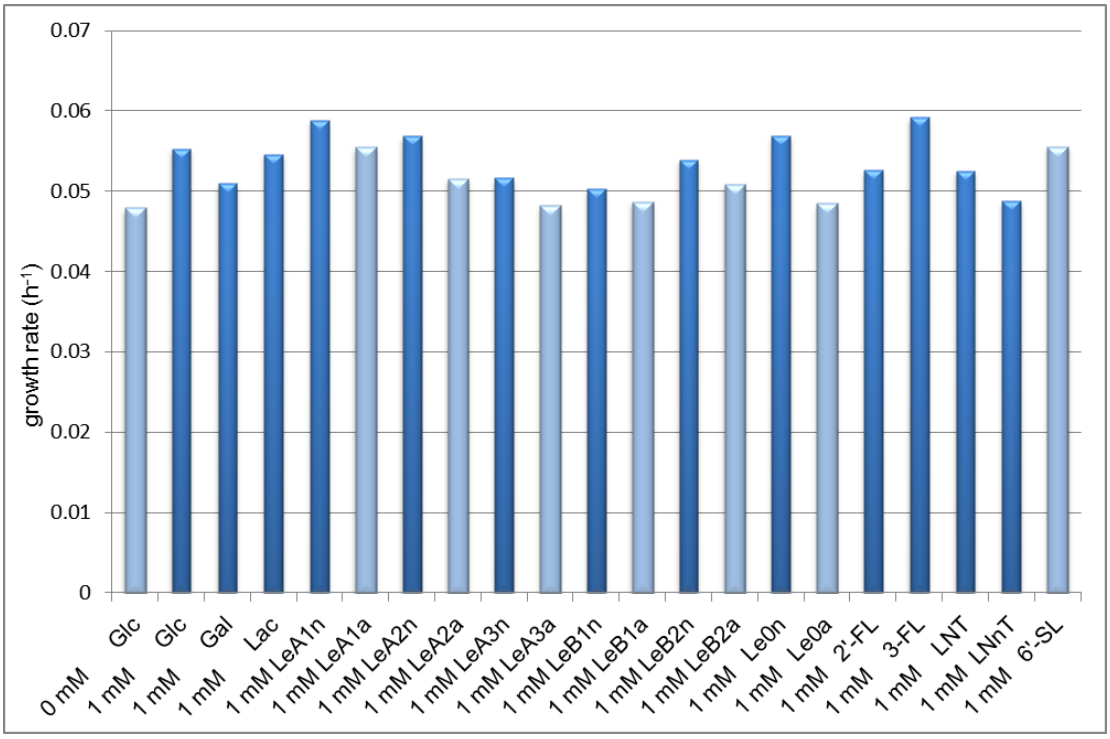


Figure 5-3(b) Comparison of the growth rate (h^{-1}) from *B. infantis* grown in *Bifidobacterium* medium (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-3(b), the growth rate from *B. infantis* grown on different substrates can be compared. The growth rates from the six neutral HMO fractions were between 0.05 and 0.06, while the growth rates from the six acidic HMO fractions were all lower than the corresponding neutral fractions, and the values were around 0.05. The growth rates from the five single HMO standards ranged from 0.049 to 0.059. The growth rates from 2'-FL and LNT were similar, the value from 3-FL was higher and the value from LNnT was lower. The reason may be that the bacteria prefer the structure of 3-FL rather than 2'-FL, and the structure of LNnT was more difficult than LNT for the bacteria to metabolize.

From the results combined with the highest OD_{500nm} and the growth rate, the following conclusions can be drawn: lactose, neutral fractions from LeA1, LeA3, LeB2, and Le0, acidic fraction from LeB2 and single standard 3-FL and LNT were substrates which seem to have beneficial effect on the growth of *B. infantis*.

5.3.4 *Bifidobacterium longum subsp. infantis vs. Bifidobacterium longum subsp. longum*

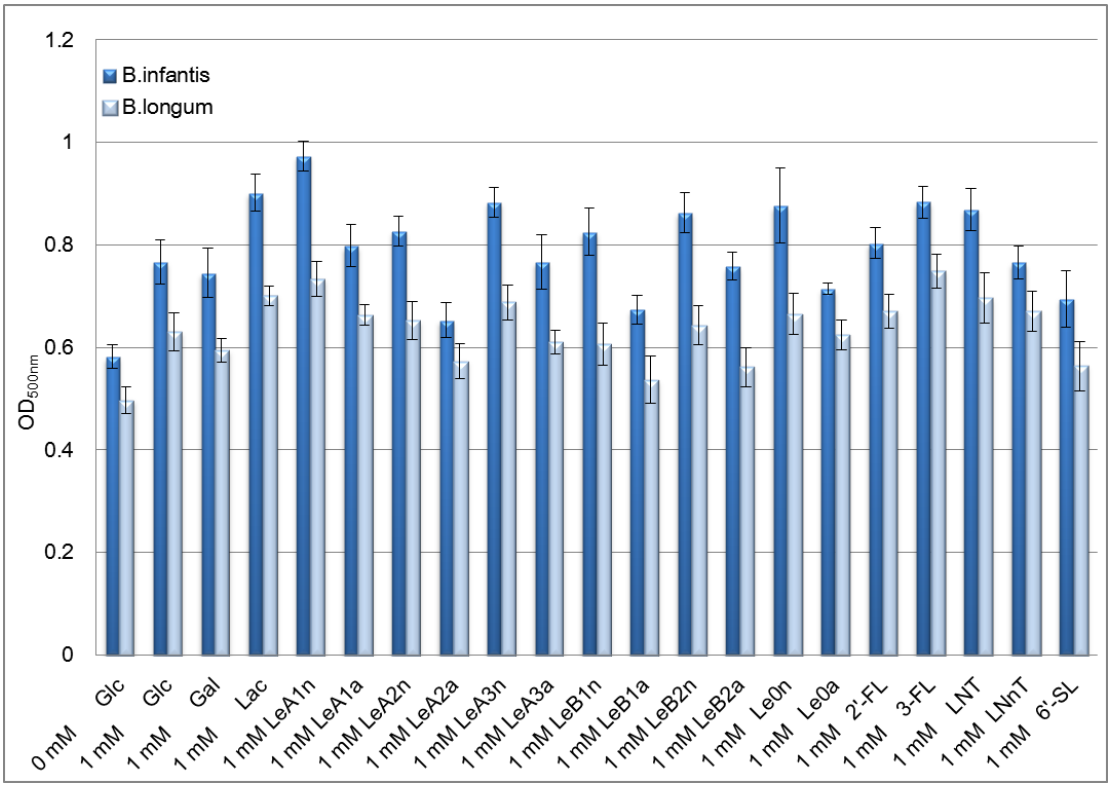


Figure 5-4(a) Comparison of the highest OD_{500nm} (Mean ± SD, n=3) of the growth from *B. infanis* vs. *B. longum* grown in *Bifidobacterium* medium (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the five different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-4(a), the highest OD_{500nm} from different substrates utilized by *B. infantis* and *B. longum* can be compared. For all the carbon substrates, the values from *B. infantis* were higher than the corresponding values from *B. longum*. The highest OD_{500nm} without substrates for both bacteria were the lowest compared with other carbon substrates. For the substrates glucose, galactose, lactose and six kinds of HMO fractions, the OD_{500nm} differences of both bacteria were similar, mostly about 0.2. For the 5 kinds of single standard, the OD_{500nm} differences between the two bacteria were smaller, mostly about 0.1.

