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RESEARCH ARTICLE

Stabilization of infant formulas against lipid oxidation: What are the key structural levers?

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Abstract

Infant follow-on formulas (IFF) mimic human milk. This study aimed to identify the key structural levers that influence oxidative stability of IFF. Representative model IFF of marketed products in term of lipid composition were formulated with varying droplet size, lipid droplet core composition, and interfacial composition using different emulsifiers (soy lecithin or dairy phospholipids [DPL]). The oxidative stability of model IFF was assessed in accelerated storage conditions. No significant stabilization effect based on the lipid droplet core composition was observed. However, the nature of the interface, influenced by the emulsifier type, had an impact. Model IFF with DPL showed no loss of tocopherols and peroxide value was up to twice lower than those with soy lecithin after 20 days. This effect was particularly pronounced for the 0.4μm droplets. These results suggest that model IFF with DPL had a greater oxidative stability, likely due to the presence of sphingomyelin and the formation of a rigidified domain at the droplet surface. Model IFF with 0.4 μm droplets stabilized with soy lecithin, especially when added to the water phase, showed a tocopherols loss twice as high as that of IFF with DPL. These results indicate that oxidative stability of IFF is more influenced by the chemical environment rather than droplet size.

Practical Application: Infant follow-on formulas (IFF) aim to ensure an adequate nutritional intake to support the proper development and infant growth. Therefore, IFF must be stable against degradation phenomena such as lipid oxidation and have a composition and structure that are as biomimetic as possible to mature breast milk. This study provides key information for the development of IFF with a lipid composition and structure that are suitable for the infant nutritional needs and have an acceptable resistance to lipid oxidation. More generally, these results can be applied to all dispersed systems in the form of oil-in-water emulsions with a similar composition.

Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; DLDC, different lipid droplet composition; DPL, dairy phospholipid; FAME, fatty acids methyl esters; HM, human milk; HMP, high melting point; IFF, infant follow-on formulas; IMP, intermediate melting point; LA, linoleic acid; LC-PUFA, long chain polyunsaturated fatty acid; LWP, lecithin in water phase; MUFA, monounsaturated fatty acid; PL, phospholipid; PUFA, polyunsaturated fatty acid; PV, peroxide value; SFA, saturated fatty acid; TAG, triacylglycerol; Tbars, thiobarbituric acid reactive substances; VMC, vitamin and mineral complex; WHO, World Health Organization.

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KEYWORDS
antioxidant, emulsifier, infant formula, lipid oxidation, lipid structure

1 | INTRODUCTION

Human milk (HM) is a complex and dynamic biofluid recognized as the reference for infant nutrition even if its composition is strongly impacted by many external factors, with one of the most influential being the mother's diet.[18,35,37] Its specific composition is adapted to the evolving nutritional needs of infants, particularly with regard to its lipid content.[17] HM provides a complex lipid profile with a majority of saturated fatty acids (SFAs) (mainly palmitic acid C16:0) that account for more than half of the total FAs. HM is also rich in polyunsaturated FAs (PUFA) including essential FAs, such as linoleic (LA, C18:2 *n*-6) and α-linolenic (ALA, C18:3 *n*-3) acids, and also provides their longer chain derivatives, namely, arachidonic (ARA, C20:5 *n*-6) and docosahexaenoic (DHA, C22:6 *n*-3) acids, which are notably involved in the infant brain development. HM is also a source of various bioactive compounds that promote optimal development and provide long-term benefits, such as a reduced risk of developing type II diabetes or obesity in adulthood.[1] The World Health Organization (WHO) recommends exclusive breastfeeding for the first 6 months, followed by mixed breastfeeding until the age of 2 years. However, 48% of infants aged under 6 months are exclusively breastfed, and only 70% of them are still breastfed up to the age of 12 months.[42] Infant follow-on formulas (IFF), which are designed for the period ranging from 6 to 12 months, are therefore widely consumed throughout the world. At the industrial scale, the lipid composition of IFF is submitted to very specific regulations that are regularly updated to align with the nutritional recommendations. For instance, the addition of DHA to IFF is mandated by the EU regulation CE 2016/127 since 2020. These regulatory changes aim to improve the composition of IFF to meet infant nutritional needs.

However, differences in composition and structure between mature HM and IFF remain.[10] The lipids in HM are mainly found as triacylglycerols (TAG) (98%) and phospholipids (PL) (1%) and have a particular structural organization in the form of milk fat globules (MFG), ranging from 0.1 to 10 µm droplet size (4 µm on average).[6,22] MFGs are composed of a core of TAG stabilized by a trilayered biological membrane composed mainly of polar lipids and proteins. However, IFFs are composed of submicron droplets (0.3–0.8 µm) stabilized by milk proteins and vegetable emulsifiers (mainly lecithin).[11] The regiodistribution of FAs on the TAG backbone also differs widely due to the use of a mixture of fats almost exclusively of vegetable origin. It has been reported that these differences in structure and composition lead to differences in the efficiency of digestion and assimilation of nutrients, especially of lipids.[4] Some studies also showed that these differences could impact the oxidative stability, with a greater resistance of HM compared to IF.[29] Various studies have focused on structural parameters that modulate oxidative stability at different levels: first, at lipid droplet core level with the FA regidistribution by formulating HM fat analogues.[44] But also depending of their esterification as PL or TAG with an increased stability attributed to PL due to the presence of the phosphate group and the polar head group.[27] In emulsions, it is now well known that the interface is the lipid oxidation initiation site. Therefore, many studies have focused on the interface properties such as the size and composition to modulate the oxidative stability.

However, to the best of our knowledge, no studies have yet proposed a multiparameter approach to screen the most efficient structural levers ranging from droplet size, core lipid composition, emulsifier source, and its mode of incorporation in model IFF having similar chemical composition.

Therefore, the present study aims at identifying structural parameters that influence oxidative stability of model IFF representative of marketed products. Additionally, particular attention is given to the biomimetic aspect related to these parameters, which may improve the nutritional profile. Model IFFs were formulated with equivalent chemical composition with a normalization of their lipid profile (similar contents in SFA, monounsaturated FA [MUFA], PUFA, DHA, ARA, vitamins E and A). The influence of the lipid structure was evaluated by adjusting the droplet size, the core lipid composition the emulsifier source, and its mode of incorporation.

2 | MATERIALS AND METHODS

2.1 | Materials

Sunflower (*Helianthus annuus*) and rapeseed (*Brassica napus*) oils were purchased in local supermarkets (Casino). High oleic sunflower oil was purchased from Cuisinor. Fungal and algae oils (ARASCO and DHASCO), dairy fat, and refined palm oil (POM) were generous gifts from DSM Nutritional products France, Corman, and Cargill, respectively. Soy lecithin and dairy PL (DPL) were kindly provided by Novastell and Corman, respectively. The skim milk powder and serum protein isolate were gifts from Ingredia. A vitamin and mineral complex (VMC), corresponds to a mixture of vitamin A (retinyl acetate, 3593 µg RA g^{-1}), vitamin C (sodium ascorbate, 81 µg g^{-1}), vitamin D (cholecalciferol, 588 mg g^{-1}), and iron (dried iron sulfate, 49 mg g^{-1}), which was kindly supplied by DSM Nutritional Products South Africa.

All analytical standards, reagents, and solvents were purchased from Sigma-Aldrich.

