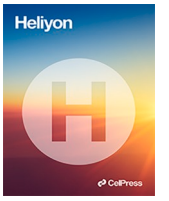
Heliyon 9 (2023) e21611

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Effects of lipids from multiple sources on glyceride composition, concentration, and structure of infant formulas benchmarked to human milk

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ARTICLE INFO

Keywords:
Human milk
Infant formulas
Glycerides
Composition analysis
Structural analysis

ABSTRACT

The important parameters affecting the nutritional properties of lipids were analyzed and compared between human milk (HM), infant formulas (IFs), mammalian milk, and substitute fat, including molecular species, fatty acid composition, glyceride content, and important structural triacylglycerols (TAGs). The molecular species of triacylglycerols with functional fatty acids were significantly different between HM and IFs, and their contents in HM were significantly higher than those in IFs. Accordingly, the evaluation scores of fatty acid composition and glyceride content in IFs were less than 50 compared to HM. Although the introduction of vegetable oils effectively improved the unsaturation of IF lipid, the excessive addition of TAGs rich in oleic and linoleic acid resulted in an imbalance of TAG composition and structure. Only 36.84 % of IFs were supplemented with structured lipids, but those still lacked *sn*-2 palmitate TAGs. The adoption of multiple lipids and novel processing technologies is required for novel IFs to match the composition, content, positional structure and spherical membrane structure of HM as closely as possible.

Abbreviations: HM, human milk; IFs, infant formulas; TAGs, triacylglycerols; rac-OPL, rac-1(3)-oleoyl-2-palmitoyl-3(1)-linoleoylglycerol; rac-POL, rac-1(3)-palmitoyl-2-oleoyl-3(1)-linoleoylglycerol; rac-PLO, rac-1(3)-palmitoyl-2-linoleoyl-3(1)-oleoylglycerol; rac-OPO, rac-1,3-dioleoyl-2-palmitoylglycerol; rac-OOP, rac-1,2-dioleoyl-3-palmitoylglycerol; DAG, diacylglycerols; OPO, 1(3)-palmitoyl-2-linoleoyl-3(1)-oleoylglycerol; OPL, 1(3)-oleoyl-2-palmitoyl-3(1)-linoleoylglycerol; CMIF, cow milk-based IF; SMIF, sheep milk-based IF; SIF, soybean-based IF; CM, cow milk; SM, sheep milk; CaM, camel milk; BM, buffalo milk; DM, donkey milk; HoM, horse milk; CSO, corn and soybean oil; CSSO, corn, soybean and sunflower oil; CSSCO, corn, soybean, sunflower and coconut oil; ALA, α-linolenic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; LA, linoleic acid; PLSDA, partial least squares discriminant analysis; SCTs, saturated triacylglycerols; MUCTs, monounsaturated triacylglycerols; PUCTs, polyunsaturated triacylglycerols; MLL, one medium-chain and two long-chain triacylglycerols; MML, two medium-chain and one long-chain triacylglycerols; MCT, medium-chain triacylglycerols; LCT, long-chain triacylglycerols.

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https://doi.org/10.1016/j.heliyon.2023.e21611
Received 28 February 2023; Received in revised form 23 October 2023; Accepted 24 October 2023
Available online 2 November 2023

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1. Introduction

Human milk (HM) is enriched in lipids, essential proteins, oligosaccharides as well as immunomodulatory and metabolic factors necessary for infant growth and development, whicha naturally reflect the developmental needs of neonates in terms of nutrients [1,2]. Given this, HM is considered the gold standard in the development of Infant formulas (IFs). Additionally, IFs are receiving increasing attention in modern society due to mounting research evidence that early dietary intake has a significant impact on the short- and long-term health of infants [3]. Each IF contains basic nutrients (proteins, fat, carbohydrates, vitamins and minerals), as well as additional substances such as prebiotics, nucleotides and even structured lipids [4]. Triacylglycerols, as the major components of HM and IFs, not only provide almost half amount of the energy required by infants undergoing fast development as well as the fatty acids essential for the healthy development of brains, vision, immune system, but also serve as a well-matched vehicle for the uptake of vitamins A, D, E, and K [5,6]. Supply of essential fatty acids as well as absorption and utilization of fatty acids and calcium are determined by TAG fatty acid composition and position [7–9]. Nonetheless, the lipid parameters of HM vary based on diet, lactation stage, individual genetics and regional provenance, while the composition of IFs mainly depends on base materials (such as raw milk, vegetable oil, structured lipids and functional lipids) and processing technology.

The glyceride composition of IFs has gone through numerous advances in recent history [3,6]. Synthetic milk adapted by Henry John Gerstenberger in 1915 was the first vegetable oil-based product with no milk fat. The first product mimicking the unique composition and structure of HM fat was released in 1986, and Betapol® has been commercially added to IFs globally since 1995. This product more closely mimics HM lipids due to the increased content of *sn*-2 palmitate TAGs, and has changed the perception of the use of palm oil in IFs, resulting in swift growth of its market share. Different from most animal fats and vegetable oils, pork fat contains saturated fatty acids at the *sn*-2 position of the triacylglycerol and is therefore widely used in the manufacturing of IFs in Japan [10]. Since the 2000s, long-chain polyunsaturated fatty acids have been prescribed as a mandatory component of IFs by law [6]. According to the history and evolution of IF glycerides, the progress of simulating HM lipids in IFs mainly revolves around the application of vegetable oils, the simulation of structured lipids, and the supplementation of functional fatty acids, aiming to more closely mimic the composition, content and structure of glycerides.

Infant formulas are mainly manufactured based on fresh milk/milk powder and mixed vegetable oils, which are partly blended with HM fat substitutes [6]. However, previous studies mainly focused on HM and IFs [5], HM and animal milk [11], vegetable oils [12] or structural lipids [13], without comprehensive analysis. Based on a previous maternal-infant cohort study of human milk lipids and referenced to other research results, the glyceride profiles of commercial IFs, mammalian milk and vegetable oils were analyzed to evaluate the current research status of IFs as well as the contributions and shortcomings of mammalian milk and vegetable oils in mimicking human milk glycerides. At the same time, human milk fat substitutes, especially structured lipids, have attracted increasing attention because their addition improves the utilization of fatty acids and calcium in infant formula, prevents the formation of calcium soap and avoids the occurrence of constipation in infants. This study focused on several structured lipids, explored the current simulation status of structural lipids, and provided data supporting the upgrading of IFs.

2. Materials and methods

2.1. Sample collection

A total of 233 H M samples (0–6 months) were donated by 61 healthy lactating mothers from city of Beijing, Liuyang, Luoyang, and Tangshan in China. Their pregnancies were full-term. The infants were born without any congenital or genetic diseases. This research was reviewed and approved by the Ethics Committee of Beijing Ditan Hospital affiliated to Capital Medical University (#2015-027-01). All study participants were informed of the purpose of this study and agreed to participate. The study was registered at clinicaltrials.gov (Registration No: NCT02658500).