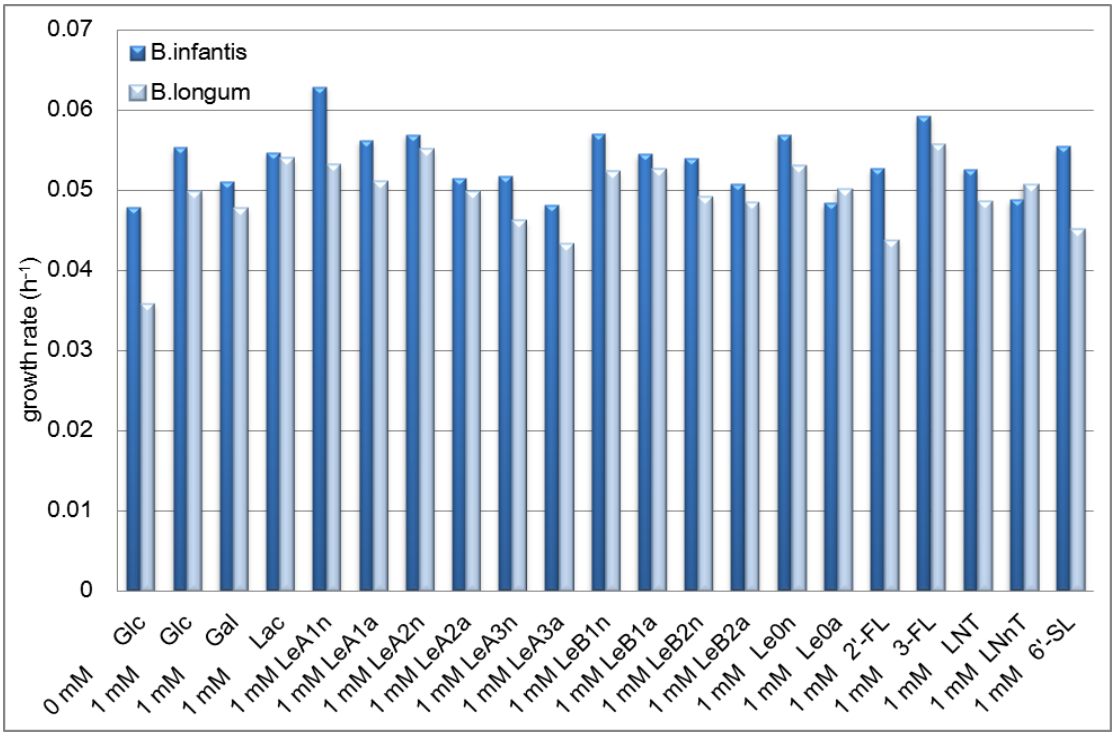


Figure 5-4(b) Comparison of the growth rate (h^{-1}) from *B. infantis* vs. *B. longum* grown in *Bifidobacterium* medium (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards.

The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-4(b), the growth rate from *B. infantis* and *B. longum* grown on different substrates can be compared. For all the carbon substrates, the values from *B. infantis* were mostly higher than the corresponding values from *B. longum* with only two exceptions: HMO fractions from Le0a and the single standard LNnT. The growth rate without substrates for *B. longum* was the lowest compared with other carbon substrates, and the value for *B. infantis* was similar to the values from HMO fractions from LeA3a, Le0a, and single standard LNnT.

From the results combined with the highest OD_{500nm} and the growth rate, the following conclusions can be drawn: the growths of B. infantis on all the carbon substrates were better the growth of *B. longum*.

5.3.5 *Bacteroides vulgatus*

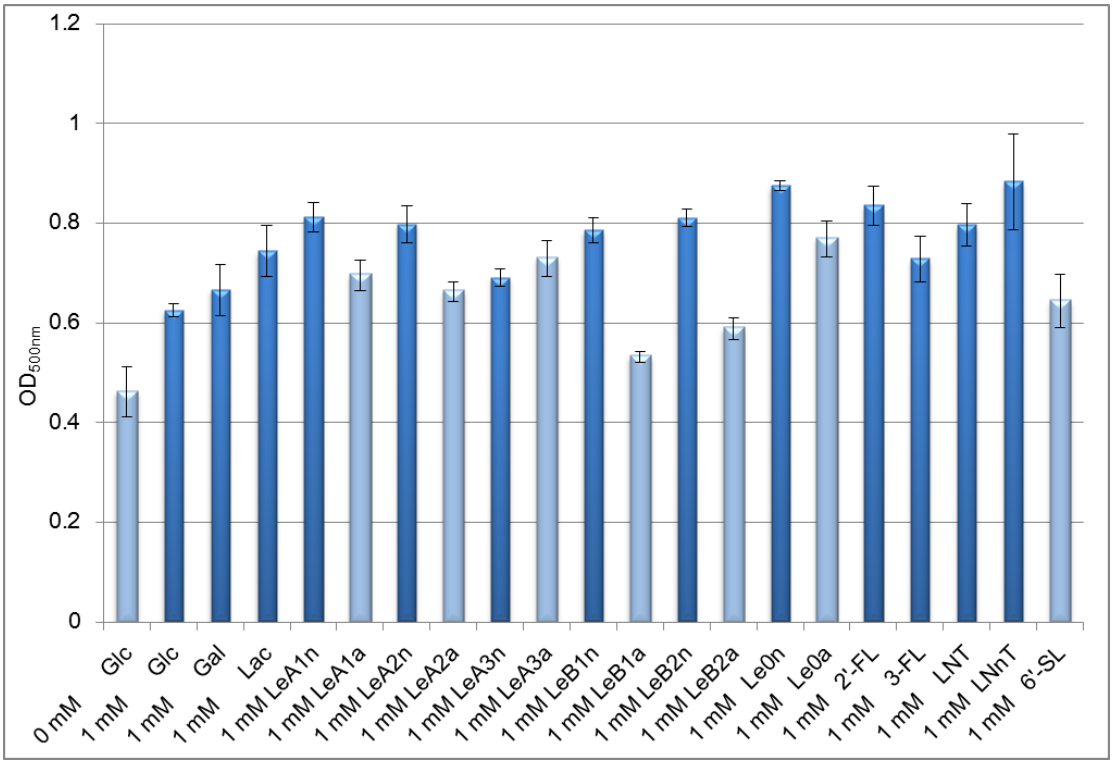


Figure 5-5(a) Comparison of the highest OD_{500nm} (Mean ± SD, n=3) of *B. vulgatus* grown in Modified Reinforced Clostridial Broth (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO

standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-5(a), the highest OD_{500nm} from different substrates utilized by *B. vulgatus* can be compared. The highest OD_{500nm} of the six kinds of neutral HMO fractions were higher than that of glucose or galactose, in which five of them were higher than that of lactose. The highest OD_{500nm} from neutral HMO fractions were mostly higher than the corresponding acidic HMO fractions with the exception of LeA3. The highest OD_{500nm} of the four kinds of neutral single HMO standards were all higher than those of glucose and galactose, in which three of them were higher than that of lactose, except 3-FL. The value from the acidic single standard 6'-SL was similar to glucose and galactose. From the comparison of the neutral HMO fractions with Lewis a, Lewis b and Lewis 0 specificity, there was no distinct difference between the Lewis patterns. From the comparison of the acidic HMO fractions with Lewis a, Lewis b and Lewis 0 specificity, the values from LeA were around 0.7, the values from LeB were between 0.5 and 0.6, the value from Le0 was about 0.8. From the results, it seems that the acidic fraction from Le0 was the best for the growth of bacteria, while the acidic fractions from LeB were not as good as the other two Lewis pattern.

