

Original Article

## Comparison of free fatty acid content of human milk from Taiwanese mothers and infant formula

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### Abstract

**Objective:** Few studies on the free fatty acid (FFA) content of milk from non-Caucasian mothers have been published. We compared the FFA concentrations in human milk (HM) from Taiwanese mothers of preterm (PTHM) and full-term infants (FTHM) and in infant formula (IF).

**Materials and methods:** Thirty-eight HM samples were collected from 23 healthy lactating mothers and 15 mothers who gave birth prematurely (range 29–35 weeks, mean 33 weeks). The regular formula and preterm infant formula (PTIF) for three brands of powdered IF were also evaluated. Milk samples were extracted and methylated for analysis by gas chromatography/mass spectrometry (GC/MS).

**Results:** Reference values for individual FFAs in breast milk from Taiwanese mothers were determined. The mean total FFAs were significantly higher in IF (21,554  $\mu\text{mol/L}$ ) and PTIF (19,836  $\mu\text{mol/L}$ ) than in FTHM (8,540  $\mu\text{mol/L}$ ) and PTHM (9,259  $\mu\text{mol/L}$ ) ( $p < 0.05$ ). Saturated FAs were predominant in all types of milk (43.1% for FTHM, 42.8% for PTHM, 45.5% for IF and 45.3% for PTIF). Monounsaturated FAs were significantly higher in IF and PTIF (42.6% and 43.9%) than in FTHM and PTHM (37.7% and 39.5%), and polyunsaturated FAs in FTHM and PTHM (20% and 18.2%) were higher than in IF and PTIF (11.9% and 10.9%). HM had a more desirable linoleic acid/ $\alpha$ -linolenic acid ratio than IF. No significant differences in individual FFAs in FTHM were observed among three lactating periods.

**Conclusion:** FFA levels in HM from Taiwanese mothers are in agreement with results for different geographically distinct populations. Nevertheless, the FFA content in IF did not meet well with HM, particularly, the excess additives of saturated and monounsaturated FAs, and the shortage of polyunsaturated FAs. The effect of variations in FFA content in IF on future unfavorable outcomes such as obesity, atopic syndrome, and less optimal infant neurodevelopment should be further investigated.

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**Keywords:** free fatty acids; human milk; infant formula; gas chromatography/mass spectrometry

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## Introduction

Breast milk is an essential and natural nutrient source for the normal growth and development of infants. The composition of breast milk is recognized as the gold standard for the manufacture of infant formula (IF). Since early 2000, long-chain polyunsaturated fatty acids (LCPUFAs) such as docosahexaenoic acid (22:6  $\omega$ 3; DHA) and eicosapentaenoic acid (20:5  $\omega$ 3; EPA) have been added to IF and the effectiveness of LCPUFA supplementation in infancy is of great interest. In particular, two LCPUFA groups have attracted special interest. Homologs of linoleic acid (18:2  $\omega$ 6; LA) of the n-6 series are precursors of arachidonic acid (20:4  $\omega$ 6; AA), and homologs of  $\alpha$ -linolenic acid (18:3  $\omega$ 3; ALA) of the n-3 series are precursors of EPA and DHA. Breast milk contains the essential FA precursors (LA and ALA) and adequate AA and DHA, and the crucial role of these FAs in central nervous system development and retinal function has been verified in many studies [1–4].

FAs in human milk, particularly LCPUFAs, contribute significantly to early infant development. According to Jensen et al, FAs provide approximately 50% of an infant's energy requirements [5]. Inadequate FA supply, especially of LCPUFAs, may have negative effects on neurologic function, visual acuity, psychomotor function, and immunological development [6–8]. There is also evidence that infants who receive DHA or EPA supplementation score significantly higher on the Bayley Psychomotor Development Index at the age of 30 months [9]. Birch and colleagues suggested that infants fed IF not fortified with DHA and/or AA exhibit lower visual acuity. This is because a reduction in DHA exposure can alter nerve cell signaling, leading to permanent changes in neural membrane function and cortical visual acuity [10]. In addition, some studies reported that atopy is associated with a higher n-6 LCPUFA status and a low n-3 LCPUFA status. Fish oil supplementation in infancy may decrease the risk of developing some manifestations of allergic disease [11,12]. Fish and fish oils are sources of long-chain n-3 PUFAs and these FAs act to oppose the actions of n-6 PUFAs [12].

