Red blood cell membrane fatty acid composition in infants fed formulas with different lipid profiles

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A B S T R A C T

Background: There is growing interest in the fatty acid composition of breast milk and substitute formulas used to replace or complement infant breastfeeding.
Aim: The aims of this study were to assess the impact of two follow-up infant formulas based on cow milk fat, vegetable oils and different docosahexaenoic (DHA) and arachidonic (ARA) acid content on red blood cell membrane fatty acid composition, and determine the percent saturated fatty acid (SFA) incorporation into the membrane.
Study design: This was a double-blind, randomized, controlled, parallel-group clinical trial. Infants received treatment or control product for at least four months before the age of six months. The control group (n = 25) received standard infant formula (FA) and the treatment group (n = 24) received the same formula supplemented with higher DHA and ARA content (FB). The reference group (n = 47) consisted of normal healthy exclusively breastfed infants.
Outcome measure: Red blood cell membrane fatty acid composition was determined by capillary gas chromatography.
Results: Ninety-six infants completed the study (FA, 25; FB, 24; reference, 47). Higher DHA content reflected higher DHA percentage in the red blood cell membrane. Breast milk and FB did not show any significant differences in DHA content. ARA percentage was higher in breastfed infants and palmitic acid percentage was higher in FB- compared with FA-fed infants.
Conclusion: DHA and palmitic acid percent distributions were higher in the red blood cell membrane of infants receiving FB. DHA percent distribution was not significantly different in FB-fed and breastfed infants. SFA percent distribution was not significantly different when comparing both formulas with breast milk.

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1. Introduction

In the last decades, there has been growing concern about the fatty acid composition of infant formulas, which are normally used when breastfeeding must be either replaced or complemented following medical advice. Breast milk is the main reference for infant feeding, since it has the best concentration of nutrients and bioactive factors [1].

The fetus, the newborn and breast-fed infants must receive sufficient amounts of long-chain polyunsaturated fatty acids (LCPUFA) to sustain optimal visual and cognitive development. In this sense, breast milk is highly recommended as a source of LCPUFA. When breastfeeding is not possible, the current recommendation is the provision of formulas with adequate docosahexaenoic acid (DHA) and arachidonic acid (ARA) levels [2].

Studies supporting the advantages of breastfeeding have focused on the concentration of LCPUFA in human breast milk, particularly DHA and ARA [3]. These two account for 20% of the brain fatty acid content and are involved in neurodevelopment, promoting healthy neuronal growth, repair and myelination [4]. At the end of pregnancy, ARA is the predominant PUFA in the brain, but DHA accretion after birth makes it the main PUFA in the adult brain, representing approximately 50–60% of the brain's dry weight [5].

Breast milk fat is better absorbed than infant formula fat. Despite similarities in their fatty acid composition, the structure of human milk triacylglycerols is different. Palmitic acid (hexadecanoic acid) is one of the main components of maternal milk, representing around 25% of the lipid profile, from which 60–85% is in sn-2 position of triacylglycerols [6,7]. Addition of palm oil (high palmitic acid content) to

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infant formulas results in a lipid composition closer to that of maternal milk. However, a critical aspect of infant formulas to mimic human milk composition is the bioavailability of nutrients, in this case, fatty acids [8–10].

Triacylglycerols (palmitic acid in sn-2 position) in human milk are digested by pancreatic lipase, which hydrolyzes them on the 1 and 3 carbons and releases saturated fatty acids (SFA), both palmitic and stearic acid, as sn-2-monoacylglycerols. These will later form mixed micelles with bile salts to be absorbed [8]. On the other hand, the greatest part of palmitic acid from palm oil used in infant formulas is present in sn-1 and sn-3 positions of triacylglycerol [11,12], becoming free fatty acid after hydrolysis. Free fatty acids bind to calcium in a process of saponification to form a non-absorbable complex that contributes to the low absorption of fat and calcium, lower weight gain and hardening of stools. The effect of palm oil has been shown in term and pre-term babies and experimental animals [12–14]. In cow milk, the concentration of palmitic acid in sn-2 position is above 45% fat [15].