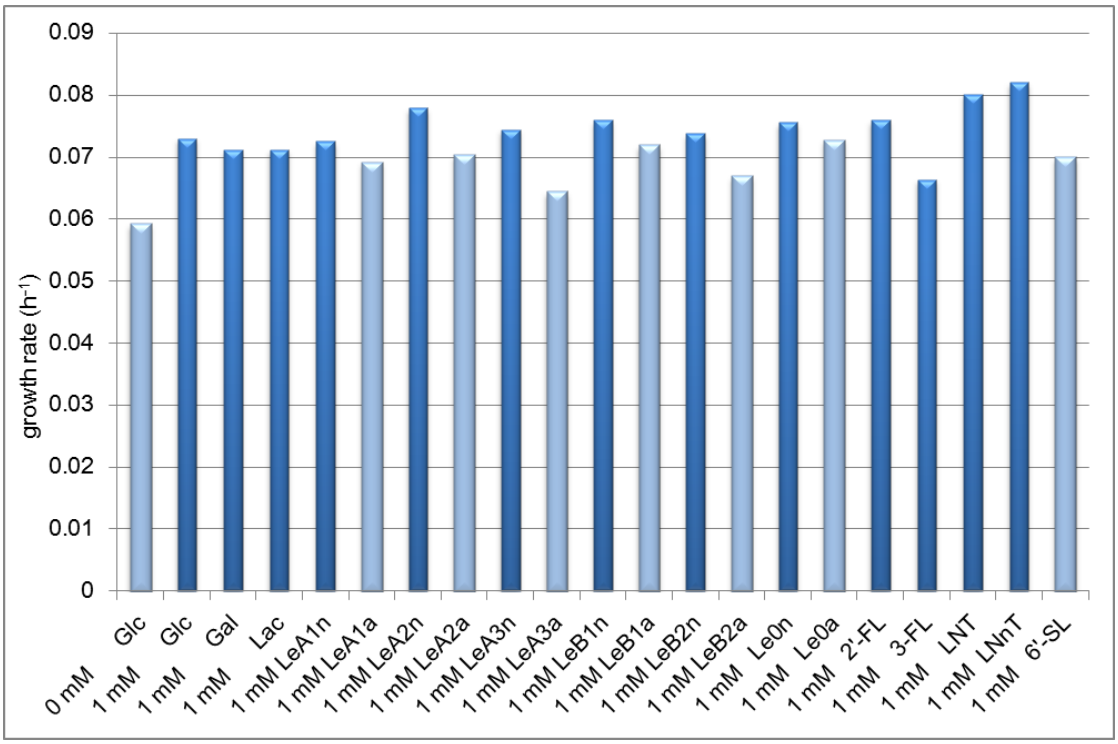


Figure 5-5(b) Comparison of the growth rate (h^{-1}) from *B. vulgatus* grown in Modified Reinforced Clostridial Broth (without glucose) with different substrates. The dark columns show the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standards. The light columns show 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-5(b), the growth rate from *B. vulgatus* grown on different substrates can be compared. The growth rates from the six neutral HMO fractions were around 0.075, which were all a little bit higher than glucose, galactose and lactose, while the growth rates from the six acidic HMO fractions were all lower than the corresponding neutral HMO fractions, the values were from 0.065 to 0.072. The growth rates from the five single HMO standards were from 0.067 to 0.082. The growth rates from 2'-FL and 3-FL were around 0.07, the value from 2'-FL was higher than 3-FL, the reason may be that the bacteria prefer to metabolize the structure of 2'-FL. The growth rates from LNT and LNnT were similar, both about 0.08. The growth rate from 6'-SL was about 0.07.

From the results combined with the highest OD_{500nm} and the growth rate, the following conclusions can be drawn: lactose, neutral fractions from LeA1, LeA2, LeB1, LeB2, LeB2 and Le0, acidic fractions from Le0, single standard 2'-FL, LNT and LNnT were substrates which seem to have beneficial effect on the growth of *B. vulgatus*.

5.3.6 *Cronobacter sakazakii*

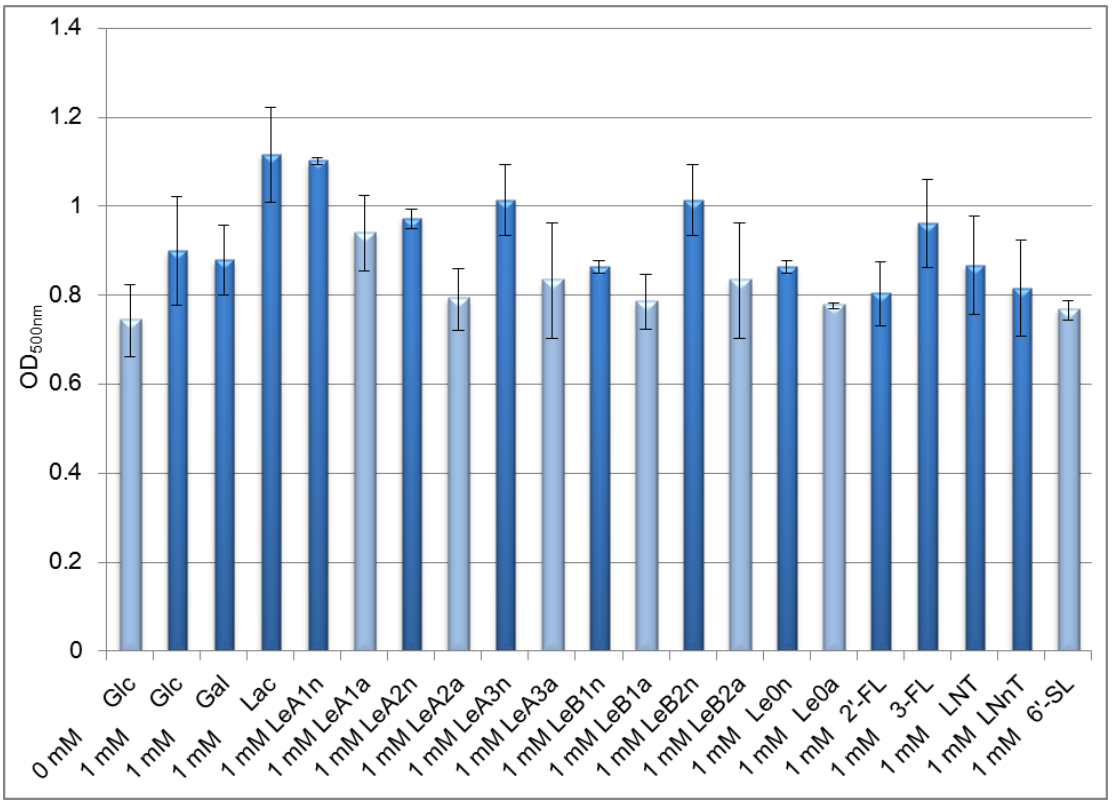


Figure 5-6(a) Comparison of the highest OD_{500nm} (Mean ± SD, n=3) of *C. sakazakii* grown in MRS medium (without glucose) with different substrates. The dark columns showed the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standard. The light columns showed 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-6(a), the highest OD_{500nm} from different substrates utilized by *C. sakazakii* can be compared. The OD_{500nm} without carbon substrate was about 0.75, was the lowest. The highest OD_{500nm} with glucose and galactose, were higher, around 0.9. The OD_{500nm} of lactose reached to about 1.1, was the highest from all the substrates. The highest OD_{500nm} of the six kinds of neutral HMO fractions were between 0.8 and 1.1, in which two of them were about 0.85, three of them were around 1.0, and the value from LeA1 was similar to lactose. The highest OD_{500nm} from acidic HMO fractions were all lower than the corresponding neutral HMO fractions, in which five of them were around 0.8, only a little bit higher than the value without carbon substrates. That means the bacteria didn't utilize the most acidic HMO

fractions. The highest OD_{500nm} of the five kinds of single HMO standards were between 0.8 and 0.95, in which 2'-FL, LNnT and 6'-SL were about 0.8, this represent that the bacteria didn't utilize the three substrates. From the comparison of the HMO fractions with Lewis a, Lewis b and Lewis 0 specificity, there was no distinct difference between the Lewis patterns.

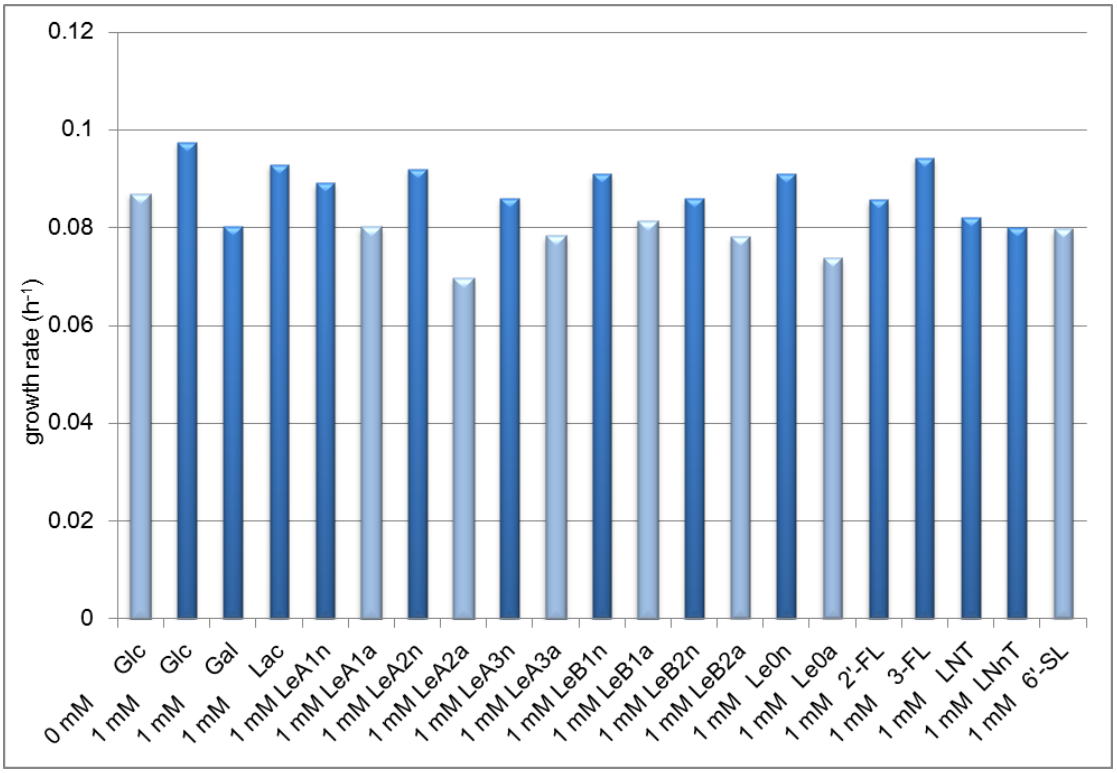


Figure 5-6(b) Comparison of the growth rate (h^{-1}) from *C. sakazakii* grown in MRS medium (without glucose) with different substrates. The dark columns showed the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standard. The light columns showed 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-6(b), the growth rate from *C. sakazakii* grown on different substrates can be compared. The growth rate without carbon substrate was about 0.085, was not the lowest compared with all the substrates. The growth rate with glucose was about 0.095, was the highest among all the substrate. The value with galactose was about 0.08, lower than without carbon substrate. The value with lactose was about 0.09. The growth rates from the six neutral HMO fractions were around 0.085, which were similar to lactose. The growth rates from the six acidic HMO

