Title page

Systematic characterization and longitudinal study reveal distinguishing features of human milk oligosaccharides in China

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Wu J, Wu S, Huo J, Ruan H, Xu X, Hao Z, Wei Y. (Pubmed indexing)

Word count: 3979; Number of Figures: 3; Number of Tables: 2

Supplementary data submitted: Supplemental Table 1 and Supplemental Figure 1-3

Running title: Features of human milk oligosaccharides in China

Abbreviations used:

2'-FL, 2'-Fucosyllactose; 3-FL, 3-Fucosyllactose; 3'-SL, 3'-Sialyllactose; 6'-SL, 6'-Sialyllactose; A-Type-6, Blood Group A tetrasaccharide; DFLNH, Difucosyllacto-N-hexaoside; DSLNT, Disialyllacto-N-tetraose; DS-L, Disialyl Lactose; FucT II, fucosyltransferase II; FucT III, fucosyltransferase III; FUT2,
fucosyltransferase 2 gene; FUT3, fucosyltransferase 3 gene; HPAEC, high-performance anion-exchange chromatography; HMOs, human milk oligosaccharides; LDFT, Lactodifucotetraose; LNDFH I, Lacto-N-difucohexaose I; LNDFH II, Lacto-N-difucohexaose II; LNFP I, Lacto-N-fucopentaose I; LNFP II, Lacto-N-fucopentaose II; LNFP V, Lacto-N-fucopentaose V; LNnDFH, Lacto-N-neodifucohexaose; LNnFP, Lacto-N-neofucopentaose; LNnH, Lacto-N-neohexaose; LNnO, Lacto-N-neooctaose; LNnT, Lacto-N-neotetraose; LNT, Lacto-N-tetraose; LNT2, Lacto-N-triose; LSTa, LS-tetrasaccharide a; LSTb, LS-tetrasaccharide b; LSTc, LS-tetrasaccharide c; p-LNNH, p-Lacto-N-neohexaose; Se+, secretors; Se-, non-secretors.

Funding disclosures: This study is a scientific project funded mainly by Quantum Hi-Tech (China) Biological Co., Ltd and partially supported by the Innovation Talent Program of Jiangmen city, Guangdong Province, China. It is the initial effort to build a HMOs database of Chinese mothers. All experimental design, implementation, and analyses were performed according to related scientific guidelines. JW, SW, JH, HR, XX, ZH, YW, are employees or advisors of Quantum Hi-Tech (China) Biological Co., Ltd.

Conflicts of interest: JW, SW, JH, HR, XX, ZH, YW, no conflicts of interest.
ABSTRACT

Background: Human milk oligosaccharides (HMOs) in breast milk contribute to the development of neonatal microbiota and immune system. However, longitudinal studies examining HMOs profiles of Chinese mothers remain scarce.

Objective: To analyze HMOs profiles including their composition, concentrations, and changes during lactation in milk of Chinese mothers.

Methods: A total of 822 milk samples from 222 mothers were collected, of which 163 mothers provided single samples. Samples from remaining 59 mothers were collected on day3, day7 and thereafter every 7 or 14 days until day168. 24 HMOs were studied using high-performance anion-exchange chromatography (HPAEC). Secretor and non-secretor status were determined based on Lewis blood types and a defined 2'-Fucosyllactose (2'-FL) threshold.

Results: 77% of the 222 mothers were secretors and 23% were non-secretors. Longitudinal study involving 59 mothers showed that the total HMOs in secretors were significantly greater than those in non-secretors during the first two weeks. Acidic HMOs decreased significantly during lactation and were similar between secretors and non-secretors. Among neutral HMOs, distinctive differences were observed. Non-fucosylated and α-1-3/4-fucosylated HMOs in non-secretors were significantly higher than those in secretors during the first month. In contrast, α-1-2-fucosylated HMOs in secretors were significantly higher than those in non-secretors throughout 168 days. In secretors, 2'-FL
levels peaked at 3.02±0.14 g/L (day3) followed by significant decreases. In
non-secretors, 2'-FL levels were fairly low throughout 168 days. Of the 24
studied HMOs, only 3-Fucosyllactose (3-FL) levels increased during lactation
in both secretor and non-secretor mothers.

**Conclusions:** Our study showed dynamic changes of 24 HMOs in secretors
and non-secretors during lactation and revealed unique features of these
HMOs profiles in the milk of Chinese mothers. Interestingly, 2'-FL levels in
secretors were found to be lower than those of western populations but higher
than those of African populations.

**Keywords:** human milk oligosaccharides; Chinese mothers; secretor;
non-secretor; longitudinal study; HPAEC; dynamic change; profile features
Teaser text

A systematic characterization and longitudinal study of HMOs in secretor and non-secretor Chinese mothers reveal overlapping and distinctive features compared to HMOs profiles around the world.

Introduction

Human milk oligosaccharides (HMOs) are the third most abundant solid component in human milk and are important natural prebiotics that contribute to healthy neonatal microbial colonization in the gastrointestinal tract (1-4). They have been shown to modulate intestinal epithelial cell function and immune responses by regulating gene expression and cytokine production (5-9). In addition, HMOs act as antibacterial agents that inhibit pathogenic microbial adhesions and reduce intestinal infections such as necrotizing enterocolitis (10-13). They also serve as a source of sialic acid, which is an essential nutrient for brain growth and cognitive development in newborns (14,15).