Breastfeeding in Taiwan has been promoted since the 1980s. According to a Taiwan Bureau of Health Promotion survey in 2004, only 29.4% of lactating mothers started exclusive breastfeeding, which is lower than in the USA (>70%) and Norway (99%). Only 13.1% of mothers were practicing restricted breastfeeding 6 months after delivery, and 21.2% of mothers were feeding children under 6 months of age with a mix of human milk (HM) and IF. This means up to 80% of young children in Taiwan rely on IF [13,14]. To evaluate the effect of food on infant growth, it is important to know the FFA content in milk. However, there have been very few studies on this topic in Taiwanese women. Clarifying the FFA content in HM and IF may help industry to manufacture IF with a composition closer to that of HM. We designed this study to compare HM from mothers with preterm and full-term infants, and to compare HM with three brands of IF in both regular and preterm infant formulations. In addition, we investigated the changes in FFA composition in HM through

the development from colostrum to transitional milk to mature milk.

## Materials and methods

Preparation of fatty acid methyl esters (FAMES) is by far the most common approach for lipid analysis [15,16]. FAMES were separated and quantified on an Agilent 5975C Inert GC/MSD system using a capillary column equipped and a flame ionization detector (Agilent Technologies, Inc., Santa Clara, CA., USA). FFAs in milk were derivatized to FAMES using 3N methanolic HCl, (MeHCl) as an acid catalyst for gas chromatography/mass spectrometry (GC/MS) analysis. Individual FFAs in HM and IF were quantified according to individual FA calibration curves, a known concentration of an internal standard and its peak area, and the internal response factor (IRF).

### Standards and reagents

All fatty acid standards, including decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2  $\omega$ 6),  $\alpha$ -linolenic acid (C18:3  $\omega$ 3),  $\gamma$ -linolenic acid (C18:3  $\omega$ 6), arachidic acid (C20:0), homo- $\gamma$ -linolenic acid (C20:3), arachidonic acid (C20:4  $\omega$ 6), eicosapentaenoic acid (20:5  $\omega$ 3), docosanoic acid (C22:0), docosahexaenoic acid (C22:6  $\omega$ 3), and tetracosanoic acid (C24:0), were purchased from Sigma (St. Louis, MO, USA). FA standard solutions (10 mL) were prepared at a concentration of 1 mM in 1:1 (vol/vol) chloroform/methanol. Aliquots of each FA standard solution were stored at  $-20^{\circ}\text{C}$ . Chloroform, methanol, and hexane were purchased from Merck (Darmstadt, Germany); 3 N MeHCl was from Supelco (Bellefonte, PA, USA), and HiPerSolv methanol was from BDH

Table 1  
Molecular weight, major ion fragments, and retention time (RT) for individual fatty acid methyl esters detected by SIM-GC/MS.

| Fatty acid                                  | Mass | Major ions                     | RT (min) |
|---|------|--------------------------------|----------|
| Decanoic acid (C10:0)                       | 186  | 74, 87, 143, 186               | 5.522    |
| Lauric acid (C12:0)                         | 214  | 74, 87, 171, 183, 214          | 6.811    |
| Myristic acid (C14:0)                       | 242  | 74, 87, 143, 199, 211, 242     | 9.367    |
| Palmitoleic acid (C16:1)                    | 268  | 55, 69, 194, 236, 268          | 12.276   |
| Palmitic acid (C16:0)                       | 270  | 74, 87, 143, 227, 239, 270     | 12.652   |
| $\alpha$ -Linolenic acid (C18:3 $\omega$ 3) | 292  | 55, 67, 79, 150, 292           | 15.243   |
| Linoleic acid (C18:2 $\omega$ 6)            | 294  | 55, 67, 81, 95, 264, 294       | 15.561   |
| Oleic acid (C18:1)                          | 296  | 55, 69, 83, 180, 222, 264, 296 | 15.738   |
| Stearic acid (C18:0)                        | 298  | 74, 87, 143, 199, 255, 298     | 16.091   |
| Arachidonic acid (C20:4 $\omega$ 6)         | 318  | 55, 67, 133, 150, 203, 318     | 18.364   |
| Eicosapentaenoic acid (20:5 $\omega$ 3)     | 316  | 55, 67, 133, 147, 201, 316     | 18.417   |
| Homo- $\gamma$ -linolenic acid (C20:3)      | 320  | 67, 79, 121, 135, 222, 320     | 18.581   |
| Arachidic acid (C20:0)                      | 326  | 55, 143, 283, 326              | 19.459   |
| Docosahexaenoic acid (C22:6 $\omega$ 3)     | 342  | 55, 67, 79, 131, 145, 342      | 22.298   |
| Docosanoic acid (C22:0)                     | 354  | 74, 87, 143, 311, 354          | 24.400   |
| Tetracosanoic acid (C24:0)                  | 368  | 74, 87, 143, 325, 368          | 27.971   |