2.2 | Formulation and preparation of model IFF

As shown in Figure 1, model IFF based on two oil mixtures with standardized lipid profiles were formulated as described in a previous work

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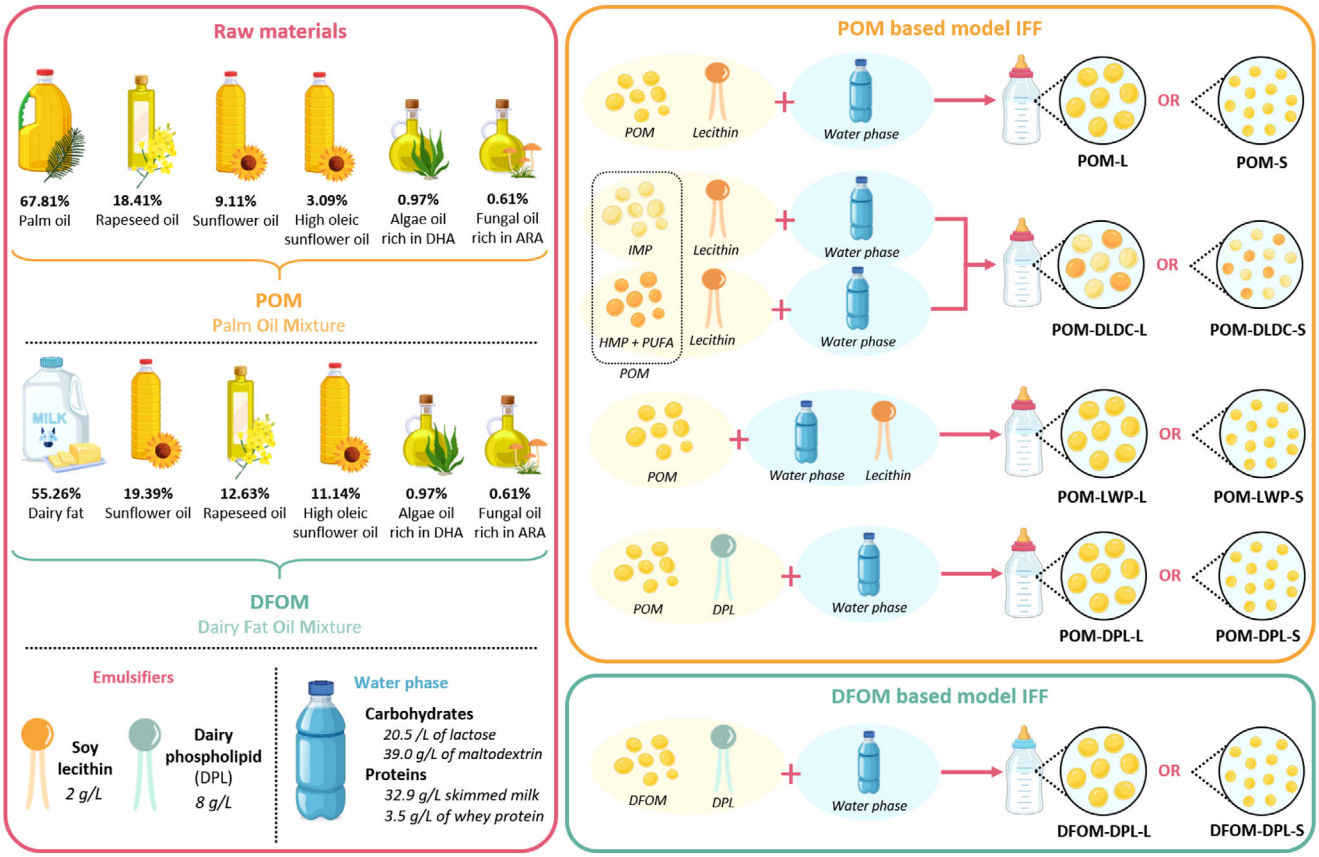

FIGURE 1 Oil and water phases composition and preparation of model infant follow-on formulas (IFF). L is used for model IFF with droplet size of 0.7 μm and S for model IFF with a droplet size of 0.4 μm. POM-L/POM-S: POM-based IFF with a droplet size of 0.7 or 0.4 μm. POM-DLDC-L/POM-DLDC-S: POM-based IFF with heterogeneous lipid droplet core composition (high and low melting point oils droplets, that is, palm with arachidonic acid (ARA) and docosahexaenoic acid (DHA) oils or intermediate melting point oils droplets, that is, sunflower, oleic sunflower, and rapeseed oils) with a droplet size of 0.7 or 0.4 μm. POM-LWP-L/POM-LWP-S: POM-based IFF with soy lecithin previously homogenized with the aqueous phase with a droplet size of 0.7 or 0.4 μm. POM-DPL-L/POM-DPL-S: POM-based IFF with a DPL as emulsifier with a droplet size of 0.7 or 0.4 μm. DFOM-DPL-L/DFOM-DPL-S: DFOM-based IFF with a DPL as an emulsifier with a droplet size of 0.7 or 0.4 μm. DFOM, dairy fat oil mixture; DLDC, different lipid droplet composition; DPL, dairy phospholipids; HMP, high melting point oil; IMP, intermediate melting point oils (rapeseed, sunflower, and high oleic sunflower oils); LWP, lecithin in water phase; POM, palm oil mixture; PUFA, polyunsaturated fatty acids.

to be representative of commercial IFF.[8] The first oil mixture was based on POM and the second on dairy fat olein (DFOM). Both were prepared by mixing oils in proportions determined by linear programming using Microsoft Excel Solver. Ten model IFF were studied, including eight based on POM and two on DFOM:

- POM-L/POM-S: POM-based IFF with a droplet size of 0.7 or 0.4 μm, respectively (L stands for large and S for large)
- POM-DLDC-L/POM-DLDC-S: POM-based IFF with heterogeneous lipid droplet composition (blend of two pre-emulsions: one with high and low melting point oils, i.e., palm with ARA and DHA oils, the other with intermediate melting point [IMP] oils, i.e., sunflower, oleic sunflower, and rapeseed oils) with a droplet size of 0.7 or 0.4 μm.
- POM-LWP-L/POM-LWP-S: POM-based IFF with assembly of two pre-emulsions: one composed of the oil phase homogenized with the aqueous phase (stabilization with proteins) and the other of soy lecithin homogenized with the aqueous phase (i.e., lecithin previously homogenized in water phase) with a droplet size of 0.7 or 0.4 μm.
- POM-DPL-L/POM-DPL-S: POM-based IFF with a DPL as emulsifier with a droplet size of 0.7 or 0.4 μm.
- DFOM-DPL-L/DFOM-DPL-S: DFOM-based IFF with a DPL as emulsifier with a droplet size of 0.7 or 0.4 μm.

Oil and water phases were prepared 24 h before model IFF formulation. The oil phases were obtained by mixing oils in proportions described in Figure 1. For POM-DLDC-L and POM-DLDC-S two premixes of oils were initially prepared, one consisting of a mixture of oils with intermediate melting points (IMPs include rapeseed, sunflower, and high oleic sunflower oils) and the other consisting of a mixture of oils with low (ARA and DHA oils) and high (HMP includes POM) melting points.

Soy lecithin was added at 2 g L^{-1} (40% PL, i.e., 0.8 g L^{-1} of PL) in the oil phase except for POM-LWP-(S and L) in which soy lecithin was

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added in the water phase. DPL was also added in the oil phase at 8 g L^{-1} (11% PL, i.e., 0.88 g L^{-1} of PL) and concomitantly increases protein content (+0.4 g/100 mL). The oil phases were flushed under nitrogen and stored in hermitically sealed brown tubes at 4°C. All model IFF were prepared using the same water phase which corresponded to a mixture of carbohydrates (20.5 g L^{-1} of lactose and 39.0 g L^{-1} of maltodextrin) and proteins (32.9 g L^{-1} of skimmed milk powder and 3.5 g L^{-1} of whey proteins). This water phase was prepared as described in a previous work[7] and stored at 4°C in a closed bottle before use. Prior to model IFF preparations, VMC was added to the oil phases (to be representative of industrial practices) at 20 mg/100 mL. Vitamins A and E contents were then normalized by adding retinol (the active form of vitamin A) and α-tocopherol to the oil phase as described in Table 1. Both phases were mixed and pre-emulsified twice for 5 min at 5000 rpm with an L5M Silverson (Silverson). Model IFF were then homogenized at two different pressures using an APV-1000 lab homogenizer (SPXFlow) to reach two droplet size. The first homogenization process corresponded to eight cycles at pressures of 100/30 bars and lead to a droplet size of 0.7 µm, the second used eight cycles at pressures of 350/40 bars and lead to a droplet size of 0.4 µm.