HMOs composition varies greatly among mothers due to factors such as genetic polymorphism found in fucosyltransferase 2 (FUT2, Se gene) and fucosyltransferase 3 genes (FUT3, Lewis gene) (16). The FUT2 gene encodes fucosyltransferase II (FucT II) and mothers can be classified as secretors (Se+) or non-secretors (Se-) based on its level of expression (17). FucT II transfers fucose to terminal galactose in an (α-1-2)-linkage (14). Its expression contributes to the diverse products of α-1-2-fucosylated HMOs in the milk of
secretor mothers, while its absence in non-secretor mothers results in little to no production of α-1-2-fucosylated HMOs (14,18-21). Therefore, concentrations of the α-1-2-fucosylated HMO 2'-FL were commonly used to define secretor and non-secretor types of mothers (20-22). The FUT3 (Lewis) gene encodes fucosyltransferase III (FucT III), which catalyzes the transfer of fucose by an (α-1-4)-linkage (17). Expression of FUT2 (Se) and FUT3 (Le) genes results in the diverse Lewis blood types. Among Le+ blood types, Le(a+b-) individuals are known Se- non-secretors while Le(a-b+) individuals are Se+ secretors. Individuals of Le(a+b+) blood type are Se\textsuperscript{weak}Le+ partial secretors (23). For Le- individuals, Le(a-b-) can be either Se+ secretors or Se- non-secretors (24,25). As a result, Le(a+b-) and Le(a-b+) Lewis blood types can be used to directly identify non-secretor and secretor types of mothers, respectively.

In addition to maternal genotype of fucosyltransferases, geography and lactation stages are also important contributors to variation in HMOs profiles (16,20,26). Different percentages of secretor mothers were reported in different regions around the world (20). In United States, the percentage of secretor type mothers was 68% in Washington State (20). In Europe, it was reported to be 76% in Spain and 79% in Sweden (20). In Africa, it was 68% in Ghana and 81% in Kenya (20). Another factor that had been shown to influence changes of HMOs was the lactation stage (26,27). Commonly, breast milk during day0-5 of lactation is considered colostrum, from day6 to day30 it is
considered transitional milk, and after 30 days, it is regarded as mature milk (28). Most colostrum contains the highest amounts of total HMOs, while in transitional and mature milk, concentrations of 2'-FL decreased but those of 3-FL increased (29).

However, to date, most research on HMOs profiles has been performed in European and American countries (27). There are limited data from Asian, especially Chinese populations regarding HMOs profiles and their dynamic changes in mothers’ milk throughout lactation. Recent advancements in high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) allowed for a more accurate and sensitive detection of different oligosaccharides in hydroxyl dissociation under alkaline conditions without derivatization (30,31). In the present study, HPAEC-PAD was used to simultaneously quantify 24 HMOs in human milk, including 17 neutral and 7 acidic HMOs. Specifically, 822 milk samples from 222 Chinese mothers were analyzed, and secretor status was defined based on 2'-FL levels. 59 of these mothers were continually sampled at day3, day7 and thereafter at intervals of 7 or 14 days in order to investigate changes of HMOs profiles throughout the lactation period. The results from Chinese mothers were compared to earlier HMOs publications worldwide to examine the variation in HMOs profiles among people of different races and geographical regions.
Materials and methods

Study design

This study was conducted from March 2015 to July 2019 with volunteers recruited mainly from Jiangmen and Guangzhou, cities of Guangdong province, P.R. China. Volunteers were healthy 23-to-39 years old mothers, all of whom signed voluntary consent forms. Study design and procedures were conducted according to working protocols, which were reviewed and advised by specialists from local hospitals and approved by the ethical committee of Quantum Hi-Tech (China) Biological Co., Ltd. Questionnaires were used to gather basic information about the mothers and infants, including maternal age, height, weight, weeks of pregnancy, mode of delivery, parity, the newborn’s birth date, height, weight, and gender. Exclusionary criteria included gestational diabetes, hypertension, cardiovascular diseases, acute infectious diseases, and/or blood transfusions within 6 months of recruitment.

The study consisted of two parts. In the first part, single samples were collected from 163 mothers within 168 days of lactation to determine the percentage of secretor mothers. In the second part, 59 mothers were similarly recruited and continually sampled until 168 days of lactation to analyze dynamic changes of HMOs profiles. Specifically, colostrum was collected on day 3 of lactation. Thereafter, milk samples were collected every 7 or 14 days from day 7 of lactation until the cessation of breast feeding with longest sampling time at day 168. Characteristics of these 59 mothers and their infants
were summarized (Supplemental Table 1). Concentrations of 24 HMOs were determined in each sample and structures of these 24 HMOs were summarized in Table 1. Based on 2'-FL concentrations in Le(a+b-) and Le(a-b+) blood types (known non-secretors and secretors, respectively), a threshold concentration of 0.2 g/L was chosen to define secretor and non-secretor types (Figure 1B). Mothers with a 2'-FL concentration greater than 0.2 g/L in their milk were identified as secretors.

Collection and storage of milk samples

Breast milk was collected between 9:00 and 13:00 with at least 2 hours of no feeding or breast pumping prior to collection. Unilateral breast milk was collected by milk pump or manual extrusion. After mixing, 5 mL of milk samples was stored in a centrifuge tube, properly labeled with collection date and donor information, and then immediately frozen at -20 °C. The frozen samples were transported in cold storage bags surrounded by ice packs to the laboratory and stored at -80 °C. HMOs analyses were performed within 6 months of collection.

Pretreatment of milk samples

In preparing for analysis, milk samples were thawed at room temperature. 1 mL of milk was mixed with 1 mL of absolute ethanol and stored at 4 °C to precipitate proteins and lipids for 60 min prior to centrifugation at 4 °C for 20 min at 10,000 r/min. After discarding the supernatant, ethanol was removed by nitrogen blow down. The remaining material was dissolved with distilled water
to 50 mL and then filtered through a 0.22 μm membrane before being stored at 
-20 °C until HMO analysis within one week.

