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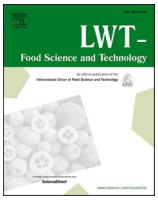

Impact of refrigeration and freezing-thawing of breast milk on *in vitro* digestibility and liposoluble vitamin bioaccessibility in breast-fed infants

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ABSTRACT

There is little information about the impact of refrigeration and freezing-thawing on breast milk digestibility under gastrointestinal conditions of healthy infants or those requiring pancreatic enzyme replacement therapy (PERT) such as infants with cystic fibrosis (CF). This study assessed the impact of refrigeration and freezing-thawing on fat and protein digestibility and liposoluble vitamin bioaccessibility of breast milk. *In vitro* digestion models mimicking both healthy infant and CF infant conditions were applied. Freezing-thawing significantly increased the fat globule particle size. For CF digestion, this change had a more negative impact when using a freezing-thawing process than when using refrigeration of breast milk, reducing lipolysis (up to 18%), proteolysis (up to 28%), and vitamin A and E bioaccessibility. Under healthy conditions, no significant effects were detected. An adequate pancreatic enzyme replacement therapy (PERT) dose (25 LU/mL of BM) would enable the same level of lipolysis (55%) as in the healthy scenario. In conclusion, breast milk is the only source of energy and nutrients for breast-fed infants, so to prevent the loss of nutrient absorption, those with CF should not be fed with frozen-thawed breast milk.

1. Introduction

Breast milk is most recommended for infant feeding, and according to the World Health Organization (WHO), babies should be exclusively breast fed for the first six months. Breast milk is composed not only of macronutrients, vitamins, and minerals, but it is also a source of non-nutritive components such as bioactive compounds (growth factors, hormones, anti-microbials, and oligosaccharides) that overall encompass all the necessary components to be the only food for newborns (Ballard & Morrow, 2013; Mitoulas et al., 2002).

Some situations impede breast-feeding. For example, premature babies are usually fed from milk banks, and mothers returning to work may prefer to extract their own milk to continue feeding their babies. To preserve breast milk in such cases, it is either refrigerated or frozen and then thawed before use (Vilar-Compte et al., 2021). The impact of these processes has been studied from bacteriological, immunological, enzymatic, and antioxidant perspectives. However, there is a research gap in regard to the effect of freezing-thawing on macronutrient digestibility.

Short-term freezing-thawing does not significantly affect the composition of milk (Gao et al., 2019), but it could affect its structure and hence nutrient digestibility (Singh, 2019). Although breast milk is a complex mixture of components, it remains stable as a colloidal emulsion. Fat globules are stabilized by a complex interfacial layer onto which different types of proteins and components are deposited. However, different freezing mechanisms could disrupt the system, including the coalescence of lipid droplets (Singh & Gallier, 2017; Tribst et al., 2020). Increased lipid droplet size resulting from coalescence and the surface area exposed to lipases are factors that have been directly linked to the extent of lipolysis (Gallier, Ye, & Singh, 2012).

Gastrointestinal conditions could also play a role in the digestibility of breast milk nutrients, as reported for different foods under conditions of pancreatic insufficiency in cystic fibrosis (CF) (Asensio-Grau et al., 2018; Asensio-Grau et al., 2019; Asensio-Grau et al., 2021; Calvo-Lerma et al., 2020; Larriba et al., 2022). In CF, non-digested lipids are known to be excreted in feces, along with fat-soluble vitamins, leading to a loss of energy and vitamin deficiency (Anthony et al., 1999).

Maldigestion of nutrients in CF affects growth and nutrition, compromising disease prognosis and survival. In fact, infants with CF are shorter and lighter than healthy babies (Koletzko & Reinhardt, 2001). The best therapeutic approach to revert maldigestion is

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pancreatic enzyme replacement therapy (PERT), which consists of taking encapsulated pancreatic enzyme supplements at every meal. The recommended dose range for breast-fed infants is 5–25 lipase units/mL of breast milk or infant formula. However, this recommended dose is supported by little scientific evidence (Turck et al., 2016). Therefore, for breast-fed infants with CF, breast milk is particularly important because it is their only source of energy and nutrients, and it may not be optimally digested.

This study assessed the impact of refrigeration and freezing-thawing of breast milk on macronutrient digestibility and liposoluble vitamin bioaccessibility under *in vitro* simulated digestion. The study aimed to show the impact of CF gastrointestinal conditions and the effect of the dose of pancreatic enzyme replacement therapy (PERT).

2. Materials and methods

2.1. Experimental design

An experiment was designed to assess the effect of breast milk processing (refrigeration *vs.* freezing-thawing), simulated intestinal conditions (healthy infant *vs.* infant with CF), and pancreatin dose (0–25 lipase units [LU] per mL of breast milk) on lipid and protein digestibility and liposoluble vitamin bioaccessibility (Table 1).

2.2. Materials

Rabbit gastric extract (RGE-15) including pepsin and lipase was acquired from Lipolytech (Marseille, France). A commercial enzyme supplement (Kreon® 5000 LU, Maylan, Ireland) was kindly donated by the *Instituto de Investigación Sanitaria La Fe*, Valencia, Spain. Kreon 5000 LU consists of microspheres with a pH sensitive coating. 100 g of gastroresistant microspheres includes 60.36 mg of porcine pancreatin equivalent to 5000 lipase units (LU), 3600 amylase units (AU), and 200 protease units (PU).