The role of SFA in milk and breast fat has been recently reviewed, with an emphasis on their relevance and benefits [16]. More recently, focusing in palmitic acid, SFA have been reported as an essential component of membrane, secretory and transport lipids, with crucial roles in protein palmitoylation and signal molecules [17]. Further, the author raises some questions, namely, why the human fetus synthesizes and accumulates 16:0, and the human mammary gland invests in specific pathways to provide the infant with 16:0, proposing that those questions are important for developmental biology, with broad implications for the nutritional care of infants that involve fatty acids that alter sources of metabolic energy, and the growing tissue and plasma lipids [17].

We analyzed the percentage of palmitic acid at sn-2 position of triacylglycerols in infant formulas from the Argentinean market that are used as breast-milk substitutes during the first six months after delivery. We found that formulas with vegetable oils as basic source of lipids had <15% of palmitic acid at sn-2 position, whereas formulas containing a blend with milk fat had 48% of palmitic acid at sn-2 position [18].

The best approach to study fatty acid incorporation into the tissues, particularly the central nervous system, is through the study of red blood cell membrane fatty acids, which are considered representative of the composition of brain cell membranes [19]. Therefore, it would be important to know whether higher DHA and ARA content in infant formulas results in higher red blood cell membrane fatty acid percentage as well as to determine the percent SFA incorporation into the membrane.

The aim of the present study was to assess the impact of two follow-up infant formulas based on cow milk fat, vegetable oils and different DHA and ARA content on red blood cell membrane fatty acid composition, and to determine the percent incorporation of SFA into the membrane.

2. Patients and methods

2.1. Study design and protocol

Healthy term infants being seen for routine follow-up appointments since the first month of life at the Health Observatory of IDIP (Instituto de Desarrollo e Investigaciones Pediátricas, La Plata's Children Hospital, Buenos Aires, Argentina) were recruited for a double-blind, randomized, controlled, parallel-group clinical trial during December 2012–November 2014.

Both gestational age and birth weight of infants were normal (>37–<42 weeks and ≥2500–<4000 g, respectively). Mothers were also healthy and >18 years of age. Written informed parental consent was obtained for each infant before inclusion.

Exclusion criteria included inadequate height-for-age and weight-for-age indices according to the World Health Organization (WHO) charts; infants with acute infections within 15 days before recruitment;

chronic diseases; antibiotic or vitamin treatment; a birth weight <2500 g; pathological fetal and neonatal history; anemia not related with nutrition; diagnosis or ongoing studies of genetic alterations; lack of written informed parental consent and mothers with chronic diseases.

The study protocol was approved by IDIP's Institutional Research Review Board and registered in the Ministry of Health of the province of Buenos Aires. It was performed in accordance with the ethical standards laid down in the 1948 Universal Declaration of Human Rights, the Nüremberg Code and the 1964 Declaration of Helsinski and successive revisions and amendments.

Participants were controlled and enrolled since the first month of life at IDIP's Health Observatory. Breastfed infants were followed-up without any intervention. At the time when mothers independently decided to cease breastfeeding and introduce infant formula, infants were randomly assigned to receive either a standard infant formula (control group, FA) or an infant formula supplemented with DHA and ARA (treatment group, FB) to enhance the levels of both fatty acids. A reference group was formed of infants who continued to be exclusively breastfed up to six months (non-randomized). Random allocation of study participants to the intervention was performed with EPIDAT 3.1 (1:1 allocation ratio). None of the two groups received complementary foods other than formula.

Physical examination included infant weight, length and head circumference measurement at baseline (age 2–4 weeks) and at monthly intervals throughout the intervention until the age of 6 months. These measurements were used to calculate height-for-age, weight-for-age and weight-for-height indices, and plotted into reference Z-score tables based on the WHO Child Growth Standard, using WHO Anthro 2007 Version.

Nutritional status was assessed using anthropometric indices of height-for-age, weight-for-age and weight-for-height; Z-scores were calculated with the EPINUT program in Epi Info (version 3.3, 2004). Stunting was defined as a height-for-age (Z-score), underweight as a weight-for-age (Z-score), and wasting as a weight-for-height (Z-score), on the basis of the WHO Child Growth Standard 2007 Version.

Infants were dropped out of the trial if they were subsequently found to have received non-compliant feeding during the intervention, or to have moved to an unidentified address. Infants on any of the two formulas had to fulfill supplementation for a minimum of 4 months before the age of 6 months and breastfeed no more than once a day. Exclusive breastfeeding had to be sustained until the age of 6 months.