**Analysis of HMOs in milk samples**

HMOs in milk samples were quantified using HPAEC-PAD with an ICS-5000+
ion chromatography detection system (Thermo Scientific, USA). This system is
comprised of a CarboPac PA-1 (4×250mm, 6.5μm) column, a CarboPac PA
(4×50mm) precolumn and a pulsed amperometric detector with a gold
electrode. The analysis was performed according to a published method with
minor modifications (29). HMOs standards were included as controls to
quantitatively calculate concentrations of 24 HMOs in milk samples. All HMOs
standards were purchased from Carbosynth, UK. Details of tested 24 HMOs
were summarized in Table 1.

**Collection and storage of blood samples**

50 μL blood samples were collected from the fingertips of volunteers using
disposable sterile needles. 2 mL of self-prepared Alsever's solution (32) was
added immediately and then transported to the laboratory in frozen storage
bags containing ice packs. Samples were stored at 4 °C and analyzed for
Lewis blood type within 24 hours.

**Pretreatment of blood samples**

Blood samples were centrifuged at 4 °C for 1 min at a speed of 1000 r/min.

After supernatants were discarded, blood cells were resuspended in 1 mL of
normal saline (0.9% NaCl solution), and centrifuged again at the above
conditions. Blood cells were resuspended to 0.5% (v/v) with normal saline for testing.

**Lewis blood type identification**

Lewis blood group was identified using an agglutination test kit of red blood cells (CE-Immundiagnostika GmbH, Germany). Procedures were performed according to manufacturer’s guidelines. Briefly, anti-Le\(^a\) and anti-Le\(^b\) sera reagents were each mixed with equal volumes of above prepared 0.5% blood cell suspension and allowed to stand at room temperature for 15 min. After centrifugation for 1 min at 1000 r/min at room temperature, the blood cells were resuspended with normal saline. Finally, 50 μL of the resuspension was placed in a 96-well plate and observed under a microscope. If agglutination occurred in the anti-serum mixture, the corresponding Le antigen was recorded as positive in the sample. The absence of agglutination was recorded as negative of that antigen.

**Statistical analysis**

Data analyses were performed using SPSS statistics software, version 17.0 (IBM Corp., NY) and GraphPad Prism V6 (GraphPad Software, Inc.). To determine significant differences of HMOs in milk from secretor and non-secretor mothers, independent-samples t test, unpaired t test and multiple t test were used accordingly. To determine significant differences at different time points during the lactation period, a one-factorial ANOVA followed by the Student–Newman–Keuls test was used. Values were expressed as Mean ±
SEM unless otherwise stated, and a \( P \) value less than 0.05 was considered significant.

Results

HMOs differences among Chinese mothers of various Lewis blood types

Among the 59 continually followed mothers, the Lewis blood types of 24 mothers were identified directly by serological tests. 14 of the 24 mothers (58%) had a Lewis blood type of \( \text{Le(a-b+)} \), 5 (21%) \( \text{Le(a+b-)} \), 4 (17%) \( \text{Le(a-b-)} \) and 1 (4%) \( \text{Le(a+b+)} \). Figure 1A showed representative HPAEC chromatograms of the 24 tested HMOs in day3 colostrum from \( \text{Le(a-b+)}/\text{Se+} \) and \( \text{Le(a+b-)}/\text{Se-} \) mothers. \( \text{Le(a-b+)} \) secretor mothers had breast milk that was rich in \( \alpha-1-2 \)-fucosylated HMOs such as LNDFH I, LDFT, 2'-FL, and LNFP I (peak 4,8,9&11) (Figure 1A). In contrast, the \( \text{Le(a+b-)} \) non-secretor mothers had little to no \( \alpha-1-2 \)-fucosylated HMOs. However, higher concentrations of \( \alpha-1-3/4 \)-fucosylated HMOs such as 3-FL and LNFP II (peak 5&7) were found in the \( \text{Le(a+b-)} \) non-secretor mother than those in the \( \text{Le(a-b+)} \) secretor mother (Figure 1A).

Figure 1B showed that 2'-FL concentrations were significantly higher in the breast milk of \( \text{Le(a-b+)} \) secretors compared to those of \( \text{Le(a+b-)} \) non-secretors. Interestingly, 2'-FL concentration at 0.2 g/L separated \( \text{Le(a-b+)} \) secretors and \( \text{Le(a+b-)} \) non-secretors with 100% accuracy compared to serological tests (analyzed by ROC statistical analysis, data not shown). Based on the 0.2 g/L 2'-FL threshold, 14 \( \text{Le(a-b+)} \) mothers and three out of four \( \text{Le(a-b-)} \) mothers
were classified as secretors, and the five Le(a+b-), one Le(a+b+), and remaining one Le(a-b-) mother were classified as non-secretors.

Representative HPAEC chromatograms of 24 tested HMOs in day3 colostrum from all five Lewis/secretor types were summarized (Supplemental Figure 1A). Interestingly, though qualified as a non-secretor (with 2'-FL concentrations lower than 0.2 g/L), 2'-FL (peak 9) in the Le(a+b+) mother was modestly produced (Supplemental Figure 1A). However, it is worth to note that such 2'-FL concentrations in the breast milk of Le(a+b+) mother were significantly higher than those of other non-secretors, but were significantly lower than those in secretors of Le(a-b+) phenotype (Supplemental Figure 1B). Therefore, the Le(a+b+) mother was classified as a partial secretor, which was also suggested by another group (23). Among non-secretor mothers, LNT concentrations in Le(a-b-) mothers were significantly higher than those in Le(a+b-) and Le(a+b+) mothers (Supplemental Figure 1C).

Distinct features of HMOs in secretor and non-secretor types of Chinese mothers during lactation

All milk samples were grouped into secretor and non-secretor types according to 2'-FL concentrations (secretors > 0.2 g/L and non-secretors < 0.2 g/L) (Figure 1B). Mothers donated samples of secretor milk were classified as secretor mothers. Among the 222 mothers, 171 (77%) were found to be secretors while 51 (23%) were non-secretors. Significant higher concentrations of 2'-FL were found in milk of secretor mothers compared to
those in non-secretor mothers (Supplemental Figure 2). Similar significant differences between secretor and non-secretor mothers were found for other α-1-2-fucosyltransferase products such as LDFT, LNDFH I and LNFP I (Supplemental Figure 2).