Pancreatin from porcine pancreas (8 x USP, P7545), bovine bile (dried, unfractionated, B3883), a free fatty acid (FFA) quantification kit (Sigma-Aldrich), palmitic acid (analytical standard), ethanol (96%, HPLC grade), methanol (≥99.9%, HPLC grade), tetrahydrofuran (≥99.9%, HPLC grade), acetonitrile (≥99.9%, suitable for HPLC, gradient grade), Triton X-100 solution, analytical grade salts (potassium chloride, sodium chloride, and sodium bicarbonate), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA, ACS reagent, 99.4%–100.6%, powder), urea (ReagentPlus®, ≥99.5%, pellets), tyrosine (≥98%, HPLC), retinol (99%, 3100 U/mg), cholecalciferol (≥98%), α-tocopherol (analytical standard), phylloquinone (analytical grade standard), and all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) and were of analytical grade.

Enzyme activities were determined as described in the Electronic Supplementary Information from the protocol provided by Brodkorb et al. (2019).

Table 1
Total contents of fat, protein, lactose, dry matter, liposoluble vitamins, and somatic cells in fresh breast milk.

Breast milk component	Contents
Fat (g/100 g)	1.66 ± 0.16
Protein (g/100 g)	1.02 ± 0.04
Lactose (g/100 g)	7.21 ± 0.08
Dry matter (g/100g)	9.71 ± 0.03
Retinol (vitamin A) (μg/100 g)	53.1 ± 1.3
Cholecalciferol (vitamin D) (μg/100 g)	0.525 ± 0.02
α-tocopherol (vitamin E) (mg/100 g)	0.363 ± 0.003
Somatic cell count (x1000 cell/mL)	19 ± 5

The data shown are mean values from independent triplicates and the standard deviation.
Somatic cell count reflects polymorphonuclear leukocytes as an estimation of the presence of mastitis.

2.3. Breast milk collection and processing

Fresh breast milk was kindly donated by a 29-year-old healthy woman (four months post-partum). Collection was previously approved by the ethical committee of *Universitat Politècnica de València* (Num. P06_24-03-2022, UPV), and informed consent was signed. The breast milk was collected between 10:00 and 12:00 a.m. with a breast pump and was stored in sterile milk collection bags. The sample was transported to the laboratory on ice within 1 h and was divided into four equal sets before experimental use.

Two breast milk sets were stored for 24 h under domestic refrigeration conditions at 4 °C. The other two breast milk sets were frozen in a domestic freezer at −18 °C for 24 h. The frozen breast milk samples were then thawed at room temperature. Refrigerated and frozen-thawed samples were allowed to reach a temperature of 25 °C for 15 min before *in vitro* digestion and analysis.

2.4. Sample characterization

2.4.1. Macronutrients

Fat, protein, lactose, and total solid contents were determined using a Milko-Scan FT6000 (Foss Electric A/S, Hillerød, Denmark). Somatic cell count (SCC) was determined using a Fossomatic 5000 counter (Foss Electric Hillerød, Denmark; Toffanin et al., 2015).

2.4.2. Liposoluble vitamins

Breast milk samples were subjected to saponification. Vitamin A (retinol, 99%, 3100 U/mg), vitamin D (cholecalciferol, ≥98%), and vitamin E (α-tocopherol, analytical standard) were extracted and quantified by liquid chromatography, as described by Hernández-Olivas et al. (2020).

2.4.3. Particle size distribution

Particle size distribution of fresh, refrigerated, and frozen-thawed milk samples was measured using a laser light scattering instrument (Mastersizer 2000; Malvern, UK). The refractive indexes were 1.458 and 1.460 for milk fat at 633 and 466 nm, respectively, and 1.33 for water. The absorption coefficient at both wavelengths was 0.0001. A quantity of 1 mL of milk was placed in the measurement cell of the apparatus (to reach 10% of obscuration), containing approximately 600 mL of water and 1 mL of 35 mM EDTA/NaOH pH 7.0 buffer to disrupt the casein micelles (Ménard et al., 2010).

2.4.4. Zeta potential

The zeta potential of milk components was measured using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Malvern, UK), as described by Zhao et al. (2019). The samples were prepared by suspending 100 μL of milk in 10 mL of buffer (20 mM imidazole, 50 mM NaCl, 5 mM $CaCl_2$, pH 7.0). The zeta potential was measured after 120 s of delay at 25 °C.

2.4.5. Microstructure

A confocal laser scanning microscope (CLSM) was used to observe the microstructure of fresh, refrigerated, and frozen-thawed breast milk samples. Fat globules (0.5 mL) were stained using 50 μL of Nile Red solution (0.45 μg/mL in acetone) and 50 μL of Fast Green FCF solution (0.01% w/v in MilliQ water). The samples were subsequently allowed to stain for at least 60 min at room temperature. Then, 10 μL of stained samples were loaded on 26 × 76 mm slides and covered with a 40 × 22 mm coverslip. Images were taken using an inverted Axio Observer Z1 Zeiss coupled to LSM 780 (Carl Zeiss, Jena, Germany) with a C-Apochromat 40x/1.20 Korr FCS M27 objective. Experiments were performed using an argon laser operating at 488 nm (emission detected between 500 and 530 nm) and a He–Ne laser operating at 561 nm

excitation wavelength (emission detected between 566 and 610 nm; Yang et al., 2021).