Infant formulas were developed and produced by Sancor CUL and had the following composition: Formula A contained (w/w) 66.81% milk fat, 33.19% vegetable oil (32.44% canola and sunflower 50/50% mixture), and 0.75% oil as source of ARA and DHA, obtained from fungi and microalgae. Formula B contained (w/w) 65.41% milk fat, 34.59% vegetable oil (33.05% canola and sunflower 50/50% mixture), 1.24% oil as source of ARA and DHA obtained from fungi and microalgae, and 0.30% oil with high DHA content from microalgae (which contains palmitic acid).

Data for maternal milk composition were taken from 32 mothers of exclusively breastfed infants. In all cases, samples were taken 3 months after delivery. Breast milk samples taken more than a week before or after the third month were discarded. Table 1 shows the fatty acid composition of supplemented formulas and the breast milk composition of 32 exclusively breastfed infants.

2.2. Breast milk sample collection

Before sample collection, mothers washed their hands and breasts with soap. Milk collection was carried out with a sterile automatic breast milk pumper with a vacuum regulator (mini electric breast pump, MEDELA Inc. USA), polystyrene suction funnels and screw-top bottles adapted to suction funnels for direct collection of milk. The bottles and the suction funnels were autoclaved before use. The milk

Table 1
Composition of supplemented formulas (SanCor CUL) and breast milk. Data are expressed in g/100 ml (*) and percent of total fatty acids (**).

Fatty acids	Formula A Control group	Formula B Treatment group	Breast milk Reference group
Total fat *	3.78	3.70	3.57
SFA* (**)	1.62 (42.86)	1.62 (43.78)	1.52 (42.64)
MUFA* (**)	1.41 (37.30)	1.37 (37.00)	1.27 (35.6)
PUFA* (**)	0.66 (17.46)	0.64 (17.30)	0.78 (22.1)
Trans* (**)	0.09 (2.38)	0.08 (2.16)	0.02 (0.72)
C10:0* (**)	0.07 (1.85)	0.05 (1.35)	0.02 (0.69)
C12:0* (**)	0.08 (2.12)	0.06 (1.62)	0.17 (4.66)
C14:0* (**)	0.27 (7.14)	0.22 (5.95)	0.21 (5.86)
C16:0* (**)	0.74 (19.58)	0.69 (18.65)	0.76 (21.42)
C18:2n6 LA* (**)	0.57 (15.32)	0.51 (13.81)	0.70 (19.64)
C18:3n3 ALA*(**)	0.095 (2.51)	0.066 (1.78)	0.02 (0.66)
C20:4n6ARA*(**)	0.0086 (0.23)	0.014 (0.38)	0.016 (0.46)
C22:6n3DHA*(**)	0.0036 (0.09)	0.010 (0.27)	0.006 (0.17)

samples suctioned with the pumper were collected in bottles (25 mL approximately) and immediately aliquoted in 10 mL tubes using sterile material, frozen and stored at −80 °C for later analysis [20,21].

2.3. Blood samples

Venous blood samples (1 mL) were taken at 6 months of age during the control visits at the Health Observatory and collected in tubes containing 5% EDTA, pH 7. Blood was centrifuged within 30 min of extraction to separate plasma from red blood cells. The pellet was washed with sodium chloride 0.9%, centrifuged three times and kept at −70 °C until processing.

2.4. Red blood cell membrane fatty acid analysis

Red blood cell membrane fatty acid composition was determined by extraction of membrane lipids with a mixture of chloroform/methanol (2/1, v/v) [22]. Partition was performed with 20% v/v distilled water. The upper methanolic phase was discarded. The remaining chloroformic phase was evaporated to dryness under a nitrogen stream. Fatty acid methyl esters (FAME) were obtained after trans-methylation by using boron trifluoride 10% methanol [23]. FAME were analyzed with gas chromatography (GC System, 7890A Series, Agilent Technologies, Wilmington, Delaware, USA); flame ionization detection (FID) was applied. A 60-m capillary column was used (DB-23; Agilent Technologies), calibrated against a standard containing 37 FAME, ranging in chain length from 4 to 24 carbon atoms (Supelco 37 Component Fame Mix; Supelco, Bellefonte—Pennsylvania, USA). Quantification was performed using the peak areas automatically retrieved from an integrator coupled to the chromatograph. The fatty acid results were expressed as percentages of total fatty acids detected.

