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Weighted analysis of 2′-fucosyllactose, 3-fucosyllactose, lacto-*N*-tetraose, 3′-sialyllactose, and 6′-sialyllactose concentrations in human milk

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ABSTRACT

Over 150 human milk oligosaccharides (HMOs) have been identified and their concentrations in human milk vary depending on Secretor and Lewis blood group status, environmental and geographical factors, lactation stage, gestational period, and maternal health. Quantitation of HMOs in human milk has been the focus of numerous studies, however, comprehensive and weighted statistical analyses of their levels in human milk are lacking. Therefore, weighted means, standard deviations, medians, interquartile ranges, and 90th percentiles for 2′-fucosyllactose (2′-FL), 3-fucosyllactose (3-FL), lacto-*N*-tetraose (LNT), 3′-sialyllactose (3′-SL) and 6′-sialyllactose (6′-SL) were calculated using random sampling and the levels of these HMOs in human milk reported in the literature. Probability distributions of the reported levels were also constructed. Although the levels reported in the published studies varied, the weighted means for 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL were calculated to be 2.58, 0.57, 0.94, 0.28, and 0.39 g/L, respectively, which are consistent with those that have been previously determined in other systematic analyses. Likely due to the use of weighting, the 90th percentiles were greater than the 95% confidence limits that have been previously calculated. Our study therefore provides accurate and important statistical data to help support the level of appropriate HMO supplementation in infant formula.

1. Introduction

Human milk oligosaccharides (HMOs) are non-digestible carbohydrates present in human milk. They are the third largest solid component present after lactose and lipids, comprising approximately 20% of the total carbohydrate fraction (Urashima et al., 2012). More than 150 different HMOs have been identified and the concentrations of the individual HMOs vary depending on Secretor and Lewis blood group status, environmental and geographical factors, lactation stage, gestational period, and maternal health (Urashima et al., 2021). The total concentration of HMOs is generally the highest in colostrum and lowest in mature milk, ranging from 20 to 25 g/L and 5–20 g/L, respectively [reviewed in (Thum et al., 2021)]. Importantly, when ingested, approximately 1% of the HMOs are absorbed while the remaining fraction serves as metabolic substrates for select microbes in the gastrointestinal tract, decoy receptors for viral, bacterial, and parasitic pathogens, bacteriostatic agents, and immune modulators [reviewed in (Bode, 2015)].

All HMOs contain lactose at the reducing end, which can be elongated with lacto-*N*-biose units (Galβ1-3GlcNAc) by a β1-3 glycosidic linkage or with *N*-acetyllactosamine units (Galβ1-4GlcNAc) by β1-3 or β1-6 glycosidic linkages, with the β1-6 glycosidic linkage introducing branching [reviewed in (Milani et al., 2017)]. HMOs can also be fucosylated via α1-2, α1-3, or α1-4 linkages or sialyllated via α2-3, or α2-6 linkages at the non-reducing end. Importantly, the combination of branching, fucosylation, and sialylation contribute to the diversity in the HMO repertoire (Milani et al., 2017).

Currently, lactating females can be classified into four groups based on the expression and activity of α1-2-fucosyltransferase (FUT2) and α1-3/4 fucosyltransferase (FUT3), which are encoded by the *Se* and *Le* genes, respectively [reviewed in (Bode, 2015)]. FUT2 transfers fucose to the terminal galactose of the oligosaccharide with a α1-2 linkage

Abbreviations: 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; LNT, lacto-*N*-tetraose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose; HMO, human milk oligosaccharide.

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whereas FUT3 transfers fucose to the subterminal N-acetyl-glucosamine of lacto-*N*-biose with a α1-3/4 linkage. Lactating females who express active FUT2 are known as "secretors' and their milk is abundant in α1-2-fucoslyated HMOs, notably 2′-fucosyllactose (2′-FL) and lacto-*N*-fucopentose I (LNFP I). Lactating females who do not express active FUT2 are known as "non-secretors" and their milk contains low or undetectable levels of α1-2-fucoslyated HMOs. In contrast, lactating females expressing FUT3 are considered Lewis-positive and their milk contains α1-3-fucoslyated and α1-4-fucoslyated HMOs, such as 3-fucosyllactose (3-FL) and LNFP II. Lactating females who do not express FUT3 are considered Lewis-negative. Although this classification system appears to be mutually exclusive, milk from non-secretor, Lewis-negative females contains low concentrations of fucosylated HMOs such as 3-FL and LNFP III, likely due to the presence of other fucosyltransferases that compensate for the lack of FUT3 (Gabrielli et al., 2011; Austin et al., 2019; Oriol et al., 1999). Additionally, milk from non-secretor females can contain higher amounts of neutral, non-fucosylated HMOs, likely due to the lack of FUT2 (Thurl et al., 2017).

As the natural sole source of nutrition, human milk contains the essential nutrients and other components, such as cytokines, immunoglobulins, lactoferrin, immune cells, metabolic hormones, and growth factors for healthy growth and development for breast-fed infants (Ballard and Morrow, 2013). Human milk and breast feeding are therefore the normative standard for infant nutrition (Section on Breastfeeding, 2012). Breast feeding and/or the ingestion of human milk, however, is not always feasible and/or adequate, forcing caregivers to rely on infant formula, which is not compositionally equivalent to human milk. As a result, achieving a better understanding of human milk composition, such as HMO content and composition, has been the focus of numerous investigations over the last two decades with the goal of bringing infant formula composition closer to that of human milk. Recently, Thurl et al. (2017) calculated mean concentrations and confidence limits for 33 neutral and acidic oligosaccharides using the statistical data reported in published studies that quantitated HMOs in human milk from different world regions. Consistent with the results reported in the primary studies, the review authors concluded that the HMO concentrations vary depending on secretor status, gestational age, and duration of lactation (Thurl et al., 2017). Soyyilmaz et al. (2021) and Thum et al. (2021) reported recently similar results for HMO concentrations in breast milk, as reported by Thurl and colleagues (Thurl et al., 2017). Additionally, they confirmed that milk from non-secretor females contains higher amounts of non-fucosylated HMOs, as well as higher amounts of α1-3 and α1-4 fucosylated HMOs. Importantly, the means calculated by Thurl et al. (2017) were not appropriately weighted, means and confidence limits for neutral HMOs in non-secretor milk were not determined, and probability distributions of the means extracted from the included studies were not reported. Weighting the statistical data reported in the published studies is of particular importance because it prevents bias and allows for a more accurate representation of the results reported across all of the published studies. Thus, although the systematic reviews conducted provide insight into the mean levels and range of HMOs in human milk (Thurl et al., 2017; Soyyilmaz et al., 2021; Parschat et al., 2021), an accurate representation of the mean concentrations in human milk and how the levels are distributed in the population are still lacking.