2.5. In vitro luminal gastrointestinal digestion

To assess differences in breast milk digestibility between healthy infants and infants with CF, two gastrointestinal digestion *in vitro* models were applied. The standardized static *in vitro* digestion method for infants published by Ménard et al. (2018) was used to represent the intestinal conditions of healthy infants (intestinal pH = 6.6, bile salts = 3.1 mM, pancreatin concentration = 230 LU/mL breast milk). Modifications were then made to this model to simulate the intestinal conditions of infants with CF (intestinal pH = 6 [Gelfond et al., 2013], pancreatin from supplement at different concentrations: 0, 5, 10, 15, 20, and 25 LU/mL of breast milk [Turck et al., 2016]).

The simulated gastric and intestinal digestion fluids were prepared according to the corresponding stock solutions (Ménard et al., 2018). The oral stage was omitted as advised for the simulation of digestion of liquid foods. *In vitro* digestion was then simulated in two stages.

For the gastric stage, 5 mL of breast milk were added to 2.9 mL of simulated gastric digestion fluid (pH 5.3) containing pepsin (268 U/mL) and gastric lipase (19 LU/mL) in a 63:37 (v:v) proportion. Tubes were head-over-heels rotated (Intelli-Mixer RM-2) at 55 rpm in a thermostatic chamber (JP Selecta SA, Barcelona) at 37 °C for 60 min. The intestinal stage was simulated by adding simulated intestinal fluid containing pancreatin and bovine bile salts to the gastric chyme in a 62:38 (v:v) proportion. Tubes remained in agitation for 60 min at 37 °C. During the digestion process, the pH was monitored to maintain the correct pH in each experimental condition. Aliquots were taken at the end of the gastric and intestinal stages of simulated digestion. After completion of digestion, samples were immediately stored at −20 °C prior to further analysis.

2.6. Nutrient digestibility and bioaccessibility

2.6.1. Lipid digestibility

The extent of lipolysis was determined as free fatty acids at the end of the gastric and intestinal stages, as described by Asensio-Grau et al. (2021). Aliquots of digested samples were added to 100 μL of pure ethanol and 150 μL Triton X® and were diluted to a volume of 0.5 and 10 mL for gastric and intestinal digesta, respectively. The amount of free fatty acids was determined using a free fatty acid quantitation kit (MAK044, Sigma–Aldrich) following the manufacturer's protocol. Absorbance was measured at 570 nm by spectrophotometer (Perkin–Elmer, Wellesley, MA, USA). Lipid digestibility was calculated according to Equation (1).

$$\textit{Extent of lipolysis}\ (\%) = \frac{\textit{g Free fatty acids in digested samples}}{\textit{g lipid in non} - \textit{digested samples}} \times 100 \qquad (1)$$

2.6.2. Protein digestibility

Protein hydrolysis was evaluated by measuring the soluble protein fraction in trichloroacetic acid (TCA), as described by Hernández-Olivas et al. (2022), with slight modifications. The soluble fraction in TCA contained small peptide and amino acid residues generated during digestion. Briefly, 36% TCA (500 μL) was added to the liquid digesta (1000 μL) to give a final concentration of 12% (w/w). Samples were then incubated at 10 °C for 15 min in an Eppendorf Thermomixer Comfort (Eppendorf AG 22331, Hamburg, Germany) and were centrifuged at 1200 g-force for 10 min (Eppendorf MiniSpin Plus, Hamburg, Germany). The supernatant was collected and diluted in 50 mM EDTA and 8 M urea buffer (pH 10). The soluble protein in TCA was determined in triplicate by measuring absorbance at 280 nm against a blank prepared with the corresponding digestive fluids of each digestion model. The ratios of supernatant to buffer (v:v) were 1:2 and 1:7 for gastric and intestinal digested samples, respectively. The extent of proteolysis (%) was calculated using a calibration curve ranging from 0 to 0.2 mg/mL using L-tyrosine as standard (Equation (2)).

$$\textit{Extent of proteolysis}\ (\%) = \frac{\textit{g TCA s.p. in the digested samples}}{\textit{g protein in undigested samples}} \times 100 \qquad (2)$$

Size exclusion chromatography (SEC-HPLC) was carried out with an HPLC system (Waters Alliance 2695) equipped with an Agilent SEC-3, 100A (7.8 mm × 300 mm). The samples were diluted and filtered using a hydrophilic syringe filter (0.45 μm PVDF membrane, CHMLAB group, Barcelona, Spain). Isocratic separation was achieved with a mobile phase consisting of 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 mL/min. Absorbance was monitored at 220 nm. The chromatograms were integrated using Empower Pro2 software (Waters). To estimate the molecular weight distribution of the peptides, elution times were compared to standard elution times: Cytochrome (12.4 kDa), insulin (5.8 kDa), bacitracin (1.4 kDa), Gly-Gly-Tyr-Arg (0.45 kDa), triglycine (0.19 kDa), and glycine (0.075 kDa).

2.6.3. Liposoluble vitamins

Saponification of the digested samples and extraction and quantification of the bioaccessible vitamins (retinol, cholecalciferol, and α-tocopherol) after intestinal digestion was performed as explained in the sample characterization section. Liposoluble vitamin bioaccessibility was calculated using Equation (3). The amount of released vitamin represented the recovered part in the bioaccessible fraction after *in vitro* digestion and the total amount of vitamin found in non-digested milk before *in vitro* digestion.

$$\textit{Vitamin bioaccessibility}\ (\%) = \frac{\textit{μg of released vitamins}}{\textit{μg of total vitamins}} \times 100 \qquad (3)$$

2.7. Statistical analysis

To study the impact of the factors (freezing-thawing, intestinal conditions, and pancreatin concentration) on lipid and protein digestibility, vitamin bioaccessibility, and changes in microbiota composition and SCFA production, one-way analysis of variance (ANOVA) was performed using Statgraphics Centurion XVIII software with a confidence level of 95% (p-value >0.05).