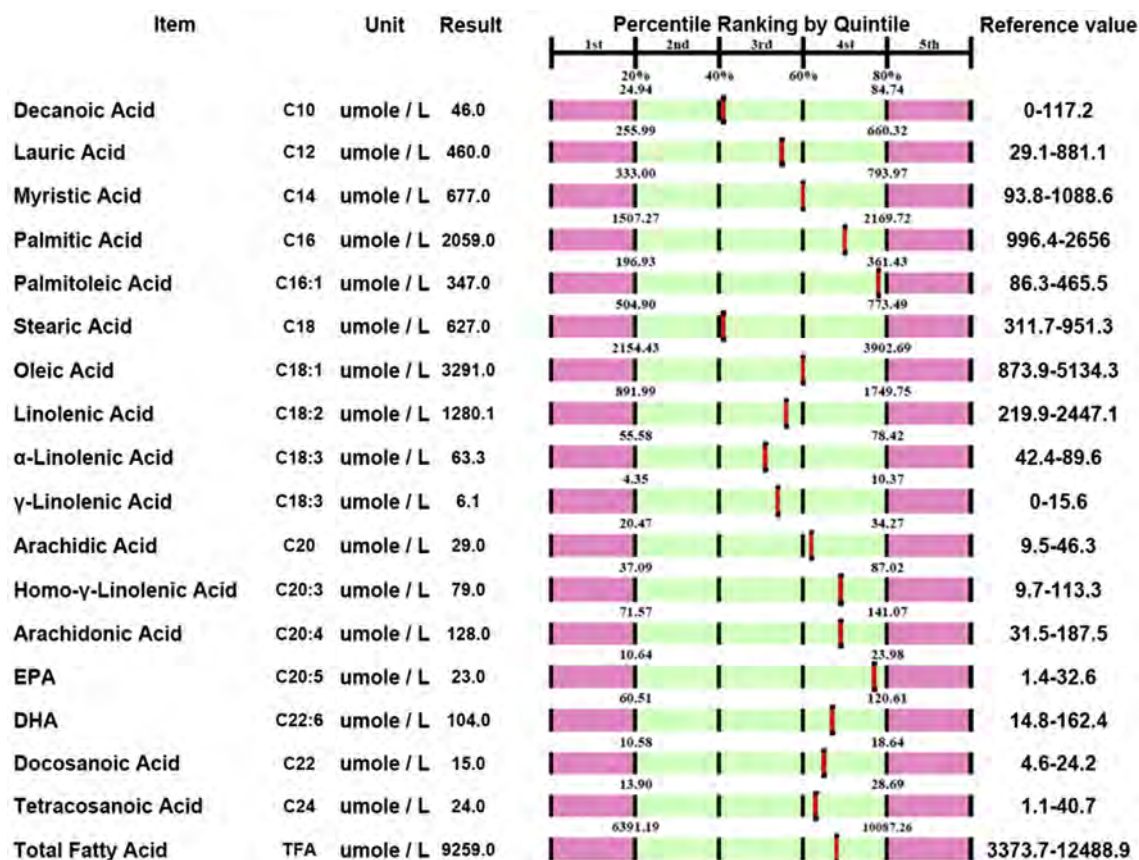


Fig. 1. Free fatty acid content of preterm compared to full-term human milk. Values for all fatty acids in preterm milk fall in the 2nd–4th quintile (between 20% and 80%) for full-term milk.