Sodium azide of 0.02% was added to all model IFF that were then aliquoted in quadruplicate into 40 mL brown tubes, with a headspace of 3.82 mL. The samples were then stored for 20 days at 40°C with 110 rpm orbital stirring using a IKA KS 4000 i-control incubator (IKA). After their sampling (0, 1, 3, 6, 9, 15, and 20 days), model IFF were flushed under nitrogen and stored at −20°C until further analysis.

2.3 | Structural characterizations of model IFF

2.3.1 | Droplet size distribution

The particle size distribution of model IFF was assessed using a Mastersizer 2000 (Malvern Instruments). Model IFF of 500 µL were placed in a measurement cell (obscuration rate between 5% and 10%). Refractive indices of 1.458 and 1.33 were used and 1500 rpm stirring was applied. The surface-weighted mean diameter ($D[3,2]$) and the distribution mode were measured.

2.3.2 | Confocal laser scanning microscopy (CLSM)

Model IFF were observed on an inverted microscope using a confocal laser scanning microscopy (CLSM) system (Leica SP8). A 40× water-immersion objective was used and three fluorescent dyes were added to 200 µL model IFF at least 10 min before observation in order to localize proteins (Fast green FCF, 6:100 v/v), nonpolar (Lipidtox, 0.2:100 v/v, λex 488 nm to λem 590 nm), or polar lipids (Rd DOPE, 1:100 v/v, λex 543 nm to λem 590 nm).[20] The images collection and analysis were performed using the Leica LAS X software.

2.4 | Chemical characterizations of model IFF

2.4.1 | Lipid oxidation monitoring by a measurement of peroxide value (PV) and thiobarbituric acid reactive substances (TBARS)

The amounts of primary oxidation compounds monitored by the measurement of the peroxide value (PV) were determined according to Ferreira da Silveira, Laguerre et al.[16]. Briefly, after an extraction on 350 µL of model IFF using 750 µL of isooctane/isopropanol (3:1 v/v), a dilution of the supernatant with methanol/butanol (3:7 v/v) was performed. Then, 2.5 µL of aqueous ammonium thiocyanate (300 mg mL^{-1}) and ferrous solution (0.144 mol L^{-1}) were added to give a final volume of 265 µL in the microplate UV star 96 well COC F-bottom (Greiner Bio-One). Each microplate was first incubated at 25°C for 10 min with a stirring of 1000 rpm in PHMP-4 microplate thermoshaker a (Grant Instruments Ltd) and then placed in an Infinite M200 microplate reader (Tecan). Absorbances were measured at 500 nm. Data acquisition was made using Magellan software (Tecan). PVs were determined using a standard calibration curve of cumene hydroperoxide and were expressed as meqO_2 kg oil^{-1}.

Secondary oxidation compounds were measured using the thiobarbituric acid reactive substances (TBARS) following the procedure as described in a previous work.[7] Briefly, 50 µL of model IFF were mixed with 200 µL of the reagent solution (150 mg mL^{-1} of trichloroacetic acid, 3.75 mg mL^{-1} of thiobarbituric acid, and 0.25 mol L^{-1} of HCL) and heated at 95°C for 15 min. Model IFF were then cooled in an ice bath for 5 min and centrifuged for 10 min at 5000 rpm using a Pico 21 centrifuge (Thermo Fisher Scientific). The absorbance of the supernatant was read with an Infinite M200 microplate, Tecan) at 532 nm with the Magellan software. TBARS were determined using a standard calibration curve of 1,1,3,3-tetramethoxypropane and were expressed as mg MDA $(kg\ oil)^{-1}$.

2.4.2 | Retinyl esters and tocopherols contents

Retinyl esters contents were determined as described in a previous work[7] by high performance liquid chromatography (HPLC) using a Thermo Scientific Ultimate 3000 HPLC system equipped with a YMC-C30 column (250 × 4.6 mm^2, YMC) and a photodiode array detector (Vanquish PDA, Thermo Scientific) with the injection method described by Ref. [31].

Tocopherols isomers (α, β, δ, and γ) were quantified according to the ISO-FDIS 9936 normalized procedure. They were extracted with a Folch mixture (chloroform/methanol 2:1 v/v) and analyzed by HPLC with an Ultimate 3000 equipped with a silica column (250 mm × 4.6 mm, i.e., 5 µm) and a fluorescence detector (Dionex). The mobile phase consisted of hexane/dioxane (97:3 v/v) in isocratic conditions with a flow rate of 1.3 mL min^{-1} and a column temperature of 25°C. Fluorescence detection was set at 296 for excitation

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TABLE 1 Model infant follow-on formulas fortification with VMC, normalization of tocopherols, and vitamin A contents and peroxides values.

	Target value	POM-L	POM-S	POM-DLDC-L	POM-DLDC-S	POM-LWP-L	POM-LWP-S	POM-DPL-L	POM-DPL-S
Supplementation (mg L^{-1})									
Retinol	–	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
Retinyl acetate	–	–	–	–	–	–	–	–	–
Tocopherol	–	9.12	9.12	9.12	9.12	9.12	9.12	9.12	9.12
VMC	–	200	200	200	200	200	200	200	200
Tocopherols contents (ppm)									
Total	676.2	694.2 ± 36.1	688.9 ± 55.6	672.0 ± 3.6	615.5 ± 11.9	651.3 ± 80.4	556.1 ± 15.4	570.8 ± 79.9	535.1 ± 58.6
α-Tocopherol	–	294.8 ± 15.5	293.4 ± 24.1	265.3 ± 2.0	224.1 ± 6.1	229.6 ± 36.6	140.8 ± 7.7	161.1 ± 35.2	122.0 ± 24.8
Vitamin A content									
Total (μg RE L^{-1})	–	493.4 ± 13.8	126.9 ± 28.7	383.3 ± 27.1	179.3 ± 31.1	440.6 ± 68.4	189.1 ± 27.4	651.2 ± 66.1	351.8 ± 85.1
Retinyl acetate (μg RA L^{-1})	849.1	566.0 ± 15.8	145.5 ± 33.0	439.6 ± 31.1	205.7 ± 35.6	505.4 ± 78.5	216.9 ± 31.4	746.9 ± 75.8	403.5 ± 97.6
PV (meqO_2 kg^{-1})									
Initial PV (day 0)	–	2.5 ± 0.4	1.9 ± 0.4	2.5 ± 0.3	2.2 ± 0.3	1.3 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	0.6 ± 0.1
Final PV (day 20)	–	10.3 ± 1.8	9.4 ± 1.2	10.9 ± 1.9	11.5 ± 1.3	11.8 ± 0.4	16.4 ± 1.9	7.1 ± 0.5	4.4 ± 0.3

Note: L is used for model IFF with droplet size of 0.7 μm and S for model IFF with a droplet size of 0.4 μm. POM-L/POM-S: palm oil mixture-based IFF with a droplet size of 0.7 or 0.4 μm. POM-DLDC-L/POM-DLDC-S: POM-based IFF with heterogeneous lipid droplet core composition (high and low melting point oils droplets, that is, palm with ARA and DHA oils or intermediate melting point oils droplets, i.e., sunflower, oleic sunflower, and rapeseed oils) with a droplet size of 0.7 or 0.4 μm. POM-LWP-L/POM-LWP-S: POM-based IFF with soy lecithin previously homogenized with the aqueous phase with a droplet size of 0.7 or 0.4 μm. *POM-DPL-L/POM-DPL-S*: POM-*based IFF with a DPL as emulsifier with a droplet size of 0.7 or 0.4 μm.* DFOM-DPL-L/DFOM-DPL-S: dairy fat oil mixture based IFF with a DPL as an emulsifier with a droplet size of 0.7 or 0.4 μm.