3. Results and discussion

3.1. Breast milk composition

The initial composition of fresh breast milk in terms of fat, protein, lactose, dry matter, and liposoluble vitamin (A, D, and E) contents is shown in Table 1. The donated breast milk had lower values of lipids than in other studies reporting average lipid contents of 3.8–3.9 g/100 mL (Fleischer Michaelsen et al., 1990). However, the values in this study fall within the range reported by Kelishadi et al. (2012) of 0.95–3.39 g/100 mL (Kelishadi et al., 2012). The protein content was at the lower limit of the normal range (1.1–3.5 g/100 mL) for breast milk (Weber et al., 2001). Notably, maternal diet determines the fat content and fatty acid composition of breast milk (Kelishadi et al., 2012). The protein content of breast milk is influenced by the time at which milk is extracted (Gidrewicz & Fenton, 2014). Although lipid content was low in the study sample, liposoluble vitamin levels were comparable with those reported in previous studies (Lammi-Keefe & Jensen, 1984; Sakurai et al., 2005; U.S. Department of Agriculture, 2019).

3.2. Impact of freezing-thawing on microstructural parameters of breast milk

The microstructure and particle size distribution of milk fat globules were first determined to support the explanation of macronutrient digestibility after refrigeration and freezing-thawing.

Fig. 1 shows the structure of the lipids (in red) and proteins (in green) contained in fresh, refrigerated, and frozen-thawed breast milk samples. The green-dyed proteins were found to be superposed on the red-dyed fat globules in all cases, showing that proteins were deposited on the fat globule external layer (Yang et al., 2021). Similar structures were found in fresh and refrigerated milk samples, with a uniform size distribution of dyed lipids and proteins. However, larger fat globules, probably as a consequence of aggregation, were observed in the

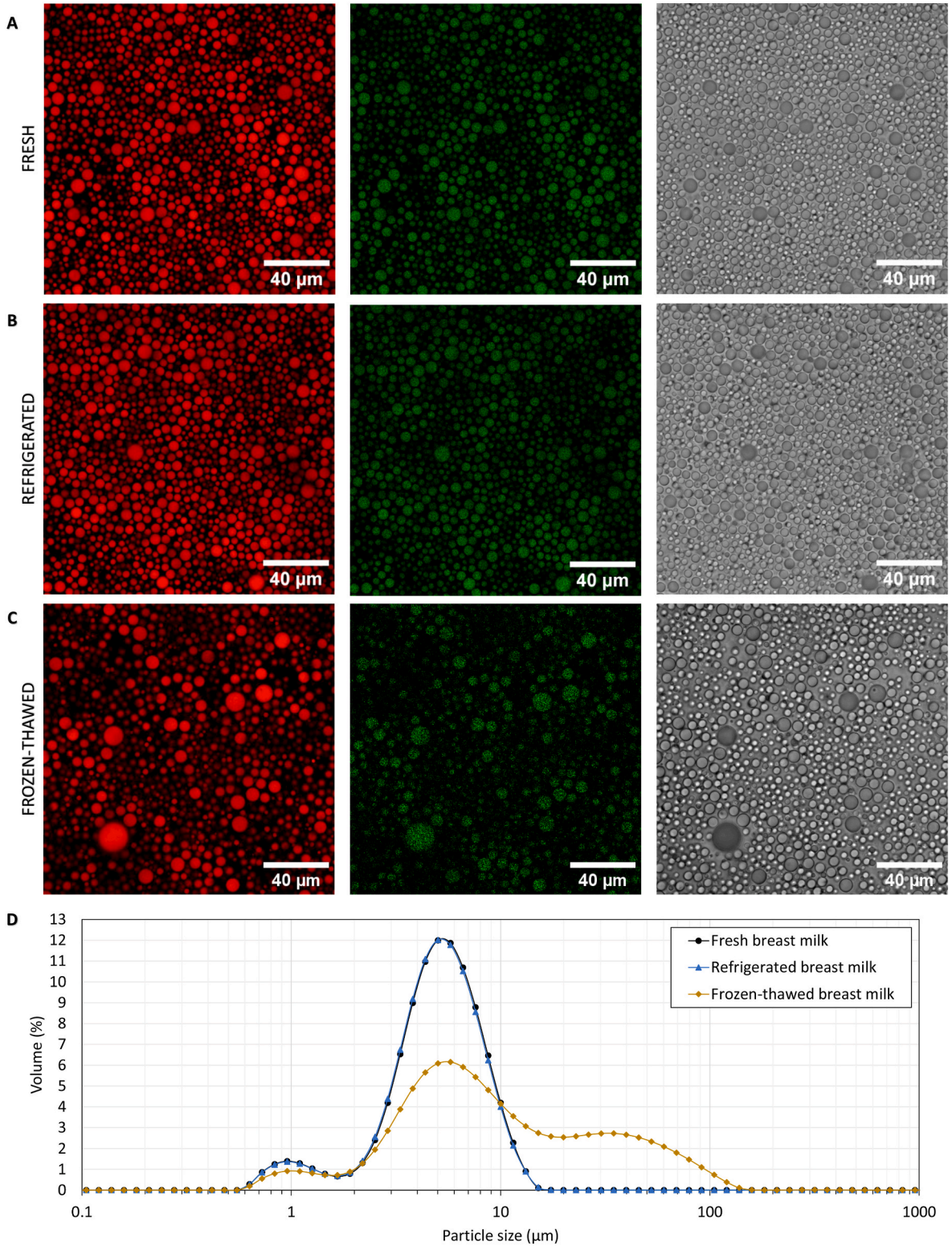

Fig. 1. Microstructure and particle size distribution of the fresh, refrigerated, and frozen-thawed breast milk fat globules obtained by confocal laser scanning microscopy (A, B, and C, respectively) and laser light scattering (D). For the microscopy, triacylglycerols from milk fat globules were labeled with Nile Red and proteins with Fast Green fluorescent dyes. Values in the particle size distribution are the means of triplicates and standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