Laboratory Supplies (Poole, UK). All solvents were of GC or HPLC grade. Working standard solutions of three different concentrations of individual FAs were prepared on the day of analysis. Heptadecanoic acid methyl ester (C17:0; purchased from Sigma) at a known concentration of 40  $\mu\text{mol/L}$  was used as an internal standard for FFA quantification and to assure the quality of GC/MS measurement. The IRF for individual FFAs ranged from 0.7 to 0.95, with the exception of palmitoleic acid, for which the IRF was 2.47.

#### Sample collection and preparation

A total of 38 HM samples were collected from 23 healthy lactating mothers after delivery of full-term infants (FTHM) and 15 mothers who delivered preterm infants (PTHM) at gestations ranging from 29 to 35 weeks (mean 33 weeks). Milk was collected in a sterile polypropylene tube (5–10 mL) at the end of a feed. FTHM samples were collected during three lactating periods to obtain samples of colostrum (0–7 days), transitional milk (8–21 days), and mature milk (>21 days). HM was frozen immediately after collection. Informed consent was obtained from human milk donors of Mackay Memorial Hospital for FFA analysis. The regular formulation of three major IF brands in Taiwan, Similac (Abbott Laboratories, Holland), Enfalac (Mead Johnson Nutritional, Holland), and S-26 (Wyeth Nutritionals, Ireland),

were selected, designated as IF-A, IF-B, and IF-C, respectively (10 samples each). The preterm formulation of the same three IF brands was also evaluated. IF samples were prepared according to the manufacturer's instructions.

All milk samples were thawed at room temperature before analysis. A well-mixed HM sample of 500  $\mu\text{L}$  was placed in a screw-top tube and 20  $\mu\text{L}$  of internal standard (C17:0) was added. FFAs were extracted in two successive steps with 5 mL of chloroform/methanol (1:1 vol/vol) and 4 mL of chloroform/water (5:3 vol/vol). The bottom organic layer was dried and 0.5 mL of 3 N MeHCl and 1 mL of HiPerSolv methanol were added for methylation at 75°C overnight. The samples were then extracted twice with hexane (1 mL); the upper layers were removed, dried under nitrogen, and dissolved in 50  $\mu\text{L}$  of hexane for GC/MS analysis.

#### GC/MS analysis

GC/MS analysis was performed on an Agilent 5975C Inert GC/MSD system in front inlet/split mode using the following parameters: split ratio, 10:1; injection volume, 1  $\mu\text{L}$ ; injection temperature, 250°C; transfer line temperature, 280°C; and carrier gas, helium at 1.0 mL/min. The full scan mode was set to a scan range of  $m/z$  40–800. The oven temperature was increased from an initial value of 50°C to 170°C at 30°C/min and held for 2 minutes, increased from 170°C to 230°C at