Infant formulas for 0–6 month old babies were bought from local supermarkets and online stores. In total, 22 commercial IFs from six countries were divided into cow milk-based (16 types), sheep milk-based (3 types) and soybean-based IFs (3 types) according to the ingredient lists. The mammalian milk (cow, sheep, camel, buffalo, donkey and horse milk) was obtained from supermarkets, the online stores of manufacturers, and animal husbandry plants. The vegetable oils and other fat-related ingredients were provided by suppliers of the IF companies. The custom-made compound vegetable oils mentioned in this study are used in the manufacturing of some of the investigated IFs to better match the characteristics of HM glycerides. Three types of custom-made compound vegetable oils were prepared: (1) corn and soybean oil mixture; (2) corn, soybean and sunflower oil mixture; (3) corn, soybean, sunflower and coconut mixture. The four structured lipids mentioned in this study were either used in IF preparation or laboratory commissioning products. OPO-8293 and OPO-B55 used in IFs were purchased from Qinhuangdao Jinhai Special Edible Oil Industry Co., Ltd. (China) and Bunge

Loders Croklaan Oil Technology Co., Ltd. (China), respectively, and the other two structured lipids were laboratory commissioning products. In the present study, 233 H M samples and 22 IFs were analyzed once, while mammalian milk, vegetable oils and structured lipids were analyzed 9 times.

2.2. Reagents and standards

All the reagents were of mass spectroscopy grade and were purchased from Thermo Fisher Scientific (USA). The TAG standards 16:0–18:1–18:1 and 16:0–18:1–18:1 (d5) were purchased from Larodan AB (Sweden) and Shanghai ZZBIO Co., Ltd. (China), respectively. The following racemic standards of TAGs were purchased from Larodan Fine Chemicals (Malmö, Sweden): rac-1 (3)-oleoyl-2-palmitoyl-3 (1)-linoleoylglycerol (rac-OPL), rac-1 (3)-palmitoyl-2-oleoyl-3 (1)-linoleoylglycerol (rac-POL), rac-1 (3)-palmitoyl-2-linoleoyl-3 (1)-oleoylglycerol (rac-PLO), rac-1,3-dioleoyl-2-palmitoylglycerol (rac-OPO), and rac-1,2-dioleoyl-3-palmitoylglycerol (rac-OOP). When TAGs were classified at the level of fatty acyl distribution, only secondary (*sn*-2) and primary (*sn*-1,3) regiopositions were considered different.

2.3. Extraction of glycerides from samples

The lipid extraction procedure was conducted according to a published protocol [14]. Briefly, the internal standard TAG 16:0–18:1–18:1 (d5) was first dissolved to 4.00 g/L in methanol-dichloromethane (1:1, v/v) containing 10 mM ammonium acetate. Then, 20 μL TAG 16:0–18:1–18:1 (d5) was added to 200 μL of HM, diluted IFs or mammalian milk. These mixtures were treated with a solution consisting of 2 mL of methanol, 900 μL dichloromethane, and 200 μL of ultrapure water and shaken for 10 s. After that, 200 μL of ultrapure water and 900 μL of dichloromethane were added and the extraction proceeded for 10 s with shaking followed by a 15 min long centrifugation at 3300×*g*. The organic phase was isolated and collected by pipetting and mixed with 2.2 mL of methanol, 600 μL of dichloromethane and 1 mL of ultrapure water followed by a 10 min long centrifugation at 3000×*g*. The aqueous phase was then mixed with 1.8 mL of dichloromethane and centrifuged at 3300×*g* for 15 min, after which the organic-phase extract was collected. The two organic phase aliquots were dried under a nitrogen gas flow and dissolved 1000 times using 1 mM ammonium acetate in methanol-dichloromethane for glyceride analysis.

The extraction liquid was further diluted 200 times, while the vegetable oils and other fat-related ingredients were directly diluted 10,000 times for glyceride analysis.

2.4. Detection conditions of glycerides

Glycerides were separated on a Phenomenex liquid chromatography column with a size of 50 × 3 mm (Kinetex® 2.6 μm C18 100 Å). The chromatographic conditions were as follows: Two microliters of samples were injected into the column, the flow speed was 0.3 mL/min, and the column temperature was set at 50 °C. Mobile phase A was a 5 mM ammonium acetate dissolved in methanol/acetonitrile/water (1:1:1, v/v/v) and mobile phase B was 5 mM AC dissolved in isopropanol. The elution gradient was published before [15].

Glycerides were detected by Q-TOF (AB Sciex, 6600) with ESI in positive ionization mode. The ESI ion source and MS were operated in a scan range of 50–1300 Da. The ion source and curtain gas pressures were 60 psi and 35 psi, respectively; declustering potential, ion source spray voltage and collision energy were 5500 V, 80 V and 10 V, respectively; ion source temperature was 650 °C.

2.5. Analysis of glycerides

The glyceride data were qualitatively and quantitatively analyzed using MSDIAL based on the fragment ions and peak areas, respectively. In this study, glycerides were detected in positive ion mode based on the *m/z* value of the precursor ions and product ions ($[M\text{-}R_nCOO]^+$, also called $[DAG]^+$, where R*n* represents the acyl radical), in which the adduct ions of TAG and diacylglycerols (DAG) were "$+NH_4$". Regression equations built with internal and external standards were used to quantify the relative content based on a published protocol [16]. The regression equation was $Y = 6.5676X$ and the coefficient of determination (R^2) was 0.9966. It should be noted that the symbol "-" indicates the fatty acid composition of lipids rather than the *sn*-1, *sn*-2 and *sn*-3 positions of glycerol.

2.6. Evaluation of the similarity of glyceride molecules

The similarities of fatty acid composition and glyceride content were assessed using a previously published evaluation model [11], with minor modifications as follows. The similarity evaluation model was established as:

$$G = 100 - \sum\left(100 \times \frac{H_i}{H_t} \times \frac{\left|H_{ih/1} - A_i\right|}{A_i}\right);$$

$$H_{ih/1} = \begin{cases} 1.3H_i\ ,\ A_i > 1.3H_i \\ A_i, 1.3H_i \geq A_i \geq 0.7H_i \\ 0.7H_i\ ,\ A_i < 0.7H_i \end{cases}$$

where, G is the similarity score, H*i* is the content of characteristic glyceride *i* in human milk, H_t is the total content of glycerides in human milk, and A_i is the content of characteristic glyceride *i* in infant formulas, mammalian milk and vegetable oils. In this study, the upper and lower limits were set to 1.3 and 0.7 times of the average relative content of HM characteristic glycerides, respectively.

2.7. Identification of important structural TAGs

The identification of OPO and OPL was conducted according to published protocols [17,18]. Regioisomeric TAG standards were mixed at different ratios and the intensity of product ions ($[DAG]^+$ fragments losing *sn*-1,2,3 fatty acyls) was quantified to plot the calibration curves, among which the ratios were 1/0, 3/1, 1/1, 1/3 and 0/1 for the OPO/OOP standard, as well as 1/0/0, 8/1/1, 14/3/3, 2/1/1, 1/1/1, 1/2/1, 3/14/3, 1/8/1 and 0/1/0 for the OPL/POL/PLO standard.