Recently, the tolerability, safety, and growth effects of an infant formula containing 2.99 g/L 2′-FL, 0.75 g/L 3-FL, 1.5 g/L lacto-*N*-tetraose (LNT), 0.23 g/L 3′-sialyllactose (3′-SL) and 0.28 g/L 6′-sialyllactose (6′-SL) were evaluated in a randomized, placebo-controlled, parallel-group clinical study conducted in healthy infants (Parschat et al., 2021). To understand how the levels of these HMOs relate to the amount of these HMOs in human milk, weighted means, standard deviations, medians, interquartile ranges, 90th percentiles, and probability distributions were calculated in the present study using statistical data reported in previous studies. Because the primary data from which the statistical data reported in these studies was not always available, random sampling of the distributions reported in each study was conducted to simulate each primary data set. The simulated data sets were then combined and means, standard deviations, medians, interquartile ranges, 90th percentiles, and probability distributions were calculated to obtain an accurate representation of the levels of 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL in human milk.

2. Materials and methods

2.1. Literature search and selection

To identify all published studies that quantitated 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL levels in human milk, all studies included in the systematic review conducted by Thurl et al. (2017) were retrieved and reviewed to determine if they met the inclusion criteria (Table 1). Literature searches were also conducted up to May 3, 2021 using PubMed and the search terms "human AND milk AND oligosaccharide", as well as "2′-fucosyllactose", "3-fucosyllactose", "lacto-*N*-tetraose", "3′-sialyllactose", and "6′-sialyllactose" to identify other studies that quantitated the HMOs in human milk. The titles and abstracts of the retrieved studies were then reviewed to determine if they met the inclusion criteria. If a study met the inclusion criteria, the full study was retrieved and reviewed to determine if the absolute amount of 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL in human milk was quantitated. If additional studies were identified during the review of the retrieved studies, they were retrieved and reviewed to determine if they also quantitated the amount of 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL in human milk.

2.2. Statistical analyses

For all included studies, the available statistical data (*n*, mean, standard deviation, range, median, interquartile range, etc.) for the HMOs were recorded in Microsoft Excel in grams per liter. For 2′-FL specifically, only data from secretor females were recorded based on the secretor status reported in the study or, if that was not provided, from the reported HMO pattern. For 3-FL, LNT, 3′-SL, and 6′-SL, all reported statistical data were used regardless of secretor status, except when combined secretor and non-secretor data was reported. If a study reported combined secretor and non-secretor data, then either the combined data or the secretor and non-secretor data was used to avoid duplicating the data. If the studies reported the HMO levels in non-numerical/graphical format only, then the mean, median, range, and/or interquartile ranges for the HMO were determined by printing enlarged graphs, measuring the axis, determining the corresponding level of the HMO using a ruler, and integrating the manual data into the spreadsheet. If a study reported only HMO levels in the milk from each participant, then the average, standard deviation, and median level for the HMOs for all the study participants were calculated and entered into the data set for that study. If a study provided the mean, standard error, and the number of participants, then the standard deviation was calculated from the standard error and the number of participants.

Table 1
Selection criteria.

Inclusion Criteria	Exclusion Criteria
• Secretor status of participants could be determined (for 2′-FL only) • Original articles from peer-reviewed journals • Absolute concentrations of single HMOs	• Animal studies • Concentrations of mixtures of HMOs • Relative concentrations of HMOs • HMO concentrations from pooled milk samples • Abstracts, monographs, review articles • Studies with data already reported

To appropriately weight the included studies, random sampling of the distributions from each study was conducted to recreate the raw data set using MATLAB, which provides 30 continuous probability distributions in which random variables can assume any value (https://www.mathworks.com/help/stats/continuous-distributions.html). Importantly, the type of continuous probability distribution used to simulate the raw data set from each study was determined based on the type of statistical data that was provided in each study. For example, if a study reported only the number of samples, mean and standard deviation, the distribution was assumed to be normal and the Normal Distribution function was used to create a synthetic data set with the same number of samples, mean and standard deviation. If a study reported the number of samples, and the maximum and minimum levels for the analyte, the Uniform Distribution function was used to create a synthetic data set from that distribution with the same maximum and minimum. The synthetic data points from all of the studies were then combined, generating a final distribution from which a mean, standard deviation, median, interquartile range, and 90th percentile was calculated.

Variability in the method was determined by repeating the random sampling for all data sets from the included studies 100 times and calculating the standard deviation of the mean, standard deviation, median, interquartile range and 90th percentiles from the 100 simulations.