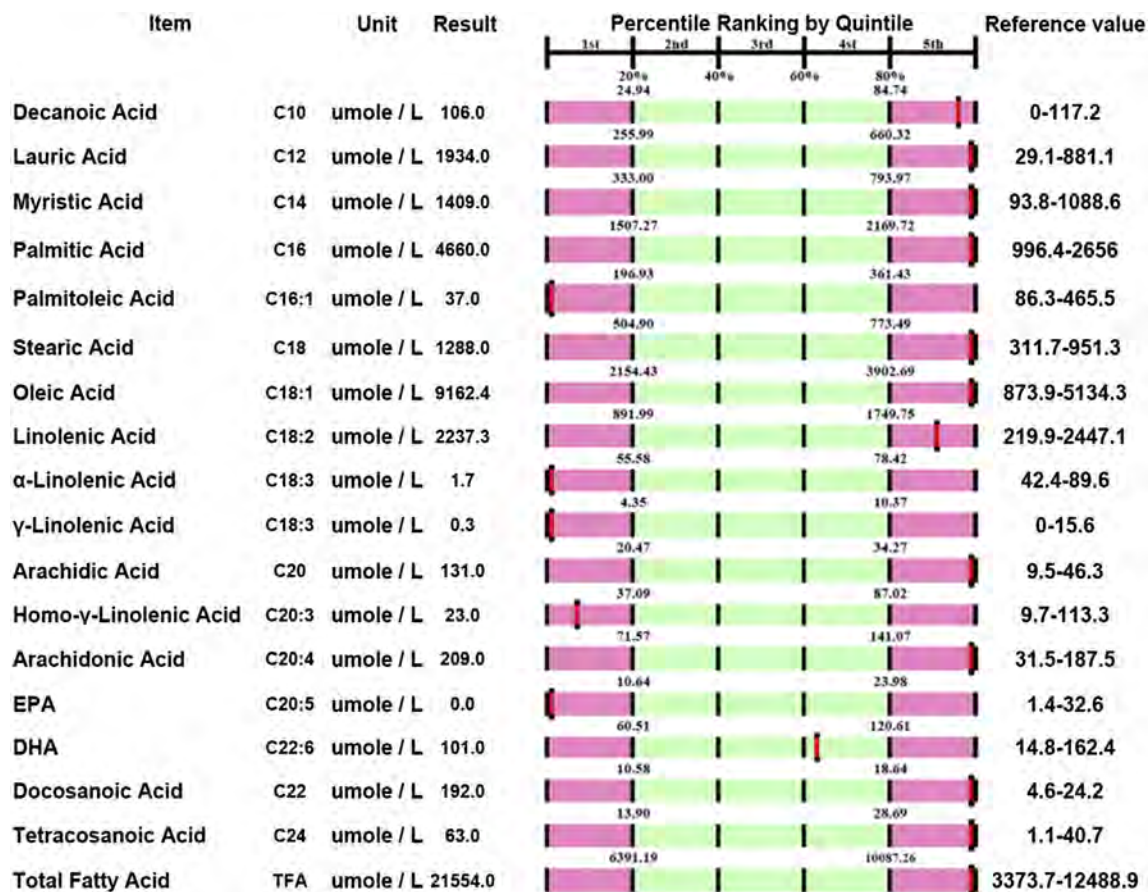


Fig. 2. Free fatty acid content of regular infant formula (IF) compared to full-term human milk (FTHM). Values for IF fall in the 5th quintile (between 80% and 100%) except for C16:1, C18:3, C20:3, and C20:5 when compared to FTHM. Docosahexaenoic acid (DHA) was added at an appropriate amount compared to FTHM and falls in the 4th quintile.

5°C/min and held for 12 minutes, and then further increased to 285°C at 12°C/min and held for 5 minutes. FFAs were separated on an Ultra Alloy-5 GC capillary column (30 m long, internal diameter 0.25 mm, film 0.25 mm; Frontier Laboratories LTD., Fukushima, Japan).

Each FA was automatically quantified according to the integrated peak area in comparison to the internal standard and calibration calculations. The internal standard was also used to assure the GC/MS measurement quality. We assumed that the calibration curve follows the linear relationship  $y = mx + b$ , where  $m$  is the relative response factor for each FAME and the  $y$  intercept is zero. The concentration of each unknown FA was calculated according to  $C_U = (A_U \times C_{IS}) / (A_{IS} \times m)$ , where  $A$  is the peak area,  $C$  is the concentration, and subscripts U and IS denote the unknown and internal standard, respectively [17].

## Results

The linear range for FAME quantification was 1–1200  $\mu\text{mol/L}$  and the linear-through-zero regression was excellent ( $r = 0.9971$ ). The within-run and between-run precision of the method was determined by assaying three samples in triplicate over a period of 3 days. The mean within-run and between-run coefficient of variation was 2.75% and 4.46%, respectively. The mean FA recovery was 92.6%.

The molecular weight, major ion fragments, and retention time for individual FAMES are listed in Table 1. Individual FFA concentrations in milk did not correspond well to a normal distribution, so FFA data are presented using percentile ranking by quintiles for a 95% reference interval. Reference values for FFAs in FTHM and PTHM from Taiwanese mothers were determined (Fig. 1). There were no significant differences in FFA concentrations between FTHM and PTHM. FFA concentrations in PTHM all fell within the 2nd–4th quintile (between 20% and 80%) for FTHM (Fig. 1). Mean FFA concentrations in regular IF and PTIF were plotted as a function of reference values for FTHM (Fig. 2) and PTHM (Fig. 3), respectively. Most of the FA concentrations in regular IF and PTIF fell within the 5th quintile (between 80% and 100%) for the corresponding reference, except for C16:1, C18:3, C20:3, and C20:5, which fell within the 1st quintile (<20%). Only DHA (C22:6) was present at an appropriate level compared to HM and fell within the 4th quintile (between 60% and 80%). There were slight variations in FA concentrations in IF and PTIF from brand to brand, but the differences were not statistically significant.