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DLDC: different lipid droplet composition; DPL, dairy phospholipid; *IFF*, infant follow-on formulas; LWP: lecithin in water phase; PV, peroxide value; RE, retinol equivalent; RA: retinyl acetate; VMC: vitamin and mineral complement.

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and 330 nm for emission. Tocopherols content was determined using standard calibration curves of each tocopherol isomers.

2.4.3 | Fatty acid composition

The FA compositions of model IFF were determined by gas chromatography (GC) after an FA methylation as described in our previous work.[7] Analyses were performed using a Focus GC (Thermo Electron Corporation) equipped with a split injector (ratio of 1/20), a CP-Cil 88 Varian capillary column (50 m× 0.25 mm with a 0.2-µm film thickness; Chrompack) with an initial temperature of 150°C to reach 225°C at 5°C min^{-1}. Final temperature (225°C) was maintained for 10 min. The injector and detector temperatures were 250 and 270°C, respectively. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. Flame ionization detector was used and FA methyl esters were identified using standard solution of methyl esters mixture. ChromCard software (version 2005, Thermo Fisher Scientific) was used to collect and analyze data.

2.5 | Statistical analysis

Results are presented as mean ± SD. Statistical analyses were conducted using R software (R.2.13.0, http://cran.r-project.org). Statistical differences were evaluated using a one-way ANOVA test and considered significant for $p < 0.05$.

3 | RESULTS

The aim of this study was to compare the impact of different chemical and physicochemical parameters on oxidative stability of model IFF. In that context, various model IFF were designed with similar FA composition and vitamin content but varying in their structure (droplet size, core lipid composition, emulsifier source, and its mode of incorporation). These model IFF were formulated in compliance with the regulations and were intended to be representative of the marketed IFF. The oxidation rates were monitored during an accelerated storage test of 20 days at 40°C through the evaluation of several indicators of lipid oxidation (PV, TBARS, FA profile evolution, and vitamin A and E contents).

3.1 | Standardization of model IFF and selection of descriptive indicators of oxidation

Model IFF size droplet distribution and structure were assessed both by granulometry and CLSM (Figure 2 and Figure S1). All model IFF show a similar monomodal droplet size distribution centered on 0.4 or 0.7 µm depending on the homogenization pressures applied. CLSM observations confirmed good structure homogeneity with a core of nonpolar lipids stabilized by proteins and amphiphilic compounds. The size droplet distribution and the CLSM observations showed that creaming occurred but did not lead to coalescence and was reversible. Therefore, before sampling an effective redispersion was achieved by repeated inversion of tubes. Creaming was similar in all model IFF and can be classically described following Stokes law.

The FA compositions of model IFF were designed to be representative of marketed products.[7] As shown in Figure 3, the linear programming used to design the various model IFF the resulted in similar initial FA profiles with equivalent SFA (ranging from 32.9% to 38.8% of total FA), MUFA (ranging from 43.3% to 46.9% of total FA), and PUFA (ranging from 17.0% to 20.3% of total FA) contents in all model IFF. The contents of LA, ALA, ARA, and DHA were also relatively equivalent and the LA/ALA ratio was 10.7 ± 0.6 on average. Total DHA and ARA content in DFOM-DPL-(S and L) is slightly higher (+0.3%), making them potentially more sensitive to oxidation.

Normalization of initial vitamin A and E contents was performed by supplementation with VMC and the addition of α-tocopherol and retinol (Tables 1 and 2). The normalization in vitamin A (retinyl acetate with the VMC and retinol) was made prior to emulsification to be representative of industrial-scale practices. All model IFF had an equivalent initial total tocopherol content (653.0 ± 67.6 ppm) (Tables 1 and 2). However, depending on the emulsification process especially the pressure applied, significant variations of the initial vitamin A content were observed. Indeed, the more drastic homogenization process (to obtain small droplets), the more important loss in vitamin A contents during the process (Figures 4 and 5).

Concerning the evaluation of lipid oxidation rates in model IFF in accelerated storage conditions, five different measurements were performed: evolution of PV, TBARS, FA profile and degradation of tocopherols and vitamin A. The measurement of primary oxidation compounds (here, PV value) is generally considered to be relevant to evaluate lipid oxidation rates at early stage of oxidation, where the formation of secondary oxidation compounds is not yet important. In our case, lipid oxidation was not advanced enough for any model IFF studied in our chosen accelerated conditions. Therefore, TBARS which is a measurement of some secondary oxidation compounds[24] did not significantly increase after 20 days at 40°C. Moreover, TBARS assay is designed to measure malondialdehyde that preferentially forms from FA containing at least three double bonds.[9] In our case, the amount of such FA especially ALA and DHA ranged from 1.7% to 2.0% of the total FA content. This amount could be too low to guarantee a relevant TBARS measurement. Similarly, the FA profile did not show any significant evolution during storage in all model IFF. This can be attributed to the fact that advanced oxidation stages were not reached during our chosen storage conditions. Finally, a correlation matrix was established to determine the most relevant indicators of the oxidation rate in our model IFF. It is also worth noting that the tocopherols presence in all model IFF induced a lag phase (up to 9 days) and that peroxidation increased when tocopherols were partially or totally consumed.

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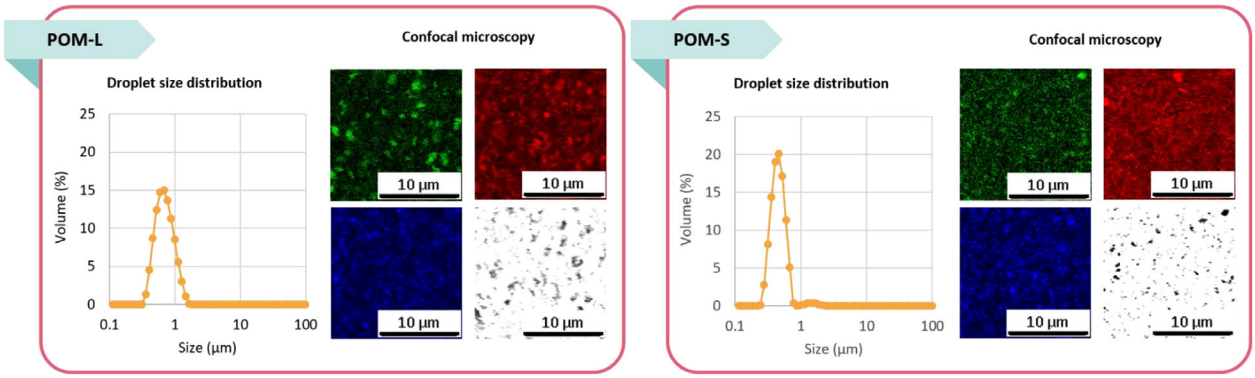

FIGURE 2 Typical droplet size distribution of model infant follow-on formulas and physical organization in palm oil (POM)-L and POM-S. Particles size distribution was assessed by laser light scattering. Confocal laser scanning micrographs were collected using a 40× water-immersion with each time four micrographs: blue colored: proteins; red colored: amphiphiles compounds; green colored: lipids; white colored: transmission light micrograph. L is used for model infant follow-on formulas (IFF) with a droplet size of 0.7 µm and S for model IFF with a droplet size of 0.4 µm. POM-L/POM-S: palm oil mixture-based IFF with a droplet size of 0.7 or 0.4 µm, respectively.