To analyze changes of HMOs profiles in secretors and non-secretors during lactation, milk samples were continually collected from 59 healthy Chinese mothers from day3 to day168 of lactation. Distinctive features of HMO composition in milk of secretor and non-secretor mothers were observed, especially for the three subtypes of neutral HMOs (non-fucosylated, α-1-3/4-fucosylated and α-1-2-fucosylated) (Figure 2A). Total HMOs in milk of secretor mothers on day3 (n=25), day7 (n=27), and day14 (n=28) of lactation were all significantly higher than those in non-secretor counterparts (n=13, 12, 14, respectively) (Figure 2B). In milk of secretors and non-secretors, levels of acidic HMOs were similar and differences of neutral HMOs concentrations contributed to the observed differences of total HMOs (Figure 2B). Among the neutral HMO subtypes, both non-fucosylated and α-1-3/4-fucosylated HMOs were consistently higher in milk of non-secretors compared to those in secretors (Figure 2C). These differences were continuously significant until day35 for non-fucosylated HMOs and until day49 for α-1-3/4-fucosylated HMOs during lactation (Figure 2C). In contrast, α-1-2-fucosylated HMOs were significantly higher in milk of secretor mothers than those in non-secretors throughout the studied 168 days of lactation (Figure 2C).
Dynamic changes of HMOs in secretor and non-secretor types of Chinese mothers during lactation

To analyze dynamic changes of HMOs in milk of secretor and non-secretor mothers, day3, 7, 21, 42, 77 and 168 of lactation were selected to statistically test the HMOs changes throughout lactation. As shown in Figure 2B, the amounts of total HMOs in secretors decreased significantly from 10.59±0.30 g/L (day3) to 4.90±0.18 g/L (day168) while in non-secretors, it decreased significantly from 7.31±0.32 g/L (day3) to 3.65±1.15 g/L (day168). For acidic HMOs, similar significant decreases in milk of secretors and non-secretors were observed during lactation (Figure 2B). For neutral HMOs significantly decreased from 8.54±0.22 g/L (day3) to 4.57±0.16 g/L (day168) in secretors, and from 5.49±0.28 g/L (day3) to 3.35±1.13 g/L (day168) in non-secretors (Figure 2B). Among neutral HMOs, non-fucosylated HMOs in secretors initially increased significantly from 1.54±0.11 g/L (day3) to 1.89±0.08 g/L (day7) but thereafter significantly decreased to 0.62±0.09 g/L (day168) (Figure 2C). In milk of non-secretors, non-fucosylated HMOs significantly decreased from 2.82±0.22 g/L at day3 to 0.66±0.20 g/L at day168 (Figure 2C). Increasing trends of α-1-3/4-fucosylated HMOs were found in both milk of secretors and non-secretors, but statistical significance was only observed in secretors (from 0.77±0.05 g/L at day3 to 1.30±0.09 g/L at day168) (Figure 2C). α-1-2-fucosylated HMOs remained low with minor changes in milk of non-secretors (Figure 2C). However, in milk of secretors, α-1-2-fucosylated
HMOs started high but decreased significantly from 6.23±0.19 g/L (day3) to 2.64±0.18 g/L (day168) (Figure 2C).

The dynamic changes of all 24 HMOs in secretors and non-secretors during lactation were shown in Figure 3 and statistical differences during lactation were compared among concentrations on day3, 7, 21, 42, 77 and 168 of lactation (Supplemental Figure 3). Similar to total acidic HMOs (Figure 2B), significant decreases were found for LSTc, 6'-SL, 3'-SL, LSTa, and DSLNT from day3 to day168, and no significant differences between secretors and non-secretors were observed for these five individual acidic HMOs during lactation (Figure 3A). Only LSTb concentrations were significantly higher in non-secretors than those in secretors until day28 of lactation (Figure 3A). However, compared to other acidic HMOs, LSTb concentrations were relatively low in both secretors (< 0.07g/L) and non-secretors (< 0.1g/L) (Figure 3A). It is worth to note that concentrations of DS-L were the lowest (< 0.04 g/L) and only detectable in a limited number of samples within first month of lactation (Figure 3A).

Among non-fucosylated HMOs, LNT was most abundant in both secretors and non-secretors (Figure 3B). In non-secretors, LNT significantly decreased from 1.69±0.17 g/L (day3) to 0.48±0.24 g/L (day168); in secretors, it initially increased significantly from 0.70±0.08 g/L (day3) to 1.05±0.06 g/L (day7) but thereafter significantly decreased to 0.43±0.08 g/L at day168 (Figure 3B). From day3 to day28 of lactation, LNT concentrations were significantly higher
in non-secretors than those in secretors, similar to results of total non-fucosylated HMOs (Figure 2C&3B). Among other non-fucosylated HMOs, LNnT and LNNO were both significantly higher in non-secretors than those in secretors during early lactation (Figure 3B). In secretors, significant decreases during lactation were found for LNT2, LNnT, p-LNnH&LNNH, and LNNO, while in non-secretors, only LNT2 and LNnT were observed to decrease significantly (Figure 3B).

