Quantification of major milk oligosaccharides in a range of formulated milk powder products using high performance liquid chromatography-multi reaction monitoring-mass spectrometry

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ABSTRACT

In formulated milks for infants and children, oligosaccharides such as galacto-oligosaccharides and/or fructo-oligosaccharides are commonly added. This paper, however, discusses the determination of the concentration in infant and follow-on formulations of the major acidic oligosaccharides: 3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL), 6′-sialyllactosamine (6′SLN) and disialyllactose (DSL) that are endogenous to bovine milk. These oligosaccharides were measured in infant, follow-on and growing up formulas purchased in the Chinese and Malaysian markets. Of these oligosaccharides, 3′SL was the most abundant, followed by 6′SL and 6′SLN, but DSL was not quantifiable due to its concentration being lower than the limit of quantitation in most samples. We observed little or no differences in the levels of 3′SL or 6′SL in Malaysian and Chinese formulas, but the levels of 6′SLN in all Malaysian samples were found to be higher than those in the samples from China.

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1. Introduction

Milk oligosaccharides are short chain polymers of 3–10 covalently linked monosaccharides. In mature human milk and bovine milk they are the most abundant carbohydrate components after lactose, making up, on average, 12–13 g L^{-1} in human milk and about 1–2 g L^{-1} in bovine colostrum before a rapid drop in mature bovine milk (Urashima et al., 2011; Urashima, Saito, Nakamura, & Messer, 2001). Human milk oligosaccharides (HMO) show greater diversity than bovine milk oligosaccharides (BMO), with more than 150 different HMO reported compared with only 46 BMO (Urashima, Hirabayashi, Sato, & Kobata, 2018; Urashima, Messer, & Oftedal, 2017). Fucosylated oligosaccharides dominate HMO both qualitatively and quantitatively, while, in contrast, BMO are dominated by sialylated species. However, HMO and BMO do share some common oligosaccharides, such as 3′- and 6′-sialyllactose, although the concentrations of these two species are significantly lower in bovine milk (Fong, Ma, & McJarrow, 2011; Ma et al., 2018). As unmodified bovine milk is not a suitable infant food, the comparison of bovine and human milk oligosaccharide concentrations is moot;

the oligosaccharide comparison should be done between human milk and infant, follow-on and growing up formula concentrations as these are what infants and toddlers consume.

The recognition of the biological benefits of milk oligosaccharides on human health, such as anti-bacterial-infection, modulation of the immune system, prevention against necrotising enterocolitis, gut maturation and brain and cognitive development (Austin et al., 2016; Jantscher-Krenn et al., 2012; Kunz & Rudloff, 2006; Martín-Sosa, Martín, García-Pardo, & Hueso, 2003; Newburg & Neubauer, 1995; ten Bruggencate, Bovee-Oudenhoven, Feitsma, van Hoffen, & Schoterman, 2014; Vandenplas, 2002), has led to increased research and understanding of the composition and levels of HMO. Although there are limited reports on oligosaccharide levels in infant formula (IF), the current data sets (Austin, Cuany, Michaud, Diehl, & Casado, 2018; Austin et al., 2016; Fong et al., 2011; Martín-Sosa et al., 2003) indicate that the levels of oligosaccharides in commercial bovine milk-based paediatric formulations are significantly lower than those in human milk and bovine milk. Currently, this functional oligosaccharide benefit gap (between human milk and infant formula) has been addressed using plant sourced fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) converted from lactose, to imitate a portion of the HMO composition found in human milk. However, over the last year or so, there has been an emergence of new bovine milk-based

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paediatric formulations fortified with human breast milk identical oligosaccharides such as 2′-fucosyllactose and lacto-N-neotetraose to help further address this gap (Marriage, Buck, Goehring, Oliver, & Williams, 2015; Puccio et al., 2017).