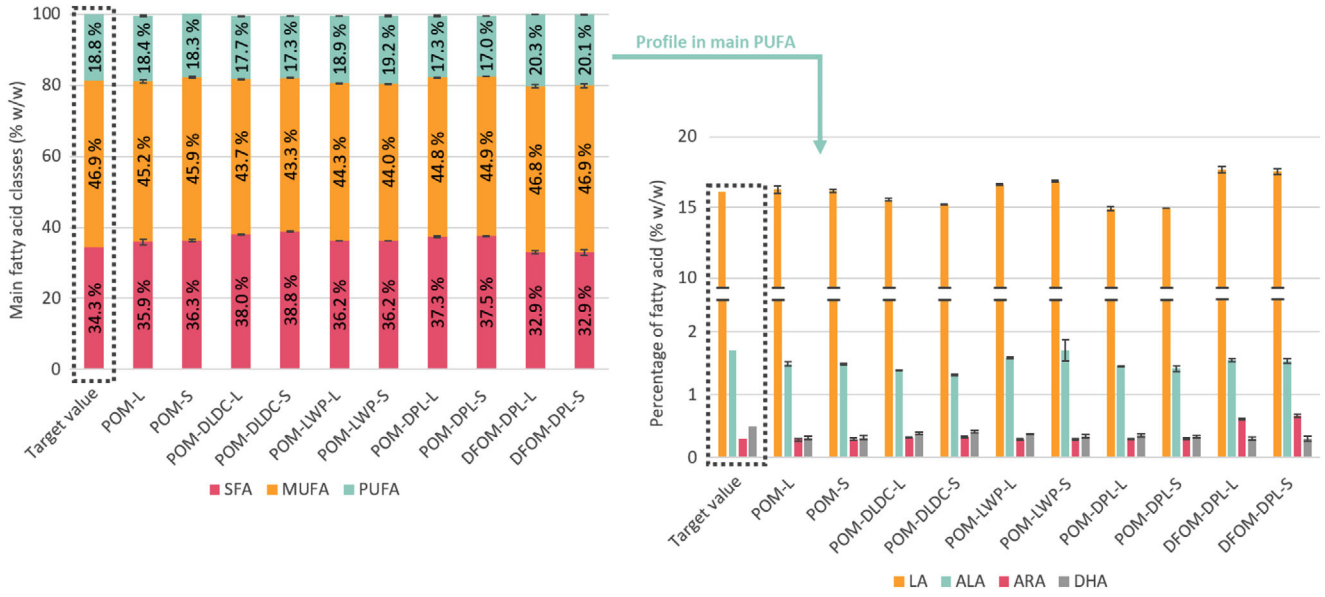

FIGURE 3 Fatty acid profiles of model infant follow-on formulas (IFF) (SFA, MUFA, PUFA, LA, ALA, ARA, and DHA) at the initial point (D0). Target values were set abiding by the regulation and to be representative of marketed infant IFF.[7] L is used for model IFF with droplet size of 0.7 µm and S for model IFF with a droplet size of 0.4 µm. POM-L/POM-S: palm oil mixture-based IFF with a droplet size of 0.7 or 0.4 µm. POM-DLDC-L/POM-DLDC-S: POM-based IFF with heterogeneous lipid droplet core composition (high and low melting point oils droplets, i.e., palm with ARA and DHA oils or intermediate melting point oils droplets, i.e., sunflower, oleic sunflower, and rapeseed oils) with a droplet size of 0.7 or 0.4 µm. POM-LWP-L/POM-LWP-S: POM-based IFF with soy lecithin previously homogenized with the aqueous phase with a droplet size of 0.7 or 0.4 µm. POM-DPL-L/POM-DPL-S: POM-based IFF with a DPL as emulsifier with a droplet size of 0.7 or 0.4 µm. DFOM-DPL-L/DFOM-DPL-S: dairy fat oil mixture-based IFF with a DPL as emulsifier with a droplet size of 0.7 or 0.4 µm. ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; DLDC, different lipid droplet composition; DPL, dairy phospholipid; LA, linoleic acid; LWP, lecithin in water phase; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; VMC: vitamin and mineral complement.

3.2 | Impact of model IFF droplets size on oxidation

In order to evaluate the impact of droplet size on model IFF oxidative stability, they were systematically homogenized at two pressures to obtain emulsions with either 0.4 or 0.7 µm droplet sizes. As presented previously, depending on the chosen emulsification process, significant variations of the initial retinyl acetate content were observed (Figures 4 and 5). In average the loss of retinyl acetate loss was twice as important for the more drastic homogenization process compared to the homogenization process that was applied to obtain larger droplets. Interestingly, this loss seems to be even more pronounced for model

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TABLE 2 Model infant follow-on formulas fortification with VMC (vitamin and mineral complement), normalization of tocopherols, and vitamin A contents and peroxides values for the additional storage test.

	Target value	POM-L	POM-S	DFOM-DPL-L	DFOM-DPL-S
Supplementation (mg L^{-1})					
Retinol	–	0.11	0.11	–	–
Retinyl acetate	–	–	–	–	–
Tocopherol	–	9.12	9.12	6.06	6.06
VMC	–	200	200	200	200
Tocopherols contents (ppm)					
Total	676.2	740.0 ± 18.6	710.5 ± 17.7	698.1 ± 33.1	703.1 ± 42.9
α-Tocopherol	–	592.3 ± 15.2	567.3 ± 13.0	628.8 ± 29.6	634.2 ± 36.1
Vitamin A content					
Total (μg RE L^{-1})	–	456.3 ± 31.8	422.3 ± 21.3	586.7 ± 62.4	491.5 ± 63.8
Retinyl acetate (μg RA L^{-1})	849.1	450.1 ± 28.1	419.8 ± 21.2	599.9 ± 70.2	537.7 ± 69.0
Retinyl palmitate (μg RP L^{-1})	–	–	–	116.8 ± 2.2	41.7 ± 6.7
PV (meqO_2 kg^{-1})					
Initial PV (day 0)	–	3.6 ± 0.5	2.8 ± 0.3	3.40 ± 0.3	3.11 ± 0.3
Final PV (day 20)	–	33.4 ± 3.6	21.8 ± 2.3	16.6 ± 1.0	4.8 ± 0.6

Note: L is used for model IFF with droplet size of 0.7 μm and S for model IFF with a droplet size of 0.4 μm. POM-L/POM-S: Palm oil mixture based IFF with a droplet size of 0.7 or 0.4 μm. POM-DPL-L/POM-DPL-S: POM based IFF with a DPL as an emulsifier with a droplet size of 0.7 or 0.4 μm.
Abbreviations: DPL, dairy phospholipid; PV, peroxide value; RA, retinyl acetate; RE, retinol equivalent.

IFF designed with soy lecithin as emulsifier, with for instance an initial retinyl acetate content up to four times lower for POM-S compared to POM-L versus twice lower for POM-DPL-S compared to POM-DPL-L (Table 1). Moreover, in the case of model IFF stabilized with soy lecithin, a higher loss of tocopherols was observed after 20 days of storage for the smaller droplet sizes with for instance 62.8% loss for POM-S against only 38.5% for POM-L. The substitution of soy lecithin with DPL in model IFF resulted in a very limited tocopherols loss after 20 days whatever the droplet size. These results suggest that the coverage of interfacial area by DPL is efficient, offering a good protection of tocopherols versus oxidation whatever the droplet size. On the contrary, with soy lecithin, the larger interfacial area obtained with smaller droplet may have been imperfectly covered by this emulsifier, resulting in a weaker protection of tocopherols. This result may also be related to the slightly higher protein content of model IFF with DPL. Concomitantly, no significant differences in the PV evolution were observed as a function of droplet size in the case of model IFF stabilized with soy lecithin (POM-(S and L) and POM-DLDC-(S and L)). However, for model IFF with a droplet size of 0.4 μm, the addition of soy lecithin to the water phase conducts to a 1.7-fold increase in PV. In the case of DPL-stabilized model IFF, the evolution of PV was significantly less important especially for the smaller droplets (4.4 ± 0.3 meqO_2 kg^{-1} for POM-DPL-S) than for the larger ones (9.4 ± 1.2 meqO_2 kg^{-1} for POM-S) after 20 days.