For the α-1-3/4-fucosylated HMOs, 3-FL, LNFP II, LDNFH II, DFLNH, and LNFP V were all found significantly higher in non-secretors than those in secretors during lactation (Figure 3C). Of all 24 tested HMOs, only the concentration of 3-FL was observed to increase during lactation. It rose from 0.60±0.11 g/L (day3) to 2.32±0.37 g/L (day154) in non-secretors, and from 0.25±0.03 g/L (day3) to 1.03±0.08 g/L (day168) in secretors, but statistical significance was only found in milk of secretors (Figure 3C). Other α-1-3/4-fucosylated HMOs such as LNFP II fluctuated in the range of 0.3 g/L to 0.9 g/L during lactation in non-secretors, while in secretors, concentrations of LNFP II were fairly low (<0.2 g/L) throughout lactation (Figure 3C). Similar results were observed for LDNFH II, DFLNH, and LNFP V in secretor and non-secretor types during lactation (Figure 3C). Among α-1-3/4-fucosylated HMOs, only LNnFP was detected higher in secretors than that in non-secretors during early lactation (Figure 3C).

Among α-1-2-fucosylated HMOs, 2'-FL and LDNFH I were significantly higher
in secretors than those in non-secretors throughout 168 days of lactation, while LNFP I and LDFT was continually significantly higher in secretors until day 91 of lactation (Figure 3D). Due to lack of α-1-2-fucosyltransferase, all tested α-1-2-fucosylated HMOs except A-Type-6 were constantly low in milk of non-secretors (Figure 3D). In contrast, three α-1-2-fucosylated HMOs in secretors started highest at the beginning of lactation with 2'-FL at 3.02±0.14 g/L (day 3), LNFP I at 1.49±0.12 g/L (day 7), and LNDFH I at 1.00±0.06 g/L (day 3), respectively (Figure 3D). These three HMOs decreased significantly to 1.28±0.10 g/L for 2'-FL, 0.40±0.09 g/L for LNFP I and 0.45±0.04 g/L for LNDFH I at day 168 of lactation (Figure 3D). Concentrations of LDFT remained relatively stable from day 3 to day 168 of lactation in secretor mothers (Figure 3D). Concentrations and dynamic changes of A-Type-6 were similar between secretors and non-secretors during lactation (Figure 3D).

**Discussion**

In our study, the prevalence of secretors was 77% among Chinese mothers, close to the 79% of secretors found by another group, which analyzed 446 milk samples from urban Chinese mothers (33). The similar results reported by these two independent studies indicate that the data are representative of the general population in China.

HMOs profiles vary greatly by geography with different percentages of secretor and non-secretor mothers reported worldwide (20). In Peru and California of United States, percentages of secretors were over 95% among Hispanic
mothers (20). In Mexico, the secretor percentage was reportedly 100% (34). In African countries like Ghana, Ethiopia and rural Gambia, less than 70% of secretors were found (20). Compared to these populations, the percentage of secretor mothers in the Chinese population is lower than those of American populations, but higher than those seen in African populations. In particular, concentrations of 2'-FL in secretor mothers also differed considerably among geographic regions. Table 2 summarized 2'-FL concentrations at different lactation time reported in Asian, western, and African populations compared to the results of Chinese mothers in the current study (22,29,35-39). 2'-FL levels were highest at the beginning of lactation despite regional differences. In Asian populations, average concentrations of 2'-FL in colostrum were 3.02 g/L in China (current study) and 2.25 g/L in Malaysia (35) (Table 2). In Europe, average 2'-FL concentrations in colostrum were 3.93 g/L and 4.13 g/L in Italian and German mothers, respectively (29,37) (Table 2). In Africa, it was 1.23 g/L in colostrum from preterm South African mothers (39), and levels of 2'-FL in preterm and term colostrum were reportedly similar (27). This indicated that in Asian populations including Chinese mothers, 2'-FL concentrations in colostrum were lower than those of European populations but higher than those in African populations. Moreover, 2'-FL concentrations were 2.35 g/L and 2.17 g/L, respectively, at day11-30 of lactation in Chinese and Singaporean mothers (36) (Table 2). In Europe, 2'-FL concentrations at the same lactation time were 3.02 g/L and 2.78 g/L in German and Italian mothers, while in
America, they were 2.87 g/L and 3.5 g/L in US and Mexican mothers (22,29,37,38) (Table 2). This further confirmed that 2'-FL concentrations in Asian populations were lower than those of western populations.

Additionally, HMOs profiles are strongly influenced by secretor and non-secretor status. A detailed analysis of HMOs profile showed significantly higher concentrations of total HMOs in secretors than in non-secretors during the first two weeks of lactation, mainly due to significantly higher concentration of neutral HMOs in secretors. Similarly, significant differences in neutral HMOs concentrations were reported during early lactation but not at day35 postpartum in western populations (37,40). No significant differences were observed for acidic HMOs between secretors and non-secretors. Among neutral HMOs, in both Chinese and western populations, α-1-2-fucosylated HMOs were significantly higher in secretors while α-1-3/4-fucosylated HMOs and the non-fucosylated HMOs were higher in non-secretors (37). These differences are more obviously significant during early lactation.

HMOs profiles are also influenced by lactation stages. The total concentration of HMOs dropped by a half in both secretor (53.7%) and non-secretor (50.1%) Chinese mothers from day3 to day168 of lactation. Similar trends of decreasing total HMOs were observed in western mothers, though total HMOs concentrations were reported higher in western populations (37). Among neutral HMOs, decreasing levels of α-1-2-fucosylated HMOs and increasing levels of α-1-3/4-fucosylated HMOs were found in both Chinese and western
populations during lactation (37). For instance, the α-1-2-fucosylated HMO 2'-FL decreased while the α-1-3/4-fucosylated HMO 3-FL increased during lactation in both populations (33,37). Interestingly, overlapping functions have been reported for these two HMOs. Studies showed that 2'-FL and 3-FL shared similar protective functions such as promoting probiotic growth and inhibiting pathogen adhesion (3,41). However, properties specific to each HMOs have also been observed. For instance, study showed that 2'-FL, but not 3-FL, inhibited infection by Campylobacter jejuni (42). Therefore, studies of HMO functions and their dynamic relationships will provide a more comprehensive understanding of human breast milk glycan and help to inform more effective design of infant milk formula.