fractions were between 0.07 and 0.08, were all lower than the corresponding neutral HMO fractions and the value without carbon substrate. The values also indicate the similar results from the highest OD_{500nm}; the bacteria didn't utilize the acidic HMO fractions. The growth rates from the five single HMO standards were from 0.08 to 0.095, in which LNT, LNnT and 6'-SL were about 0.08, 2'-FL was about 0.085, similar to no carbon substrate. 3-FL was the highest, about 0.095. The reason may be that 2'-FL, LNT, LNnT and 6'-SL were no the growth substrate, and may inhibit the growth of the bacteria.

From the results combined with the highest OD_{500nm} and the growth rate, the following conclusions can be drawn: glucose, lactose and 3-FL were substrates which seem to have beneficial effect on the growth of *C. sakazakii*. The acidic HMO fractions from LeA2, LeA3, LeB1, LeB2 and Le0, the single standard 2'-FL, LNT, LNnT and 6'-SL were substrates which seem to have no effect or inhibit effect on the growth of *C. sakazakii.*

5.3.7 *Akkermansia muciniphila*

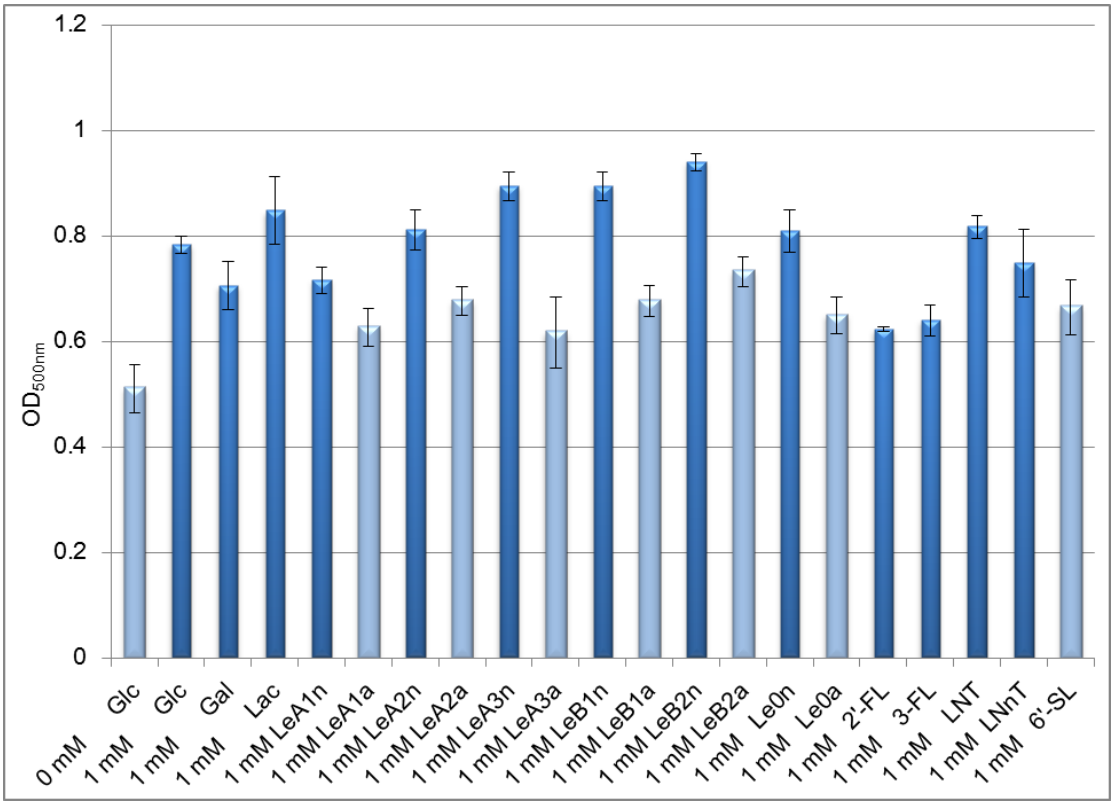


Figure 5-7(a) Comparison of the highest OD_{500nm} (Mean ± SD, n=3) of the growth from *A. muciniphila* grown in Modified Reinforced Clostridial Broth (without glucose) with different substrates. The dark columns showed the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standard. The light columns showed 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-7(a), the highest OD_{500nm} from different substrates utilized by *A. muciniphila* can be compared. The highest OD_{500nm} without carbon substrate was about 0.5, was the lowest. The highest OD_{500nm} with glucose and galactose, were higher, between 0.7 and 0.8. The OD_{500nm} of lactose was even higher, about 0.85. The highest OD_{500nm} of the six kinds of neutral HMO fractions were between 0.7 and 0.95, in which three of them were above 0.9, higher than lactose, one of them was about 0.8, was lower than glucose. The highest OD_{500nm} from acidic HMO fractions were from 0.6 to 0.75, all lower than glucose and the corresponding neutral HMO fractions. The highest OD_{500nm} of the five kinds of single HMO standards were between 0.6 and

0.8, in which 2'-FL and 3-FL were about 0.6, LNT and LNnT were around 0.8. The reason may be that the bacteria prefers the structure of LNT and LNnT than 2'-FL and 3-FL. The value of 6'-SL was about 0.65. From the comparison of the HMO fractions with Lewis a, Lewis b and Lewis 0 specificity, there was no distinct difference between the Lewis patterns. For the neutral HMO fractions, the values of the two Lewis b seem to have benefit effect compared with most other Lewis patterns. For the acidic HMO fractions, the values from the all the six samples were similar.

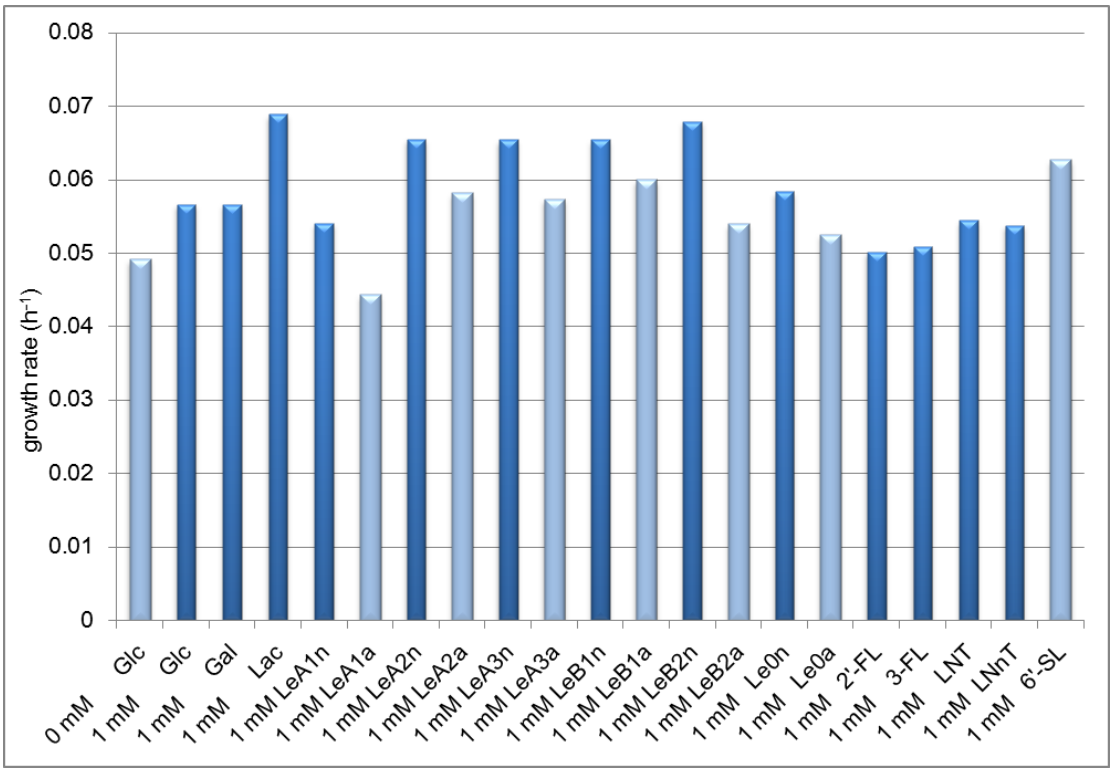


Figure 5-7(b) Comparison of the growth rate (h^{-1}) of the growth from *A. muciniphila* grown in Modified Reinforced Clostridial Broth (without glucose) with different substrates. The ark columns showed the neutral saccharides, including the 1 mM glucose, 1 mM galactose, 1 mM lactose, and six different neutral HMO fractions, four kinds of neutral single HMO standard.The light columns showed 0 mM glucose (for control) and the acidic saccharides, including the six different acidic HMO fractions and one kind of single acidic HMO standard.