2.8. Statistical analyses

The OmicStudio tools (https://www.omicstudio.cn/tool) were used to comparatively analyze glyceride species. The glyceride contents were determined at a molecular resolution by multiple testing in MetaboAnalyst (https://www.metaboanalyst.ca/). The differences of glyceride profiles between IFs, mammalian milk, vegetable oils and HM were compared in SPSS Statistics Software 22.0 (IBM Corp., USA), using one-way ANOVA with Tukey's multiple tests or Tamhane's T2 in case of unequal variance. The independent-samples *t*-test was employed to analyze paired samples. Results were presented as means ± standard deviations or percentages. Differences with *P*-values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Molecular species of glycerides in HM, IFs, mammalian milk and vegetable oils

In this study, the molecular species of the identified glycerides accounting for less than one-third of the total samples were excluded from the analysis and discussion in order to reflect the more general situation. There were 94, 97, 72, 43, 82, 115, 101, 135, 61, 109, 110, 101 and 125 different glyceride species in HM, cow milk-based IF (CMIF), sheep milk-based IF (SMIF), soybean-based IF (SIF), cow milk (CM), sheep milk (SM), camel milk (CaM), buffalo milk (BM), donkey milk (DM), horse milk (HoM), corn and soybean oil (CSO), corn, soybean and sunflower oil (CSSO), as well as corn, soybean, sunflower and coconut oil (CSSCO), respectively (Supplementary Table 1). These results indicated that there are differences between IFs, mammalian milk, vegetable oils and HM in the richness of glyceride species. The molecular species of glycerides in common between IFs, mammalian milk, vegetable oils and HM are shown in Fig. 1. The total counts of species in common between CMIF, SMIF, SIF, CM, SM, CaM, BM, DM, HoM, CSO, CSSO, CSSCO and

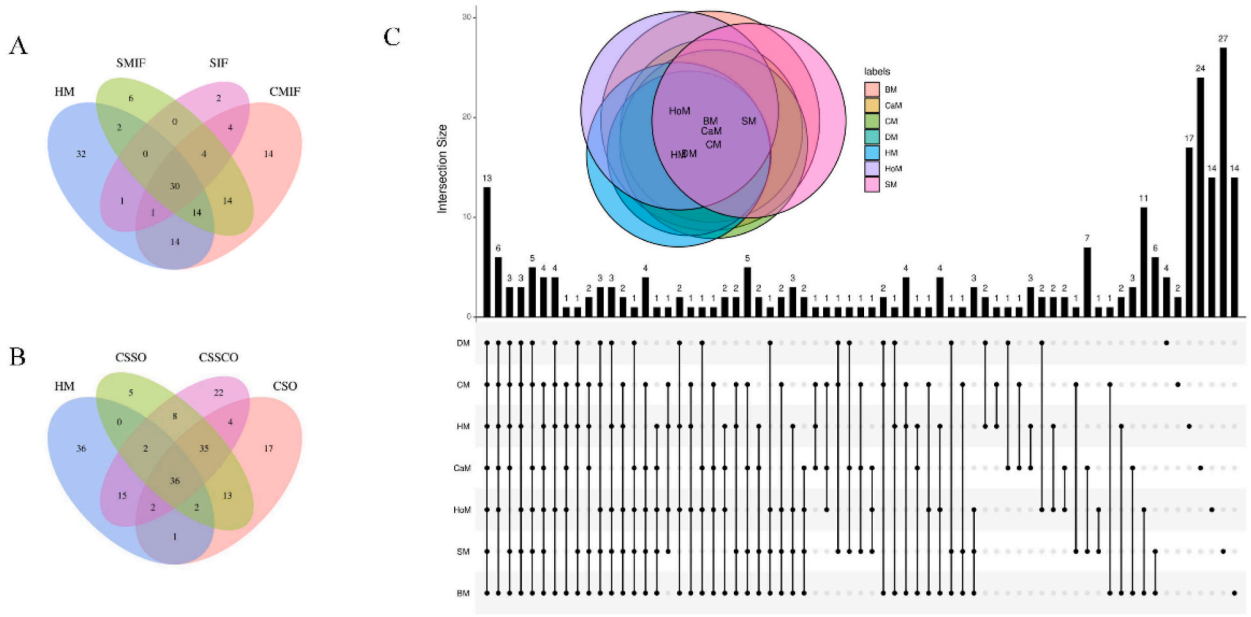

Fig. 1. Venn and Upset diagrams of IFs (A), vegetable oils (B), other mammalian milk (C) and HM. The upset plot (C) consists of two parts: the lower part indicates that the vertically connected filled dark circles corresponds to an intersection between the data sets of different mammalian milk labeled on the left, and the top vertical bar chart shows the intersection numbers. HM: human milk; CMIF, cow milk-based IF; SMIF, sheep milk-based IF; SIF, soybean-based IF; CSO, corn and soybean oil; CSSO, corn, soybean and sunflower oil; CSSCO, corn, soybean, sunflower and coconut oil; CM, cow milk; SM, sheep milk; CaM, camel milk; BM, buffalo milk; DM, donkey milk, HoM, horse milk.

HM were 59, 46, 32, 49, 40, 39, 67, 38, 52, 41, 40, and 55, respectively (Supplementary Table 1). The glyceride species found in CMIF, SMIF, SIF, CM, SM, CaM, BM, DM, HoM, CSO, CSSO and CSSCO also found in HM accounted for 60.82 %, 63.89 %, 74.42 %, 59.76 %, 34.78 %, 38.61 %, 49.63 %, 62.23 %, 47.71 %, 37.27 %, 39.68 % and 44.00 % of each respective total (Supplementary Table 1). According to the comparison of different mammalian milk types, DM possessed the highest ratio of common glycerides with HM but the number of common molecular species number was the lowest. SM, CaM and HoM were not superior to BM and CM in either the ratio or number of shared molecular species with HM. Considering only the molecular species of glycerides, BM and CM may be better raw materials for IF. Among vegetable oils, CSSCO may be a better option because the glyceride composition of CSSCO was significantly better than those of CSO and CSSO according to the numbers and ratios of glycerides in common with HM. The addition of coconut oil made mixed vegetable oil more similar to HM in glyceride molecular species. CMIF was better in terms of molecular species because it contained dozens more glyceride species in common with HM than SMIF, although the ratios of glycerides in common with HM were similar between CMIF and SMIF. Notably, standardized IFs containing vegetable oil and similar surrogate ingredients are more similar to human milk in terms of lipid species than mammalian milk. The ratios of saturated to unsaturated fatty acids were 0.7–1:1 in HM and 3–4:1 in the milk from other mammals, such as sheep, buffalo and cow [11,12,19]. In the manufacturing of IF, vegetable oil, skim milk powder and other ingredients are mixed with raw milk to adjust the composition of fatty acids so that it is more similar to HM. Vegetable oils enriched with unsaturated fatty acids (such as corn oil with 84.57 %, soybean oil with 83.09 %, sunflower oil with 84.57 %, etc.) [20] are always compounded and added to IFs to ensure a fatty acid composition consistent with HM. Furthermore, only 32 glycerides in SIF (accounting for about one-third of the total glycerides in HM) were the same as those found in HM.