In summary, earlier studies on the changes in HMOs profiles during lactation in Chinese populations remain scarce. The current longitudinal study attempts to fill that void through intensive sampling from the same cohort of secretor and non-secretor Chinese mothers during lactation. 24 HMOs were systematically analyzed and their dynamic changes over the lactation period were reported in details. By comparing these results with HMOs results worldwide, geography and race are found to greatly influence HMOs profiles. Importantly, more comprehensive studies with larger sample sizes and broader inclusion of Chinese mothers from other regions of China are still needed to understand other factors influencing HMOs profiles such as diets and lifestyles. The current research project is part of an on-going research initiative aimed to build
a HMOs database of Chinese mothers and to obtain a more concrete, fine-grained comparison of results around the world. Hopefully, better knowledge on the nutritional and protective properties of HMOs in human milk will provide a strong foundation of scientific principles for guiding personalized products and precision nutrition.

Acknowledgments

The authors would like to thank Xinyu Wei and Karen Barnes for critical reading of the manuscript.

Authors’ contributions: YW, ZH and SW designed research; JW, JH and HR conducted research; JW and XX analyzed data and wrote the manuscript. YW held major role in finalizing the concepts and contents. All authors read and approved the final manuscript.
References

1. Oliveira DL, Wilbey RA, Grandison AS, Roseiro LB. Milk oligosaccharides: A review. Int J Dairy Technol 2015;68:305-21.

2. Ruiz-Moyano S, Totten SM, Garrido DA, Smilowitz JT, German JB, Lebrilla CB, Mills DA. Variation in consumption of human milk oligosaccharides by infant gut-associated strains of Bifidobacterium breve. Appl Environ Microbiol 2013;79:6040-9.

3. Yu ZT, Chen C, Kling DE, Liu B, McCoy JM, Merighi M, Heidtman M, Newburg DS. The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. Glycobiology 2013;23:169-77.

4. Zivkovic AM, German JB, Lebrilla CB, Mills DA. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4653-8.

5. Newburg DS. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. J Anim Sci 2009;87:26-34.

6. Triantis V, Bode L, van Neerven R. Immunological effects of human milk oligosaccharides. Front Pediatr 2018;6:190.

7. Zehra S, Khambati I, Vierhout M, Mian MF, Buck R, Forsythe P. Human milk oligosaccharides attenuate antigen-antibody complex induced chemokine release from human intestinal epithelial cell lines. J Food Sci 2018;83:499-508.
8. Donovan SM, Comstock SS. Human milk oligosaccharides influence neonatal mucosal and systemic immunity. Ann Nutr Metab 2016;69 Suppl 2:42-51.

9. He Y, Liu S, Kling DE, Leone S, Lawlor NT, Huang Y, Feinberg SB, Hill DR, Newburg DS. The human milk oligosaccharide 2'-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation. Gut 2016;65:33-46.

10. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr 2005;25:37-58.

11. Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferranante L, Capretti R, Orazio G. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: Escherichia coli, Vibrio cholerae, and Salmonella fyris. Pediatric Research 2006;59:377-82.

12. Jantscher-Krenn E, Lauwaet T, Bliss LA, Reed SL, Gillin FD, Bode L. Human milk oligosaccharides reduce Entamoeba histolytica attachment and cytotoxicity in vitro. Br J Nutr 2012;108:1839-46.

13. Bode L. Human milk oligosaccharides in the prevention of necrotizing enterocolitis: a journey from in vitro and in vivo models to mother-infant cohort studies. Front Pediatr 2018;6.

14. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 2012;22:1147-62.

15. ten Bruggencate SJ, Bovee-Oudenhoven IM, Feitsma AL, van Hoffen E,
Schoterman MH. Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. Nutr Rev 2014;72(6):377-89.

16. Azad MB, Robertson B, Atakora F, Becker AB, Subbarao P, Moraes TJ, Mandhane PJ, Turvey SE, Lefebvre DL, Sears MR, et al. Human milk oligosaccharide concentrations are associated with multiple fixed and modifiable maternal characteristics, environmental factors, and feeding practices. J Nutr 2018;148:1733-42.

17. Mantovani V, Galeotti F, Maccari F, Volpi N. Recent advances on separation and characterization of human milk oligosaccharides. Electrophoresis 2016;37:1514-24.

18. Blank D, Gebhardt S, Maass K, Lochnit G, Dotz V, Blank J, Geyer R, Kunz C. High-throughput mass finger printing and Lewis blood group assignment of human milk oligosaccharides. Anal Bioanal Chem 2011;401:2495-510.

19. Elwakiel M, Hageman JA, Wang W, Szeto IM, van Goudoever JB, Hettinga KA, Schols HA. Human milk oligosaccharides in colostrum and mature milk of Chinese mothers: Lewis positive secretor subgroups. J Agric Food Chem 2018;66:7036-43.

20. McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, Kamau-Mbuthia EW, Kamundia EW, Mbugua S, Moore SE, et al. What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. Am J Clin Nutr 2017;105:1086-100.

21. Smilowitz JT, O'Sullivan A, Barile D, German JB, Lonnerdal B, Slupsky CM.
The human milk metabolome reveals diverse oligosaccharide profiles. J Nutr 2013;143:1709-18.

22. Goehring KC, Kennedy AD, Prieto PA, Buck RH. Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PLoS One 2014;9:e101692.

23. Henry S, Mollicone R, Fernandez P, Samuelsson B, Oriol R, Larson G. Homozygous expression of a missense mutation at nucleotide 385 in the FUT2 gene associates with the Le(a+b+) partial-secretor phenotype in an Indonesian family. Biochem Biophys Res Commun 1996;219:675-8.

24. Ameno S, Kimura H, Ameno K, Zhang X, Kinoshita H, Kubota T, Ijiri I. Lewis and Secretor gene effects on Lewis antigen and postnatal development of Lewis blood type. Biol Neonate 2001;79:91-6.