frozen-thawed micrographs. This finding echoes those of Zhang et al. (2022), who reported aggregated milk fat globules after freezing breast milk at −18 °C and thawing at different temperatures of 4 °C, 25 °C, and 45 °C. When coalescence occurred, the lipidic surface area increased. Therefore, an impact on nutrient digestion should be expected given that previous studies have reported a direct correlation between fat globule size and extent of lipolysis (Calvo-Lerma et al., 2019).

Fig. 1D shows the particle size distribution of the fresh, refrigerated, and frozen-thawed breast milk samples. The microscopy assessment showed that fresh and refrigerated milk samples did not change in terms of their particle size distribution or their $D_{4,3}$, $D_{3,2}$, d(0.1), d(0.5), and d (0.9) values (Table 2), assuming that no changes were due to the 24-h refrigeration of the breast milk fat globules. However, significant differences were found in all particle size parameters of the frozen-thawed breast milk sample. A bimodal distribution was observed in fresh and refrigerated milk samples, with particles ranging from 2 to 20 μm in diameter and, to a lesser extent, particles of about 1 μm. The breast milk fat globules typically had a diameter of 3–9 μm and were mainly composed of a hydrophobic core including triacylglycerols (TAG). This core was enveloped by a triple layer of amphipathic compounds forming the milk fat globule membrane. These compounds included phospholipids, proteins, enzymes, and cholesterol (Abrahamse et al., 2012). However, frozen-thawed breast milk had a trimodal distribution, with large particles (ranging from 20 to 100 μm). This finding offers further evidence that the milk fat globules increased in size due to coalescence or agglomeration.

The different household storage conditions seemed to alter the breast milk structure and particle size. This impact was especially evident during freezing. Breast milk is commonly stored at temperatures between −15 and −20 °C in household refrigerators or between −60 and −80 °C in breast milk banks. Zhang et al. (2022) noted that, under the freezing conditions of breast milk banks, fewer changes occur in milk structure and components. The lower degree of fat globule aggregation could be the result of a faster and more powerful phase change process. However, these conditions entail a high storage cost and are uncommon at the domestic level.

The apparent zeta potential of the assessed breast milk samples (Table 2) showed that refrigeration had no effect when compared with the fresh breast milk sample. However, the freezing-thawing process significantly ($p < 0.05$) decreased the zeta potential value by around 12%. Michalski et al. (2005) reported zeta potential values of −7.8 ± 0.1 mV, which are in accordance with the results of the current study. However, decreased apparent zeta potential values have been reported for cow's milk (−13.5 mV) and homogenized commercial cow's milk (−20 Mv; Michalski et al., 2002).

Given the lack of differences in terms of structure between fresh and refrigerated breast milk samples (Table 2), the subsequent analytical determinations focused on the differences between the refrigerated and frozen-thawed samples.

Table 2
Parameters evaluated in breast milk after refrigeration and freezing-thawing.

	Fresh	Refrigerated	Frozen-thawed
$D_{3,2}$ (μm)	3.52 ± 0.05	3.50 ± 0.03	5.05 ± 0.09[b]
$D_{4,3}$ (μm)	5.03 ± 0.05[a]	4.97 ± 0.03[a]	6.9 ± 0.9[b]
d(0.1)	2.24 ± 0.05[a]	2.22 ± 0.04[a]	2.55 ± 0.05[b]
d(0.5)	4.79 ± 0.03[a]	4.70 ± 0.02[a]	7.47 ± 0.15[b]
d(0.9)	8.20 ± 0.08[a]	8.13 ± 0.03[a]	46 ± 3[b]
Apparent zeta potential (mV)	−5.85 ± 0.04[a]	−5.9 ± 0.5[a]	−6.7 ± 0.6[a]

Values are means of triplicates and standard deviation. $D_{4,3}$ is the volume weighted mean.

3.3. Influence of processing and intestinal conditions on nutrient digestibility

The extent of lipolysis of the digested refrigerated and frozen-thawed breast milk samples appears in Fig. 2. In healthy infant conditions, the breast milk had a lipolysis extent of 34.0 ± 0.9 and 32 ± 3 g FFA/100 g of fat for the refrigerated and frozen-thawed samples, respectively. Although lower values were observed after freezing-thawing, there were no significant differences ($p > 0.05$). The digestion of dietary lipids and the absorption of lipolysis products in infant digestion is important and could be altered by the particle size and structure of milk fat globules (Calvo-Lerma et al., 2019). Larger fat globules have a smaller interfacial area, so there will be less lipase physical interaction with the fat globules, which leads to a decreased rate and extent of lipid digestion (Bourlieu et al., 2015; Sun et al., 2023). This mechanism could explain the significantly lower lipolysis extents found in the frozen-thawed samples in some of the CF experimental conditions.