As shown in Figs. 1C and 17 molecular species of glycerides in HM were not found in other types of mammalian milk, among which the TAG54:3 (18:0–18:1–18:2), TAG56:8 (18:2–18:2–20:4), TAG58:8 (18:1–18:1–22:6), TAG58:9 (18:1–18:2–22:6) and TAG58:10 (18:2–18:2–22:6) were characterized by high content and/or functional fatty acids, and were reported to be critical nutrients for the growth and development of infants [14]. However, vegetable oils are often deficient in some organic acids including α-linolenic acid (ALA), arachidonic acid (AA) and docosahexaenoic acid (DHA), so they cannot completely supplement all the glycerides deficient in mammalian milk types, especially in terms of the ALA, AA and/or DHA-containing TAGs. According to the nutrition labels, linoleic acid (LA), ALA, AA and DHA were added to almost all of the investigated IFs, but the LA, ALA, AA and/or DHA-containing TAGs identified in HM, such as TAG52:6 (18:1–18:2–18:3), TAG56:7 (18:1–18:2–20:4), TAG56:8 (18:2–18:2–20:4), TAG58:8 (18:1–18:1–22:6), TAG58:9 (18:1–18:2–22:6) and TAG58:10 (18:2–18:2–22:6), were not found in the IFs. Twenty-two commercial IFs had different fatty acid compositions in their AA and/or DHA-containing TAGs, including TAG56:7 (16:0–18:1–22:6), TAG58:8 (18:0–18:2–22:6), TAG56:8 (16:0–20:4–20:4), TAG62:8 (20:4–20:4–22:0) and TAG64:8 (20:4–22:2–22:2). In addition, the AA or DHA supplements used in IFs were analyzed and compared, and a striking difference was also detected in the fatty acid compositions of AA and/or DHA-containing TAGs between HM and AA or DHA supplements. Thus, AA or DHA supplements can affect the fatty acid composition of AA and/or DHA-containing TAGs in IFs. Teng et al. [21] found that longer *sn*-2 fatty acid chain length can cause a stronger steric-hindrance effects and consequently inhibit the binding of gastric lipase to *sn*-3 fatty acids. Additionally, long-chain triacylglycerols (LCTs) containing saturated long-chain fatty acids on the *sn*-1/3 positions were less digestible than their positional isomers with unsaturated long-chain fatty acids on the *sn*-1/3 positions. The AA and/or DHA-containing TAGs in IFs possess saturated

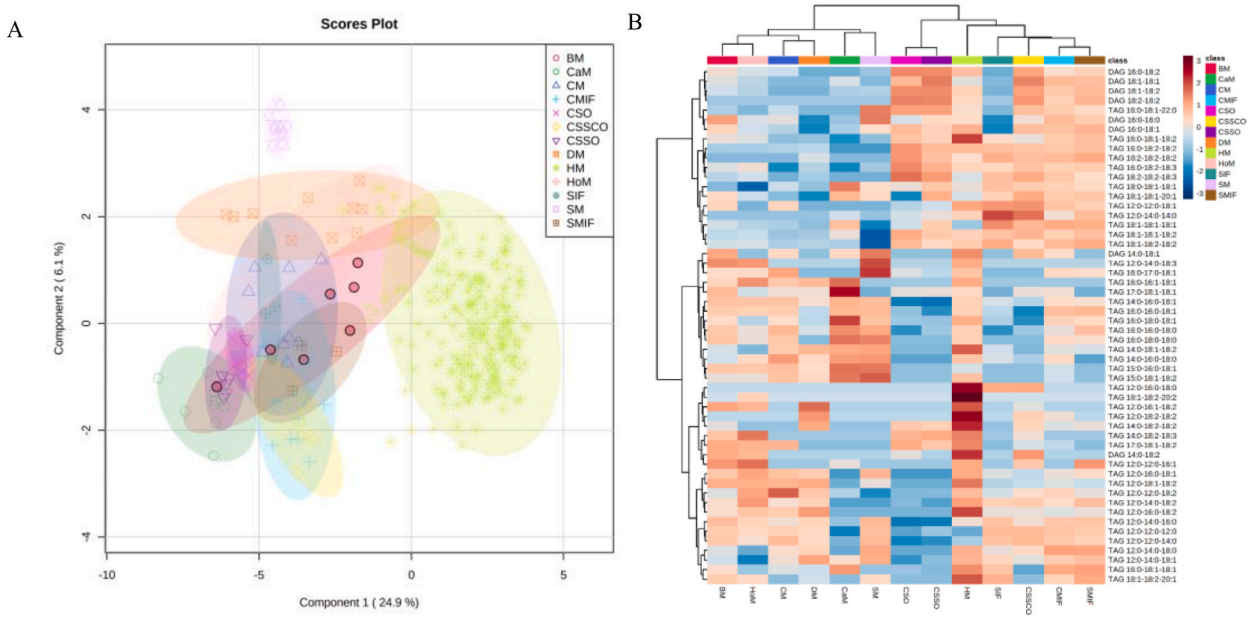

Fig. 2. Partial least squares discriminant analysis (A) and hierarchical clustering coupled with heatmap analysis (B) of glycerides in HM, IFs, other mammalian milk and vegetable oils.

long-chain fatty acids with higher content and longer chain length than those in HM, which may indicate a poorer digestibility of IFs than HM in *vivo*. Previous studies have also shown that HM and IFs had DHA-containing TAGs with a different distribution molecular weights and species, which might affect the nutritional profile of IFs [22]. Some TAGs can not only be broken down into energy sources and simple building blocks, but also provide compounds which directly participate in neonatal tissue development and growth. For example, DHA contained in TAGs can be transported across the blood-brain barrier in the form of lysophosphatidylcholine with the help of a specific protein transporter [22]. However, the differences between the TAGs containing AA and/or DHA in HM and IFs may lead to changes in digestibility that affect subsequent metabolism and ultimately manifest as differences in lipid nutrient availability between HM and IFs.