Together with a need to address the gap, there is also a need to understand better the concentrations of oligosaccharides present in infant formula powders currently commercially available as a basis for new formulations. In this study, a validated high-performance liquid chromatography-mass spectrometry method was used to measure the major sialylated bovine milk oligosaccharide concentrations in a range of infant formulas purchased from supermarkets in China and Malaysia.

2. Materials and methods

2.1. Standards and chemicals

The oligosaccharide standards 3′-sialylactose (3′SL), 6′-sialyllactose (6′SL), 3′-sialyllactosamine (3′SLN) and 6′-sialyllactosamine (6′SLN) (all >95% purity) were obtained from Dextra Laboratories Ltd. (Reading, Berkshire, UK), while disialyllactose (DSL; >90% purity) was obtained from Carbsynth (Compton, Berkshire, UK). All solvents used (Merck, Darmstadt, Germany) were of liquid chromatography grade, except for chloroform, which was of analytical grade (ethanol stabilised).

2.2. Preparation of chromatography standards

Oligosaccharide standards were rehydrated with Milli-Q water to give individual stock solutions of 1 mg mL^{-1}. Since it is present in bovine milk in only very low amounts (Fong et al., 2011), 3′SLN was used as the internal standard. For use, 3′SLN was first diluted with Milli-Q water to a concentration of 100 μg mL^{-1}, then further diluted with 95% acetonitrile to 4 μg mL^{-1}. Oligosaccharide standard stock solutions were serially diluted in 95% acetonitrile to give a 10-point mixed calibration solution from 0.02 μg mL^{-1} to 10 μg mL^{-1} for 3′SL, 6′SL and 6′SLN, and from 0.04 μg mL^{-1} to 20 μg mL^{-1} for DSL. All the calibration solutions contained 3′SLN internal standard at 2 μg mL^{-1}. All solutions were stored at −30 °C.

2.3. Samples

A range of infant formula (stage 1), follow-on milk powder (stage 2) and growing-up milk powder (stage 3) samples from a number of brands/manufactures were purchased from supermarkets in China and Malaysia, from 2015 to 2017, and shipped to Fonterra Research and Development Centre (Palmerston North, New Zealand) for analysis. In total, 36 samples were purchased from China (32 products from 17 different brands, 17 stage 1, 5 stage 2 and 14 stage 3) and 20 were purchased from Malaysia (14 products from 7 different brands, 4 stage 1, 8 stage 2 and 6 stage 3). All products were manufactured from bovine milk derived ingredients as the base powder and non-dairy ingredients, such as vegetable oil and minerals. Two samples, labelled as A and B, from Malaysia were formulated for anti-diarrhoea needs; the results obtained from these samples are included in discussions below, but were excluded from the statistical analysis of the data from the rest of the samples to avoid bias.

It should be noted that while the formulas were purchased in these respective countries, China and Malaysia, all the Malaysian formulas were imported into the country, whereas 20% of the powders purchased in China were locally manufactured products, but using local and imported ingredients. Thus, the comparison of local and imported formulas for their BMO levels were not made.

2.4. BMO extraction

All sample powders were reconstituted to 10% (w/v). A further five-fold dilution with Milli-Q water was made to all reconstituted samples and 100 μL 3′SLN (100 μg mL^{-1}) internal standard was added to 0.4 mL of each sample. The sample/internal standard mixture was extracted using the protocol described by Fong et al. (2011) that, in brief, is as follows. To each 0.4 mL sample, 2 mL chloroform/methanol (2:1) was added. The mixtures were vortexed briefly and centrifuged (2000×g for 1 h). The top phase was transferred to a 5 mL volumetric flask. The bottom phase was re-extracted with 0.5 mL 50% methanol with brief vortexing and centrifugation (2000×g for 1 h). The top phase was pooled to the 5 mL volumetric flask, and made up to the mark with 95% acetonitrile.