These results suggest that the droplet size cannot be considered individually to anticipate the resulting model IFF oxidative stability. Indeed, the chemical environment at the interface probably has a more significant influence than the droplet size alone on the oxidative stability.

3.3 | Impact of homogenous or heterogeneous droplet core composition

The impact of the droplet core composition (homogeneous or heterogeneous) on the oxidative stability was evaluated by comparing reference model IFF, that is, POM-(S and L) with model IFF in which the individual core composition of the droplets was heterogeneous (PUFA and HMP oils droplets or IMP oils droplets), that is, POM-DLDC-(S and L) (comparison of Figure 4a,b). The results show no significant differences in the PV evolution after 20 days and reach on average 10.5 ± 0.9 meqO_2 kg^{-1}. The tocopherols loss was similar but higher for model IFF with a droplet size of 0.4 μm (62.8% and 65.3% of total tocopherol loss for POM-S and POM-DLDC-S, respectively) compared to the one with a size of 0.7 μm (38.5% and 42.6% of total tocopherol loss for POM-L and POM-DLDC-L, respectively). According to the tocopherols isomers composition, the tocopherols loss was mainly attributed to a loss of α-tocopherol that was the second most abundant isomer after the γ-tocopherol in these model IFF. Significant differences in retinyl acetate content were measured after processing, but levels after storage were equivalent.

3.4 | Impact of the interfacial composition

3.4.1 | Impact of emulsifier type

Lipid oxidation is well known to occur at the lipid/water interface in emulsified system.[3] Therefore, the interface composition and

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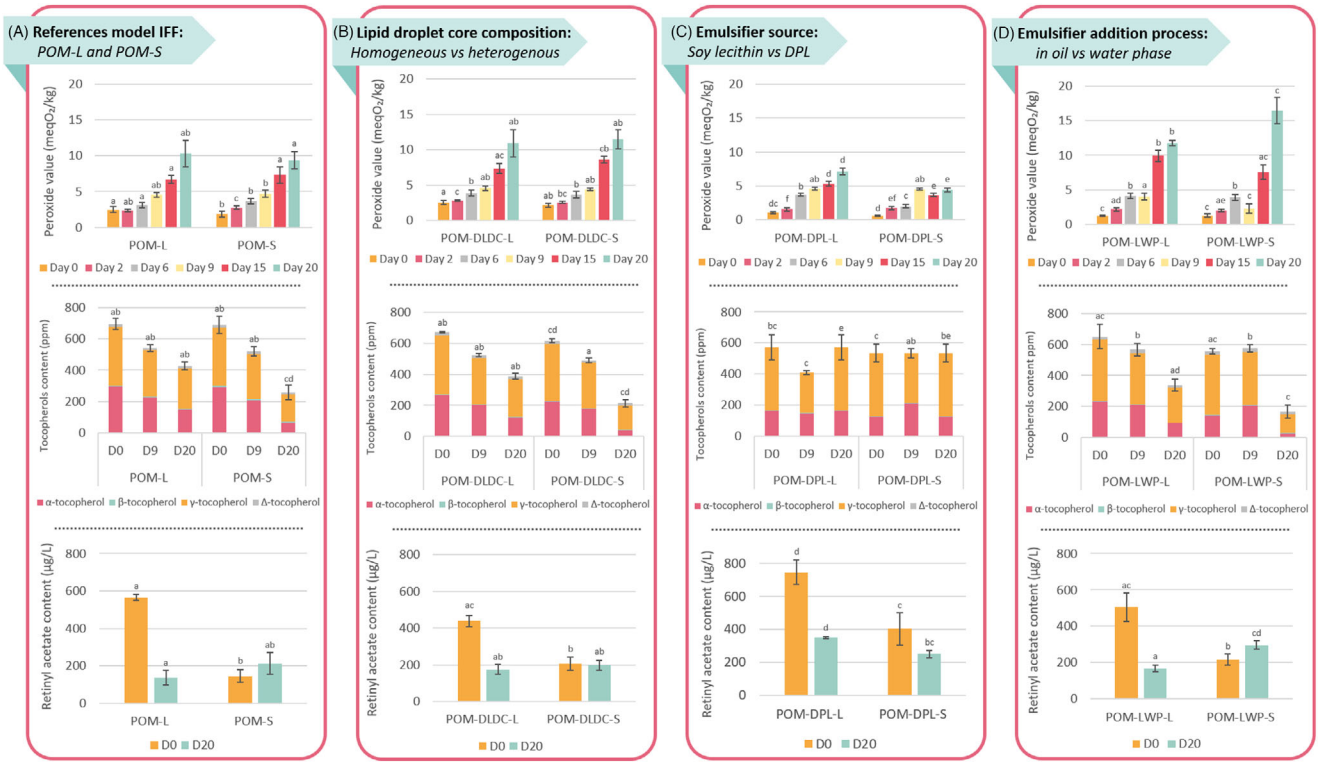

FIGURE 4 Peroxide values, tocopherols, and retinyl acetate contents evolution (a) in the reference model infant follow-on formulas (IFF) (palm oil mixture [POM]-S and POM-L) and depending on (b) the lipid droplet core composition (homogeneous or heterogeneous), (c) the source of emulsifier (soy lecithin or dairy phospholipid), and (d) the emulsifier addition process (in the oil or water phase) during accelerated storage conditions. Oxidative stability is systematically compared with model IFF references: POM-S and POM-L. The different letters indicate a significant difference ($p < 0.05$) between two model IFF at a given time. L is used for model IFF with droplet size of 0.7 µm and S for model IFF with a droplet size of 0.4 µm. POM-L/POM-S: POM-based IFF with a droplet size of 0.7 or 0.4 µm. POM-DLDC-L/POM-DLDC-S: POM-based IFF with heterogeneous lipid droplet core composition (high and low melting point oils droplets, i.e., palm with arachidonic acid [ARA] and docosahexaenoic acid [DHA]) oils or intermediate melting point oils droplets (i.e., sunflower, oleic sunflower, and rapeseed oils) with a droplet size of 0.7 or 0.4 µm. POM-LWP-L/POM-LWP-S: POM-based IFF with soy lecithin previously homogenized with the aqueous phase with a droplet size of 0.7 or 0.4 µm. POM-DPL-L/POM-DPL-S: POM-based IFF with a DPL as emulsifier with a droplet size of 0.7 or 0.4 µm. DPL, dairy phospholipid; DLDC, different lipid droplet composition; LWP: lecithin in water phase; VMC: vitamin and mineral complement.

physicochemical properties are crucial regarding oxidation rates and are directly correlated with the type of the emulsifier used. Therefore, model IFF that prepared either with soy lecithin, that is, POM-(S and L) or DPL, that is, POM-DPL-(S and L) were compared to evaluate the impact of the emulsifier source on the oxidative stability. According to PV evolution, model IFF stabilized with a DPL were significantly more stable than the ones stabilized with soy lecithin (comparison of Figure 4a,c). This tendency was even more pronounced for the small droplets size with PV of 4.4 ± 0. 3 $meqO_2\ kg^{-1}$ for POM-DPL-S after 20 days versus 9.4 ± 1.2 $meqO_2\ kg^{-1}$ for POM-S. In parallel, the total tocopherols content remains constant after 20 days for model IFF with DPL as an emulsifier, whereas losses of 62.8% for POM-S and 38.5% for POM-L were observed. As expected, these results show that the type of emulsifier modulates the oxidative stability. In our case, DPL demonstrates superior protection against lipid oxidation compared to soy lecithin. Moreover, retinyl acetate losses were significantly lower when using DPL.