Focusing on the specific infant digestion model (healthy *vs.* CF), significantly lower lipolysis values were detected under CF conditions at all enzymatic supplement doses. The exception was 25 LU/mL of breast milk, for which equivalent lipolysis to the standard pancreatin concentration in healthy infant conditions was observed. Therefore, a dose of 25 LU/mL of breast milk achieved the same extent of lipolysis as in healthy conditions. In contrast, the results for the 20 and 15 LU/mL doses were not significantly different from those for the control. Therefore, the recommended dose would seem to be in the range 15–25 LU/mL.

Notably, the total extent of lipolysis in the studied samples was low (60% or less in all cases). This finding contrasts with those of previous studies reporting lipolysis values of around 80% for breast milk samples under *in vitro* digestion simulation (Cheong et al., 2018; Pan et al., 2021). The difference could be that, in this study, the breast milk sample had a lower lipid content than is standard, which has been linked to a decreased extent of lipolysis in hydrocolloid systems (Calvo-Lerma et al., 2018).

Focusing on the gastric stage, significantly higher extents of lipolysis were detected under CF conditions. In the CF model, pancreatin was supplied by an encapsulated pancreatic enzyme supplement. Therefore, partial degradation of the encapsulation system could have led to the release of pancreatic lipase in the gastric stage. The overall low values of gastric lipolysis may be related to endogenous maternal bile salt-stimulated lipase (BSSL) in breast milk, which has been reported to delay lipase action (Liu et al., 2021).

Proteolysis was also assessed for the same range of doses of pancreatic enzyme supplements. The results appear in Fig. 3, along with the extent of proteolysis in the healthy infant model. At the end of gastric digestion, the infant standardized gastrointestinal conditions had values of around 6%, with no effect of refrigeration versus freezing-thawing. However, under CF conditions, increasing the dose of the pancreatic enzyme supplement was associated with a slight but non-significant ($p > 0.05$) increase in gastric proteolysis. However, after intestinal digestion, increased proteolysis values were detected. Depending on the enzyme dose, the values ranged from 5.2% to 12.8% and 4.7%–9.3% for refrigerated and frozen-thawed breast milk samples, respectively. Maximum protein digestibility was detected at 20 LU/mL of breast milk for the refrigerated breast milk sample and at 15 LU/mL for the frozen-thawed breast milk sample. Thus, refrigeration or freezing-thawing and the dose of the pancreatic enzyme supplement significantly affected breast milk protein digestion.

Proteolysis was also evaluated by SEC-HPLC after the gastric and intestinal digestion of refrigerated and frozen-thawed breast milk. Fig. 4A shows that more than 95% of non-digested breast milk proteins had a relative size distribution of >12.5 kDa. After gastric digestion, proteins were broken down into smaller peptides. In healthy infant conditions (230 LU/mL breast milk) and CF conditions without pancreatic supplement (0 LU/mL milk), larger peptides decreased to

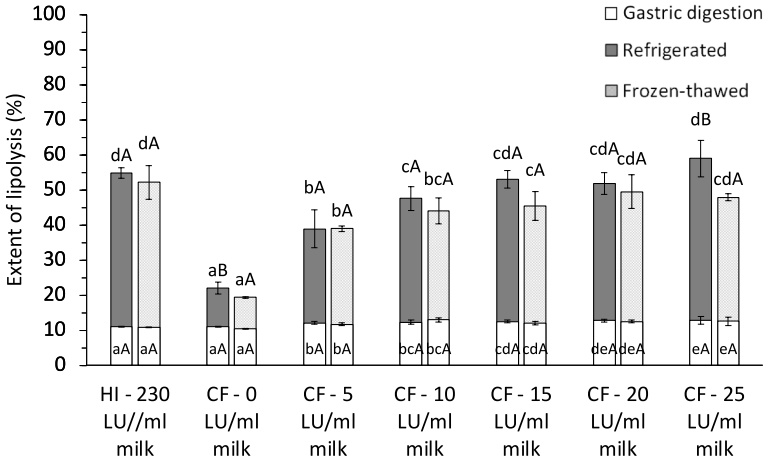

Fig. 2. Extent of lipolysis (g FFA/100 g fat) of refrigerated and frozen-thawed breast milk under healthy infant (HI) conditions (230 LU/mL breast milk) and cystic fibrosis using different pancreatin doses (0, 5, 10, 15, 20, and 25 LU/mL of breast milk). Lowercase letters denote differences between gastrointestinal conditions in the same milk sample. Uppercase letters describe significant differences between thermal conservation processes (refrigeration or freezing-thawing).

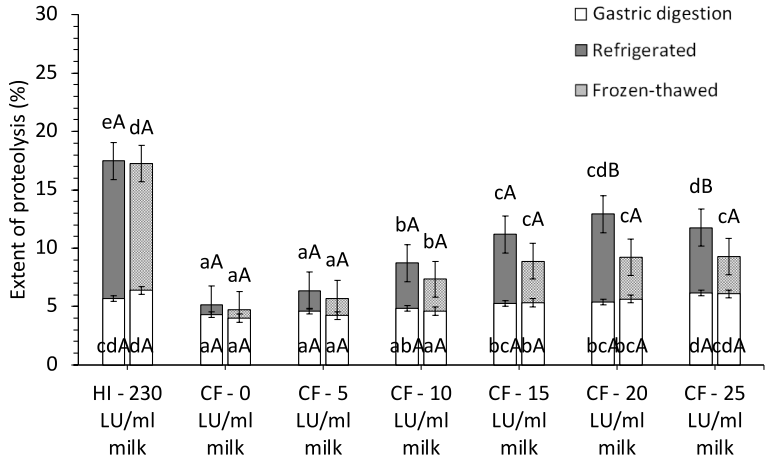

Fig. 3. Extent of proteolysis after gastric and intestinal *in vitro* digestion of refrigerated and frozen-thawed breast milk samples. Healthy infant (HI) gastrointestinal conditions were those used by Ménard et al. (2018). Tyrosine was used as standard and represents the protein fraction solubilized in trichloroacetic acid (TCA). Lowercase letters denote differences between gastrointestinal conditions in the same milk sample. Uppercase letters denote significant differences between thermal conservation processes (refrigeration or freezing-thawing).