3. Results

3.1. 2′-Fucosyllactose

Sixty-five published studies, either cited by (Thurl et al., 2017) or obtained from searches of PubMed, were reviewed to determine if absolute levels of 2′-FL were quantitated in human milk and the secretor status of the participants was defined (Gabrielli et al., 2011; Austin et al., 2019; Wang et al., 2020; Goehring et al., 2014; Chaturvedi et al., 1997; Song et al., 2002; Sumiyoshi et al., 2003a; Morrow et al., 2004; Musumeci et al., 2006; Sjögren et al., 2007; Leo et al., 2009; Albrecht et al., 2010; Albrecht et al., 2011; De Leoz et al., 2012; Galeotti et al., 2012; Totten et al., 2012; Kottler et al., 2013; Galeotti et al., 2014; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; Wang et al., 2015; Austin et al., 2016; Williams et al., 2017; Xu et al., 2017; Nijman et al., 2018; Isganaitis et al., 2019; Moossavi et al., 2019; Jorgensen et al., 2020; Mank et al., 2020; Borewicz et al., 2020; Binia et al., 2021; Coppa et al., 1999; Nakhla et al., 1999; Erney et al., 2000; Chaturvedi et al., 2001; Asakuma et al., 2008; Thurl et al., 2010; Coppa et al., 2011; Smilowitz et al., 2013; Hong et al., 2014; Van Niekerk et al., 2014; Aakko et al., 2017; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Saben et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Oliveros et al., 2021; Plows et al., 2021; Saben et al., 2021; Wang et al., 2021; Ma et al., 2018b). Because 2′-FL production is dependent on the expression of FUT2 and the milk from non-secretor females does not contain 2′-FL [reviewed in (Bode, 2015)], 2′-FL levels from only secretor females were included in the analysis. Twenty-nine were excluded for at least one of the following reasons: 1) reporting of relative quantitative data; 2) failure to report secretor status; or 3) reporting of results from another publication (Chaturvedi et al., 1997; Song et al., 2002; Sumiyoshi et al., 2003a; Morrow et al., 2004; Musumeci et al., 2006; Sjögren et al., 2007; Leo et al., 2009; Albrecht et al., 2010; Albrecht et al., 2011; De Leoz et al., 2012; Galeotti et al., 2012; Totten et al., 2012; Kottler et al., 2013; Galeotti et al., 2014; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; Wang et al., 2015; Austin et al., 2016; Williams et al., 2017; Xu et al., 2017; Nijman et al., 2018; Isganaitis et al., 2019; Moossavi et al., 2019; Jorgensen et al., 2020; Mank et al., 2020; Borewicz et al., 2020; Binia et al., 2021; Ma et al., 2018b) (Supplemental Table 1). From the remaining 36 studies, statistical data for 134 distributions with a total of 6797 data points were extracted and recorded (Supplemental Table 2) (Gabrielli et al., 2011; Austin et al., 2019; Wang et al., 2020; Goehring et al., 2014; Azad et al., 2018; Coppa et al., 1999; Nakhla et al., 1999; Erney et al., 2000; Chaturvedi et al., 2001; Asakuma et al., 2008; Thurl et al., 2010; Coppa et al., 2011; Smilowitz et al., 2013; Hong et al., 2014; Van Niekerk et al., 2014; Aakko et al., 2017; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Saben et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Oliveros et al., 2021; Plows et al., 2021; Saben et al., 2021; Wang et al., 2021). Twelve of these 36 studies were included in the systematic review by (Thurl et al., 2017). One hundred random sampling simulations of the distributions provided in each study yielded a mean of 2.58±0.012, a standard deviation of 1.46±0.017, a median of 2.56±0.054 and a 90th percentile of 4.32±0.028 g/L (Table 2). The interquartile range was 1.14–3.89 g/L. A representative probability distribution of one random sampling simulation shows that the 2′-FL levels are normally distributed (Fig. 1A). The increased frequency of the values close to zero was due to one study that quantitated 2′-FL concentrations in 268 milk samples from secretors females, ranging from 0.04 to 0.28 g/L based on estimations from graphical data (Wang et al., 2020).

3.2. 3-Fucosyllactose

Sixty-five published studies, either cited by (Thurl et al., 2017) or obtained from searches of PubMed, were reviewed to determine the absolute levels of quantitated 3-FL in human milk (Gabrielli et al., 2011; Austin et al., 2019; Wang et al., 2020; Chaturvedi et al., 1997; Song et al., 2002; Sumiyoshi et al., 2003a; Morrow et al., 2004; Newburg et al., 2004; Sjögren et al., 2007; Albrecht et al., 2010; Albrecht et al., 2011; De Leoz et al., 2012; Galeotti et al., 2012; Totten et al., 2012; Kottler et al., 2013; Galeotti et al., 2014; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; AustinDe Castro et al., 2016; Williams et al., 2017; Xu et al., 2017; Isganaitis et al., 2019; Moossavi et al., 2019; Azad et al., 2018; Jorgensen et al., 2020; Mank et al., 2020; Borewicz et al., 2020; Binia et al., 2021; Coppa et al., 1999; Nakhla et al., 1999; Erney et al., 2000; Chaturvedi et al., 2001; Asakuma et al., 2008; Thurl et al., 2010; Coppa et al., 2011; Smilowitz et al., 2013; Van Niekerk et al., 2014; Aakko et al., 2017; McGuire et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Saben et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Plows et al., 2021; Saben et al., 2021; Wang et al., 2021; Underwood et al., 2015; Thurl et al., 1996; Leo et al., 2010; Bode et al., 2012; Bao et al., 2013; Kuhn et al., 2015; Ma et al., 2018a; Tonon et al., 2019; Lodge et al., 2020). Fifteen studies were excluded for at least one of the following reasons: 1) reporting of relative quantitative data or 2) reporting of results from another publication (Morrow et al., 2004; Albrecht et al., 2010; Albrecht et al., 2011; De Leoz et al., 2012; Galeotti et al., 2012; Totten et al., 2012; Kottler et al., 2013; Galeotti et al., 2014; Isganaitis et al., 2019; Moossavi et al., 2019; Jorgensen et al., 2020; Mank et al., 2020; Binia et al., 2021; Saben et al., 2020; Underwood et al., 2015) (Supplemental Table 3). Although the expression of FUT3 is required for 3-FL production, 3-FL is present in the milk from those lacking FUT3, indicating that other fucosyltransferases may compensate FUT3 deficiency (Gabrielli et al., 2011; Austin et al., 2019; Oriol et al., 1999). Therefore, 3-FL levels in the milk of secretor and non-secretor females were included in the analysis from the 50 studies included in the analysis (Gabrielli et al., 2011; Austin et al., 2019; Wang et al., 2020; Chaturvedi et al., 1997; Song et al., 2002; Sumiyoshi et al., 2003a; Newburg et al., 2004; Sjögren et al., 2007; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; AustinDe Castro et al., 2016; Williams et al., 2017;

Table 2
Random sampling results for the human milk oligosaccharides.