From Figure 5-7(b), the growth rate from *A. muciniphila* grown on different substrates can be compared. The growth rate without carbon substrate was about 0.05, was not the lowest compared with all the substrates. The growth rate with

glucose and galactose were nearly the same, about 0.057. The value with lactose was about 0.07, was the highest among all the substrates. The growth rates from the six neutral HMO fractions ranged from 0.053 to 0.068, in which three of them were about 0.065, higher than glucose and galactose, one of them was about 0.068, similar to lactose. The growth rates from the six acidic HMO fractions were all lower than the corresponding neutral HMO fractions, mostly between 0.05 and 0.06, with exception of LeA1, which was only about 0.045, was the lowest among all the substrate. The growth rates from the five single HMO standards were from 0.05 to 0.062, in which 2'-FL and 3-FL were about 0.05, LNT and LNnT were about 0.055, 6'-SL was the highest, about 0.62, higher than the values from glucose and galactose.

From the results combined with the highest OD_{500nm} and the growth rate, the following conclusions can be drawn: glucose, lactose, neutral HMO fractions from LeA2, LeA3, LeB1, LeB2 and Le0, the single standard LNT and LNnT were substrates which seem to have beneficial effect on the growth of *A. muciniphila*.

5.3.8 Comparison of ΔOD from different microorganisms

In order to compare the effects of different carbon substrates on the growth of different microorganisms, ΔOD from the highest OD_{500nm} on different carbon substrates and the highest OD_{500nm} without carbon substrate were evaluated. Table 5-1 shows the highest OD_{500nm} and ΔOD from without and with different carbon substrates on different microorganisms. For easier comparison, the substrates were divided into four groups: the first group contained mono- and di-saccharides, the second group was the neutral HMO fractions, the third group was the acidic HMO fractions, and the last group was the single HMO standards. For all the seven bacteria, the results from the first group of different microorganisms were similar. The ΔOD values from glucose and galactose were mostly ++, only the values from *B. longum* were +. The values from lactose were from +++ to ++++, with the exception of *C. sakazakii*. The results from the second group showed that the carbon substrates LeA1n, LeA3n, LeB2n and Le0 had beneficial effects on most of the microorganisms.

The results from the third group showed that the acidic HMO fractions didn't have beneficial effects on microorganism as neutral HMO fractions. The values from LeA1a, LeA3a and LeB2a had relatively better effects than the other carbon substrates. The results from the last group showed that 3-FL and LNT had beneficial effects on most of the microorganisms. From the comparison of seven microorganisms, the results showed that most HMO fractions and single HMO standards had beneficial effects *L. lactis*, *L. acidophilus*, *B. infantis* and *B. vulgatus.* For *A. muciniphila*, lactose and the neutral HMO fractions had beneficial effects, but the acidic HMO fractions and most single HMO standards didn't have beneficial effects. For *B. longum* and *C. sakazakii*, only lactose, few of HMO fractions showed beneficial effects.

Table 5-1 The highest OD_{500nm} and ΔOD from without and with different carbon substrates on different microorganisms

	L. lactis	ΔOD	*L. acidophilus*	ΔOD	*B. infantis*	ΔOD	*B. longum*	ΔOD	*B. vulgatus*	ΔOD	*C. sakazakii*	ΔOD	*A. muciniphila*	ΔOD
0 mM Glc	0.410		0.356		0.578		0.497		0.462		0.744		0.511	
1 mM Glc	0.592	++	0.542	++	0.751	++	0.630	+	0.626	++	0.899	++	0.784	+++
1 mM Gal	0.554	++	0.458	+	0.722	++	0.595	+	0.667	+++	0.879	++	0.706	++
1 mM Lac	0.653	+++	0.616	+++	0.920	++++	0.701	+++	0.745	++++	1.117	++++	0.848	++++
1 mM LeA1n	0.850	++++++	0.657	++++	0.974	++++	0.969	++++	0.813	++++	1.103	++++	0.716	+++
1 mM LeA2n	0.509	+	0.658	++++	0.787	+++	0.652	++	0.798	++++	0.972	+++	0.812	++++
1 mM LeA3n	0.805	++++	0.647	++++	0.952	++++	0.688	++	0.691	+++	1.016	+++	0.895	++++
1 mM LeB1n	0.679	+++	0.639	++++	0.826	+++	0.826	++++	0.786	++++	0.874	++	0.892	++++
1 mM LeB2n	0.858	++++++	0.669	++++	0.938	++++	0.643	++	0.811	++++	1.014	+++	0.940	++++
1 mM Le0n	0.862	++++++	0.635	+++	0.877	++++	0.666	++	0.876	++++	0.863	++	0.809	++++
1 mM LeA1a	0.776	+++++	0.599	+++	0.798	+++	0.798	++++	0.696	+++	0.939	++	0.627	+
1 mM LeA2a	0.612	+++	0.550	++	0.628	+	0.573	+	0.663	+++	0.791	+	0.676	++
1 mM LeA3a	0.738	++++	0.560	+++	0.832	+++	0.611	+	0.729	+++	0.833	+	0.617	+
1 mM LeB1a	0.562	++	0.531	++	0.674	+	0.674	++	0.532	+	0.785	+	0.678	++
1 mM LeB2a	0.708	+++	0.604	+++	0.827	+++	0.562	+	0.589	++	0.833	+	0.733	+++
1 mM Le0a	0.631	+++	0.573	+++	0.714	++	0.625	++	0.769	++++	0.777	+/-	0.651	++
1 mM 2'-FL	0.620	+++	0.699	++++	0.804	+++	0.671	++	0.836	++++	0.804	+	0.623	+
1 mM 3-FL	0.743	++++	0.582	+++	0.884	++++	0.749	+++	0.729	+++	0.962	+++	0.641	++
1 mM LNT	0.654	+++	0.687	++++	0.940	++++	0.697	+++	0.797	++++	0.867	++	0.817	++++
1 mM LNnT	0.511	+	0.553	++	0.826	+++	0.671	++	0.884	++++	0.816	+	0.749	+++
1 mM 6'-SL	0.823	+++++	0.686	++++	0.771	++	0.564	+	0.644	++	0.766	+/-	0.665	++

Note: +/-: <0.04; +: ≥0.04 and <0.12; ++: ≥0.12 and <0.20; +++: ≥ 0.20 and < 0.28; ++++: ≥ 0.28 and <0.36; +++++: ≥0.36 and < 0.44; +++++: ≥ 0.44 and < 0.52.

5.3.9 Effect of HMOs on growth of microorganisms in comparison to previous studies

Human milk oligosaccharides have been considered to be beneficial for growth of infants' intestinal microorganisms for a long time. Recently, scientists used *in vitro* studies to investigate the effect of HMOs on intestinal microorganisms. In many studies the focus was on proofing the beneficial effects of HMOs on the growth of *Bifidobacteria*, especially *B. infantis* (Chichlowski *et al.*, 2011). In further studies other *Bifidobacteria* species, such as *B. longum*, *B. breve*, *B. bifidum*, *B. adolescentis* were used (Ward *et al.*, 2007). In addition, some other intestinal microorganisms, such as *L. lactis*, *L. acidophilus*, *L. gasseri* (*Lactobacillus gasseri*), *E. coli*, *S. thermophiles* (*Streptococcus thermophilus*), *B. vulgatus*, *B. fragilis* (*Bacteroides fragilis*) (Marcobal *et al.*, 2010; Ward *et al.*, 2004) were also studied for growth effects of HMOs. The carbon substrates for the studies were the whole HMO fractions. Glucose, galactose, lactose, or some specific oligosaccharides, such as GOS, FOS, arabino-oligosaccharides have also been studied for comparison of effects on microorganisms although the latter oligosaccharides are not a component of human milk.