approximately 75%. However, as observed with the TCA soluble protein method, extra proteolytic activity was registered in CF conditions. This extra activity could be ascribed to the partially released proteases from the pancreatic supplement during gastric digestion. During this stage, the progressive increase of pH has been shown to cause partial disintegration of the coating of the pancreatic enzyme supplement (Hartmann et al., 2022). This interpretation is supported by the higher proteolytic activity registered with increasing doses of pancreatic supplement.

At the intestinal stage in healthy infant gastrointestinal conditions, Fig. 4B does not reveal differences in the peptide size distribution of the digested breast milk under any thermal process. However, when no supplement was added to the simulation at the lowest dose of pancreatin (5 LU/mL milk), slight differences were found. The rest of the doses appeared not to be affected by the choice of freezing-thawing or refrigerating. When increasing the pancreatic supplement dose, increased hydrolysis into smaller peptides was observed. For infants with CF, a peptide profile similar to that of healthy infants is desirable. For example, if undigested protein fractions enter the distal colon for bacterial fermentation, undesirable metabolites (e.g., ammonia, amine, N-nitroso compounds, phenol, and sulfide) may be generated. Allergic reactions or indigestion symptoms (such as diarrhea) could also occur (Zhang et al., 2022). A similar peptide size distribution to that found in healthy infants was achieved after digestion using an enzyme supplement dose of 15 LU/mL milk, suggesting that this dose would be the optimal dose of proteolysis, even for frozen-thawed breast milk.

3.4. Effect of refrigeration or freezing-thawing and intestinal conditions on liposoluble vitamin bioaccessibility

Impaired lipid digestion in CF has been associated with the loss of liposoluble vitamin absorption because they are excreted along with the unabsorbed fat fraction (Rayner, 1992). Liposoluble vitamin deficiencies are related to a detriment in nutritional status and disease prognosis. A deficiency of vitamin A (retinol) can lead to chronic lung disease and may increase vulnerability to infection. A lack of vitamin D (cholecalciferol) can decrease bone mineralization and lead to impaired

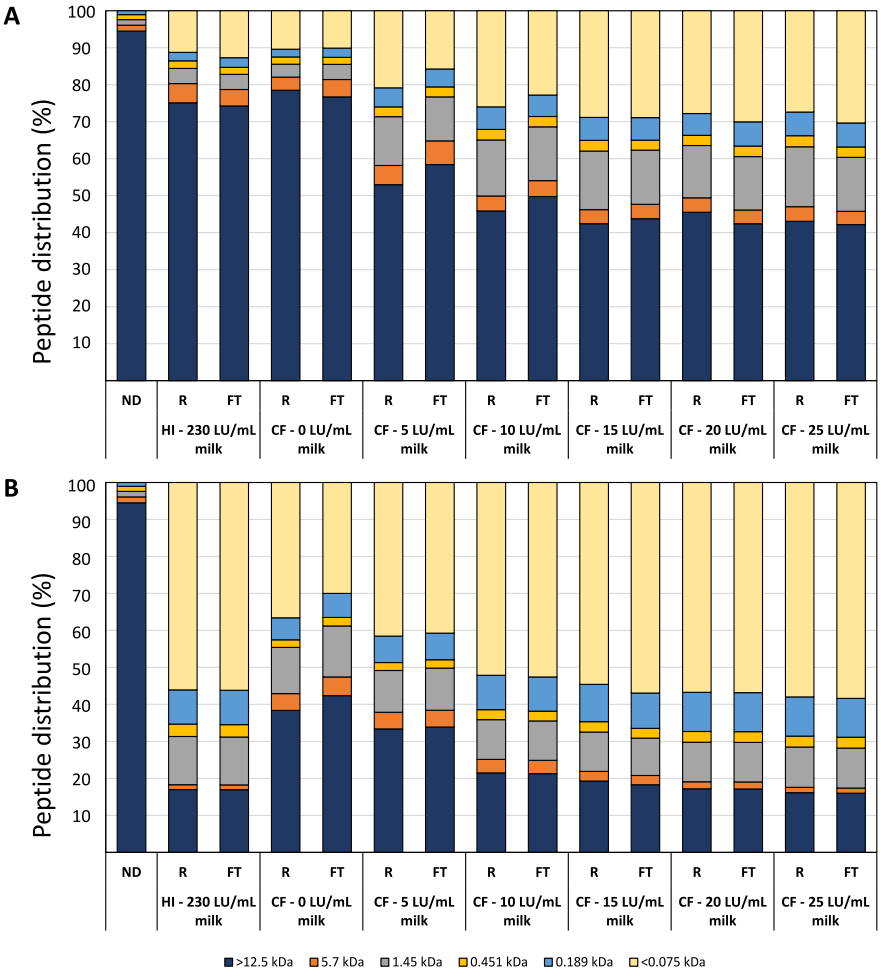

Fig. 4. Relative size distribution of peptides of refrigerated (R) and frozen-thawed (FT) breast milk proteins after gastric (A) and intestinal (B) *in vitro* digestion under healthy infant conditions (HI – 230 LU/mL breast milk) and different doses of pancreatic supplements (0, 5, 10, 15, 20, and 25 LU/mL milk) for infants with cystic fibrosis (CF).