	2′-FL	3-FL	LNT	3′-SL	6′-SL
Number of Studies Retrieved	65	65	68	63	54
Number of Studies Excluded	29	15	20	15	13
Number of Studies Included	36	50	48	48	41
Number of Distributions	134	170	150	190	187
Number of Subjects	6797	9372	8205	8031	7780
Mean ± SD from 100 simulations (g/L)	2.58 ± 0.012	0.57 ± 0.0045	0.94 ± 0.039	0.28 ± 0.0021	0.39 ± 0.002
SD ± SD from 100 simulations (g/L)	1.46 ± 0.017	0.67 ± 0.0062	3.3 ± 0.61	0.29 ± 0.0092	0.32 ± 0.002
Median ± SD from 100 simulations (g/L)	2.56 ± 0.054	0.32 ± 0.0045	0.82 ± 0.0057	0.23 ± 0.0018	0.33 ± 0.003
90%-tile ± SD from 100 simulations (g/L)	4.32 ± 0.028	1.4 ± 0.18	1.76 ± 0.016	0.49 ± 0.0057	0.77 ± 0.0072
Interquartile Range (g/L)	1.14–3.89	0.057–1.1	0.35–1.5	0.10–0.42	0.09–0.65

Abbreviations: 2′-FL – 2′-fucosyllactose; 3-FL – 3-fucosyllactose; LNT – lacto-*N*-tetraose; 3′-SL – 3′-sialyllactose; 6′-SL – 6′-sialyllactose; SD – standard deviation.
[a] 2′-FL levels in milk from secretor females only.