2.5. High performance liquid chromatography-mass spectrometry quantification

The high performance liquid chromatography-mass spectrometry (HPLC-MS) separation conditions were as described in Fong et al. (2011), except that an Agilent 1100 series HPLC system (Agilent, Santa Clara, CA, USA) coupled to a 6500 QTRAP mass spectrometer (AB SCIEX Pte Ltd., Framingham, MA, USA) was used for analysis of the oligosaccharides. The different classes of oligosaccharides were separated on a Luna HILIC column (150 mm × 2.1 mm, 3 μm, Phenomenex, Torrance, CA, USA) (see Fig. 1).

The mass spectrometer was optimised to measure 4 major BMO (3′SL, 6′SL, 6′SLN and DSL), as well as 3′SLN, using multiple reaction monitoring (MRM) in negative ion mode. Instrument settings were: electrospray voltage, −4500 V; source temperature, 500 °C; declustering potential (DP), −100 V; entrance potential, −10 V; collision cell exit potential (CXP), −15 V. The curtain gas, and ion source gases 1 and 2 were set at 20 psi, 85 psi and 15 psi, respectively. Details of the optimised collision energy for each oligosaccharide and their monitored fragments are shown in Table 1.

Table 1
Parent and daughter ions used in multi reaction monitoring scan in negative mode.[a]

Oligosaccharide	Parent ion (*m/z*)	Daughter ion (*m/z*)
3′SL	632.000	289.950
	632.000	408.100
6′SL	632.000	289.950
	632.000	408.100
3′SLN	672.900	289.900
	672.900	572.000
6′SLN	672.900	289.900
	672.900	572.000
DSL	923.200	289.900
	461.100	289.900

[a] Abbreviations are: 3′SL, 3′sialyllactose; 6′SL, 6′sialyllactose; 3′SLN, 3′sialyllactosamine; 6′SLN, 6′sialyllactosamine; DSL, disialyllactose. Collision energy was 38.00 V in all cases.

2.6. Statistical analysis

All statistical analysis was carried out using Minitab (Release 17.2.1, 2015; Minitab Inc., State College, PA, USA). Tukey's pairwise comparisons were carried out to compare the results among different groups and between countries where $p < 0.05$ was considered to be significantly different.

3. Results and discussion

### 3.1. Repeatability, limit of detection and limit of quantitation

Analysis of an IF was repeated (n = 18) over 3 individual days for the repeatability test. The coefficients of variation (CV%; Table 2) for 3′SL and 6′SL (6.7% and 4.8%, respectively) were lower than those for 6′SLN and DSL (11.1% and 11.8%, respectively); this was not unexpected, since 6′SLN and DSL were present at lower concentrations than 3′SL and 6′SL, thus explaining the higher CV%.

Extended calibration curves with 16 points from $0.0012\ \mu g\ mL^{-1}$ to $20\ \mu g\ mL^{-1}$ for DSL and from $0.0006\ \mu g\ mL^{-1}$

to $10\ \mu g\ mL^{-1}$ for the other sialyl-oligosaccharides were prepared serially with 95% acetonitrile to test the limit of detection (LOD) and limit of quantitation (LOQ) for each component in the system (Table 2). Whilst the linear range of detection for 3′SL, 6′SL and 6′SLN was 0.024—100 ng loaded on the column (with the LOQ being the lower value of the range), that for DSL was 0.195—100 ng.