3.2. *Fatty acid profiles of glycerides from HM, IFs, mammalian milk and vegetable oils*

To characterize the differences of glycerides in HM, IFs, mammalian milk and vegetable oils, partial least squares discriminant analysis (PLSDA) and hierarchical cluster analysis was conducted based on the relative contents and molecular species of glycerides (Fig. 2). The PLSDA revealed significant differences between HM and IFs, mammalian milk and vegetable oils. The clustering correlation of HM with IFs was better than with CSO, CSSO, or mammalian milk. Compared with CSO and CSSO, CSSCO is more suitable for the adjustment of lipids in IFs according to the cluster analysis. The hierarchical clustering between BM and HoM, CM and DM, as

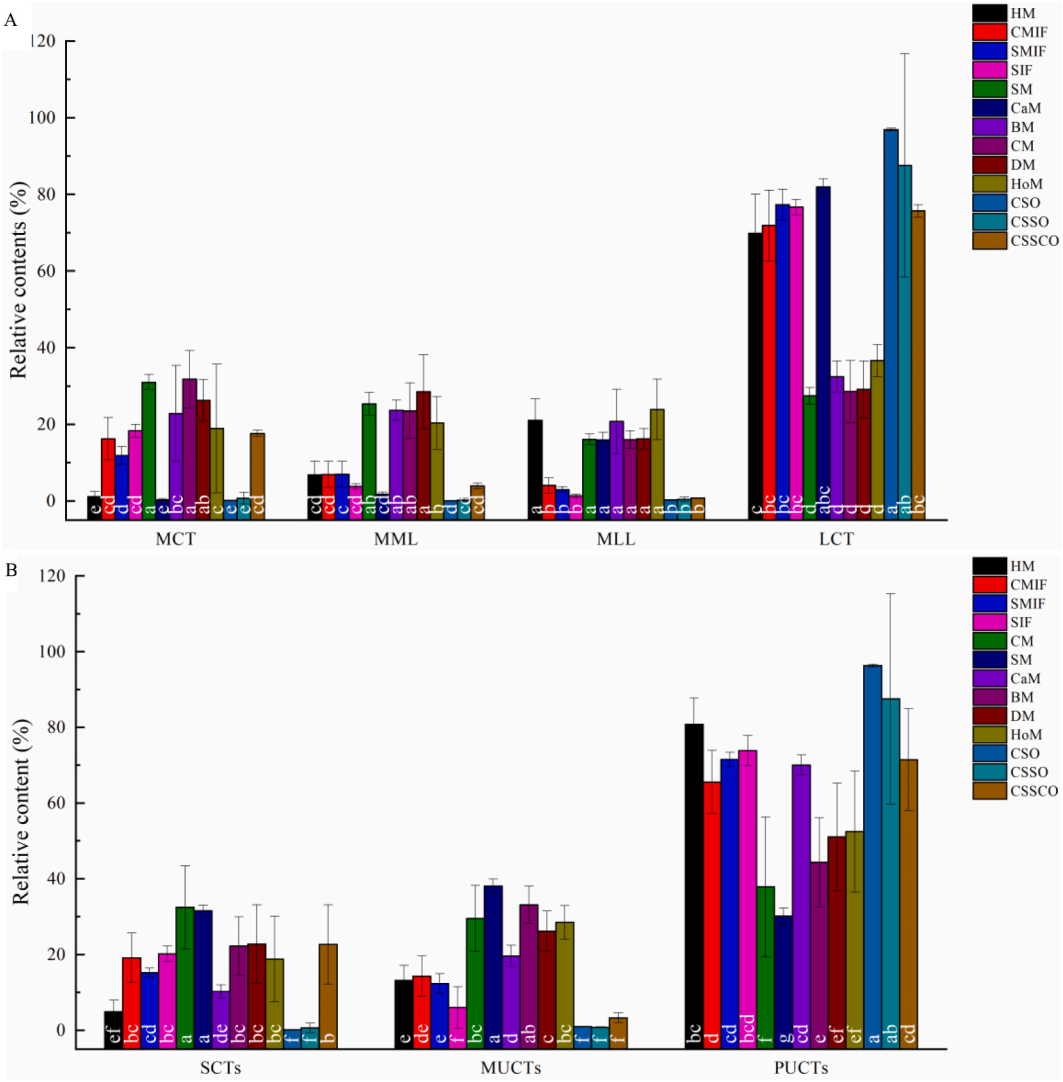

Fig. 3. The relative contents of four types of TAGs containing medium- and long-chain fatty acids (A); the distribution of TAGs with different saturation levels in HM, IFs, other mammalian milk and vegetable oils (B). MCTs, medium-chain triacylglycerols; MML, two medium-chain and one long-chain triacylglycerols; MLL, one medium-chain and two long-chain triacylglycerols; LCTs, long-chain triacylglycerols; SCTs, saturated triacylglycerols; MUCTs, monounsaturated triacylglycerols; PUCTs: polyunsaturated triacylglycerols.

well as CaM and SM was closer than those with HM, IFs and vegetable oils. The contents of glycerides containing stearic, oleic, and/or linoleic acid in mammalian milk were significantly lower than those in HM, which could be supplemented by adding vegetable oils. The content of characteristic glycerides in HM was generally higher than that in IFs, indicating that both the glyceride species and content should be mimicked more closely in HM substitutes.

The hierarchical cluster analysis revealed many differences in TAGs with medium chain fatty acids between HM and IFs, mammalian milk and vegetable oils. Further, to analyze the differences of the chain length of fatty acids in TAGs, the TAGs from HM, IFs, mammalian milk and vegetable oils were divided into the four types of medium-chain triacylglycerols (MCTs), two medium-chain and one long-chain triacylglycerols (MML), one medium-chain and two long-chain triacylglycerols (MLL) and LCTs, after which their relative contents and differences were characterized as shown in Fig. 3A. The relative contents of MCTs, MML, MLL, and LCTs in HM were 1.13 ± 1.30 %, 6.80 ± 3.57 %, 21.02 ± 5.66 %, and 69.89 ± 10.12 %, respectively. Thus, the content of LCTs was the highest, followed by those of MLL and MML. The differences between HM and IFs were mainly found in the TAGs containing medium-chain fatty acids, and their main forms in HM and IFs were MLL (about 21.02 %) and MCTs (11.85–18.33 %), respectively, which was in line with published studies [23,24]. This might be caused by the differences of TAGs in HM and raw mammalian milk, because the relative contents of MCTs accounted for approximately 18.91–31.78 % of the total TAGs in mammalian milk (except for CaM), compared to only 1.13 % in HM. Compared with HM, mammalian milk except for CaM was rich in TAGs containing medium-chain fatty acids (28.98 % vs. 63.12–72.36 %). In the stomach, gastric lipases were found to selectively release *sn*-3 medium-chain fatty acids [25,26], which can enter the liver via the hepatic portal vein for β-oxidation independent of carnitine-mediated activation as well as be used to resynthesize TAGs [27,28]. Additionally, the structured TAGs, namely MLM (containing medium-chain fatty acids on the *sn*-1/3 positions and long-chain fatty acids on the *sn*-2 position) are designed and used widely to enhance the energy intake of patients