3.4.2 | Impact of the emulsifier partition between the lipid and the water phases

The oxidation rate in emulsions can be also related to the processing conditions.[28] Thus, the way the emulsifier is added during emulsification process plays an important role as it will determine the potential presence of emulsifier micellar or colloidal structures. These particular structures are known to play an important role on mass transport phenomena in lipid oxidation.[23,40] They could hypothetically be carriers of antioxidants, prooxidant, or oxidation compounds in emulsion. The oxidative stability of POM-(S and L) in which soy lecithin was added to the oil phase prior to emulsification processing was compared to POM-LWP-(S and L) in which the same emulsifier was added to the water phase also prior to emulsification processing. Depending on the addition process, it is expected that the emulsifiers are more prone to be localized at the interface or to form colloidal structures in the emulsions. The comparison of Figure 4a,d shows that the incorporation of soy lecithin in the water phase (POM-LWP-(S and

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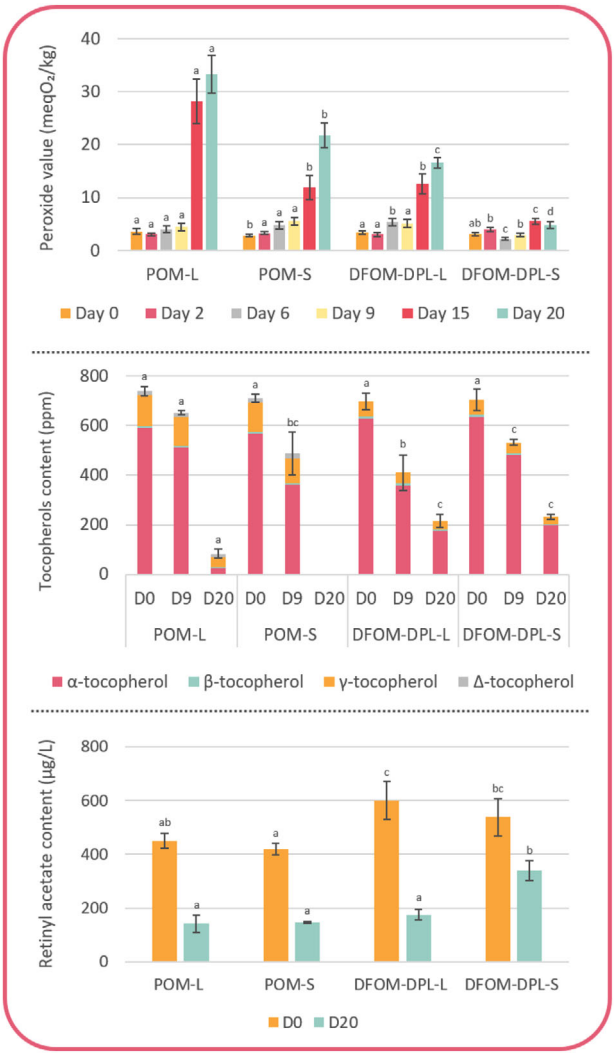

FIGURE 5 Impact of introduction of dairy lipids (dairy fat olein and dairy phospholipids) in model infant follow-on formulas (IFF) composition on peroxide value, tocopherols, and retinyl acetate contents. Different letters indicate a significant difference ($p < 0.05$) between two model IFF at a given time. L is used for model IFF with droplet size of 0.7 µm and S for model IFF with a droplet size of 0.4 µm. Palm oil mixture (POM)-L/POM-S: POM-based IFF with a droplet size of 0.7 or 0.4 µm. POM-DPL-L/POM-DPL-S: POM-based IFF with a DPL as emulsifier with a droplet size of 0.7 or 0.4 µm. DPL, dairy phospholipid; VMC, vitamin and mineral complement.

L)) leads to a higher PV evolution after 20 days whatever the droplet size. Similarly, the tocopherols loss was significantly higher when the emulsifier was added in the water phase. This loss was even more important for small droplets (70.2% loss for POM-LWP-S vs. 48.2% for POM-LWP-L).

3.5 | Effect of dairy lipids introduction in model IFF

An additional storage test was performed to further investigate the impact on oxidative stability of the substitution of POM by dairy lipids (including DPL and DFOM) (Figure 5). Despite a faster oxidation kinetics than for the previous model IFF (resulting in higher PV and tocopherol degradation after 20 days of storage for POM-(S and L), model IFF based on dairy lipids were more stable than the ones with POM. PVs were indeed significantly lower, especially for the smaller droplets for which PV were 9.5 times lower in comparison with model IFF made from POM (4.8 ± 0.6 and 21.8 ± 2.3 meqO_2 kg^{-1} for DFOM-DPL-S and POM-S, respectively). Moreover, the tocopherols loss in DFOM-DPL-(S and L) was less important than POM-(S and L) with a loss up to 1.5 times lower. The difference in oxidative stabilities with the prior model IFF studied could be attributed on the fact that in these additional model IFF, α-tocopherol was the most abundant tocopherol isomers (85% of the total tocopherols). However, in the prior model IFF, the most abundant isomer was the γ-tocopherol and represented at least 54% of the total tocopherols. The post-processing retinyl acetate content was significantly higher in DFOM-(S and L) and a lower loss after storage was measured in DFOM-DPL-S.

4 | DISCUSSION

Results presented in the current study showed that the studied model IFF have an overall good oxidative stability despite the addition of LC-PUFA (in particular DHA and ARA). Among the structural levers that we evaluated regarding their impact on oxidative stability, the most influential being the interface nature that can be modulated depending on the type of the used emulsifier (in our case soy lecithin vs. DPL). On the opposite, the droplet size and their core composition homogeneity have a limited impact which is mostly related to the chemical environment, especially to the presence of colloidal vesicles.

4.1 | Droplet size alone has no strong impact on oxidative stability over the range of model IFF sizes tested

Our results show that the droplets size (0.4 or 0.7 µm) impacts mainly the initial vitamin A content with a more important loss when model IFF were designed with smaller droplets. This observation is most likely explained by higher shearing forces that lead to higher local heating of vitamin A molecules favoring their oxidative degradation during process.

Over the droplet sizes range tested no strong effect on oxidative stability was observed considering this parameter alone illustrated for instance by POM-(S and L). The hypothesis most often encountered in literature is that smaller droplets oxidized faster due to their larger specific surface area in comparison with larger droplets. However, it is difficult to distinguish between the effect of droplet size and that of the chemical environment. In fact, homogenization conditions not only modulate droplet size but also have an impact on other crucial parameters involved in the oxidation reaction rates, such as the emulsifier partitioning with more or less adsorption at the interface.[39] The present study shows that the type of emulsifier and the chemical

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environment determine stability rather than the droplet size. There are many contradictory results in the literature about the effect of droplet size. Some studies demonstrate that decreasing droplet sizes resulted in increased oxidation,[26] whereas others show that there was no effect of droplet size on lipid oxidation initiation or propagation,[14] or the opposite, an increased stability with a decreasing of the droplets size.[25] This dissensus supports our hypothesis that other factors, such as the interface composition, are more important than the total surface area itself.

From a nutritional point of view, droplet size has a very strong impact on the bioaccessibility of lipid compounds with accelerated lipolysis kinetics for smaller droplet sizes and a structure less close to that of HM.[4,5] Moreover, processing conditions to obtain smaller droplet may result in a loss of added vitamin A in model IFF has seen in the present study.