The mean total FFA content in regular IF and PTIF was 21,554 and 19,836  $\mu\text{mol/L}$ , respectively, which was significantly higher than the content in FTHM (8,540  $\mu\text{mol/L}$ ) and PTHM (9,259  $\mu\text{mol/L}$ ) ( $p < 0.05$ ). For the sum of saturated

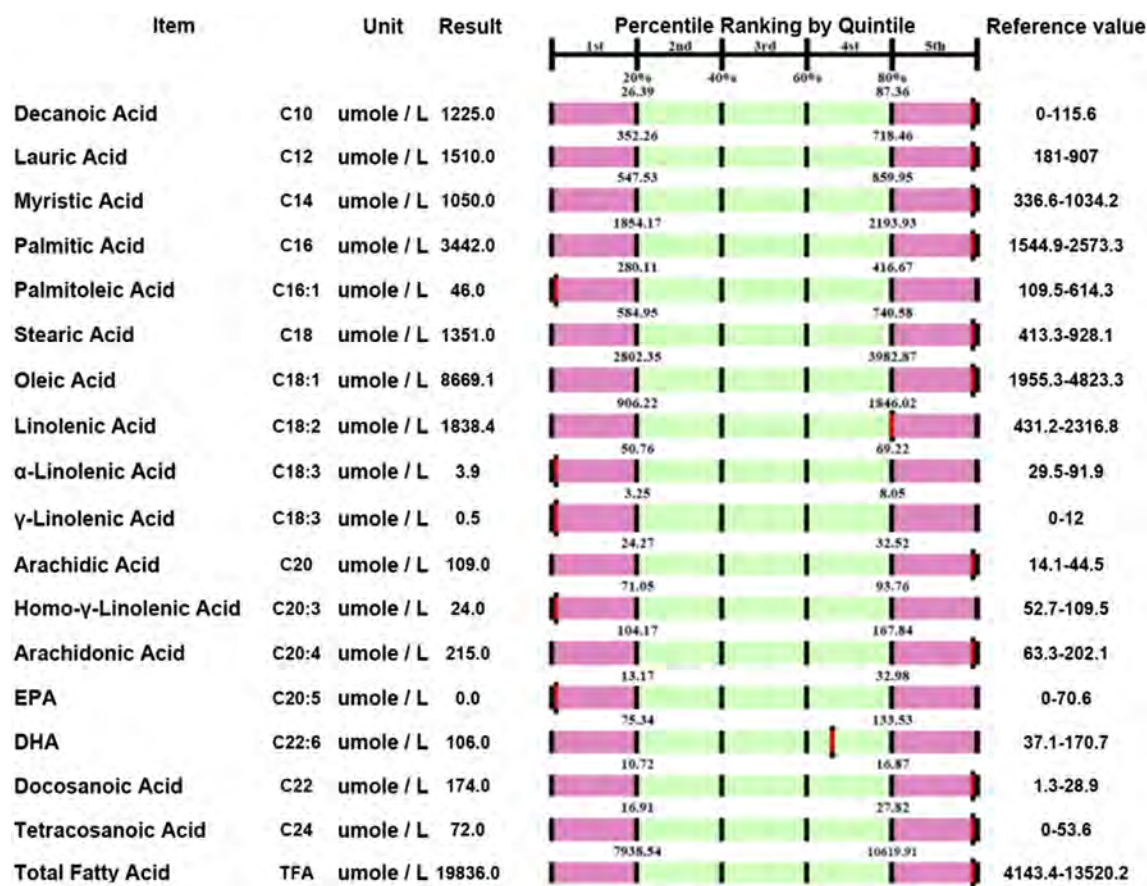


Fig. 3. Free fatty acid content of preterm infant formula (PTIF) compared to preterm human (PTHM) milk. Values for PTIF fall in the 5th quintile (between 80% and 100%) except for C16:1, C18:3, C20:3, and C20:5 when compared to PTHM.