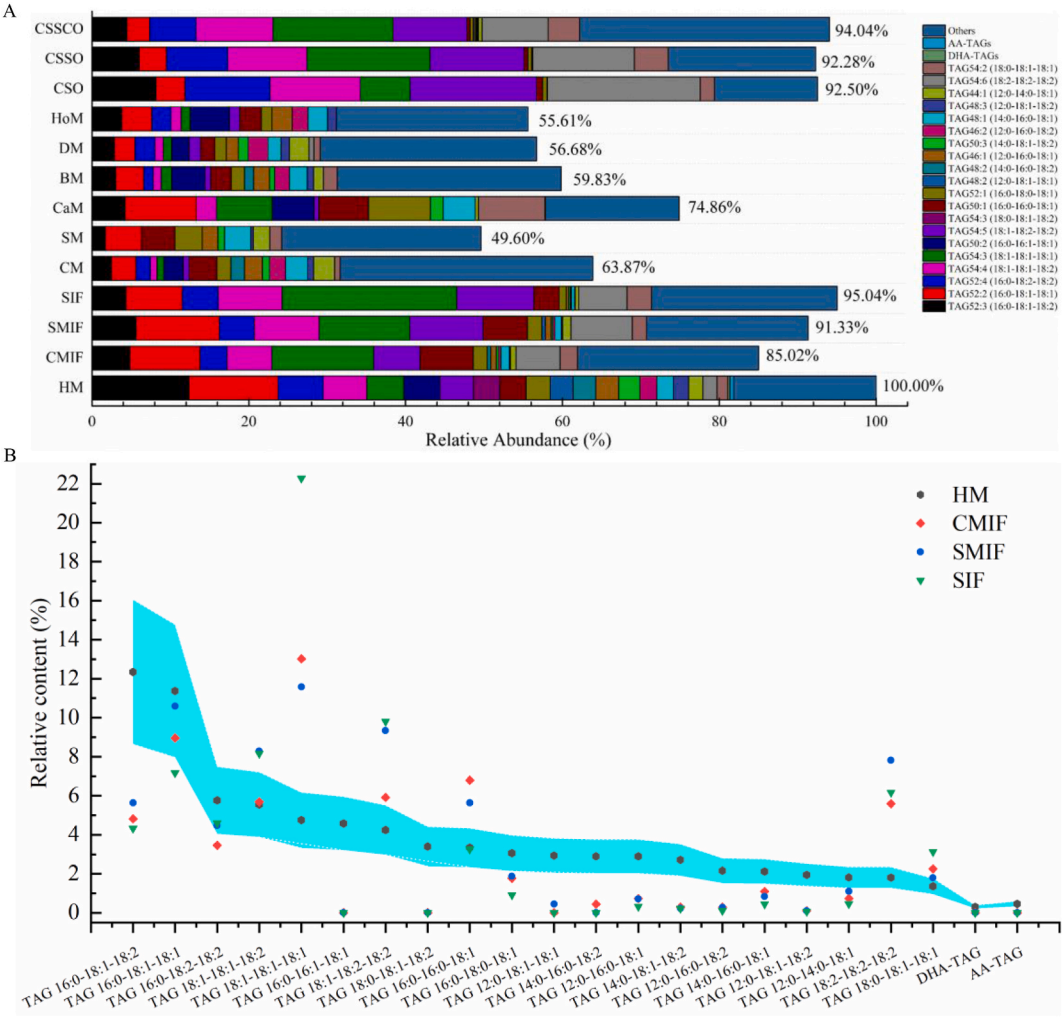

Fig. 4. Relative contents (%) of the common glycerides in IFs, other mammalian milk and vegetable oils compared to HM as the gold standard, defined as 100 % (A). The number of glycerides at the top of the horizontal columns indicates the percentage of glycerides in common with HM. Distribution of important glycerides in HM and IFs (B). The blue area indicates the range of HM glycerides (B).

with pancreatic insufficiency [29]. Therefore, mammalian milk rich in TAGs containing medium-chain fatty acids seems to be a good choice of raw material for IFs in terms of digestion and energy supply, especially for premature infants and those with low birth weight [24]. However, the higher digestion and faster energy supply induced by the TAGs containing medium-chain fatty acids is more likely to lead to early obesity in formula-fed infants. The health benefits of fatty acid chain length distribution in HM still need further research. Compared with other mammalian milk, CaM and HM had the most similar profiles of MCTs, MML, MLL and LCTs, because CaM and HM were the only group with no differences in the relative content of the four types of TAGs (Fig. 3A).

It is evident that an increase of medium-chain fatty acids in mammalian milk is accompanied by the increase of saturated fatty acids (Fig. 3). The content of long-chain glycerides in CSO and CSSO was originally over 90 %, while the contents of MCTs and MML in CSSCO increased significantly with the addition of coconut oil, since the latter contains approximately 78 % medium-chain fatty acids [20]. Mammalian milk, particularly CM and SM, had significantly higher relative contents of saturated and monounsaturated TAGs, but lower relative content of polyunsaturated TAGs compared with HM (Fig. 3B). Therefore, the addition of vegetable oils could effectively alleviate the problem that the saturation of mammalian fat is significantly higher than that of HM. However, palmitic acid at *sn*-2 position accounts for about 40–50 % in mammalian milk, compared to only 0.82–14.89 % in vegetable oils [20,30]. Therefore, mammalian milk has a significant structural advantage in providing sn-2 palmitic TAGs compared with vegetable oils. Very low concentrations of AA- and/or DHA-containing TAGs were found in CM and CaM, while none were found in vegetable oils. The relative contents of TAGs containing AA and/or DHA were lower in IFs than those in HM. The TAGs containing AA and/or DHA are firstly hydrolyzed into free DHA and monoacylglycerol, then absorbed as TAGs in chylomicrons, and eventually absorbed by adipose tissue, heart and liver [31]. It was observed that high DHA and AA consumption might promote the postnatal growth of preterm infants [32]. Moreover, the supplementation of AA and DHA was found to reduce blood glucose and liver fat levels, the deposition of type I collagen in the liver, and the concentration of MCP1 in white adipose tissue [33], indicating that it may ameliorate cardiovascular diseases and metabolic disorders. Therefore, the addition of TAGs containing AA and/or DHA to IFs should be appropriately increased to fully support the growth and development of infants, or potentially even prevent disease.

As discussed in section 3.1, almost all of the investigated IFs were supplemented with AA and DHA according to their nutrition labels. Therefore, vegetable oils, AA and DHA were supplemented to augment the content of unsaturated fatty acids in IFs, among which vegetable oils mainly increased the contents of oleic and linoleic acid, while AA and DHA supplements were used to augment the contents of functional fatty acids at the same time.

3.3. Similarity of glycerides based on HM as the gold standard

The fatty acid composition of TAGs can affect their positional distribution, which in turn changes the intestinal digestion of lipid and consequently influences the biological value of TAGs [21]. The fatty acid composition of glycerides in IFs, mammalian milk and vegetable oils were different from that of HM. For the purposes of this study, the fatty acid compositions and contents of glycerides in HM were considered as a gold standard, and were defined as 100 %. When the glycerides identified in IFs, mammalian milk or vegetable oils were the same with those identified in HM, the relative contents of these glycerides in their total glycerides were summed. The glycerides that were among the top 20 %, AA- and/or DHA-containing glycerides and the remaining glycerides identified in HM are presented in Fig. 4. The contents of glycerides in mammalian milk similar to HM was in the order CaM > CM > BM > DM > HoM > SM. Notably, the investigated vegetable oils generally possessed a higher relative content of glycerides than mammalian milk. After the addition of vegetable oils and other human milk fat substitutes, IFs had higher relative contents of glycerides. However, it is insufficient to evaluate the quality of IFs only by comparing the relative content of the common glycerides with HM. As shown in Fig. 4, the relative contents of TAG54:3 (18:1–18:1–18:1), TAG54:5 (18:1–18:2–18:2) and TAG54:6 (18:2–18:2–18:2) in IFs and vegetable oils were 1.34–4.69 times higher than those in HM. Although the relative contents of these glycerides in IFs increased after the addition of vegetable oils, the proportion of each glyceride was different from that in HM. The proportions of different species of glycerides are also an important quality indicator. Therefore, the deviations of glycerides relative content from IFs, mammalian milk and vegetable oils compared to HM were analyzed, and the total deviation degree was calculated as the sum of relative content differences of the 94 H M-characteristic species of glycerides.