immune response. Vitamin E (α-tocopherol) deficiency may cause hemolytic anemia (Henriksen et al., 2006). Table 3 presents the results for the bioaccessibility of liposoluble vitamins from refrigerated and frozen-thawed breast milk samples after intestinal digestion. In healthy infant conditions, the bioaccessibility of vitamins from refrigerated breast milk was 63%, 84%, and 65% for retinol, cholecalciferol, and α-tocopherol, respectively. The freezing-thawing process significantly affected ($p < 0.05$) vitamin E by reducing its bioaccessibility by 8%. Under CF experimental conditions, freezing-thawing, compared to refrigerating, achieved the same bioaccessibility of liposoluble vitamins as in healthy conditions for almost all doses.

4. Conclusions

The study showed that freezing-thawing significantly affected breast milk structure, including increasing the fat globule particle size. These changes had a significant negative effect in CF digestion conditions but not in healthy infant digestion conditions. The results suggest that infants with CF being fed frozen-thawed breast milk will have reduced lipolysis, proteolysis, and vitamin A and E bioaccessibility. The study also assessed the dose of pancreatic enzyme replacement therapy (PERT). The results showed that 25 LU/mL of breast milk would enable equivalent lipolysis (55%) to that achieved under healthy infant conditions.

In conclusion, freezing-thawing of breast milk did not change macronutrient digestibility in healthy breast-fed infant digestion. However, it may have a negative impact in breast-fed infants with CF. This finding may be explained by the different physical properties of the samples under these two treatments in the CF digestion environment. This study also provides the first evidence that the correct dose of pancreatic enzyme supplements in cases of infants with CF can lead to equivalent lipolysis as in healthy subjects. However, the same is not true for proteolysis, at least for the dose range assessed in this study. The results of the study could lead to the early development of evidence-based criteria to recommend the dose of pancreatic enzyme replacement therapy (PERT) in breast-fed infants with CF. These results could also encourage the avoidance of freezing as a preservation method for breast milk for infants with CF.

CRediT authorship contribution statement

Ever Hernández-Olivas: Methodology, Investigation, Resources, Writing – original draft. **Andrea Asensio-Grau:** Conceptualization, Methodology, Investigation, Resources. **Joaquim Calvo-Lerma:** Conceptualization, Software, Investigation, Writing – original draft, Writing – review & editing. **Ana Heredia:** Conceptualization, Investigation, Writing – review & editing. **Ana Andrés:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Table 3
Liposoluble vitamin (retinol, cholecalciferol, and α-tocopherol) bioaccessibility after intestinal *in vitro* digestion of refrigerated and frozen-thawed breast milk samples under healthy infant (HI) and cystic fibrosis (CF) intestinal conditions.

Liposoluble vitamin	Intestinal condition	Bioaccessibility (%)	
		Refrigerated	Frozen-thawed
Retinol	HI - 230 LU/mL milk	63 ± 4^{bcA}	58 ± 4^{bcA}
	CF - 0 LU/mL milk	49 ± 2^{aA}	46 ± 3^{aA}
	CF - 5 LU/mL milk	56 ± 6^{abA}	55 ± 5^{bA}
	CF - 10 LU/mL milk	60 ± 2^{bA}	58 ± 4^{bcA}
	CF - 15 LU/mL milk	62 ± 2^{bA}	59 ± 4^{bcA}
	CF - 20 LU/mL milk	70 ± 4^{cA}	61 ± 5^{bcA}
	CF - 25 LU/mL milk	65 ± 4^{bcA}	62 ± 5^{cA}
Cholecalciferol	HI - 230 LU/mL milk	84 ± 2^{abA}	82.9 ± 0.8^{abA}
	CF - 0 LU/mL milk	82.0 ± 1.3^{aA}	80.5 ± 0.6^{aA}
	CF - 5 LU/mL milk	82.6 ± 1.2^{aA}	83 ± 2^{abA}
	CF - 10 LU/mL milk	84.33 ± 1.05^{abA}	85 ± 2^{abA}
	CF - 15 LU/mL milk	84 ± 3^{abA}	84.2 ± 1.2^{abA}
	CF - 20 LU/mL milk	87 ± 4^{abA}	87.0 ± 1.3^{bA}
	CF - 25 LU/mL milk	88.5 ± 1.2^{bA}	86.39 ± 1.10^{bA}
α-tocopherol	HI - 230 LU/mL milk	65 ± 2^{bcB}	57 ± 5^{bA}
	CF - 0 LU/mL milk	43 ± 4^{aA}	50.7 ± 0.6^{aB}
	CF - 5 LU/mL milk	46 ± 5^{aA}	51 ± 5^{abA}
	CF - 10 LU/mL milk	50 ± 4^{abA}	54 ± 3^{abA}
	CF - 15 LU/mL milk	58 ± 4^{bA}	58 ± 5^{bA}
	CF - 20 LU/mL milk	67 ± 4^{cA}	61 ± 6^{bA}
	CF - 25 LU/mL milk	64 ± 3^{bcB}	57 ± 2^{bA}

The data shown are mean values from independent triplicates and the standard deviation. Lowercase letters ^{ab} denote differences among gastrointestinal conditions. Uppercase letters ^{AB} denote significant differences between refrigerated and frozen-thawed breast milk samples.

Declaration of competing interest

Authors have no conflict of interest to declare.

Data availability

Data will be made available on request.

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