4.2 | Substitution of soy lecithin by DPL improves oxidative stability of model IFF

In emulsified systems, the interface area is known to be one of the main site of lipid oxidation phenomena.[3,40,41] Our study shows that, for an equivalent lipid composition, the substitution of soy lecithin by DPL in model IFF improves their oxidative stability (illustrated by the comparison of POM-DPL-(S and L) and POM-(S and L)). A first hypothesis to explain this result is that the thickness and density of the interface is more important for model IFF formulated with DPL. Indeed, DPL provides very long chains SFA (beyond C20:0) that create rigid domains at the interface which may form a steric protective barrier at the interface limiting the prooxidant effects of water-dissolved molecules such as metals. DPL also provides different PL species such as sphingomyelin which are known to have an antioxidant effect themselves or have synergistic effect with tocopherols.[38] The efficiency of the synergy could be related to the number of amino groups, which may regenerate tocopherols after a series of rearrangement reactions.[2] Another hypothesis that could explain these results is that the normalization of PL content has concomitantly slightly increased the protein content for model IFF with DPL compared to soy lecithin that can exhibit antioxidant properties.[32]

The results obtained in our study show that in presence of DPL the smaller the droplet size, the greater the stability. Moreover, the addition of DPL concomitantly increases protein content. Similar results were obtained in the study of[25] in which fish oil and/or rapeseed oil were homogenized with milk at different temperatures and pressures to obtain droplets from 0.5 to 1.36 μm ($D[3,2]$). The authors show that oxidative stability was improved for smaller droplet sizes and hypothesized that the adsorption of proteins and their rearrangement at the interface was more important in the case of smaller droplets, enabling an interface more fully covered and facilitating the contact with amino groups.

The results showed that stability was even enhanced when DPL was used in combination with dairy fat. Dairy fat provides short and medium saturated chains and minor lipids such as cholesterol and other complex lipids (glycerophospholipids or sphingolipids) that are suitable for infant nutrition. In addition, the organization of its FA on the TAG backbone, with long-chain SFA and LC-PUFA mainly esterified in *sn-2* position, leads to better lipid absorption by avoiding the formation of insoluble soaps (calcium palmitate) which are directly excreted.[13] This organization is also suspected of influencing oxidative stability, with a protective effect for PUFA in the central position, as they are less accessible.[43] Complexifying the chemical composition by introducing dairy lipids is more relevant to mimic HM, and therefore more adapted to the needs of infants.[10] Moreover, despite a slightly lower oxidative stability than POM-DPL-S, droplets of 0.7 μm would be closer to the average size of MFG and avoid overprocessing leading to significant losses of vitamin A in particular.

4.3 | The addition of lecithin to the water phase in model IFF may favor lipid oxidation

Our results show that the presence of colloidal structures in the water phase illustrated by POM-LWP-(S and L) was not inert regarding lipid oxidation and result in a decrease of the stability compared to POM-(S and L). Lipid oxidation depends not only on the amount of PUFA but also on the overall reactivity and interactions of the many molecules involved in lipid oxidation, and their location and mobility in the emulsion. More and more evidences show that colloidal structures such as the one studied in POM-LWP-(S and L) play a crucial role in mass transport phenomena in lipid oxidation pathway.[21,40] According to literature, micelles and colloidal structures may have two antagonistic effects regarding lipid oxidation in emulsified systems. They could act as antioxidant reservoirs, leading to a better diffusion of these molecules at the interface area and thus limiting oxidation. On the contrary, colloidal structure may serve as carriers of oxidation products, leading to their transfer from one droplet to another and promoting the oxidation. The results obtained in the present study seem to agree with the second pathway, as we observed that the additions of lecithin to the water phase diminish oxidative stability of model IFF. Moreover, this decrease in stability could also be attributed to the fact that the addition of lecithin in the water phase concomitantly affects the interfacial composition, by reducing its concentration at the oil–water interface. Consequently, interface coverage and the steric barrier would be lower. This would thus favor contacts between oxidizing compounds (transition metals or oxidation products such as hydroperoxides) and consumption of lipid-soluble antioxidants such as tocopherols leading to their fast degradation. Moreover, PL and tocopherols are known to have synergistic effect that favor the regeneration of tocopherols.[12,19,34] The lack of lecithin at the interface due to colloidal structure formation may also limit this synergy effect and could also explain the more important sensitivity to oxidation of POM-LWP-(S and L) compared to POM-(S and L). Finally, due to functional groups such as phosphates and amines, lecithin can also act as metal chelators that contribute to limit the prooxidant effects of iron or copper ions. The lower concentration of lecithin at the interface in POM-LWP-(S and

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L) may also limit this effect at the oxidation initiation site, which could explain their higher susceptibility to oxidation. However, the transition metals in their free form are located in the water phase in direct contact with the colloidal structures, this hypothesis seems limited.

4.4 | The individual core lipid droplets composition in a population is difficult to control and stabilize

MFG core composition of HM is heterogeneous, with the smallest globules containing a higher content of medium-chain SFA (C8–C12) and lower amount of long-chain SFA than the largest globules.[15] LC-PUFAs are therefore "encapsulated" in large globules surrounded by long-chain SFA, with high melting point leading to a partial surface crystallization.[30] Instinctively, these parameters could impact the oxidative stability due to the formation of a lipid shell protecting LC-PUFA (steric barrier). In our study, the individual composition of the lipid core droplet in POM-DLDC-(S and L) was expected to be heterogeneous to mimic the HM as closely as possible. Our results show no significant effect on oxidative stability. However, Okubanjo, Loveday et al.[33] studied the oxidative stability of oil-in-water emulsions by forming a lipid shell and demonstrated that structuring the interface with solid lipids improved oxidative stability. The authors suggest that this kind of system leads to limited contact between prooxidant and oxidizable compounds. Similar results were obtained by Salminen, Helgason et al.[36] who found that the thicker the crystallized shell, the better the oxidative stability. These findings were attributed to a reduced diffusion and mobility of prooxidants compounds and oxygen at the interface.

Contrary to the previous studies, our results show no significant effect of the lipid core heterogeneity. One hypothesis to explain this contradictory result would be that a homogenization of the composition of the lipid droplet cores occurred during storage. Physical instability, possibly due to the stirring and reduction in viscosity caused by storage at 40°C, could favor collisions between droplets and concomitantly the transfer of lipids between droplet cores. Further investigations will be necessary to determine whether the individual lipid core droplet can be controlled in a such complex food matrix and used to delay lipid oxidation.

5 | CONCLUSIONS

This study showed that the oxidative stability of model IFF depends on their chemical composition and can be modulated according to different structural levers. Based on the tocopherol degradation kinetics and PV evolution, the most representative indicators of oxidation in our systems, the results showed that in a such complex food matrix it is difficult to manage the individual lipid core droplet composition. The impact of droplet size on lipid oxidation in IFF is limited and highly dependent on other factors such as the chemical environment, and in particular the nature of the interface. The interface quality could be modulated according to the source of emulsifier with a more important oxidative stability when soy lecithin is substituted by DPL. Moreover, the presence of colloidal structures in IFF has an impact on stability, probably by promoting the diffusion of oxidized compounds or limiting the synergy with tocopherols. Thus, considering both issues related to lipid oxidation and those related to nutrition, IFF enriched in LC-PUFA and incorporating dairy lipids may be a reliable strategy to guarantee oxidative stability and an adequate nutritional profile.

AUTHOR CONTRIBUTIONS

Methodology; investigation; formal analysis; writing—review and editing: Mathilde Cancalon. *Methodology; validation, writing—review and editing*: Nathalie Barouh and Youna Hemery. *Resources*: Bruno Baréa. *Writing—review and editing*: Erwann Durand. *Supervision; methodology; validation, writing—review and editing*: Claire Bourlieu-Lacanal. *Supervision; writing—review and editing*: Pierre Villeneuve.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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