L. acidophilus and *L. lactis* are considered as very important intestinal microorganisms in infants. The study from Marcobal *et al.* (2010) showed that *L. lactis* grew on HMOs with ΔOD_{600nm} of 0.124, and *L. acidophilus* grew on HMOs with ΔOD_{600nm} of 0.290 in the chemical ZMB medium. In our study, the medium for *L. lactis* and *L. acidophilus* was modified MRS medium. The ΔOD_{500nm} for *L. lactis* was from 0.1 to 0.4. The ΔOD_{500nm} for *L. acidophilus* was from 0.15 to 0.35. Therefore, we confirmed that both bacteria can grow on HMOs to some extent although slightly different growth conditions (basic medium, concentrations of HMO fractions, wavelength used for spectrophotometer) have been used. More relevant in comparison of the growth studies is the fact that Marcobal *et al.* (2010) used the whole HMO fraction whereas in our study, the HMOs were separated into the neutral and acidic Lewis specific fractions, or single HMO standard. The substrates were investigated individually. There are also some studies on the growth of *Lactobacillus*

on some other specific oligosaccharides, which can also be compared to the results from HMOs. Kaplan and Hutkins (2000) investigated the metabolism of Fructo-oligosaccharides with several *Lactobacilli*, such as *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus lactis and Lactobacillus casei* using MRS-FOS agar medium or broth, and found that *L. acidophilus* could grow on MRS-FOS medium with the OD_{625nm} about 1.6, *L. plantarum* could also grow on MRS-FOS. The results indicated that *L. acidophilus* is a FOS fermenter. Al-Tamimi *et al.* (2006) investigated the fermentation of arabino -oligosaccharides by gut bacteria and found that *Lactobacilli* could change the amount of arabinose slightly and changed arabinan and FOS significantly. Barrangou *et al.* (2003) showed that *L. acidophilus* could utilize fructo-oligosaccharide and described the gene locus involved in transport and catabolism of FOS compound. These studies showed that *L. acidophilus* could metabolize some oligosaccharides, like FOS. Although the structures of FOS, arabino -oligosaccharides have no structural similarities with HMOs, they support growth of *L. acidophilus*. In our study, we found that *L. acidophilus* could grow on glucose, galactose, lactose and some components of HMOs.

Since *Bifidobacterium* species are considered as very important infant intestinal microorganisms, there were many studies on *B. infantis* and other *Bifidobacterium* species. Ward *et al.* (2006) compared the growth of *B. infantis* and *L. gasseri* on HMOs as a sole carbon substrate, and found that *B. infantis* could grow vigorously but *L. gasseri* grew poorly. The research group then investigated several *Bifidobacteria* species, *B. infantis*, *B. longum*, *B. breve*, *B. bifidum* and *B. adolescentis* (Ward *et al.*, 2007). The results indicated growth capabilities on HMOs; *B. infantis* grew well on HMOs as a sole carbon source, *B. bifidum* also grew on HMOs, *B. longum* and *B. breve* could grow only a little bit on HMOs, *B. adolescentis* did not grow on HMOs. LoCascio *et al.* (2007) did several studies on the consumption of HMOs. They found that *B. infantis* preferentially consumes small mass oligosaccharides (DP<7), and grew very well on HMOs with consumption of nearly all available substrates. But other

Bifidobacteria, such as *B. longum*, *B. adolescentis*, *B. breve* and *B. bifidum*, showed low or only moderate growth ability on HMOs (LoCascio *et al.*, 2009). In 2010, they used comparative genomic hybridizations (CGH) and determined that HMO utilization gene regions are highly conserved across all *B. infantis* bacteria capable of growth on HMOs and have been changed in *B. longum* that can't grow on HMOs (LoCascio *et al.*, 2010). These studies are in agreement with the result from our study that found that *B. infantis* metabolized all kinds of HMO fractions and grow well, but *B. longum* could only metabolize some sorts of HMO fractions and grew not as well as *B. infantis*. Similarly, Marcobal *et al.* (2010) also found that the best growth occurred with *B. infantis* (ΔOD_{600nm} of 0.52 on HMOs), compared to the other bacteria in the study. The ΔOD_{500nm} in our study (between 0.1 and 0.4) was also higher than that of most other bacteria. The difference of OD values can be explained by different growth condition, concentration of HMOs and testing wavelength. Asakuma *et al.* (2011) studied the consumption of human milk oligosaccharides by *B. infantis*, *B. longum*, *B. bifidum* and *B. breve*. This was the first published study that reveals consumption of each single HMO during growth. The growth curves showed difference to our study in the time needed to reach the highest OD by *B. infantis*. The faster growth of *B. infantis* in the study of Asakuma *et al.* (2011) may be due to a higher concentration of HMO fractions used and the medium difference. The analysis of substrate consumption by *B. infantis* and *B. longum* were similar in both studies showing that *B. infantis* degraded all the single HMO fractions tested, and for *B. longum*, the degradation of the HMO fractions only occurred to some extent. As *Bifidobacteria* are unique microorganism particularly for the newborn infant, there are also many studies on the growth of *Bifidobacteria* on other oligosaccharides, such as FOS or GOS. In the study of Kaplan and Hutkins (2000), several species of *Bifidobacteria* were analyzed. With FOS as growth substrate, they found that *B. adolescentis* (OD_{625nm} of 1.3), *B. breve* (OD_{625nm} of 1.7), *B. infantis* (OD_{625nm} of 1.8), and *B. longum* (OD_{625nm} of 1.4) grew well whereas *B. bifidum* could not grow well on FOS. The results fit our study that *B. infantis* grew better on glucose, galactose, lactose and HMOs than *B. longum*. Al-Tamimi *et al.*

(2006) found that arabinan, arabinose and FOS could support the growth of *Bifidobacteria*. Vernazza *et al.* (2006) compared the carbohydrate preference of five *Bifidobacteria* species using several kinds of carbohydrates. *B. adolescentis* was not able to utilize xylo-oligosaccharide (XOS), lactitol or maltodextrin; *B. infantis* exhibited some growth on all of the substrates; *B. lactis* could not utilize lactitol or high-molecular weight inulin; one *B. longum* could not ferment low molecular weight inulin or HP, maltodextrin or lactitol. Another *B. longum* could ferment even small concentrations of these substrates. Although the carbohydrates in the study of Al-Tamimi *et al.* (2006) were different to our study, their results indicated good growth of *B. infantis* on many kinds of carbon substrate, an observation we also found withB. infantis growing best on almost all substrates including HMOs. Amaretti *et al.* (2007) investigated the kinetics and metabolism of *B. adolescentis* growing on glucose, galactose, lactose, and Galacto-oligosaccharides (GOS), and found that *B. adolescentis* could metabolize galactose best, followed by lactose and GOS. Gopal *et al.* (2001) studied two kinds of lactic acid bacteria, *Bifidobacterium lactis* and *Lactobacillus rhamnosus*, they found that *B. lactis* could utilize GOS as selective substrates.

B. vulgatus is also common gut-related microorganism. Rotimi and Duerden (1981) investigated the *Bacteroides* species in the normal neonatal faecal flora. They isolated 144 *Bacteroides* and found that *B. vulgatus* (46%) and *B. thetaiotaomicron* (30%) were the predominant species. All the 66 isolates of *B. vulgatus* fermented glucose, lactose, sucrose, rhamnose and xylose. As a typical species of *Bacteroides, B. vulgatus* were also studied further. In the study of Marcobal *et al.* (2010), very good growth of *B. vulgatus* was determined (ΔOD_{600nm} of 0.66) on whole HMO fractions. Only *Bacteroides fragilis* was able to grow even better then *B. vulgatus* on HMOs. The ΔOD_{500nm} of *B. vulgatus* in our study was from 0.1 to 0.42, which was also almost the maximum value compared to other bacteria. In another study from Marobal *et al.* (2011), they investigated several kinds of *Bacteroides* occurring in the infant gut and found that all the five kinds of *Bacteroides* species, *B. fragilis, B. caccae, B. vulgatus,*

B. ovatus and B. stericoris grew in the presence of HMOs. The growth of *B. fragilis*, *B. vulgatus* and *B. caccae* were similarly well with the OD_{600nm} higher than 0.9. The OD_{500nm} in our study was from 0.64 to 0.87, which was also with high value. The differences between our study and the former study were the growth condition (medium and concentration of HMOs) and the used wavelength. From the data of these studies, we conclude that *B. vulgatus* grows very well on HMOs.