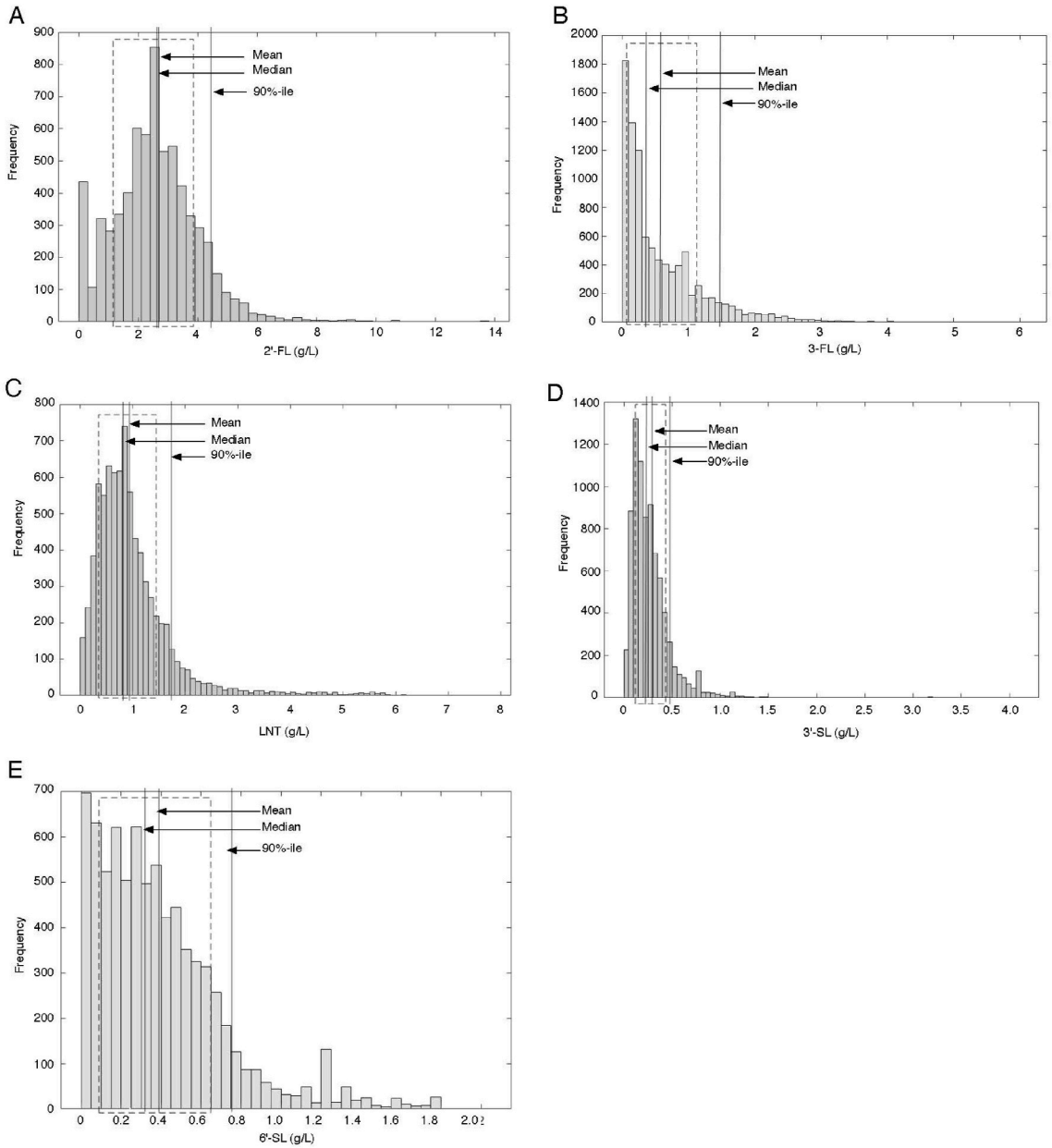

Fig. 1. Human Milk Oligosaccharide Distributions. A. Distribution of reported 2′-FL levels in the milk of secretor females. B. Distribution of reported 3-FL levels in the milk of secretor and non-secretor females. C. Distribution of reported LNT levels in the milk of secretor and non-secretor females D. Distribution of reported 3′-SL levels in the milk of secretor and non-secretor females. E. Distribution of reported 6′-SL levels in the milk of secretor and non-secretor females. Means, medians, and 90th percentiles are highlighted with arrows. The hatched boxes denote the interquartile range.

Xu et al., 2017; Azad et al., 2018; Borewicz et al., 2020; Coppa et al., 1999; Nakhla et al., 1999; Erney et al., 2000; Chaturvedi et al., 2001; Asakuma et al., 2008; Thurl et al., 2010; Coppa et al., 2011; Smilowitz et al., 2013; Van Niekerk et al., 2014; Aakko et al., 2017; McGuire et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Plows et al., 2021; Saben et al., 2021; Wang et al., 2021; Thurl et al., 1996; Leo et al., 2010; Bode et al., 2012; Bao et al., 2013; Kuhn et al., 2015; Ma et al., 2018a; Tonon et al., 2019; Lodge et al., 2020). Ten of these 50 studies were included in the systematic review conducted by (Thurl et al., 2017). Statistical data from 170 distributions with a total of 9372 data points were extracted and recorded (Supplemental Table 4). One hundred random sampling simulations of the distributions provided in each of the studies yielded a 3-FL mean of 0.57±0.0045, a standard deviation of 0.67±0.0062, a median of 0.32±0.045 and a 90th percentile of 1.4 ±0.18 g/L (Table 2). The interquartile range was 0.057–1.1 g/L. A representative probability distribution of one random sampling simulation shows that the 3-FL levels are skewed toward higher levels because the mean is higher than the median (Fig. 1B).

3.3. Lacto-*N*-tetraose

Sixty-eight published studies, either cited by (Thurl et al., 2017) or obtained from searches of PubMed, were reviewed to determine the absolute levels of quantitated LNT in human milk (Gabrielli et al., 2011; Austin et al., 2019; Wang et al., 2020; Chaturvedi et al., 1997; Song et al., 2002; Sumiyoshi et al., 2003a; Morrow et al., 2004; Newburg et al., 2004; Sjögren et al., 2007; Leo et al., 2009; Albrecht et al., 2010; Albrecht et al., 2011; De Leoz et al., 2012; Galeotti et al., 2012; Totten et al., 2012; Kottler et al., 2013; Galeotti et al., 2014; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; Wang et al., 2015; AustinDe Castro et al., 2016; Williams et al., 2017; Nijman et al., 2018; Isganaitis et al., 2019; Moossavi et al., 2019; Azad et al., 2018; Jorgensen et al., 2020; Mank et al., 2020; Borewicz et al., 2020; Binia et al., 2021; Coppa et al., 1999; Nakhla et al., 1999; Asakuma et al., 2008; Thurl et al., 2010; Smilowitz et al., 2013; Hong et al., 2014; Van Niekerk et al., 2014; Aakko et al., 2017; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Saben et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Plows et al., 2021; Saben et al., 2021; Underwood et al., 2015; Bode et al., 2012; Bao et al., 2013; Kuhn et al., 2015; Ma et al., 2018a; Tonon et al., 2019; Lodge et al., 2020; Kunz et al., 1999; Olivares et al., 2015; Sarkozy et al., 2020). Twenty studies were excluded for at least one of the following reasons: 1) reporting of relative quantitative data; 2) LNT levels were quantified with lacto-*N*-neotetraose (LNnT); or 3) reporting of results from another publication (Wang et al., 2020; Morrow et al., 2004; Albrecht et al., 2010; Albrecht et al., 2011; De Leoz et al., 2012; Galeotti et al., 2012; Totten et al., 2012; Kottler et al., 2013; Galeotti et al., 2014; Wang et al., 2015; Isganaitis et al., 2019; Moossavi et al., 2019; Jorgensen et al., 2020; Mank et al., 2020; Borewicz et al., 2020; Binia et al., 2021; Ayoub Moubareck et al., 2020; Saben et al., 2020; Underwood et al., 2015; Tonon et al., 2019) (Supplemental Table 5). Although Lewis blood group antigen genotype of the female can affect the LNT levels in their milk, the expression of FUT2 or FUT3 is not required for LNT production [reviewed in (Bode, 2015)]. Therefore, LNT levels in the milk of secretor and non-secretor females were included in the analysis. From the 48 studies included in the analysis, statistical data for 150 distributions with a total of 8205 data points were extracted and recorded (Gabrielli et al., 2011; Austin et al., 2019; Chaturvedi et al., 1997; Song et al., 2002; Sumiyoshi et al., 2003a; Newburg et al., 2004; Sjögren et al., 2007; Leo et al., 2009; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; AustinDe Castro et al., 2016; Williams et al., 2017; Nijman et al., 2018; Azad et al., 2018; Coppa et al., 1999; Nakhla et al., 1999; Asakuma et al., 2008; Thurl et al., 2010; Smilowitz et al., 2013; Hong et al., 2014; Van Niekerk et al., 2014; Aakko et al., 2017; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Plows et al., 2021; Saben et al., 2021; Bode et al., 2012; Bao et al., 2013; Kuhn et al., 2015; Ma et al., 2018a; Lodge et al., 2020; Kunz et al., 1999; Olivares et al., 2015; Sarkozy et al., 2020) (Supplemental Table 6). Eleven of the 48 studies were included in the systematic review conducted by (Thurl et al., 2017). One hundred random sampling simulations of the distributions provided in each of the studies yielded a LNT mean of 0.94±0.0039, a standard deviation of 3.30±0.61, a median of 0.82±0.0057 and a 90th percentile of 1.76±0.016 g/L (Table 2). The interquartile range was 0.35–1.5 g/L. A representative probability distribution of one random sampling simulation shows that the LNT levels are skewed toward higher levels because the mean is higher than the median (Fig. 1C).