25. Totten SM, Zivkovic AM, Wu S, Nguyen U, Freeman SL, Ruhaak LR, Darboe MK, German JB, Prentice AM, Lebrilla CB. Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. J Proteome Res 2012;11:6124-33.

26. Spevacek AR, Smilowitz JT, Chin EL, Underwood MA, German JB, Slupsky CM. Infant maturity at birth reveals minor differences in the maternal milk metabolome in the first month of lactation. J Nutr 2015;145:1698-708.

27. Thurl S, Munzert M, Boehm G, Matthews C, Stahl B. Systematic review of the concentrations of oligosaccharides in human milk. Nutr Rev...
28. Billeaud C. Breast milk substitutes: changing ideas. J Food Sci Eng 2018;6:231-6.

29. Coppa GV, Pierani P, Zampini L, Carloni I, Carlucci A, Gabrielli O. Oligosaccharides in human milk during different phases of lactation. Acta Paediatr Suppl 1999;88:89-94.

30. Coppa GV, Gabrielli O, Zampini L, Galeazzi T, Ficcadenti A, Padella L, Santoro L, Soldi S, Carlucci A, Bertino E, et al. Oligosaccharides in 4 different milk groups, Bifidobacteria, and Ruminococcus obeum. J Pediatr Gastroenterol Nutr 2011;53:80-7.

31. Gabrielli O, Zampini L, Galeazzi T, Padella L, Santoro L, Pella C, Giuliani F, Bertino E, Fabris C, Coppa GV. Preterm milk oligosaccharides during the first month of lactation. Pediatrics 2011;128:e1520-31.

32. Rasmusen BA. Blood groups in sheep. I. the X-Z system. Genetics 1958;43:814-21.

33. Austin S, De Castro CA, Benet T, Hou Y, Sun H, Thakkar SK, Vinyes-Pares G, Zhang Y, Wang P. Temporal change of the content of 10 oligosaccharides in the milk of Chinese urban mothers. Nutrients 2016;8:346.

34. Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O’Ryan ML, Ruiz-Palacios G, Hilty MD, Pickering LK, Prieto PA. Variability of human milk neutral oligosaccharides in a diverse population. J Pediatr Gastroenterol Nutr 2000;30:181-92.
35. Ma L, McJarrow P, Jan Mohamed HJB, Liu X, Welman A, Fong BY. Lactational changes in the human milk oligosaccharide concentration in Chinese and Malaysian mothers’ milk. Int Dairy J 2018;87:S2083259822.

36. Sprenger N, Lee LY, De Castro CA, Steenhout P, Thakkar SK. Longitudinal change of selected human milk oligosaccharides and association to infants’ growth, an observatory, single center, longitudinal cohort study. PLoS One 2017;12:e171814.

37. Thurl S, Munzert M, Henker J, Boehm G, Muller-Werner B, Jelinek J, Stahl B. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. Br J Nutr 2010;104:1261-71.

38. Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, Newburg DS. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 2001;11:365-72.

39. Van Niekerk E, Autran CA, Nel DG, Kirsten GF, Blaauw R, Bode L. Human milk oligosaccharides differ between HIV-infected and HIV-uninfected mothers and are related to necrotizing enterocolitis incidence in their preterm very-low-birth-weight infants. J Nutr 2014;144:1227-33.

40. Hong Q, Ruhaak LR, Totten SM, Smilowitz JT, German JB, Lebrilla CB. Label-free absolute quantitation of oligosaccharides using multiple reaction monitoring. Anal Chem 2014;86:2640-7.

41. Weichert S, Jennewein S, Hufner E, Weiss C, Borkowski J, Putze J,
Schroten H. Bioengineered 2'-fucosyllactose and 3-fucosyllactose inhibit the adhesion of Pseudomonas aeruginosa and enteric pathogens to human intestinal and respiratory cell lines. Nutr Res 2013;33:831-8.

42. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. Campylobacter jejuni binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. J Biol Chem 2003;278:14112-20.
Table 1. Glycan structures of tested HMOs

| HMOs | GLYCAN | STRUCTURE |
|------|--------|-----------|
| LNnDFH | Lacto-N-neodifucohexaose | Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc |
| LNDFH II | Lacto-N-difucohexaose II | Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc |
| DFLNH | Difucosyllacto-N-hexaose | Galβ1-4(Fucα1-3)GlcNAcβ1-6[Galβ1-3(Fucα1-4)GlcNAcβ1-3]Galβ1-4Glc |
| LNDFH I | Lacto-N-difucohexaose I | Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc |
| 3-FL | 3-Fucosyllactose | Galβ1-4(Fucα1-3)Glc |
| A-Type-6 | Blood Group A tetrasaccharide | GlcNAcβ1-3(Fucα1-2)Galβ1-4Glc |
| LNFP II | Lacto-N-fucopentaose II | Fucα1-4(Galβ1-3)GlcNAcβ1-3Galβ1-4Glc |
| LDFT | Lactodifucotetraose | Fucα1-2Galβ1-4(Fucα1-3)Glc |
| 2'-FL | 2'-Fucosyllactose | Fucα1-2Galβ1-4Glc |
| LNT2 | Lacto-N-triose | GlcNAcβ1-3Galβ1-4Glc |
| LNFP I | Lacto-N-fucopentaose I | Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc |
| LNNT | Lacto-N-neotetraose | Galβ1-4GlcNAcβ1-3Galβ1-4Glc |
| LNnFP | Lacto-N-neofucopentaose | Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc |
| LNFP V | Lacto-N-fucopentaose V | Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc |
| p-LNnH* | p-Lacto-N-neohexaose | Galβ1-4GlcNAcβ1-3Galβ1-4Glc |
| LNN* | Lacto-N-neohexaose | Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc |
| LNT | Lacto-N-tetraose | Galβ1-3GlcNAcβ1-3Galβ1-4Glc |
| LNnO | Lacto-N-neoctaose | Galβ1-4GlcNAcβ1-3Galβ1-4Glc |
| LSTc | LS-tetrasaccharide c | NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc |
| 6'-SL | 6'-Sialyllactose | NeuAcα2-6Galβ1-4Glc |
| 3'-SL | 3'-Sialyllactose | NeuAcα2-3Galβ1-4Glc |
| LSTa | LS-tetrasaccharide a | NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc |
| LSTb | LS-tetrasaccharide b | NeuAcα2-6(Galβ1-3)GlcNAcβ1-3Galβ1-4Glc |
| DSLNT | Disialyllacto-N-tetraose | NeuAcα2-6(NeuAcα2-3Galβ1-3)GlcNAcβ1-3Galβ1-4Glc |
| DS-L | Disialyl Lactose | NeuAcα2-8NeuAcα2-3Galβ1-4Glc |