The scores of the similarity evaluation were 42.05, 41.57, 19.07, 28.82, 16.13, 26.20, 38.37, 36.79, 36.33, 12.00, 7.13 and 7.89 for CMIF, SMIF, SIF, CM, SM, CaM, BM, DM, HoM, CSO, CSSO and CSSCO, respectively (Supplementary Table 1). These values indicated that CMIF was a better HM substitute than SMIF or SIF. Similarity, BM, DM and HoM were better than CM and CaM as raw milk. By contrast, the glycerides of vegetable oils have a lower similarity with HM, and can only compensate for a part of the deficiency of unsaturated fatty acids (e.g., oleic acid, linoleic acid) in mammalian milk. The similarity evaluation also suggested that there was still a significant difference between HM substitutes and authentic HM in terms of glycerides. One of the effective methods to improve the similarity evaluation is to improve the species of characteristic HM lipids in IFs by coordinating their corresponding proportions and contents through the combination of multiple lipid sources. Therefore, more abundant sources of oil or fat such as lard and algal oil should be considered for mimicking the lipids of HM.

3.4. Structural analysis of glycerides in HM and IFs

The major TAGs in HM were TAG52:2 (16:0–18:1–18:1) and TAG52:3 (16:0–18:1–18:2), mainly present in the form of 1 (3)-palmitoyl-2-linoleoyl-3 (1)-oleoylglycerol (OPO) and 1 (3)-oleoyl-2-palmitoyl-3 (1)-linoleoylglycerol (OPL). The positional structure of TAGs cannot be directly identified using the lipid analysis method adopted in this study, but the distribution of fatty acyl groups on the glycerol skeleton can be analyzed indirectly according to the different dissociation tendencies of fatty acyl groups at the *sn*-2 and

sn-1,3 positions of TAGs using mass spectrometric analysis [14,34]. In fact, *sn*-2 fatty acyls require a greater energy for ionization than *sn*-1,3 fatty acyls, presenting higher fragment abundance of *m/z* at $[OP]^+$ than at $[OO]^+$ as well as at $[OP]^+$ and $[PL]^+$ than at $[OL]^+$ in the MS/MS fragmentation spectra of OPO and OPL as shown in Fig. 5A and C, respectively. The standard curves and characteristic ion fragments of OPO and OPL are shown in Fig. 5B and D.

Pancreatic lipases activated by bile salts preferentially hydrolyze *sn*-1 and *sn*-3 ester bonds of TAGs, producing two free fatty acids and one monoglyceride containing an *sn-2* fatty acid [35]. Palmitic acid (C16:0) or other saturated fatty acids at the *sn*-2 position of TAGs facilitate fatty acid absorption, and prevents calcium loss, while also benefiting the intestinal microbiota, bone strength and wellbeing of infants [19,36]. Furthermore, saturated fatty acids are more likely to be absorbed in the intestines in the form of *sn-2* monoglycerides than in the form of free fatty acids, because the absorption of saturated free fatty acids is highly affected by their melting points (63 °C for palmitic acid and 70 °C for stearic acid), which are higher than the human body temperature [37]. However, palmitic acid is partially esterified at the *sn*-2 position in mammalian milk, but mainly at the *sn*-1 and *sn*-3 positions in vegetable oils [20,38]. Hence, the ingredients in IFs are structurally different from HM, and even the employment of vegetable oils compromises the structural benefits of TAGs from mammalian milk. In addition, the content of palmitic acid at the *sn*-2 position in HM is significantly higher than that in mammalian milk [39]. Therefore, OPO, OPL or other *sn*-2 palmitate TAGs are usually used to adjust the structure of glycerides in IFs.

In this study, the investigated IFs were divided into OPO-containing IF (OPO-IF) and those without OPO according to product labels, among which OPO-IFs only accounted for 36.84 %. As shown in Fig. 6, the relative contents of TAG52:2 (16:0–18:1–18:1) in HM (12.48 %) and OPO-IF (12.91 %) were significantly higher than those in IFs without OPO (8.10 %). In addition, the content of OPO accounted for TAG52:2 (16:0–18:1–18:1) were 78.12, 68.97 and 40.39 % in HM, OPO-IF and IF without OPO, respectively, indicating that the supplementation of OPO improved the structure and content of TAG52:2 (16:0–18:1–18:1) in IFs. Furthermore, the differences in the content of TAG52:3 (16:0–18:1–18:2) between HM and IFs with or without added OPO (13.55 % vs. 5.01–5.80 %) were highly significant, while OPL accounted for 81.24, 54.43 and 41.76 % of the TAG52:3 (16:0–18:1–18:2) in HM, OPO-IF and IF without OPO, respectively. These results suggested that the supplementation of OPO had no significant effect on the content of TAG52:3 (16:0–18:1–18:2) in IFs, but slightly improved its structure due to the small amount of OPL contained in OPO. Thus, even though the content of OPO in OPO-IFs was equal to that of HM, the abundance of *sn*-2 palmitate TAGs was much lower than that in HM [40]. It has been proved that the OPO (*sn*-2 palmitate)-supplemented IFs are more conducive to infant growth than conventional IFs [41], but the difference in structured lipids between HM and OPO (*sn*-2 palmitate)-supplemented IFs might also lead to differences in their nutritional properties.