2.5. Milk fatty acid analysis

Fatty acids were extracted from 500 μL of milk with a mixture of chloroform/methanol (v/v, 2:1) [22]. After lipid extraction, the chloroform phase was evaporated under nitrogen current; fatty acids were saponified with potassium hydroxide in 10% methanol and acidified with hydrochloric acid to neutralize. FAME were extracted twice with n-hexane and obtained after trans-methylation of total lipids by using boron trifluoride 10% methanol [23] for 1 h at 80 °C. They were separated and quantified as previously described for the red blood cell membrane.

2.6. Sample size

Sample size was estimated to obtain a 90% power and a 95% confidence interval and to detect differences of 0.9 in DHA values between the control and the treatment formulas, assuming a standard deviation

0.94 for DHA [24]. Thus, the required sample size was 25 infants per group, totalling 75 infants.

2.7. Statistical analyses

Data were processed with SPSS, version 18.0 for Window. Red blood cell membrane fatty acids were grouped as follows: SFA, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C24:0; monounsaturated (MUFA), C16:1n7, C18:1n9 cis and trans, C20:1n9, C22:1n9, C24:1n9; PUFA were divided into Omega 6 (C18:2n6 cis and trans, C18:3n6, C20:2n6, C20:3n6, C20:4n6, C22:2n6) and Omega 3 (C18:3n3, C:20:3n3; C20:5n3, C22:6n3).

The normality of each variable was evaluated with the Shapiro–Wilk test. Means and standard deviations were used for comparisons between groups. ANOVA was used to assess the effects of the dietary groups. Between-pair comparisons were performed with corrected p-value by Scheffé method.

In all cases, results with a significance level of $p < 0.05$ were considered statistically significant. Fatty acid levels were reported as percentage of total fatty acids analyzed.

3. Results

Of the 109 infants enrolled, 96 completed the study. Infants whose mothers chose not to provide breast milk were randomly assigned to receive a standard formula (control group [FA]; n = 29) or formula with higher DHA and ARA content (treatment group [FB]; n = 28), while infants who continued to be exclusively breastfed constituted the reference group (n = 47). During the trial, 13 infants were dropped out (Fig. 1). Sample characteristics and nutritional condition after the intervention are described in Table 2. We did not observe statistically significant differences among groups.

Table 3 shows the percentage of red blood cell membrane fatty acids according to diet.

The percentage of palmitic acid was higher in FB-fed infants compared with FA-fed ones. Conversely, the percentage of stearic acid was higher in FA-fed infants. Total SFA were similar in both groups. No significant differences were found when comparing both formulas with breast milk.

The percentage of MUFA was significantly higher in FA-fed compared with FB-fed and breastfed infants. Differences between FB and breast milk were not significant.

The percentage of ω3 PUFA and DHA was markedly higher in infants fed FB compared with FA (control). We could not detect differences between FB and breastfed infants (reference).

Finally, the percentage of ω6 PUFA was similar in FA-, FB- and breastfed infants. However, ARA percentage was lower in FB-fed infants as compared with the control and the reference groups.

4. Discussion

In this study, we evaluated the impact of two infant formulas based on cow milk fat, vegetable oil and different DHA and ARA content on red blood cell membrane fatty acid composition, and determined the percent SFA incorporation into the membrane. As a reference, we included a group of exclusively breastfeeding infants and also assessed maternal milk composition. To our knowledge, there are no previous studies in our region reporting the impact of type of feeding on red blood cell membrane fatty acid composition in healthy, well-nourished infants.