3.2. 3′-Sialyllactose concentrations

Unsurprisingly, since it is the major sialyl-oligosaccharide in bovine milk, 3′SL was the major oligosaccharide component in the bovine milk-based formulations, comprising about 65—78% of all oligosaccharides measured. The concentration of 3′SL ranged from $136.3\ \mu g\ g^{-1}$ to $668.5\ \mu g\ g^{-1}$ powder for the samples purchased from China and from $195.6\ \mu g\ g^{-1}$ to $520.9\ \mu g\ g^{-1}$ powder for those purchased from Malaysia (excluding the two anti-diarrhoeal samples) across the 3 stages (Fig. 2A). The mean values (±standard deviation) for the concentration of 3′SL for products of each stage purchased from China and Malaysia are
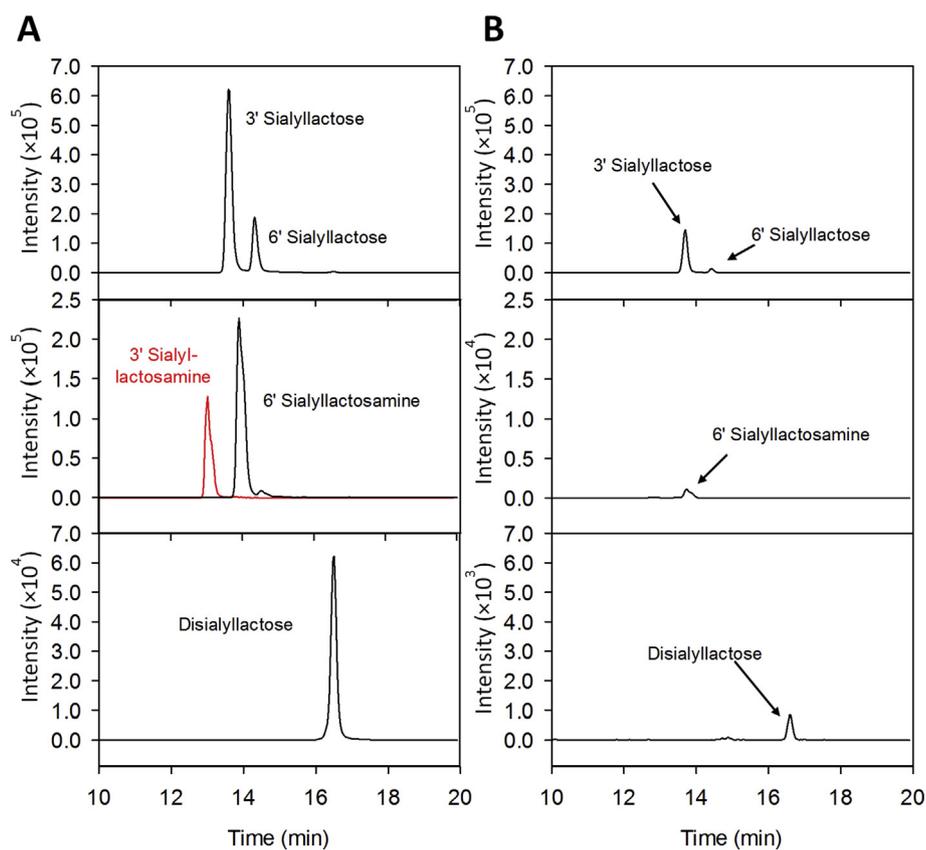

Fig. 1. Typical chromatograms of major oligosaccharides in (A) the bovine sourced standards and (B) formulated milk powder products; 3′siallyllactosamine (red) is used as the internal standard.

Table 2
The LOD and LOQ of each oligosaccharide and repeatability of the determination.[a]

Oligo-saccharide	LOD (ng)	LOQ (ng)	Linear range (ng)	R^2	Repeatability (CV%; n = 18)
3′SL	0.006	0.024	0.024—100	0.99994	6.7%
6′SL	0.012	0.024	0.024—100	0.99990	4.8%
6′SLN	0.012	0.024	0.024—100	0.99984	11.1%
DSL	—	0.195	0.195—100	0.99944	11.8%