3.4. 3′-Sialyllactose

Sixty-three published studies, either cited by (Thurl et al., 2017) or obtained from searches of PubMed, were reviewed to determine the absolute levels of quantitated 3′-SL in human milk (Gabrielli et al., 2011; Austin et al., 2019; Albrecht et al., 2010; Albrecht et al., 2011; Galeotti et al., 2012; Totten et al., 2012; Kottler et al., 2013; Galeotti et al., 2014; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; AustinDe Castro et al., 2016; Williams et al., 2017; Nijman et al., 2018; Isganaitis et al., 2019; Moossavi et al., 2019; Azad et al., 2018; Jorgensen et al., 2020; Mank et al., 2020; Borewicz et al., 2020; Binia et al., 2021; Coppa et al., 1999; Thurl et al., 2010; Smilowitz et al., 2013; Hong et al., 2014; Van Niekerk et al., 2014; Aakko et al., 2017; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Saben et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Plows et al., 2021; Saben et al., 2021; Wang et al., 2021; Underwood et al., 2015; Thurl et al., 1996; Leo et al., 2010; Bode et al., 2012; Kuhn et al., 2015; Ma et al., 2018a; Tonon et al., 2019; Lodge et al., 2020; Kunz et al., 1999; Shen et al., 2000; Bao and Newburg, 2008; Bao et al., 2007; Martín-Sosa et al., 2003; Sumiyoshi et al., 2003b; Asakuma et al., 2007; Sakaguchi et al., 2014; Monti et al., 2015). Fifteen studies were excluded for at least one of the following reasons: 1) reporting of relative quantitative data; 2) reporting of pooled milk; or 3) reporting of results from another publication (Albrecht et al., 2010; Albrecht et al., 2011; Galeotti et al., 2012; Totten et al., 2012; Kottler et al., 2013; Galeotti et al., 2014; Isganaitis et al., 2019; Moossavi et al., 2019; Jorgensen et al., 2020; Mank et al., 2020; Binia et al., 2021; Saben et al., 2020; Underwood et al., 2015; Shen et al., 2000; Bao and Newburg, 2008) (Supplemental Table 7). From the 48 studies included in the analysis, statistical data for 190 distributions with a total of 8031 data points were extracted and recorded (Gabrielli et al., 2011; Austin et al., 2019; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; AustinDe Castro et al., 2016; Williams et al., 2017; Nijman et al., 2018; Azad et al., 2018; Borewicz et al., 2020; Coppa et al., 1999; Thurl et al., 2010; Smilowitz et al., 2013; Hong et al., 2014; Van Niekerk et al., 2014; Aakko et al., 2017; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Plows et al., 2021; Saben et al., 2021; Wang et al., 2021; Thurl et al., 1996; Leo et al., 2010; Bode et al., 2012; Kuhn et al., 2015; Ma et al., 2018a; Tonon et al., 2019; Lodge et al., 2020; Kunz et al., 1999; Bao et al., 2007; Martín-Sosa et al., 2003; Sumiyoshi et al., 2003b;

Asakuma et al., 2007; Sakaguchi et al., 2014; Monti et al., 2015) (Supplementary Table 8). Eleven of the 48 studies were included in the systematic review conducted by (Thurl et al., 2017). One hundred random sampling simulations of the distributions provided in each of the studies yielded a 3′-SL mean of 0.28±0.0021, a standard deviation of 0.29±0.0092, a median of 0.23±0.0018 and a 90th percentile of 0.49 ±0.0057 g/L (Table 2). The interquartile range was 0.10–0.42 g/L. A representative probability distribution of one random sampling simulation shows that the 3′-SL levels are skewed toward higher levels because the mean is higher than the median (Fig. 1D).