* p-Lacto-N-neohexaose (p-LNnH) and Lacto-N-neohexaose (LNnH) were not separated well in our HPAEC system. Thus, their concentrations were given as the sum of p-LNnH+LNnH.
Gal=Galactose, Glc=Glucose, GlcNAc=N-Acetyl-glucose, Fuc=Fucose, NeuAc=N-acetyl-neuraminic acid.
Table 2. Comparison of mean 2'-FL concentrations in secretor mothers of different regions around the world.

| days of lactation | China (this study) | Malaysia /Singapore (36) | Germany (37) | Italy (29) | USA (22) | Mexico (38) | South Africa* (39) |
|-------------------|------------------|-------------------------|-------------|------------|---------|------------|-------------------|
|                   | n=39             | n=26/34                 | n=21        | n=18       | n=13/15 | n=20       |
| 0 – 4             | 3.02             | 2.25/-                  | 4.13        | 3.93       | -       | 1.23       |
| 5 – 10            | 2.54             | -                       | 3.37        | 3.02       | -       | -          |
| 11 – 30           | 2.35             | -2.17                   | 3.02        | 2.78       | 2.87/3.50 | -          |
| 31 – 60           | 1.96             | 1.29/1.76               | 2.82        | 1.84       | -       | -          |
| 61 – 100          | 1.56             | -                       | 2.59        | 2.46       | -       | -          |

*Data of 2'-FL concentration in South Africa were from mothers with premature delivery.

(n) indicates reference numbers accordingly.
Figure 1 HMOs in milk samples of Le(a+b-) non-secretors and Le(a-b+) secretors.

(A) Representative HPAEC chromatograms of 24 HMOs in milk samples of Le(a+b-) non-secretor (Se-) and Le(a-b+) secretor (Se+) mothers. Arrows indicated peak 9 of 2'-FL. 1. LNnDFH, 2. LNDFH II, 3. DFLNH, 4. LNDFH I, 5. 3-FL, 6. A-Type-6, 7. LNFP II, 8. LDFT, 9. 2'-FL, 10. LNT2, 11. LNFP I, 12. LNnT, 13. LNnFP, 14. LNFP V, 15. p-LNnH&LNnH, 16. LNT, 17. LNnO, 18. LSTc, 19. 6'-SL, 20. 3'-SL, 21. LSTa, 22. LSTb, 23. DSLNT, 24. DS-L. (B) 2'-FL concentrations in milk of Le(a+b-) non-secretor (Se-) and Le(a-b+) secretor (Se+) mothers. Dash lines indicated 0.2 g/L, respectively. **** P< 0.0001.
Figure 2 Concentration changes of HMOs in milk of secretors and non-secretors during lactation.

(A) Overview of HMO subtype changes in secretors and non-secretors during lactation. Acidic HMOs are calculated as LSTc + 6'-SL + 3'-SL + LSTa + LSTb + DSLNT + DS-L. Non-fucosylated HMOs are calculated as LNT2 + LNnT + p-LNnH + LNnH + LNT + LNnO. α-1-2-fucosylated HMOs are calculated as LNDFH I + A-Type-6 + LDFT + 2'-FL + LNFP I. α-1-3/4-fucosylated HMOs are calculated as LNnDFH + LNDFH II + DFLNH + 3-FL + LNFP II + LNnFP + LNFP V. Comparison of dynamic changes of HMO concentrations (B) and neutral HMO subtypes (C) in secretors (Se+) and non-secretors (Se-) during lactation. All values are means ± SEMs. “a”, “b”, ... and “x”, “y”, ... indicated differences among different time points in secretors and non-secretors, respectively. # indicated statistical significance between secretors and non-secretors.
non-secretors at the same lactation time. $P < 0.05$ was considered significant.
Figure 3 Dynamic changes of 24 HMOs in milk of secretors and non-secretors during lactation.

Comparison of dynamic changes of (A) acidic, (B) non-fucosylated, (C) α-1-3/4-fucosylated and (D) α-1-2-fucosylated HMOs in secretors (Se+) and
non-secretors (Se-) during lactation. Acidic HMOs include LSTc, 6'-SL, 3'-SL, LSTa, LSTb, DSLNT, and DS-L. Non-fucosylated HMOs include LNT2, LNnT, p-LNnH&LNnH, LNT and LNnO. α-1-3/4-fucosylated HMOs include LNnDFH, LNDFH II, DFLNH, 3-FL, LNFP II, LNnFP, and LNFP V. α-1-2-fucosylated HMOs include LNDFH I, A-Type-6, LDFT, 2'-FL, and LNFP I. All values are means ± SEMs. “a”, “b”, ... and “x”, “y”, ... indicated differences among different time points in secretors and non-secretors, respectively. # indicated statistical significance between secretors and non-secretors at the same lactation time. 

$P < 0.05$ was considered significant.