[a] Abbreviations are: 3′SL, 3′sialyllactose; 6′SL, 6′sialyllactose; 6′SLN, 6′sialyllactosamine; DSL, disialyllactose; LOD, limit of detection; LOQ, limit of quantitation; R^2, coefficient of determination; CV, coefficient of variance. LOD and LOQ are presented as the amount (ng) loaded on column in this system.
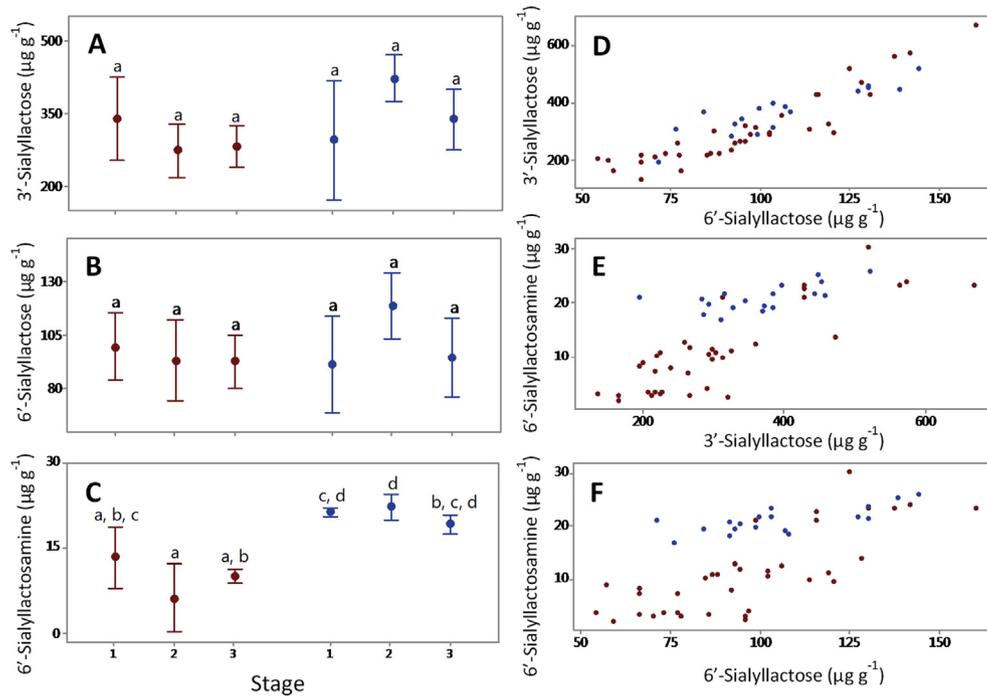
Fig. 2. The distributions of (A) 3′-sialyllactose (3′SL), (B) 6′-sialyllactose (6′SL) and (C) 6′-sialyllactosamine (6′SLN) in the samples across 3 stages (1, infant formula; 2, follow-on milk powder; 3, growing-up milk powder) from both China (red symbols) and Malaysia (blue symbols) and correlations between (D) 3′SL and 6′SL, (E) 6′SLN and 3′SL and (F) 6′SLN and 6′SL. In panels A–C data points with different letters above are significantly different ($p < 0.001$).

given in Table 3. The large variation within each stage for both regions is likely due to the number of different brands and formulations. Statistically, the 3′SL results for all stages from both countries are not significantly different ($p = 0.128$). Interestingly, of the two anti-diarrhoeal formulations from Malaysia, sample A contained no detectable 3′SL while sample B contained only 19.7 μg g^{-1} 3′SL.

3.3. 6′-Sialyllactose concentrations

The average 6′SL concentrations measured across all 3 stages of products purchased from both counties were not significantly different ($p = 0.186$), although the mean value for 6′SL concentration for Malaysian stage 2 samples (119.4 ± 18.4 μg g^{-1}) appeared slightly higher than that for the all other samples (Fig. 2B). The 6′SL concentration ranged from a low of 57.0 μg g^{-1} to high of 160.1 μg g^{-1} (Table 3), making up 17.7%–32.2% of the total oligosaccharides measured in this study. Among the 2 anti-diarrhoea products, no 6′SL was detected in sample A while 5.52 μg g^{-1} 6′SL was detected in sample B.