C. sakazakii is an opportunistic pathogen mainly associated with infections in neonates, outbreaks of a rare form of infant meningitis, necrotizing enterocolitis (NEC), bacteraemia and neonate deaths (Baumgartner *et al.*, 2009; Iversen and Forsythe, 2004). To proof the hypothesis that HMOs have prebiotic effects that can support the growth of beneficial bacteria and inhibit undesired microorganisms, we investigated the effect of HMOs as well as of glucose, galactose and lactose on the growth of *C. sakazakii* on. In a previous study, Joseph *et al.* (2013) focused on the sialic acid utilization by *C. sakazakii*, and found that *C. sakazakii* was the only *Cronobacter* species that was able to use sialic acid as a carbon source for growth. In our study, we didn't use sialic acid; however our results indicated that *C.sakazakii* could grow on some of HMO fractions, mainly neutral fractions. *C. sakazakii* didn't show growth on 6'-SL. The reason may be that *C. sakazakii* doesn't contain the enzyme which can hydrolyze 6'-SL into sialic acid and lactose. This comparison indicates that *C. sakazakii* seems to prefer simple sialic acid compared to sialylated HMOs. Growth tests of *C. sakazakii* on other HMO fractions of our study showed poor growth. This may indicate that HMOs support growth of beneficial bacteria much stronger than those of potentially pathogenic bacteria.

A. muciniphila is a human intestinal mucin-degrading bacterium; it can be isolated from faeces in anaerobic medium containing gastric mucin as the sole carbon. Mucins are composed of a peptide core rich in serine and threonine residues that is decorated via *O*- or *N*-*glycosidic* bonds by oligosaccharides, which are composed of one or some of four primary sugars (*N*-acetylglucosamine, *N*-acetylgalactosamine, galactose and fucose) and are terminated by sialic acids or sulfate groups. Mucus can

serve as a barrier to protect the underlying epithelium from the attachment of pathogens and also as a source of nutrients for commensal bacteria. Previous studies with *A. muciniphila* found that it could grow on a limited number of sugars, including N-acetylglucosamin, N-acetylgalactosamin and glucose, but only when a protein source was provided (Derrien *et al.*, 2004). There are also some studies on *A. muciniphila* and their effects on glucose homeostasis, and on intestinal epithelium in diet-induced obesity (Everard *et al.,* 2013; Shin *et al.*, 2014). But there are no researches on the effect of HMOs on the growth of *A. muciniphila*. Due to the importance of *A. muciniphila* and the structure relationship between mucin and oligosaccharides, we studied on the growth of *A. muciniphila* using glucose, galactose, lactose and HMO fractions. We selected the Modified Reinforced Clostridial Broth as the growth medium, and found that it grew well on glucose, lactose, most neutral HMO fractions, LNT and LNnT.

5.4 Analysis of substrates in the medium before and after cultivations

From the analysis of substrates in the medium before and after cultivations from *B. infantis* and *B. longum*, the following conclusion can be drawn: *B. infantis* could metabolize almost all the carbon substrates, including mono-, di-saccharides, neutral and acidic HMO fractions, and single HMO standards. *B. longum* could only metabolize part of the HMO fractions, or metabolized the components to some extent. The results showed that the HMOs had beneficial effects on *B. infantis*, but not on *B. longum*. This observation indicates a prebiotic effect of HMOs on *B. infantis* but not on *B. longum.* An explanation could be that *B. infantis* contains more suitable enzymes to hydrolyze HMO fractions. This can also explain the phenomenon that *B. infantis* is dominant in infants, whereas *B. longum* exist later in the adulthood as well.

5.5 Analysis of metabolic products of cultivation

From the analysis of metabolic products after cultivation of *B. infantis* and *B. longum*, the following conclusion can be drawn: both microorganisms metabolize the carbon substrates and mainly produce lactic acid, acetic acid and some formic acid. The amounts of metabolic products from *B. infantis* were mostly larger than those from *B. longum*. This result fits the growth curves and the analysis of carbon substrates of the two microorganisms. Again the difference metabolic products can be explained by different set of enzymes to hydrolyze the HMO fractions in the two microorganisms. The results can also well explain the beneficial effects of HMOs on *B. infantis*.

There were also some former studies on the fermentation of *Bifidobacteria*. Early in 1968, de Vries and Stouthamer studied on the fermentation of glucose, lactose, galactose, mannitol, and xylose by *Bifidobacteria*, and proved that acetate, lactate, ethyl alcohol, and formate were the main fermentation products. Recently, Rossi *et al.* (2005) studied on the fermentation of FOS by *Bifidobacteria* and found that lactic and acetic acids were the major products of FOS fermentation. Pokusaeva *et al.* (2011) concluded the carbohydrate metabolism in *Bifidobacteria* and demonstrated that *Bifidobacteria* produce lactic acid as one of their main fermentation end products, so that they were often included in the lactic acid bacteria. Kajiwara *et al.* (2002) researched on the effect of GOS, FOS and honey on the growth of five human intestinal *Bifidobacterium spp.*, including *B. longum* and *B. infantis*. They also analyzed the fermentation products of lactic and acetic acid by HPLC. These results of fermentation products fit to our study in which we also found that lactic acid and acetic acid are the main products, and formic acid is also one of the products.

6. Summary

Human milk oligosaccharides (HMOs), as the third largest components in human milk, are thought to be important for the development of infants for various reasons. Previous *in vitro* studies support the idea that HMOs help to build up the human gut microbiota in infants. It has been shown that some of the intestinal microorganisms found in feces of infants grow on single HMOs as sole carbon substrate. However, so far, the growth effect of specific HMO fractions whose pattern is strongly influenced by the mothers Lewis blood group and secretor status on gut bacteria has not been investigated yet.

In this study, HMO fractions were isolated from human milk samples with known Lewis blood group specificity (Lewis a, Lewis b and Lewis negative). Human milk was first delipidated and deproteinized followed by an exclusion of lactose by Sephadex G25 Chromatography. The separation of HMO fractions into neutral (n) and acidic (a) fractions was achieved by FPLC-anion exchange chromatography. Resulting fractions and, in addition, single HMO standards as well as glucose, galactose and lactose were used for growth study of microorganisms.

The HMO composition of the samples and the Lewis specificity were analyzed by HPAEC-PAD in combination with MALDI-TOF-MS. Fourteen fractions with Lewis a-, Lewis b- or Lewis-negative specificity and five single HMOs (2'-FL, 3-FL, LNT, LNnT and 6'-SL) were tested for their properties to function as growth substrate. According to previous studies and the existence of intestinal microorganisms in infants' gut, we selected seven kinds of intestinal microorganisms for the study: *Lactococcus lactis subsp. lactis, Lactobacillus acidophilus, Bifidobacterium longum subsp. infantis, Bifidobacterium longum subsp. longum, Bacteroides vulgatus, Cronobacter sakazakii and Akkermansia muciniphila.* The growth studies were performed under anoxic condition using specific medium with 1 mM carbon substrates instead of glucose. Growth curves were measured by OD_{500nm} value using spectrophotometer. The

carbon substrates in the medium were analyzed by RP-HPLC and the metabolic products (fatty acids) were analyzed by HPLC.

From growth studies of *L. lactis* and *L. acidophilus* on glucose, galactose and lactose, we found that *L. acidophilus* grew on glucose, galactose and lactose; *L. lactis* grew on glucose, low concentration of galactose or lactose. From growth studies of seven microorganisms on 1 mM glucose, galactose, lactose, neutral or acidic HMO fractions and single HMO standards, we found that *L. lactis* grew well on lactose and on Lewis fractions LeA1n, LeA3n, LeA3a, LeB2n as well as on 3-FL, LNT and 6'-SL; *L. acidophilus* grew well on lactose, LeA1n, LeA2n, LeA3n, LeB1n, LeB2n, LeA1a, LeB2a, single 2'-FL, 3-FL and 6'-SL; *B. infantis* grew well on most of the carbon substrates, *B. longum* could only grow well on some of the substrates. *B. vulgatus* grew well on lactose, LeA1n, LeA2n, LeB1n, LeB2n, LeB2n, Le0n, Le0a, 2'-FL, LNT and LNnT. *C. sakazakii* could grow on glucose, lactose and 3-FL, but most carbon substrates seem to have no effect nor inhibitory effect on the growth. *A. muciniphila* grew well on glucose, lactose, LeA2n, LeA3n, LeB1n, LeB2n, Le0n, LNT and LNnT. Most neutral HMO fractions had better growth effects than acidic HMO fractions on bacterial growth.