Our results show that the percentage of ω3 PUFA and DHA in the red blood cell membrane was markedly higher in infants fed FB compared with FA (control). We could not detect differences between FB and breastfed infants (the reference). Our results also agree with those of other authors [25] in the sense that higher DHA content reflects higher DHA percentage in the red blood cell membrane. Comparison of FB-
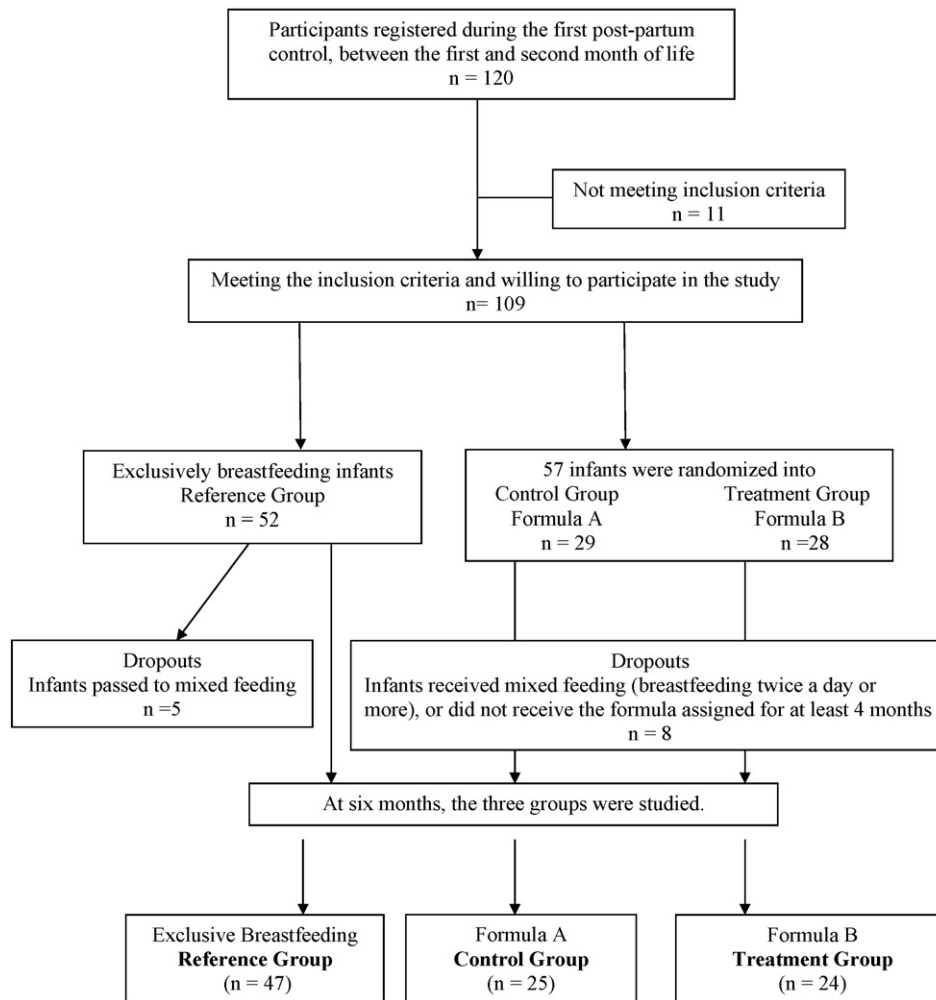
Fig. 1. Flow chart of participant registration, randomization and follow-up.

with FA-fed infants showed that FB presented a higher DHA percentage in the red blood cell membrane. However, ARA percentage was lower.

The same results were reported by Miller et al. [26] when comparing formulas supplemented or not with DHA and ARA, probably due to their competitive nature because, when both are available, the membrane will preferably incorporate DHA [26]. However, DHA and ARA percentage was higher in the red blood cell membrane of exclusively breastfed infants as compared with infants fed with either formula. The weakness in Miller's study is concerned with the lack of measurement of fatty acid levels in breast milk. Therefore, diets with a high omega-3/omega-6 ratio result in increased omega-3 and DHA contents in red blood cells [27,28].

Regarding palmitic acid, its percentage in the red blood cell membrane was higher in FB- compared with FA-fed infants, and its content was higher in FB, probably because the source of DHA contains palmitic acid, which might finally increase the total percentage of this fatty acid. However, stearic acid was higher in FA-fed infants and consequently, total

SFA were similar in both groups. No significant differences were found in SFA percentage when comparing both formulas with breast milk.

A recent report determined the effect of an infant formula supplemented with a mixture of dairy lipids and plant oils on the erythrocyte membrane omega-3 fatty acid profile in full-term infants [25]. However, the study does not include a reference group of exclusively breastfed infants, which is one of the strengths of our research, together with the fact that we also studied the lipid composition of maternal milk.

Hoffman et al. [24] studied the percent distribution of red blood cell membrane fatty acids in infants fed two formulas with different PUFA content. The same as in our study, they found a higher percentage of DHA in infants fed the formula with greater PUFA content; the authors also reported that the formula with lower DHA content had a greater percentage of precursors (LA-ALA), not sufficient to equal the amount produced by the pre-formed DHA. These authors neither presented a reference group nor compared their results with breast milk.