The contents of OPO and/or OPL structured lipids in the four products were compared and analyzed. The relative content of OPO in one product was 11.36 %, and the following three TAGs were TAG54:6 (18:2–18:2–18:2), TAG54:5 (18:1–18:2–18:2) and TAG54:3 (18:1–18:1–18:1), respectively, none of which are *sn-2* palmitate TAGs. By contrast, the relative content of OPO was 25.59 % and the following two lipids were *sn*-2 palmitate TAGs (11.60 % of OPL and 12.17 % of TAG 16:0–16:0–18:1) in another OPO product. Among the two other structural lipid products, the five highest TAG types were *sn*-2 palmitic TAGs with similar composition to HM, and the most abundant molecule was OPL followed by OPO, making these HM fat substitutes more effective in improving the structure and fatty acid composition of IF lipids composed of mammalian milk fat and vegetable oils, especially for Chinese babies, since the content

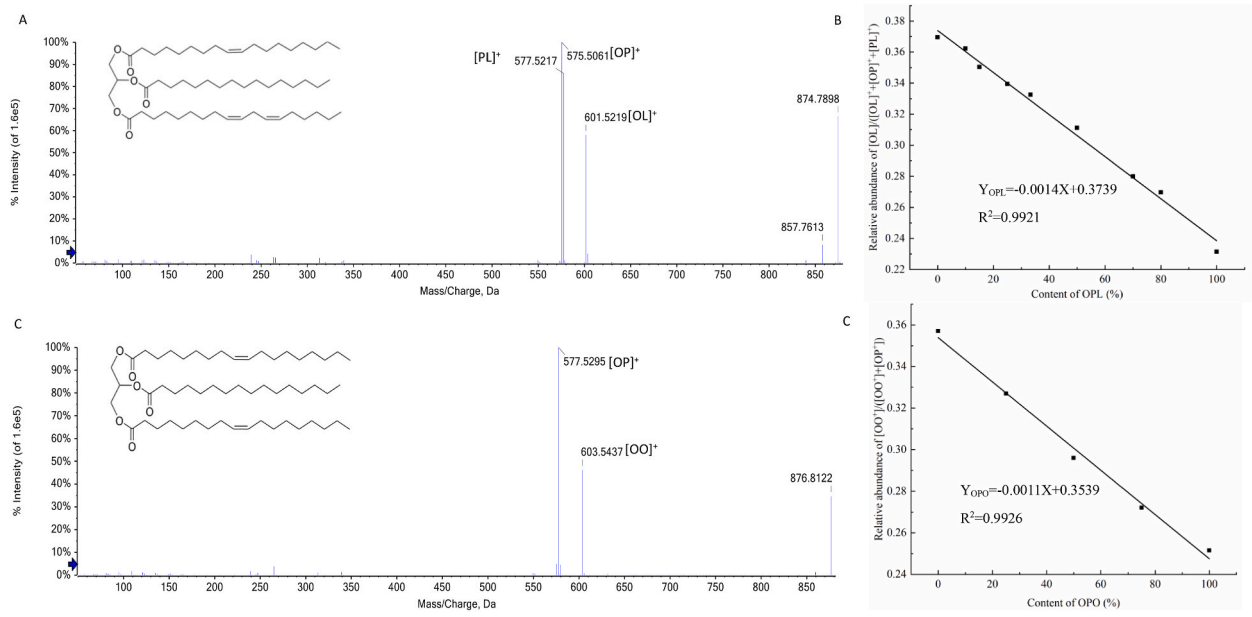

Fig. 5. (A and C) MS/MS fragments of OPL and OPO in positive ion mode; (B) calibration curve for the quantification of OPL in a mixture of OPL, POL and PLO standards; (D) calibration curve for the quantification of OPO in a mixture of OPO and OOP standards.

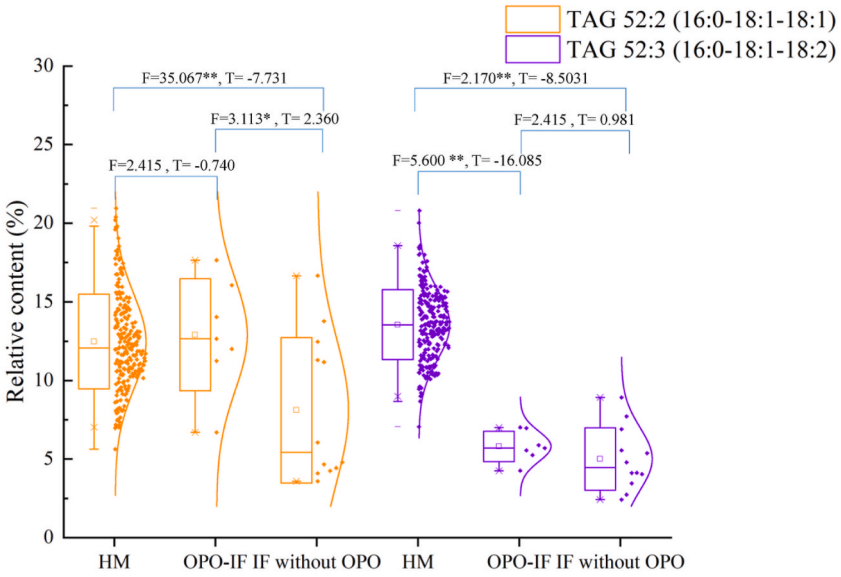

Fig. 6. Relative contents of TAG52:2 (16:1–18:1–18:1) and TAG52:3 (16:1–18:1–18:2) in HM, OPO-IF and IF without OPO. *: $P < 0.05$, **: $P < 0.01$.

of OPL in Chinese HM is generally higher than that of OPO [14]. Additionally, the results showed that OPO and OPL were the predominant regioisomers of TAG52:2 (16:0–18:1–18:1) and TAG54:3 (18:1–18:1–18:1) in four structured lipid products, with the proportions ranging from 62.47 to 87.54 %. Therefore, the fatty acid position and TAG composition of HM fat substitutes should be considered in order to continuously improve the quality of OPO/OPL or *sn*-2 palmitate TAG products, resulting in IFs with a more similar composition, structure and content of TAGs to that of HM.

4. Conclusions

The composition, content, and positional structure of glycerides in HM were significantly different from those in milk-based IFs with the addition of vegetable oils and HM fat substitutes. In detail, the fatty acid compositions of DHA-containing TAGs were different between HM and IFs, since HM possesses a higher relative contents of TAGs containing functional fatty acids. Additionally, even though structured lipids were added to the IFs, the content of *sn*-2 palmitate TAGs in IFs was still lower than those in HM. This helps explain why the nutritional properties of IFs do not perfectly match HM even though they are supplemented with functional fatty acids and structural glycerides. In order to better simulate HM, it is necessary to mimic the composition, content and structure of HM lipids to improve the biological properties of HM fat substitutes (i.e., improving the functional fatty acid composition and content of *sn*-2 palmitate TAGs). The introduction of vegetable oils improved the saturation of milk fat TAGs, but compromised their structural advantages to a certain extent. We should therefore consider the structural advantages of mammalian fat compared with vegetable oils and appropriately limit their supplementation in IFs. In addition, this study also analyzed the characteristics of mammalian milk and vegetable oils, providing a scientific basis for the manufacturing of IFs adapted to different needs.

Ethics statement

This research was reviewed and approved by the Ethics Committee of Beijing Ditan Hospital affiliated to Capital Medical University (#2015-027-01). All study participants were informed of the purpose of this study and agreed to participate. The study was registered at clinicaltrials.gov (Registration No: NCT02658500).

Data availability statement

The data associated with our study has not been deposited into a publicly available repository. Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No. 32072191), Daxing District Major Scientific and Technological Achievements Transformation Project (Grant No. 2020006), Beijing Science and Technology Plan (Grant No. Z201100002620005), National Key R&D Program of China (Grant No. 2021YFD2100700), and Beijing Innovation Team of Dairy Industry Technology Systems.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21611.

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