3.4. 6′-Sialyllactosamine concentrations

6′SLN made up only 0.6–7.3% of the total oligosaccharides measured in this study. The levels of 6′SLN ranged from 2.0 μg g^{-1} to 30.2 μg g^{-1} and 16.9 μg g^{-1} to 25.9 μg g^{-1} for the China and Malaysia sourced powders, respectively (Fig. 2C). China sourced products displayed a much greater spread of the 6′SLN in stage 1 and 2 samples, whereas significantly ($p < 0.001$) higher levels of 6′SLN were found in IFs purchased from Malaysia (16.9–25.9 μg g^{-1}) compared with those purchased from China (2.4–13.8 μg g^{-1}), a finding for which we have found no explanation. Interestingly, the 6′SLN levels in the two anti-diarrhoea products were essentially the same and, at 18.82 μg g^{-1} for sample A and 18.89 μg g^{-1} for sample B, were consistent with that measured in all other powders from all stages.

3.5. Disialyllactose

Only 20 of the 36 samples purchased from China had quantifiable DSL, which ranged from 15.73 μg g^{-1} to 43.02 μg g^{-1} powder

Table 3
The oligosaccharide concentrations of the formulated milk samples across 3 stages from China and Malaysia.[a]

Country	Stage	Number of samples	3′SL (μg g^{-1})	6′SL (μg g^{-1})	6′SLN (μg g^{-1})	DSL (μg g^{-1})
China	1	17	339.8 ± 166.8	100.1 ± 31.4	13.3 ± 10.4	21.5 ± 4.9
China	2	5	273.8 ± 43.7	93.5 ± 15.5	6.2 ± 4.8	24.7 ± 5.3
China	3	14	267.7 ± 73.4	92.9 ± 21.6	10.1 ± 2.0	27.3 ± 7.4
Malaysia	1	4	295.0 ± 78.3	91.3 ± 14.3	21.3 ± 0.5	nd
Malaysia	2	8	421.7 ± 57.7	119.4 ± 18.4	22.2 ± 2.8	nd
Malaysia	3	6	338.7 ± 59.7	95.0 ± 17.6	19.2 ± 1.6	nd

[a] Stages are: 1, infant formula; 2, follow-on formula; 3, growing up formula. Abbreviations are: 3′SL, 3′sialyllactose; 6′SL, 6′sialyllactose; 6′SLN, 6′sialyllactosamine; DSL, disialyllactose. Data (in μg g^{-1} powder) are the mean ± standard deviation for each sample group. For DSL, the data for the Chinese samples are from the 20 of the 36 samples in which DSL was detected; for the Malaysian samples DSL was not detected (nd), with the exception of one stage 2 sample that was found to contain a DSL concentration of 60.89 μg g^{-1} powder.

(Table 3); for all others the DSL levels were below the LOQ (about 0.195 ng powder on column). For those purchased from Malaysia, only 1 of the 18 samples (excluding the 2 anti-diarrhoea products) had a quantifiable level of DSL (60.89 μg g^{-1}). The levels of DSL for rest of the samples, including the 2 anti-diarrhoea products, were below the LOQ.

3.6. Correlations and comparisons

A significant correlation between 3′SL and 6′SL ($p = 0.906$) was found, as well as positive correlations between 3′SL and 6′SLN ($p = 0.769$) and between 6′SL and 6′SLN ($p = 0.679$) (Fig. 2D–F). This is possibly a true reflection of their relationship in the source materials as there are normally not controlled. The ratios of these oligosaccharides have been shown to be relatively consistent through the bovine lactation period (McJarrow & van Amelsfort-Schoonbeek, 2004), and so higher levels of one in the final formulation analysed here will be reflected by higher levels of the other two. This consistency in the bovine milk source is in contrast to the human milk lactation period where the 3′SL to 6′SL ratio changes (Austin et al., 2016).

Although higher concentrations of 3′SL, 6′SL and 6′SLN were found stage 2 products purchased in Malaysia, concentrations of these oligosaccharides were high in stage 1 products purchased from China. This may due to local regulation of formulation, such as whey proportion, or other reasons unknown.

There are limited literature data on BMO levels in existing IFs. Two older studies (Martín-Sosa et al., 2003; Wang, Brand-Miller, McVeagh, & Petocz, 2001) reported IF contained either lower levels or no sialyl oligosaccharides compared with human milk by measuring oligosaccharide-bound sialic acid, which is a crude measurement of sialyl-oligosaccharides. No direct value of oligosaccharides in IF was reported in these studies. More recently two New Zealand sourced IFs (Fong et al., 2011) were found to contain BMO at the lower end of the concentration range observed in this survey.