FAs (C10:0–C24:0) as a percentage of total FFAs, no significant differences were observed (43.1% for FTHM, 42.8% for PTHM, 45.5% for IF, and 45.3% for PTIF; Fig. 4). There were also no significant differences for long-chain monounsaturated FAs (LCMUFA; C16:1 and C18:1) and long-chain polyunsaturated FAs (LCPUFA; C18:2, C18:3, C20:3, C20:4, C20:5, and C22:6) as a percentage of total FFAs. The relative LCMUFA content was 37.7%, 39.5%, 42.6%, and 43.9%, and the relative LCPUFA content was 20.0%, 18.2%, 11.9%, and 10.9% for FTHM, PTHM, regular IF, and PTIF, respectively (Fig. 4). Total concentrations of each group of FAs are shown in Fig. 5. Essential FAs, particularly LA, ALA, and their eicosanoid products such as AA, EPA, and DHA, were abundant in HM, but less so in IF and PTIF (Fig. 6). The LA/ALA ratio in FTHM and PTHM from Taiwanese mothers was 17.3 and 20.2, respectively, whereas the ratio for IF and PTIF was greater than the recommended ratio. FFA concentrations in HM did not significantly differ among colostrum and transitional and mature milk.

## Discussion

The total FFA content in IF was much higher than in HM; however, the saturated FA, LCMUFA, and LCPUFA content as a percentage of total FFAs did not significantly differ among the IF and HM samples. The saturated FA content in IF and

PTIF was more than twice as high as in FTHM and PTHM (Fig. 5). Whether or not high levels of saturated FAs in IF will result in infant obesity is a controversial issue. According to Burdette et al, neither breastfeeding nor timing of the introduction of complementary foods was associated with adiposity at 5 years of age [18]. By contrast, van Dijk et al concluded that consumption of a diet high in saturated FAs results in proinflammatory obesity-linked gene expression, whereas consumption of a diet high in LCMUFAs leads to a more anti-

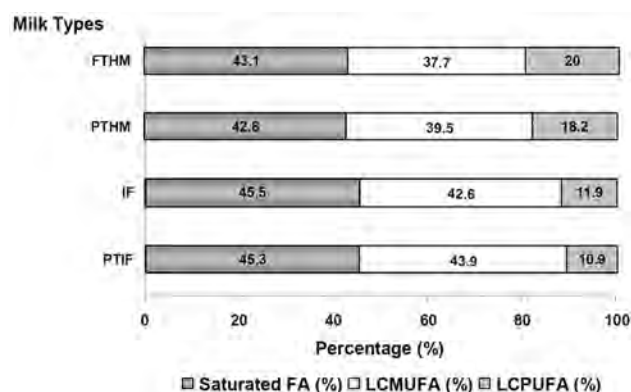


Fig. 4. Percentage saturated fatty acids (FAs), long-chain monounsaturated FAs (LCMUFAs) and long-chain polyunsaturated FAs (LCPUFAs) among total free FFAs in full-term human milk (FTHM), preterm human milk (PTHM), regular infant formula (IF) and preterm infant formula (PTIF).

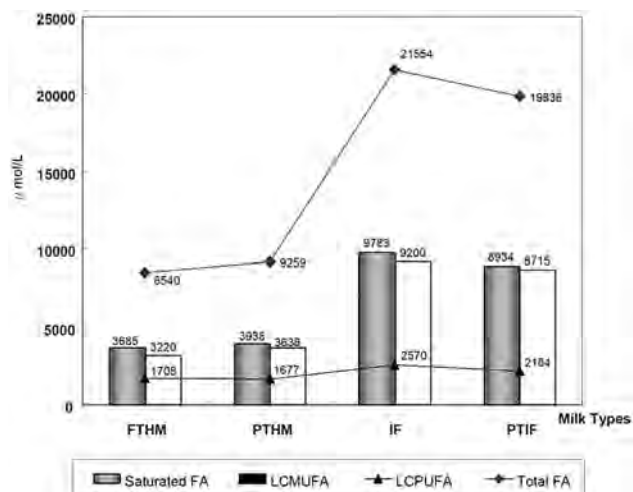


Fig. 5. Saturated fatty acid (FA), long-chain monounsaturated FA (LCMUFA), long-chain polyunsaturated FA (LCPUFA), and total free FA content in full-term human milk (FTHM), preterm human milk (PTHM), infant formula (IF) and preterm infant formula (PTIF).

inflammatory result [19]. Investigation of whether the significantly higher saturated FA content of IF and PTIF will lead to future unfavorable outcomes such as obesity should be the subject of long-term studies.