3.5. 6′-Sialyllactose

Fifty-four published studies, either cited by (Thurl et al., 2017) or obtained from searches of PubMed, were reviewed to determine the absolute levels of quantitated 6′-SL in human milk (Gabrielli et al., 2011; Austin et al., 2019; Albrecht et al., 2010; Albrecht et al., 2011; Totten et al., 2012; Kottler et al., 2013; Spevacek et al., 2015; AustinDe Castro et al., 2016; Nijman et al., 2018; Isganaitis et al., 2019; Moossavi et al., 2019; Azad et al., 2018; Jorgensen et al., 2020; Mank et al., 2020; Borewicz et al., 2020; Binia et al., 2021; Coppa et al., 1999; Asakuma et al., 2008; Thurl et al., 2010; Smilowitz et al., 2013; Hong et al., 2014; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Saben et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Oliveros et al., 2021; Plows et al., 2021; Saben et al., 2021; Underwood et al., 2015; Thurl et al., 1996; Leo et al., 2010; Ma et al., 2018a; Tonon et al., 2019; Lodge et al., 2020; Kunz et al., 1999; Shen et al., 2000; Bao and Newburg, 2008; Bao et al., 2007; Martín-Sosa et al., 2003; Sumiyoshi et al., 2003b; Sakaguchi et al., 2014; Monti et al., 2015). Thirteen studies were excluded for at least one of the following reasons: 1) reporting of relative quantitative data; 2) reporting of pooled milk; or 3) reporting of results from another publication (Albrecht et al., 2010; Albrecht et al., 2011; Totten et al., 2012; Kottler et al., 2013; Isganaitis et al., 2019; Moossavi et al., 2019; Jorgensen et al., 2020; Mank et al., 2020; Binia et al., 2021; Saben et al., 2020; Underwood et al., 2015; Shen et al., 2000; Bao and Newburg, 2008) (Supplemental Table 9). From the 41 studies included in the analysis, statistical data for 187 distributions with a total of 7780 data points were extracted and recorded (Gabrielli et al., 2011; Austin et al., 2019; Spevacek et al., 2015; AustinDe Castro et al., 2016; Nijman et al., 2018; Azad et al., 2018; Borewicz et al., 2020; Coppa et al., 1999; Asakuma et al., 2008; Thurl et al., 2010; Smilowitz et al., 2013; Hong et al., 2014; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Ayoub Moubareck et al., 2020; Berger et al., 2020; Ferreira et al., 2020; Lagström et al., 2020; Lugli et al., 2020; Seferovic et al., 2020; Torres Roldan et al., 2020; Wu et al., 2020; Oliveros et al., 2021; Plows et al., 2021; Saben et al., 2021; Thurl et al., 1996; Leo et al., 2010; Ma et al., 2018a; Tonon et al., 2019; Lodge et al., 2020; Kunz et al., 1999; Bao et al., 2007; Martín-Sosa et al., 2003; Sumiyoshi et al., 2003b; Sakaguchi et al., 2014; Monti et al., 2015) (Supplemental Table 10). Eleven of the 48 studies were included in the systematic review conducted by (Thurl et al., 2017). One hundred random sampling simulations of the distributions provided in each of the studies yielded a mean of 0.39±0.002, a standard deviation of 0.32±0.002, a median of 0.33±0.003 and a 90th percentile of 0.77±0.0072 g/L for 6′-SL (Table 2). The interquartile range was 0.09–0.54 g/L. A representative probability distribution of one random sampling simulation shows that the 6′-SL levels are skewed toward higher levels because the mean is higher than the median (Fig. 1E).

4. Discussion

In this systematic review, weighted means, standard deviations, medians, 90th percentile concentrations, interquartile ranges, and probability distributions were calculated for the levels of 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL in human milk, assuming the methods used to quantitate these HMOs in the included studies are equivalent. Although the weighted means calculated here are similar to those calculated in previous studies (Thurl et al., 2017; Soyyilmaz et al., 2021) (<1.5 fold difference), the weighting and inclusion of 3-FL and LNT milk levels from non-secretor females in our analysis provide a more accurate representation of the amount of these HMOs in human milk. The probability distributions also show that the levels of LNT, 3-FL, 3′-SL, and 6′-SL in human milk are not normally distributed, with levels reaching 3- to 6-fold greater than their means. The lack of normality for these HMOs is likely due to a combination of the kinetics of their production in human milk over the course of lactation, including samples from term and pre-term infants, the timepoints at which the milk samples were collected during the studies, and the compliance of the milk donors to the protocol over the course of each clinical study. Importantly, although variability in the levels exist, the standard deviations calculated from the 100 random sampling simulations show that the variability is not large enough to affect the calculated means, standard deviations, medians, 90th percentiles, and interquartile ranges for the population. Thus, it is likely that the means calculated here and by Thurl et al. and Soyyilmaz et al. approximate the true means for 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL in human milk.

Until recently standard infant formulas have lacked HMOs due to the inability to manufacture them at commercial scale. To fill this inadequacy, infant formula manufacturers have resorted to supplementing their infant formulas with artificially-produced, non-human milk oligosaccharides, such as galactooligosaccharides (GOS), polydextrose, oligofructose, long-chain inulin, and fructooligosaccharides (FOS), to mimic the prebiotic activities of human milk (Salminen et al., 2020). Chemically- and microbially-produced 2′-FL, 3-FL, LNT, lacto-*N*-neotetraose (LNnT), 3′-SL, 6′-SL, and difucosyllactose are now commercially available and have been determined to be safe for use in infant formulas in the United States, European Union, and other jurisdictions (US FDA, 2014e, 2015b, 2016b, 2018a, 2018b, 2019b, 2019a, 2019c, 2020b, 2020a, 2020f, 2020e, 2020d, 2021a, 2021b; EFSA Panel on Dietetic Products et al., 2015; EFSA Panel on Dietetic Products and Nutrition and Allergies, 2015; EFSA Panel on Nutrition et al., 2019; EFSA Panel on Nutrition et al., 2020a; EFSA Panel on Nutrition et al., 2020b; EFSA Panel on Nutrition et al., 2021; EFSA Panel on Nutrition and Novel Foods and Food Allergens, 2019). However, because infant formula is the only alternative to human milk during the initial 4–6 months of life and is consumed during a sensitive period of development, the addition and/or use of novel, non-nutritive ingredients, such as the manufactured HMOs, brings to light key questions regarding not only safety, but also the appropriate level of supplementation.