The lowest levels of oligosaccharides were found in the anti-diarrhoeal formulations which is likely due to the low proportion, and the type, of dairy ingredients used in these formulations. The ingredient lists for these products showed they contained banana starch or hydrolysed corn starch as the main base powder, whereas the naturally low in BMO dairy derived ingredients, such as potassium caseinate, milk protein concentrate and whey protein concentrate were minor components.

The concentrations of 3′SL, 6′SL and 6′SLN in bovine milk were reported as 35–55 mg L^{-1}, 3.6–9.6 mg L^{-1}, and less than 0.01 mg L^{-1} respectively (Fong et al., 2011; McJarrow & van Amelsfort-Schoonbeek, 2004). When reconstituted at 130 g L^{-1} (the average of manufacturers' recommendations), the concentrations of these three oligosaccharides in the formulated products are approximate 34.8–54.8 mg L^{-1}, 12.1–15.5 mg L^{-1} and 0.8–2.9 mg L^{-1} of 3′SL, 6′SL and 6′SLN, respectively, across all 3 stages from both markets. The levels of 3′SL in both bovine milk and formulated products are very comparable, however, the levels of 6′SL and 6′SLN in bovine milk are significantly lower than those in formulated products. These differences, as well as the large BMO variability observed across all formulated products, are likely to be due to differences in the formulation variation, manufacture processes and variations in ingredients such as skim milk power and whey proteins. Furthermore, it has been reported that BMO do vary over the course of lactation (McJarrow & van Amelsfort-Schoonbeek, 2004), hence will also have an impact on the final BMO levels in the IFs and follow-on products, especially in countries where seasons have a significant impact on milk composition.

The concentrations range of 3′- and 6′-sialyllactose oligosaccharides are ranging from 79 to 157.6 mg L^{-1} and from 41.1 to 365.4 mg L^{-1}, respectively, in human milk (HM) across 1–12 month lactation (Austin et al., 2016; Ma et al., 2018; Nijman et al., 2018). The concentrations of these acidic oligosaccharides are significantly higher than those in formulated products mentioned in this study. The 6′SLN is found in relatively low concentration in human milk, ranging from 0.7 to 5.9 mg L^{-1} (Ma et al., 2018). In this study, the concentrations of 6′SLN in the formulated milk powder products were at the lower end of HM range. The total BMO (3′SL, 6′SL, 6′SLN and DSL) concentration ranges in these formulated products (0.03–0.13 g L^{-1} when reconstituted at 130 g L^{-1}) were significantly lower than the total sialyl-HMOs concentration range (0.13–0.48 g L^{-1}) reported (Austin et al., 2016; Ma et al., 2018) in human milk.

In summary, although the IFs or bovine milk had oligosaccharides in common with HBM (3′SL, 6′SL, 6′SLN, DSL), the levels were typically lower. The concentrations of these oligosaccharides in the formulated milk powder products should be within the HBM range. Here, we determined and reported the concentrations of the major oligosaccharides in a range of formulated milk powder products currently available on the market from both China and Malaysia. These data can be used to calculate enrichment levels required to move formulated paediatric sialyl-oligosaccharide levels into the HBM range.

4. Conclusion

The sialyl-oligosaccharide compositions measured in the formulated milk powder products is reflective of their bovine milk source ingredients, with 3′SL the major oligosaccharide. The concentration of these formulated sialyl-oligosaccharides is significantly lower than that in human milk.

Oligosaccharides are recognised as important bioactive components in human milk, and have been added into IF in the form of GOS and FOS equivalents; however, these are structurally very dissimilar to the naturally existing HMOs. The results of this study provide a picture of the oligosaccharide status in existing IFs, so that HMO can be fortified into IF at levels that similar to those in HBM to help ensure normal growth and development of the infant.

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