Table 2
Characteristics and nutritional condition of infants enrolled in the study 4 months after receiving FA, FB or breast milk.

	Formula A Control group	Formula B Treatment group	Breast milk Reference group	P-value
Sex (male)	56%	54.2%	40.4%	0.245
Age (months)	6.10 ± 1.18	6.32 ± 0.33	6.34 ± 0.59	0.326
Weight (g)	7782.04 ± 1081.98	7937.71 ± 814.50	7678.09 ± 970.59	0.838
Z-score weight/height	0.74 ± 2.64	0.48 ± 0.87	0.37 ± 0.99	0.710
Z-score height/age	−0.27 ± 3.14	−0.21 ± 1.09	−0.41 ± 1.02	0.811
Z-score weight/age	0.09 ± 1.38	0.16 ± 0.89	−0.04 ± 0.9	0.977

Table 3
Red blood cell membrane fatty acid composition in the study groups.

Fatty acid	Mean ± SD						ANOVA P-value
	Breast milk Reference group	d/tn	Formula A Control group	d/tn	Formula B Treatment group	d/tn	
C16:0 Palmitic	22.05 ± 3.00[b]	47/47	20.71 ± 2.11[c]	25/25	24.64 ± 3.59[b,c]	24/24	<0.0001
C18:0 Estearic	19.94 ± 3.04[a]	47/47	20.90 ± 2.09[b]	25/25	17.83 ± 3.64[a,b]	24/24	0.002
C18:1n9 cis	13.6 ± 2.70[a1,a2]	47/47	15.52 ± 2.51[a1]	25/25	15.53 ± 2.79[a2]	24/24	0.003
C20:4n6 ARA	20.55 ± 2.25[c]	47/47	19.87 ± 2.11[b]	25/25	17.42 ± 3.79[b,c]	24/24	<0.0001
C22:6n3 DHA	4.97 ± 1.14[c]	47/47	3.36 ± 1.29[b,c]	25/25	4.55 ± 1.60[b]	24/24	<0.0001
SFA	42.86 ± 4.14	47/47	42.82 ± 3.90	25/25	43.40 ± 6.70	24/24	ns
MUFA	16.83 ± 3.12[c]	47/47	19.86 ± 3.16[a,c]	25/25	17.75 ± 2.55[a]	24/24	<0.0001
ω3 PUFA	4.98 ± 1.14[c]	47/47	3.52 ± 1.29[a,c]	25/25	4.55 ± 1.60[a]	24/24	<0.0001
ω6 PUFA	35.24 ± 4.06	47/47	34.50 ± 2.37	25/25	34.34 ± 5.09	24/24	ns
Total PUFA	40.22 ± 4.07	47/47	38.01 ± 2.99	25/25	38.89 ± 5.42	24/24	ns

[a], [a1], [a2] $p \leq 0.05$, [b] $p < 0.01$, [c] $p \leq 0.0001$ between-pair comparison with corrected p-value by Scheffé method.
d = detected, tn = total n, ns = not significant.

Our study showed that the formulas supplemented with DHA and ARA were similar to those reported in the study by Hoffman et al. [24]. However, the formula used in the Miller et al. study [26] had higher PUFA content. The Codex recommendation for standard infant formulas suggests DHA supplementation not >0.5% of total fat content, without proposing a minimal percentage [29].

A limitation of our study was the lack of infants fed formulas containing only vegetable oils, since they present a very low percentage of palmitic acid at sn-2 position, which could impact on the percentage incorporated by the red blood cell membrane.

5. Conclusions

Comparison of two infant formulas based on cow milk fat, vegetable oil and different DHA and ARA content and assessment of their impact on red blood cell membrane fatty acid composition showed that percent distributions of DHA and palmitic acid were higher in the formula with greater supplementation (FB). DHA percentage was not significantly different in FB-fed and breastfed infants, and SFA percentage in the red blood cell membrane was not different when comparing both formulas with breast milk.

Conflicts of interest

Dimas Vicentin and Graciano Magrini are members of Sancor CUL Department of Development, Innovation and Research. Horacio F Gonzalez has a contract with the mentioned company.

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