In order to analyze the carbon substrates before and after cultivation, 2-AA was used for labeling and RP-HPLC was used for quantification. From the analysis of substrates, we found that *B. infantis* could metabolize almost all the carbon substrates, including mono-, di-saccharides, HMO fractions, and single HMO standards. *B. longum* could only metabolize some kinds of the HMO fractions, or metabolized some components to some extent.

From the analysis of metabolic products using HPLC (analysis for fatty acid detection), we found that both *B. infantis* and *B. longum* consumed the carbon substrates, including mono-, di-saccharides, HMO fractions and single HMOs; they mainly produced lactic acid, acetic acid and some formic acid. The amounts of fatty acids produced by *B. infantis* were mostly larger than those from *B. longum*.

In conclusion, HMOs can positively affect the growth of intestinal microorganisms. *B. infantis* and *B. vulgatus* grew well on almost all the HMO fractions. *B. longum*, *L. lactis*, *L. acidophilus* grew well on some of HMO fractions. *C. sakazakii* could grow on only a few carbon substrates whereas single HMOs and Lewis specific fractions seem to have no effect. *A. muciniphila* grew well on most of the neutral HMO fractions. The results indicated that most neutral HMO fractions had better growth effects than acidic HMO fractions on bacterial growth. In addition, there were no distinct differences between the effects of the various Lewis blood group specific neutral HMO fractions.

7. ZUSAMMENFASSUNG

Aus verschiedenen Gründen wird angenommen, dass humane Milcholigosaccharide (HMOs), die am dritthäufigsten in der Milch vertretenen Komponenten, bei der Entwicklung des Säuglings eine wichtige Rolle spielen. Frühere Studien belegen, dass HMOs am Aufbau der Darmmikrobiota des Säuglings beteiligt sind. In Zellkulturstudien konnte gezeigt werden, dass einige der intestinalen Mikroorganismen, die in Säuglingsfaeces vorkommen, bei Zugabe von bestimmten HMOs als einzigem Kohlenstoff (C)-Substrat wachsen. Bisher wurde jedoch die Auswirkung spezifischer HMO-Fraktionen, deren Muster stark durch die Lewis-Blutgruppe und den Sekretorstatus der Mutter beeinflusst wird, noch nicht auf das Wachstum von Darmbakterien hin untersucht.

In der vorliegenden Studie wurden HMO-Fraktionen aus Humanmilchproben mit bekannter Lewis-Blutgruppen-Spezifität (Lewis a, Lewis b und Lewis negativ) isoliert. Nachdem die Milch zunächst entfettet und entproteinisiert worden war, wurde anschließend mittels Sephadex G25 Chromatographie die Lactose entfernt. Mittels FPLC-Anionenaustauschchromatographie wurden die HMO-Fraktionen in neutrale und saure Fraktionen getrennt. Die so entstandenen Fraktionen, einzelne HMO-Standards sowie Glucose, Galactose und Lactose wurden für die Untersuchung des Wachstums der Mikroorganismen benutzt.

Die HMO-Zusammensetzung der Proben und die Lewis-Spezifität wurden mittels HPAEC-PAD in Kombination mit MALDI-TOF-MS analysiert. Vierzehn Fraktionen mit Lewis a-, Lewis b- oder Lewis-negativ-Spezifität und fünf einzelne HMOs (2'-FL, 3-FL, LNT, LNnT und 6'-SL) wurden auf ihre Eignung als Wachstumssubstrat untersucht. Entsprechend früheren Studien und den im Säuglingsdarm vorhandenen Mikroorganismen wurden sieben intestinale Mikroorganismen für unsere Studie ausgewählt: *Lactococcus lactis subsp. lactis, Lactobacillus acidophilus, Bifidobacterium longum subsp. infantis, Bifidobacterium longum subsp. longum, Bacteroides vulgatus, Cronobacter sakazakii* und *Akkermansia muciniphila*. Die

Wachstumsstudien wurden unter Anwendung eines spezifischen Mediums mit 1 mM verschiedener Kohlenstoff-Substrat anstelle von Glucose unter anoxischen Bedingungen durchgeführt. Die Wachstumskurven wurden anhand des OD_{500nm} Wertes mittels Spektrophotometer gemessen, die C-Substrate im Medium anhand von RP-HPLC und die metabolischen Produkte (Fettsäuren) mittels HPLC analysiert.

Wachstumsstudien an *L. lactis* und *L. acidophilus* mit Glucose, Galactose und Lactose zeigten, dass *L. acidophilus* erwartungsgemäß mit Glucose, Galactose und Lactose wächst, *L. lactis* wuchs mit Glucose und mit geringen Konzentrationen von Galactose oder Lactose. Anhand von Wachstumsstudien an sieben Mikroorganismen mit 1 mM Glucose, Galactose, Lactose, neutralen und sauren HMO-Fraktionen und einzelnen HMO-Standards konnte festgestellt werden, dass *L. lactis* gut auf Lactose und auf den Lewis-Fraktionen LeA1n, LeA3n, LeA3a, LeB2n wie auch auf 3-FL, LNT und 6'-SL wuchs; *L. acidophilus* wuchs gut auf Lactose, LeA1n, LeA2n, LeA3n, LeB1n, LeB2n, LeA1a, LeB2a, single 2'-FL, 3-FL und 6'-SL; *B. infantis* zeigte auf den meisten C-Substraten ein gutes Wachstum, *B. longum* nur mit einigen der Substrate. *B. vulgatus* wuchs mit Lactose, LeA1n, LeA2n, LeB1n, LeB2n, LeB2n, Le0n, Le0a, 2'-FL, LNT und LNnT gut. *C. sakazakii* konnte mit Glucose, Lactose und 3-FL wachsen, die meisten C-Substrate jedoch scheinen keine Auswirkungen auf das Wachstum zu haben. *A. muciniphila* zeigte ein gutes Wachstum auf Glucose, Lactose, LeA2n, LeA3n, LeB1n, LeB2n und Le0n, LNT und LNnT.

Um Kenntnisse zur gezielten Verwertung von Kohlenstoffsubstraten durch einzelne Mikroorganismen zu erhalten, wurden Aliquots der jeweiligen Mono-, Di-, oder Oligosaccharide vor und nach der Kultivierung mittels 2-AA markiert und anschließend mittels RP-HPLC quantifiziert. Dabei zeigte sich, dass *B. infantis* praktisch alle Kohlenstoffsubstrate metabolisiert hat, einschliesslich Mono- und Di-sacchariden, HMO-Fraktionen sowie einzelnen HMO-Standards. *B. longum* dagegen metabolisierte nicht alle getesteten HMO-Fraktionen bzw. verwertete manche Substrate nur in geringerem Mengen.

Die Analyse der metabolischen Produkten, gemessen an dem Auftreten von Fettsäuren, ergab, dass beide Mikroorganismen die eingesetzten Monosaccharide, Dissaccharide, HMO-Fraktionen und einzelne Standards verwerteten. Dabei wurden hauptsächlich Milchsäure, Essigsäure und Ameisensäure gebildet, wobei die produzierten Mengen bei *B. infantis* deutlich größer als bei *B. longum* waren.

Schlussfolgernd kann festgestellt werden, dass HMOs das Wachstum intestinaler Mikroorganismen positiv beeinflussen können. *B. infantis* und *B. vulgatus* zeigten mit fast allen HMO-Fraktionen ein gutes Wachstum, *B. longum, L. lactis, L. acidophilus* auf einigen. *C. sakazakii* konnte nur auf wenigen Kohlenstoff-Substraten wachsen, während einzelne HMOs und Lewis-spezifische Fraktionen keine Auswirkungen zu haben scheinen. *A. muciniphila* wies auf den meisten der neutralen HMO-Fraktionen ein gutes Wachstum auf, nicht aber auf sauren Oligosacchariden. Die Ergebnisse zeigten, dass die meisten neutralen HMO-Fraktionen das Wachstum der Mikroorganismen eher förderten als saure HMO-Fraktionen. Zwischen den verschiedenen Lewis-Blutgruppen-spezifischen HMO-Fraktionen gab es hinsichtlich der Auswirkungen keine deutlichen Unterschiede.

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Erklärung

Ich erkläre: Ich habe die vorgelegte Dissertation „Human milk oligosaccharides (HMOs) and their effects on intestinal microorganisms“ selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Gießen, den 16.08.2015 Ke Yue

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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