Infant formula ingredients must be safe and not impede the ability of the formula to deliver the nutritional components required for growth and development. Safety is demonstrated using adequately controlled toxicology and clinical studies (Institute of Medicine, 2004). An additional consideration during the safety evaluation of HMOs is their limited absorption. The bulk of HMOs pass through the gastrointestinal tract where they are either metabolized by the microbiota or excreted in the feces. They are largely unavailable systemically as demonstrated by the small amounts that have been detected in the plasma and urine of infants consuming human milk and/or HMO-containing infant formulas (Marriage et al., 2015; Goehring et al., 2014; Ruhaak et al., 2014; Dotz et al., 2014). Moreover, clinical studies conducted in infants have shown that the absorption of 2′-FL from a 2′-FL-containing infant formula is similar to that from human milk demonstrating that the formula matrix does not change bioavailability of the HMO. Ingestion of formulas containing 2′-FL or a mixture of 2′-FL and LNnT are well-tolerated and

do not impair growth (Marriage et al., 2015; Goehring et al., 2016; Storm et al., 2019; Nowak-Wegrzyn and Reyes, 2019; Puccio et al., 2017; Kajzer and Oliver, 2016; Riechmann et al., 2020). More recently, a clinical study conducted showed that the ingestion of an infant formula containing 2.99 g/L 2′-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3′-SL and 0.28 g/L 6′-SL was well-tolerated and did not impair growth (Parschat et al., 2021). Taken together, these data indicate that the absorption of HMOs from HMO-containing infant formula is similar to their absorption from human milk, and when ingested alone or in combination up to 5.75 g/L, are safe and do not interfere with the ability of infant formula to support normal growth.

The concept of appropriate intake is one that has been defined by the Institute of Medicine (IOM) for nutrients (vitamins, minerals, macronutrients, and energy) using Dietary Reference Intakes (DRIs), which are a common set of reference values that are based on scientifically grounded relationships between nutrient intakes and indicators of adequacy, as well as the prevention of chronic diseases in healthy populations. Dietary Reference Intakes include the Estimated Average Requirement (EAR), the Recommended Dietary Allowance (RDA), the Adequate Intake (AI), and the Tolerable Upper Intake Level (UL). Although the DRIs have not been developed for novel ingredients such as artificially-produced HMOs and non-human milk oligosaccharides, there has been a significant paradigm shift from the concept of RDAs that existed prior to 1990, which focused primarily on reducing incidence of diseases of deficiency. Dietary Reference Intakes now embrace the concept of optimizing health and the IOM emphasizes that there is a distribution of requirements within a population associated with this concept (Institute of Medicine, 2004). Today, IOM compares the distribution of intakes to the distribution of requirements and inferences are then made about the degree of adequacy in deriving the DRIs. Moreover, IOM has opined that the assessment of dietary intakes should be used as only one part of the assessment, and the results must be kept in context. Nutrient intake data should always be considered in combination with other information, such as anthropometric measurements, biochemical indices, diagnoses, clinical status, and other factors (Institute of Medicine, 2004). Therefore, dietary adequacy or appropriateness for novel ingredients should utilize these principles, be evaluated based on the totality of evidence, not on dietary intake data alone, and answer the question “adequate for what?”

The safety of food ingredients must accommodate the distribution of intake, which includes heavy consumers (those at the 90th and 95th percentiles of intake), because a varied distribution of intake occurs in the free-living population (Alger et al., 2013; Ververis et al., 2020). Currently, a variety of non-digestible carbohydrate ingredients are used at levels that approximate the highest levels found in human milk. Specifically, GOS is added to infant formula at levels up to 8.0 g/L, based on the highest amount of complex oligosaccharides reported (5–8 g/L) (Kunz et al., 2000) and supporting toxicology and clinical studies (US FDA, 2008, 2009a, 2009b, 2010, 2014b, 2014c, 2014d, 2014a, 2015a, 2016c, 2017, 2018c, 2020c; Commission Implementing Regulation (EU) 2017/2470). Likewise, *N*-acetyl-D-neuraminic acid (also referred to as sialic acid) has been determined safe for use in infant formulas up to 50 mg/L, based on the highest amount found in mature milk (10–60 mg/L) (US FDA, 2016a). The use levels of artificially produced HMOs, however, are in stark contrast to this approach. The artificially produced forms of 2′-FL, 3-FL, LNT, LNnT, 3′-SL, 6′-SL, and a mixture of 2′-FL and difucosyllactose in infant formula is currently allowed in the United States only when the level of supplementation corresponds to the mean levels found in human milk. Yet, all of these HMOs have been demonstrated to be safe for ingestion at levels that exceed the highest levels found in human milk in numerous toxicology and neonatal piglet tolerance studies. Importantly, the risk assessments that support the safe use of these HMOs in infant formula have not addressed the question of whether supplementation at the mean levels is the most appropriate for the majority of infants consuming the formula.

Over the last two decades, the role of HMOs in infant health has been the subject of extensive research. Human milk oligosaccharides are the third largest solid component in human milk and are believed to play a pivotal role in balancing the intestinal microbiota, imparting antimicrobial effects, developing the intestinal barrier, and modulating immune response (Moubareck, 2021). Our results show that the levels of 2′-FL, 3-FL, LNT, 3′-SL, and 6′-SL vary widely and define their distribution in the general population Considering the overarching goal of matching infant formula composition as closely as possible to human milk, the “appropriate” supplementation levels should accommodate the variability of the HMO levels found in human milk. Moreover, supplementation above the mean levels in human milk is not only appropriate, but safe, as demonstrated in extensive toxicology, tolerance, and clinical studies that directly support the safety of the HMOs, as well as other similar prebiotic ingredients.

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Institutional review board statement

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Data availability statement

All data used in this study was extracted from published studies.

CRediT authorship contribution statement

Dietrich B. Conze: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Claire L. Kruger:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Jennifer M. Symonds:** Data curation, Writing – review & editing. **Robert Lodder:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Yannik B. Schönknecht:** Writing – review & editing. **Manki Ho:** Writing – review & editing. **Sami M. Derya:** Writing – review & editing. **Julia Parkot:** Writing – review & editing. **Katja Parschat:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Manki Ho, Sami M. Derya, Yannik B. Schönknecht, Julia Parkot and Katja Parschat are employees of Chr. Hansen HMO GmbH. Dietrich Conze, Claire Kruger, Robert Lodder, and Jennifer Symonds were contracted by Chr. Hansen HMO GmbH to conduct this study.

Appendix A. Supplementary data

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