HM from women who consume a Western diet generally contains 10–17% LA, 0.8–1.4% ALA, 0.3–0.7% AA, and 0.1–0.5% DHA [20,21]. We found relative concentrations of 15.6% LA, 0.9% ALA, 1.3% AA, and 1.2% DHA, which were all close to the optimal proportions of LCPUFAs as a primary source for proper development of the central nervous system in infancy. The LA and LAL levels in our study were similar to those found in HM from Spanish and German mothers [22,23]. However, the LA/ALA ratio was 17.3 and 20.2 for

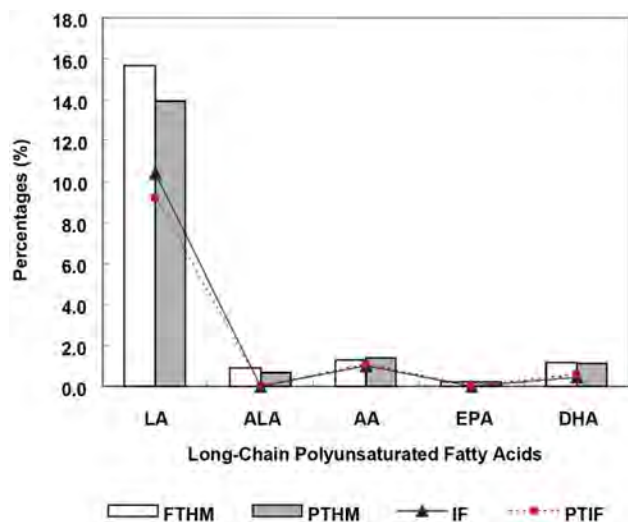


Fig. 6. Percentage long-chain polyunsaturated fatty acids (LCPUFAs) (linoleic acid, LA;  $\alpha$ -linolenic acid, ALA; arachidonic acid, AA; eicosapentaenoic acid, EPA; and docosahexaenoic acid, DHA) full-term human milk (FTHM), preterm human milk (PTHM), infant formula (IF) and preterm infant formula (PTIF).

FTHM and PTHM, respectively, both of which were higher than the recommended LA/ALA ratio of 5–15 proposed by Thiombiano-Coulibaly et al [24]. The slightly higher LA and lower ALA levels might be closely related to the dietary habits of Taiwanese women [25]. The DHA content in HM from Taiwanese women was lower than in HM from Chinese women (2.8%) [26] but similar to the content in HM from Japanese women (1.1%) [27]. Because Taiwan is an ocean nation, this finding can be explained by higher DHA intake from fish and seafood by Taiwanese women compared to women from North America and Europe. According to Wu et al, the FA composition of human milk varies during lactation [25]. Our data are similar to their findings; in particular, LCPUFA concentrations decrease as lactation progresses. However, differences in FA variations were not statistically significant.

Mean LA, ALA, AA, and DHA concentrations measured in IF and PTIF were 10.4% and 9.3%; <0.1% and 0.2%; 1.0% and 1.1%; and 0.48% and 0.54%, respectively. Because relatively low amounts of LCPUFAs, particularly ALA, are added to IF, the LA/ALA ratio was far higher than the recommendation of 5–20 [28]. For IF-fed babies, the imbalance between LA and ALA in IF may affect endogenous DHA and AA synthesis and leads to lower plasma phospholipid DHA and AA concentrations, which might affect visual function [29]. Notably, EPA, which might have the same influence on DHA production, was not detected in either IF or PTIF.

In conclusion, the FFA content was higher in IF than in HM, but short or no additive of several LCPUFAs, like ALA, GLA, and EPA. Whether the significantly higher saturated FA and lower LCPUFA content in IF and PTIF leads to future unfavorable outcomes such as obesity, atopic syndrome, and less optimal neurodevelopment in mature and premature infants should be further investigated. Breast milk is an essential and natural nutrient source for normal growth and development of infants. The nutrient content of breast milk is recognized as the gold standard for IF manufacture.

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